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Epigenetic Modifiers to Augment the Immunogenicity of Chronic Lymphocytic Leukemia

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by

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Abstract

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Cancer cells employ a litany of immunosuppressive and immunevasive strategies to avoid detection and elimination by the various arms of the innate and adaptive immune system. Many hematologic and solid tumors progressively develop a specialized microenvironment which promotes tissue restructuring inflammation while masking the immune signature of the tumor cells themselves. Chronic lymphocytic leukemia, a malignancy of mature B lymphocytes must constantly balance on the precipice of immune recognition. A mature antigen presenting cell themselves, CLL clonal growth is dependent on the very interactions which, if effective, could potentially lead to their demise. To circumvent this, CLL clones set up unique signatures which promote immune recognition yet provide diversionary signals to the remaining immune armament resulting in profound immune dysfunction.

While the aforementioned immune dysfunction is widespread, the B cell and T cell repertoire are severely impaired during leukemic progression. The lack of healthy B cells due to displacement by malignant B cells results in the obvious loss of an important antigen presenting cell as well as antibody-based immunity. Additionally, deficient interactions with T cells result in anergy and the preponderance of improperly polarized T lymphocytes which are impotent to eliminate both pathogens and leukemic cells. The

result of such severe immune dysfunction is chronic infection and progressive disease which is the primary cause of death in CLL patients.

Our research was focused on the premise that alleviating immune dysfunction and providing immunotherapeutic tools will significantly benefit CLL therapy. To this end we developed methods to improve the cellular interaction between CLL cells and T cells a critical step towards improving the antigen presentation capacity of the diseased B cell repertoire. We also identified a therapeutic strategy which can revert the anergic or improperly polarized state of T cells already in circulation allowing those cells to more effectively perform the effector functions necessary to fight pathogenic attack and malignant transformation. Finally, we identified a number of novel targets in CLL which could be used in a vaccinate-induce method to license the elimination of CLL cells by the patient's adaptive immune system. To achieve our goals we utilized a relatively new class of drugs called epigenetic modifiers which specifically alter the chromatin structure resulting in novel genetic signatures which are heritable over cellular generations. The unique properties of these drugs allow for the elicitation of suppressed genetic programs which, when properly controlled, have the potential to reassert healthy lymphocyte functions.

Our studies provide a comprehensive therapeutic initiative which, by simultaneously alleviating the major causes of immune dysfunction in addition to facilitating the use of novel active immunotherapeutic strategies could potentially impact clinical therapy for CLL.

Chapter One:

Introduction

Tumor Immunology Overview

Our view of tumor immunology underwent a dramatic change in the early 90's given the surprising observations that most of the antigens expressed by tumor cells were not necessarily neo-antigens uniquely present in cancer cells, but rather tissue-differentiation antigens also expressed in normal cells (Rosenberg 1995; Boon and van der Bruggen 1996). These unexpected findings led to the hypothesis that perhaps the greatest obstacles for harnessing the immune system against tumors were the complex mechanisms that establish T-cell tolerance against "self" tumor antigens. Experimental evidence supporting the above hypothesis was indeed provided by the Bogen's and Levitsky's groups who independently demonstrated that antigen-specific CD4+ T-cells were rendered unresponsive during tumor growth in vivo (Bogen 1996; Staveley-O' Carroll, Sotomayor et al. 1998). Following the initial report of this phenomenon termed "tumor-induced anergy", several studies showed that this state of T-cell unresponsiveness also occurs during the growth of hematologic or solid tumors expressing model or true tumor antigens (Morgan, Kreuwel et al. 1998; Cuenca, Cheng et al. 2003; Overwijk, Theoret et al. 2003), during the progression of spontaneously arising tumors (Willimsky and Blankenstein 2005), and more importantly during the progression of human cancers (Lee, Yee et al. 1999; Noonan, Matsui et al. 2005). This different view of tumor immunity also raised the bar for cancer immunotherapy, since the barrier imposed by

immune tolerance must be broken in order for the immune system to effectively recognize and eliminate tumors expressing mainly “self” antigens.

More recently, several studies have provided important insights into the cellular and molecular mechanisms of tolerance induction and the composition and in vivo fate of the tolerized population(Rabinovich, Gabrilovich et al. 2007). For instance, at the cellular level, bone-marrow (BM) derived antigen-presenting cells (APCs) and regulatory T-cells (Tregs) have been implicated as playing a central role in tolerance induction. At the molecular level, signaling pathways involved in the regulation of pro- or anti-inflammatory pathways in the APC have been shown to influence the ability of these cells to determine T-cell priming versus T-cell tolerance. Among those pathways, Stat3, c-kit, SOCS-1 and the zinc-finger A20 molecule have emerged as enticing molecular targets in APCs to overcome the remarkable barrier that tolerance to tumor antigens has imposed to cancer immunotherapy(Cheng, Wang et al. 2003; Wang, Cheng et al. 2005; Evel-Kabler, Song et al. 2006; Song, Evel-Kabler et al. 2008).

A continued effort to identify molecular mechanism(s) regulating pro and/or anti-inflammatory pathways in the APC would likely unveil additional targets to circumvent tumor-induced tolerance. Indeed, in recent years significant emphasis has been devoted to mechanistically understand regulation of pro-inflammatory/anti-inflammatory genes in its natural setting, the chromatin substrate. Although several mechanisms influence chromatin flexibility to allow dynamic changes in gene expression, chromatin modifications by acetylation/deacetylation of histone tails resulting in transcriptionally active or inactive chromatin play an important role in regulation of gene transcription, including genes involved in the inflammatory response(Foster, Hargreaves et al. 2007).

Similarly, DNA methylation status at the level of gene promoters also influences the transcriptional activity of inflammatory genes(Bruniquel and Schwartz 2003; Baguet and Bix 2004; Foster, Hargreaves et al. 2007). Of note, inhibition of DNA methyltransferase 1 (DNMT1) leading to hypomethylation of previously silenced chromatin has been associated not only with enhanced gene transcription but also with de-repression of antigens that could potentially serve as targets for cancer immunotherapy(Sigalotti, Fratta et al. 2004; Roman-Gomez, Jimenez-Velasco et al. 2007; Natsume, Wakabayashi et al. 2008). The latter observations are particularly relevant for tumors arising from antigen-presenting cells such as B-cell lymphomas and myeloid malignancies. In these cells, their antigen presenting capabilities could be enhanced via epigenetic manipulation with histone deacetylase inhibitors and/or hypomethylating agents that provide the double advantage of inducing de novo expression of tumor antigens such as cancer testis antigens (CTA), and enhance the expression of pro-inflammatory mediators resulting in an environment conducive to T-cell activation rather than T-cell tolerance. Importantly, re-activation of tumor antigens not previously seen by the immune system confers the additional opportunity to therapeutically target antigens that are less likely to induce T-cell tolerance. Alternatively, the de novo expression of these tumor antigens in response to epigenetic modifiers might provide a temporal window to effectively harness T-cell immune responses with vaccination strategies before T-cell tolerance towards these antigens is ultimately established.

Tumor Antigens

Three tenants of a good antigen

When identifying the classical tumor vaccine antigen there are three critical characteristics that have the potential to dictate overall vaccine efficacy.

I. The antigen must be of high antigenicity (Selvaraj, Yerra et al. 2008).

II. The antigen should be expressed in a significant percentage of the patient population, and

III. The antigen must be expressed in tumor cells and required for tumor survival.

Despite the seemingly obvious nature of these criterion, to date there have been few cancer vaccines that have satisfied all three. Indeed, most of the targeted tumor antigens, including cancer-testis antigens, fail to satisfy one or more of these criteria. One of the primary reasons that these three factors are so difficult to fulfill is because they might be working against each other. Take for instance, an antigen that is highly antigenic and is expressed on premalignant cancer cells. In this scenario there are two possible outcomes for the developing tumor clone: it could either succumb to immune cell recognition and be eliminated, or it could actively suppress antigenicity utilizing a variety of escape mechanisms. Take again, the case where a particular tumor associated antigen (TAA) is expressed in a large percentage of tumors. Given the vast heterogeneity inherent in the mammalian immune repertoire it is likely that such an antigen would maintain adequate central tolerance (and thus low antigenicity), otherwise the immune system would have already detected and eliminated cells expressing that particular antigen. Nevertheless, the fact that the tumor has progressed to a detectable level indicates the generation of significant peripheral tolerance. Finally, lets examine NY-

ESO-1, perhaps the most clinically studied member of the CTA family (Gnjatic, Nishikawa et al. 2006) with more than 30 clinical trials performed in cancer patients. Vaccines and immunotherapies targeting NY-ESO-1 assume that the antigen might be expressed within 100% of the tumor cells, a lofty goal given that NY-ESO-1 is not considered necessary for the survival of the malignant clone(Nicholaou, Ebert et al. 2006). In addition, the silencing mechanism for NY-ESO-1 is known to be promoter hypermethylation, providing a relatively simple immune escape mechanism(Hong, Kang et al. 2005; Sigalotti, Coral et al. 2005). Finally, in light of recent reports of T-regulatory cell generation and the emergence of tolerogenic APCs, it is clear that peripheral T-cell tolerance generated by the tumor may be insurmountable using current modalities targeting NY-ESO-1(Gnjatic, Altorki et al. 2009). Taken together, while our fundamental knowledge of tumor antigen expression and regulation has progressed significantly, our antigen choices and therapeutic modalities are moving to a much slower pace.

Cancer Testis Antigens

Cancer-testis antigens represent a large family of proteins with restricted expression to the germ-line and trophoblast. These antigens have been also found to be aberrantly expressed in certain solid and hematologic malignancies(Scanlan, Simpson et al. 2004). This abnormal expression, it has been postulated, may confer the special function of "gametogenesis" to the developing malignancy, a theory reminiscent of the "trophoblastic theory of cancer" proposed by John Beard over a century ago(Gurchot 1975). According to this theory, CTA expression may provide a fitness advantage to cancer cells like independent assortment of chromosomes, limitless replicative capacity,

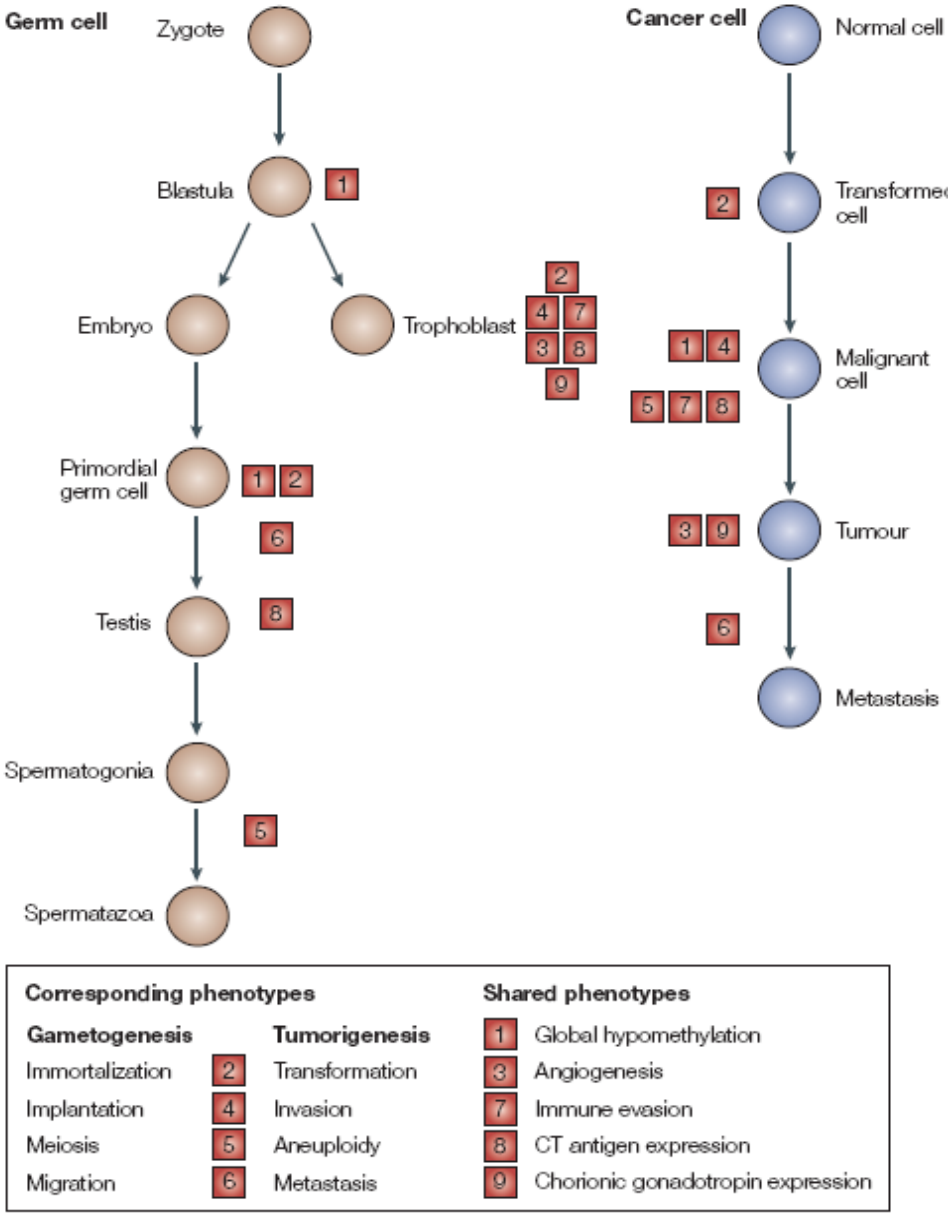

Figure 1: Shared characteristics of germ cells and cancer cells. Activation of the gametogenic programme (shown by brown cells) might contribute to properties of tumour formation and progression (shown by blue cells). Corresponding features between cancer cells and those in the germ cell/gamete/trophoblast differentiation pathways include: immortalization (involved in transformation), invasion, induction of meiosis (can lead to aneuploidy) and migration (contributes to metastasis). Shared phenotypes between germ cells and cancer cells include demethylation, angiogenesis induction, downregulation of the major histocompatibility complex (immune evasion), and expression of chorionic gonadotropin. The numbers (1–9) indicate gametogenesis- and tumorigenesis-related phenotypic traits and the stages at which these events occur. *Figure and legend copied from* (Simpson, Caballero et al. 2005).

tissue invasiveness, and increased motility (Figure 1) (Simpson, Caballero et al. 2005).

MAGE-A1 is the founding CTA member discovered almost two decades by T. Boon and coworkers using a very laborious process that characterize the antigen specificity of tumor-infiltrating cytotoxic T-lymphocytes (TILs)(van der Bruggen, Traversari et al. 1991). Since then, more streamlined approaches for antigen discovery have been developed including the serological identification of antigens by recombinant expression cloning method (SEREX), differential gene expression analysis, high throughput immunoblot, and bioinformatics methods (Hubank and Schatz 1994; Sahin, Tureci et al. 1995; Scanlan, Gordon et al. 2002; Hoeppner, Dubovsky et al. 2006; Dubovsky, Albertini et al. 2007). As a result of this concerted effort to identify, characterize, and classify CTAs, we now recognize 44 CTA families comprising over 89 potential tumor antigens.

Epigenetic regulation of CTAs

CTA mRNA expression has been centrally linked to DNA methylation patterns within both the germ lineage and the cancer cell(Karpf and Jones 2002; Simpson, Caballero et al. 2005; Wischnewski, Pantel et al. 2006). Two specific cytosine methylation changes occur in human malignancy, genome-wide hypomethylation and the eventual hypermethylation of specific promoter regions generally associated with tumor suppressor genes(Feinberg 1988; Feinberg, Gehrke et al. 1988; De Smet, De Backer et al. 1996). The former is of greater relevance to the expression of CTAs since it is believed that genome-wide hypomethylation is an early step in carcinogenesis and has the capacity to release chromatin constriction on a multitude of genetic elements(Woloszynska-Read, James et al. 2007). CTAs which reside on the X chromosome (CT-X antigens) are

considered especially susceptible to epigenetic control in this manner, given the well understood processes of meiotic sex chromosome inactivation (MSCI) and meiotic silencing of unsynapsed chromatin (MSUC)(Scanlan, Gure et al. 2002; Turner 2007). An additional class of autosomal CTAs has been linked to the expression of Brother of the Regulator of Imprinted Sites (BORIS). Located on chromosome 20q13.2, a locus frequently amplified in cancer, and expressed predominantly in the testis, BORIS, is both a CTA and a possible oncogene(Klenova, Morse et al. 2002; Loukinov, Pugacheva et al. 2002). BORIS is considered a paralog of, and antithesis to, the CCCTC-binding factor (CTCF) which is primarily responsible for maintaining methylation insulator boundaries(Loukinov, Pugacheva et al. 2002; Woloszynska-Read, James et al. 2007).

Immunotherapeutic interest in CTAs

Since their discovery, the CTA genes have been primarily investigated for their ability to elicit anti-tumor immune responses. The high antigenicity of CTAs is due to their restricted expression pattern. The testis and trophoblast are considered immunologically privileged tissues. In the case of germ cells, the lack of major histocompatibility complex (MHC) class I molecules effectively blinds peripheral tolerogenic mechanisms(Kowalik, Kurpisz et al. 1989). In addition, the presence of the blood-testis barrier and the complete absence of CTA expression within the thymus serve to prevent CTA-specific tolerance at both the central and peripheral levels(Scanlan, Simpson et al. 2004). This low basal tolerance and frequent overexpression in tumors makes CTA ideal candidates for anti-tumor vaccines or active immunotherapies.

Much of the fervor surrounding CTAs comes as a result of the encouraging clinical trial obtained with vaccines targeting these antigens. NY-ESO-1 and the MAGE

family of antigens have been indeed evaluated in phase I and II clinical trials as anti-tumor vaccines(Marchand, Weynants et al. 1995; Marchand, van Baren et al. 1999; Jager, Gnjatic et al. 2000; Coulie, Karanikas et al. 2002; Jager, Karbach et al. 2006). Although tumor regressions have been observed in patients with metastatic melanoma, the most frequent result was generation of robust anti-CTA CD4 and CD8 T-cell responses (Marchand, Weynants et al. 1995; Marchand, van Baren et al. 1999; Jager, Gnjatic et al. 2000; Jager, Nagata et al. 2000; Coulie, Karanikas et al. 2002; Chen, Jackson et al. 2004; Davis, Chen et al. 2004; Huarte, Karbach et al. 2004; Kruit, van Ojik et al. 2005; van Baren, Bonnet et al. 2005; Jager, Karbach et al. 2006). A recent study conducted by Gnjatic et. al. brought to light the fact that, in addition to robust cytolytic T-lymphocytes, CTA based vaccination also induces CD4+CD25hi T-regulatory cells that potentially blunt clinical responses(Gnjatic, Altorki et al. 2009). One possible workaround could be the use of humanized monoclonal antibody anti-CTLA4 therapy, which in clinical trials has been found to elicit robust NY-ESO-1 reactivity and improve the balance of regulatory (Treg) to activated CD4 T-cells(Liakou, Kamat et al. 2008; Fong, Kwek et al. 2009). Notably, recent evidence supports the spontaneous generation of CTA-specific CD4 T-cells with cytotoxic capabilities in patients treated with anti-CTLA4 (Huarte, Karbach et al. 2004). This finding is specially promising given that a number of the CTAs were discovered using SEREX which provides indirect evidence of CD4 T-cell recognition. It is plausible therefore that an intact and functional repertoire of CD4 T-cells specific to CTAs might exist in many cancer patients(Dubovsky, McNeel et al. 2009).

Immunological Effects of Epigenetic Modifiers

Demethylating Agents

Demethylating agents, or DNA methyltransferase inhibitors (DNMTi), were initially investigated in the late 60's and early 70's as purine analogs with the potential to block DNA and/or RNA synthesis(Sorm and Vesely 1968; Vesely, Cihak et al. 1968; Vesely, Cihak et al. 1970). It was not until the 1980's that the differentiating capacity of these drugs was truly appreciated(Jones and Taylor 1980). It was later found that 5-aza-2'-deoxycytidine (5A2) and 5-aza-2'-cytidine (5AC) were non-competitive inhibitors of DNMT1, eliciting the development of a thioether bond at C-6 covalently linking the enzyme to the DNA strand and functionally inactivating it(Ferguson, Vertino et al. 1997). DNMT1 is considered the primary methyltransferase enzyme responsible for propagating the methylome to daughter cells after mitotic division(Luczak and Jagodzinski 2006). Thus, inhibition of DNMT1 results in the hypomethylation of previously silenced chromatin which is believed to directly facilitate cytotoxicity in cancer cells. More specifically, critical tumor suppressor proteins such as p16 and p15INK4B are postulated to induce abrupt cell cycle arrest after treatment with DNMTi(Claus, Almstedt et al. 2005). Moreover, the ability of specific inhibitors, namely 5-azacytidine (5AZA) to interrupt RNA synthesis gives them anti-metabolite characteristics as well.

While demethylation of the promoter region is the most studied phenomenon associated with DNMTi there are numerous proposed mechanisms by which they may enable the reactivation of genes. DNMTi also effectively inhibit Methyl-CpG binding domain (MBD) proteins which serve as mediators of transcriptional repression(Hendrich and Bird 1998). MBDs recruit transcriptional co-repressors such as histone deacetylases and Sin3a to the chromatin facilitating the assembly of repressive chromatin structures

onto the DNA(Jones, Veenstra et al. 1998; Ng, Zhang et al. 1999). An additional mechanism results due to the allosteric inhibition of transcriptional repressor domains located within DNMT1, 3a, and 3b which normally facilitate chromatin constriction similar to MBDs(Fuks, Burgers et al. 2000; Robertson, Ait-Si-Ali et al. 2000; Rountree, Bachman et al. 2000; Bachman, Rountree et al. 2001). More notably, the mechanisms attributed to DNMTi have unveiled previously unknown links between DNA methylation, histone acetylation, and chromatin constriction.

Demethylating Agents and CTA Expression

From an immunotherapeutic perspective DNMTi have received attention due to their ability to enhance or induce CTA expression in cancer(Weber, Salgaller et al. 1994). Evidence generated in multiple laboratories indicates that DNMTi-induced CTA expression may be cancer specific in nature(Vatolin, Abdullaev et al. 2005; Dubovsky and McNeel 2007). These data has been confirmed in studies conducted by Karpf and collaborators using microarray analysis of normal epithelial cells and cancer cells exposed to 5A2(Karpf and Jones 2002; Karpf, Lasek et al. 2004). In addition, studies conducted by our group indicate that, in human malignancy, expression of CTAs can persist for weeks to months after treatment with DNMTi(Dubovsky and McNeel 2007). The molecular mechanisms which underlie the cancer-specificity and longevity of CTA expression post treatment have yet to be fully elucidated; however the prevailing hypothesis involves cancer-specific induction of BORIS expression following DNMTi treatment. It has been found that the BORIS promoter becomes increasingly hypomethylated as cancer progresses(Woloszynska-Read, James et al. 2007). A preexisting hypomethylated state could conceivably lead to BORIS expression in a

cancer-specific manner following limited treatment with DNMTi. Since BORIS competes for the same 11ZF binding site as CTCF, once BORIS is expressed it may perpetuate the expression of CTAs post DNMTi treatment(Loukinov, Pugacheva et al. 2002; Vatolin, Abdullaev et al. 2005). Supporting this concept, tumor suppressor proteins unrelated to CTAs are re-silenced by promoter methylation following halt of DNMTi treatment. In contrast, CTAs themselves remain hypomethylated, implicating that a master regulator is induced which can specifically perpetuate CTA hypomethylation(Hong, Kang et al. 2005).

Immunomodulatory activity of Demethylating Agents

Equally valuable from an immunotherapeutic perspective is the recapitulation of the interferon (IFN) pathway in cancer cells treated with DNMTi. Epigenetic suppression of the IFN response has been shown to be a frequent and deleterious result of malignancy (Lee and O'Neill 1987; Reid, Merigan et al. 1992; Karpf, Peterson et al. 1999; Katzenellenbogen, Baylin et al. 1999; Lu, Au et al. 2000; Morris, Spangler et al. 2000; Liang, Gonzales et al. 2002; Kulaeva, Draghici et al. 2003; Reu, Bae et al. 2006; Reu, Leaman et al. 2006). In an attempt to identify expression signatures which represent signal transduction pathways it was discovered that the expression of IFN pathway genes is an indirect effect of 5A2 treatment(Karpf, Peterson et al. 1999). Induction of the IFN pathway can increase tumor immunosurveillance and antigenic peptide presentation. In addition, our group has shown that the costimulatory profile of certain hematologic malignancies can be rescued using DNMTi, corroborating data obtained by Coral et. al. in melanoma (Coral, Sigalotti et al. 1999; Dubovsky, McNeel et al. 2009). Our results, albeit demonstrated in a B-cell malignancy, point to the possibility of induce and/or

augment the antigen-presenting capabilities of tumor cells leading to enhanced priming of antigen-specific immune responses(Dubovsky, Wang et al. 2009). In addition, DNMTi-induced IFN signaling and costimulatory expression may further increase the level of CTA peptide presentation amongst cancer cells aiding in the formation of a robust anti-cancer immune response.

Histone Deacetylase Inhibitors

Histone Deacetylases (HDACs)

Chromatin structure is determined by covalent and non-covalent modifications of the DNA itself (DNA methylation, destabilization of double-strand) or by changes in proteins associated to DNA (acetylation, methylation, and phosphorylation). One of these DNA-associated proteins is histones, who are targets for numerous covalent and non-covalent modifications that ultimately affect the status of the chromatin structure. Acetylation of positive amino acids is one of the covalent modifications on histones. This enzymatic reaction mediated by histone acetyltransferases (HATs) results in transcriptionally active chromatin. Conversely, removal of the acetyl group mediated by histone deacetylases leads to histone hypoacetylation and transcriptionally inactive chromatin. Thus, histone acetylation patterns have a central role in modulating chromatin accessibility, gene expression and, as a result, cellular phenotype.

In the progression of cancer, hypoacetylation results in the silencing of selected genes which would otherwise decrease the clonotypic survival advantage(Liu, Kuljaca et al. 2006). HDACs comprise a family of enzymes recruited by co-repressors or by multi-protein transcriptional complexes to gene promoters where they regulate transcription

Figure 2: Human histone deacetylases enzymes (HDACs). the class I (HDAC1, 2, 3 and 8), class IIa (HDAC4, 5, 7 and 9), class IIb (HDAC6 and 10) and class IV (HDAC11) HDACs with the various structural/functional domains listed. The capacity of structurally diverse HDACi to inhibit the activity of different HDAC classes or specific HDACs is also shown. *Figure and legend copied from* (Bolden, Peart et al. 2006).

through chromatin modification without directly binding DNA. Approximately twenty HDACs have been identified, and they are divided into four principal classes (Figure 2) (Bolden, Peart et al. 2006). Class I HDACs include HDAC1, 2, 3 and 8, class II HDACs include HDAC4, 5, 6, 7, 9 and 10, class III HDACs are members of the sirtuin family of HDACs, among which yeast Sir2 is the founding member, and Class IV, represented by HDAC11, the newest HDAC discovered (Bolden, Peart et al. 2006). Histones are the main substrates for the enzymatic activity of HDACs. However, several studies have shown that HDACs also deacetylate non-histone proteins such as p53, E2F1, RelA, YY1, TFIIE, BCL6 and TFIIF. In addition each member is also capable of exerting a variety of other cellular functions related to their deacetylase activity, tissue expression profile, cellular compartment distribution, stage of cellular differentiation and/or pathophysiological conditions (Glozak, Sengupta et al. 2005; Minucci and Pelicci 2006). As an example, HDAC6 is mainly localized in the cell cytoplasm and it is uniquely endowed with tubulin deacetylase activity(Hubbert, Guardiola et al. 2002). This HDAC is a key regulator of cytoskeleton, cell migration and cell-cell interaction(Valenzuela-Fernandez, Cabrero et al. 2008) and, in immune cells plays a role in the organization of the APC/T-cell immune synapse(Serrador, Cabrero et al. 2004). Similarly, HDAC11, the newest member of the HDAC family, is a transcriptional repressor of IL-10 gene expression in antigen-presenting cells(Villagra, Cheng et al. 2009) and, it has been found to be tissue-restricted and uniquely expressed in the brain, kidney, testis and abnormally over-expressed in certain types of leukemias and lymphomas(Gao, Cueto et al. 2002).

HDAC inhibitors

HDACs are the target of several structurally diverse compounds known as HDAC inhibitors (HDACi)(Marks, Richon et al. 2000). Of note, these compounds were used as inhibitors long before a clear understanding of the role of specific HDACs in normal and/or transformed cells began to emerge. Existing HDACi can induce cytodifferentiation, cell cycle arrest and apoptosis of transformed cells (Marks, Richon et al. 2000; Bolden, Peart et al. 2006). Clinical use of HDACi have shown potent and specific anti-cancer activities partially due to the upregulation of cyclin-kinase inhibitor p21, induction of proaptotic BCL2 family proteins, repression of angiogenic factors such as vascular endothelial growth factor (VEGF), and inhibition of transcription factors such as nuclear factor kappa B (NF-κB) (Sambucetti, Fischer et al. 1999; Richon, Sandhoff et al. 2000; Chen, Fischle et al. 2001; Kim, Kwon et al. 2001; Ruefli, Ausserlechner et al. 2001; Chen, Mu et al. 2002; Deroanne, Bonjean et al. 2002; Peart, Tainton et al. 2003; Yeung, Hoberg et al. 2004; Hoberg, Popko et al. 2006) Of note, one compound, SAHA, is the first HDAC inhibitor approved by the FDA for the treatment of patients with cutaneous T-cell lymphomas(Mann, Johnson et al. 2007; Marks and Breslow 2007). In spite of these important therapeutic advances, the underlying target(s) and/or mechanism(s) mediating the antitumor activity of HDACi in human malignancies remain to be fully elucidated.

The regulatory role of particular HDACs and the therapeutic use of HDACi is not restricted to cancer since several studies have also shown that HDACs play a role in autoimmune diseases (Bhavsar, Ahmad et al. 2008), inflammatory regulation (Blanchard and Chipoy 2005), central nervous system disorders(Kazantsev and Thompson 2008), and during development (Haberland, Montgomery et al. 2009). Supporting this concept,

HDACi have emerged as a potential therapeutic tools for the treatment of autoimmune diseases (Bhavsar, Ahmad et al. 2008) (Mishra, Brown et al. 2001), cystic fibrosis (Rubenstein and Zeitlin 1998), muscular dystrophy (MDX) (Minetti, Colussi et al. 2006) and in regulation of immune tolerance (Tao and Hancock 2007). In summary, the increasingly recognized participation of HDACs in regulation of several cellular functions, make the ever-expanding variety of HDACi amongst the most promising drugs currently under investigation (Glozak and Seto 2007).

HDACi as immunomodulatory drugs

In contrast to the rapidly increasing knowledge of the role of HDACs in cancer biology and the use of HDACi in cancer and other pathological conditions, little is still know about the role of specific HDACs in immune cells and the functional consequences of their inhibition by HDACi. Of note, while some studies have highlighted the ability of particular HDACi to augment inflammatory and antitumor responses, other studies have shown the opposite, ie., HDACi display anti-inflammatory properties and can ameliorate the severity of graft-versus-host disease (GVHD) and autoimmune disorders. Among the former studies, seminal work performed by T. Tomasi's lab demonstrated that treatment of immune cells and some tumor cells with tricostatin A (TSA) induce the expression of MHC class I and II, the costimulatory molecules CD80, CD86, and CD40, the immunoproteosome subunits LMP2 and LMP7 and the peptide loading machinery TAP1 and TAP2(Magner, Kazim et al. 2000; Magner and Tomasi 2000; Khan, Magner et al. 2004; Tomasi, Magner et al. 2006; Khan, Magner et al. 2007; Khan, Gregorie et al. 2008; Khan and Tomasi 2008). These results indicate that this particular HDACi can functionally replace the histone acetyltransferase activity of CIITA, CBP, p300, and

PCAF, thus allowing MHC class II activation. In addition, our group recently investigated the antigen-presenting capabilities of malignant B-cells from patients with chronic lymphocytic leukemia (CLL) and found that LAQ824, a member of the hydroxamic acid analog family of HDACi, was capable of significantly increase the APC function of malignant B-cells resulting in increased priming of T-cells (Dubovsky, Wang et al. 2009). Furthermore, we have recently found that treatment of professional APCs with LBH589, SAHA, and LAQ824 (all belonging to the hydroxamic acid analog family) inhibit the secretion and transcription of immunosuppressive factors such as IL-10 while simultaneously increasing the production of the pro-inflammatory cytokines IL-12p70, IL-1α, IL-1β, IL-2, GM-CSF, TNFα, and RANTES in a dose dependent manner (Wang et al. Unpublished data). Taking together, the positive immunological effect of these particular HDACi might play to the advantage of CTA-based immunotherapy and as such studies combining CTA based vaccine modalities with HDACi are currently ongoing in our lab.

In contrast with the above results, evidence also exists linking some classically studied HDACi with immunosuppressive effects. For instance, HDACi can inhibit the production of inflammatory mediators such as tumor necrosis factor-α, interleukin-1, and interferon-γ in the setting of graft-versus host disease, an outcome with direct clinical benefit for the treatment of this complication in patients undergoing an allogenic bone marrow transplant (Reddy, Maeda et al. 2004; Li, Zhao et al. 2008). Of note, the anti-inflammatory properties of HDACi did not impacted upon graft-versus leukemia (GVL) effect, which was surprisingly found to be enhanced in these studies.

The seemingly contradictory effects of HDACi upon inflammatory responses could be a reflection of several factors, ie. the pan-HDAC inhibitory effect of the compounds currently in use, resulting in a myriad of cellular effects that likely would differ based on HDACs expression in particular immune cells, the stage of cellular differentiation and/or activation at the time of exposure to HDACi, and the dose and/or length of exposure to HDACi in vitro and or in vivo among others. Complicating this picture even further, there is now evidence that the role of specific HDACs in immune cells goes beyond their initially described effects on histones and now encompass more complex regulatory functions, like the propensity for certain HDACs to directly acetylate, and thereby regulate, key immunologically relevant transcription factors such as STAT1, STAT3, and NFκB (Chen, Fischle et al. 2001; Nusinzon and Horvath 2003; Yuan, Guan et al. 2005).

Given the above scenario, dissection of the relevant mechanism(s)/target(s) involved in the divergent inflammatory effects of pan-HDACi would be challenging and difficult to achieve. To try to overcome this obstacle, we have recently pursued a different approach in which we have first identified the role of specific HDACs in the transcriptional regulation of a particular inflammatory/anti-inflammatory gene. Following this step, we have next pursued mechanistic studies leading to the evaluation of the immunological effects of more specific HDACi targeting particular HDACs. Under this approach, we recently over-expressed or knocked-down specific HDACs in antigen-presenting cells and determined the expression of IL-10, cytokine that plays a central role in tolerance induction and regulation of inflammatory responses. We have found that among all the HDACs evaluated, HDAC11 by interacting at the chromatin level with the

distal region of the IL-10 promoter, down-regulates IL-10 transcription in murine and human APCs(Villagra, Cheng et al. 2009). In addition, we found that over-expression of HDAC6 induce the opposite effect, i.e. transcriptional activation of IL-10 gene expression (Villagra et al. Manuscript in preparation). The significance of these findings lies at several levels: First, it has provided a physiological role for HDAC11, the newest member of the HDAC family, with previous unknown function. Secondly, HDAC11 and HDAC6 by inducing dynamic changes at the chromatin level regulate the expression of IL-10, (and perhaps other genes involved in the inflammatory response), effect that might explain -at least in part- the plasticity of the APC to determine T-cell activation versus T-cell tolerance. Third, HDAC11 and HDAC6 represent novel molecular targets for more specific HDACi to potentially influence immune activation versus immune tolerance, a critical decision with significant implications not only for cancer immunotherapy but also for the transplantation and autoimmunity fields.

Histone acetylation and T-lymphocyte polarization

Of equal immunologic importance is the proper polarization of anti-cancer immune reactivity. HDACi have shown divergent effects on this front as well. For instance, studies conducted by Edens et. al. suggest that TSA is particularly potent at inducing T-cell anergy (Edens, Dagtas et al. 2006). A potential mechanism is that excessive expression of p21 inhibits the proliferation of responder lymphocytes (Jackson, DeLoose et al. 2001; Gilbert, Boger et al. 2005). In contrast, we have recently found that the HDACi LAQ824 can reverse antigen-specific anergy, rendering previously tolerized T-cells functional (Wang et al. Manuscript in preparation). Furthermore, studies in malignant CLL cells have shown that disruption of histone deacetylase enzymes can shift

the polarization of anti-cancer CD4+ lymphocyte responses from Th2 to Th1(Dubovsky, Wang et al. 2009). These differential effects are likely related to differences in the inhibition profile of the HDACi used.

Several groups have recently demonstrated an increased frequency and potency of CD4+CD25hi T-regulatory cells after treatment with HDACi(Tao and Hancock 2007; Reilly, Thomas et al. 2008; Samanta, Li et al. 2008; Wang, Lee et al. 2009). Additional evidence suggests that HDACi can inhibit the autoimmune component of a variety of diseases including systemic lupus erythematosus, concanavalin A induced hepatitis, experimental autoimmune encephalomyelitis, rheumatoid arthritis, and colitis (Leoni, Zaliani et al. 2002; Chung, Lee et al. 2003; Reilly, Mishra et al. 2004; Camelo, Iglesias et al. 2005; Glauben, Batra et al. 2006; Leng, Gries et al. 2006). These results are likely related to the inhibition of HDAC 9, which was recently shown to deacetylate key lysine amino acids in the forkhead domain of FOX-P3 reducing the functional capacity of T-regulatory cells(Tao and Hancock 2007). Reminiscent of our studies of the role of particular HDACs in APCs, these studies in T-cells shed light on the differential roles of specific HDACs and serve to direct future efforts at generating more specific HDACi which could directly benefit immunotherapy and minimize off-target immunosuppressive effects.

Crosstalk between HDAC activity and DNA methylation

Although the biochemical pathways which facilitate DNA acetylation are distinct from those which regulate DNA methylation, evidence supports a relationship between the two systems that plays a role in modulating gene repression programming(Cedar and Bergman 2009). This recently unveiled link between DNA methylation and the histone

code led investigators to induce CTA expression in malignant cells using HDACi. It has now been demonstrated in both solid and hematologic malignancies that CTA expression can be achieved using a variety of clinically relevant HDACi (Weiser, Ohnmacht et al. 2001; Fradet, Picard et al. 2005; Wischnewski, Pantel et al. 2006; Dubovsky, Wang et al. 2009; Oi, Natsume et al. 2009). At the same time it became clear that combination of HDACi and DNMTi might served to synergistically activate CTA expression in cancer(Weiser, Guo et al. 2001; Dubovsky, Wang et al. 2009; Oi, Natsume et al. 2009). The mechanism(s) underlying this synergism was shown to be concordant DNA hypomethylation and histone acetylation of CTA promoter regions, namely those within the MAGE CTA family(Claus, Almstedt et al. 2005; Wischnewski, Pantel et al. 2006). Thus, the combination of HDACi and DNMTi is a promising venue for enhancing CTA-based immunotherapeutic approaches.

The "vaccinate-induce" model

One critical lesson learned from vaccine against microorganisms is that successful vaccination requires delivery of the vaccine and the induction of long lasting immunological memory prior to pathogenic challenge ("preventive vaccination") (O'Donnell and DeWolf 1995; Plotkin 1999). Whereas this ideal scenario once seemed impossible in the setting of therapeutic cancer vaccines, the advent of epigenetic modifiers has brought to light a novel immunotherapeutic strategy. A refreshed perspective on active immunotherapy incorporates the classical tenants of cancer-specificity and high antigenicity with the novel concept of forced antigen expression by using epigenetic modifiers. This particular immunotherapeutic modality that our group has termed "vaccinate-induce" is supported by recent studies by Guo et. al. who

demonstrated that de novo induction of the murine CTA, P1A, using the demethylating agent 5A2 can be combined with adoptive immunotherapy to achieve therapeutic benefit (Guo, Hong et al. 2006; Coral, Sigalotti et al. 2007). This approach resulted not only in the expansion of P1A specific cytolytic T-cells but also in the reduction of metastatic 4T1 mammary tumor nodules in tumor bearing mice. In parallel to the Guo's report, a plethora of studies have recently identified CTAs which were inducible in particular malignancies, drastically expanding the list of potential vaccine antigen candidates (Wischnewski, Pantel et al. 2006; Dubovsky and McNeel 2007; Adair and Hogan 2009; Oi, Natsume et al. 2009). Shortly thereafter, a group from Nagoya University in Japan published similar results in an orthotopic glioma model (Natsume, Wakabayashi et al. 2008). A key scientific feature of this study was the utilization of the human CTA, NY-ESO-1, further demonstrating the relevance of such an approach to human malignancy. Most recently, Richard Morgan's group, in collaboration with David Schrump and Steven Rosenberg demonstrated similar NY-ESO-1 based cytolytic ability in an in vitro system using a variety of epithelial malignancies; promising precursory evidence for future evaluation in the clinical setting (Wargo, Robbins et al. 2009). The significant advancement of this study was the evaluation of a combination DNMTi/HDACi therapy, namely 5A2 and depsipeptide. The results indicated that there was a significant advantage, in terms of CTL IFNγ production, to 5A2/DP therapy in pancreatic, ovarian, and glioblastoma cancers.

As it stands, there are no published reports supporting the hypothesis that vaccination, as apposed to adoptive transfer, will potentiate similar effects. However, in preliminary experiments we have found that the in vivo combination of HDACi with a

vaccination strategy resulted in tumor rejection, an effect that was not seen in animals treated with HDACi or vaccine alone. In addition, this approach was associated with very low toxicity to normal tissues reaffirming the safety of epigenetic therapy combined with active immunotherapy. Needless to say, studies which focus efforts on diversifying the immunotherapeutic approaches that can accompany epigenetic modifier treatment are currently underway. On an additional note, the vast majority of studies thus far have focused on solid tumors, however our laboratory has recently generated data which supports the use of a vaccinate-induce therapy in the treatment of hematologic malignancies.

The potential benefits of such vaccinate-induce therapeutic model combining vaccines with epigenetic modifiers are listed below:

I. De novo induction of CTAs represents a means to avoid peripheral tolerogenic mechanisms induced by the tumor
II. Use of CTAs avoids central tolerance
III. Forced CTA expression inhibits the rapid generation of tumor escape variants
IV. Induction of CTA expression expands the patient population for which a particular vaccine strategy might be effective
V. Intratumoral heterogeneity can be overcome via tumor-wide epigenetic induction, and
VI. Immunomodulatory effects of epigenetic modifiers (ie. increased signal one, increased signal 2 or co-stimulatory molecules and increased signal 3 or pro-

inflammatory mediators) would likely enhance CTA presentation to the immune system.

Chronic lymphocytic leukemia

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 (Krackhardt, Witzens et al. 2002; Jemal, Siegel et al. 2008; Ouillette, Erba et al. 2008; Ramsay, Johnson et al. 2008). Even with today's therapies, CLL remains incurable, and patients almost invariably succumb to the disease (Cheson 1994; Rossmann, Lewin et al. 2002). The classical clinical staging system developed by Rai and Binet has been able to predict the long-term survival of patients with CLL but has failed to predict the specific disease course in patients with early-stage CLL (Rai, Sawitsky et al. 1975; Binet, Auquier et al. 1981). Recent advances in cytogenetic and biomarker discovery are helping to better predict disease progression and survival in such heterogenic patient populations. Cytogenetic analysis by fluorescence in situ hybridization (FISH) is widely used to identify the more common chromosomal aberrations, primarily deletions in 13q14, 11q23 (ATM), 17p13 (p53), 6q, and trisomy 12. Deletion of 17p13 is associated with chemotherapy resistance, rapid disease progression, and inferior survival (Dohner, Stilgenbauer et al. 2000). In addition, the mutational statuses of the Ig heavy chain, ZAP70, and CD38 expression are important prognostic factors for CLL, which can be used to differentiate patients with poor prognosis (Dohner, Stilgenbauer et al. 2000; Zenz, Mertens et al. 2008). First-line treatments with purine analogs such as fludarabine or pentostatin in combination with alkylating agents and or monoclonal antibodies against CD20 can result in high response

rates, but not a clear survival benefit (Kay, Rai et al. 2006). Furthermore, this approach is not considered curative, and the inevitable relapse is typified by immune dysfunction, myelosupression, and outgrowth of chemotherapy-resistant CLL clones (Kater, van Oers et al. 2007).

B-cell dysfunction

In healthy individuals B-lymphocytes are proficient stimulators of antigen specific Th1 T-cell responses capable of eliminating virally infected or neoplastic cells via direct cellular cytotoxicity (Mosmann and Coffman 1989). However, in CLL an increasingly immunosuppressive phenotype enables the malignant B-cell to evade immune detection(Krackhardt, Witzens et al. 2002; Scrivener, Goddard et al. 2003; Mellstedt and Choudhury 2006; Horna and Sotomayor 2007). Mounting evidence points to specific defects in the antigen presenting cell (APC) functions including decreased self-peptide presentation, improper T-cell synapse formation, deficient costimulation, and diversionary cytokine signaling.

Although the distinct molecular mechanisms which elicit CLL-induced immunosupression remain unclear, recent evidence supports the notion that chromatin constriction of critical immunostimulatory factors may be involved(Rush, Raval et al. 2004; Chen, Raval et al. 2009). Moreover, promoter hypermethylation and histone hypoacetylation are primary mechanisms for silencing highly antigenic protein products which promote the immune recognition of malignant cells. Novel strategies have been suggested which may supplant immunosupression with effective cancer-antigen presentation leading to robust T-cell activation and prolonged killing(Khan, Magner et al. 2004; Khan, Magner et al. 2007; Wierda and Kipps 2007). The hallmarks of such

strategies hinge upon functionally increasing the APC capacity of CLL cells by reducing the levels of immunosuppressive factors, generating a robust response against a non-tolerized cancer-specific antigen, and properly polarizing the resulting primed T-cells. Recent studies have demonstrated increased expression of MHC class I class II, immunoproteosome subunits, peptide processing machinery, pro-apoptotic molecules such as APO2L/TRAIL, and various costimulatory molecules after treatment with a histone deacetylase inhibitor (HDACi) leading to enhanced cancer-specific immune responses(Borden 2007; Khan, Gregorie et al. 2008; Khan and Tomasi 2008). Additionally, both HDACi and DNA methyltransferase inhibitors (DNMTi), such as 5-aza-2'-deoxycytidine (5A2), have proven capable of inducing long lasting, cancer-specific, expression of a highly antigenic class of proteins, termed cancer-testis antigens (CTAs)(Dubovsky, McNeel et al. 2009). Published studies demonstrate that this effect alone may potentiate effective anti-cancer immune responses(Guo, Hong et al. 2006). . Furthermore, a role for epigenetic modifiers in the regulation of pro-inflammatory cytokines has also been demonstrated(Sailhamer, Li et al. 2008; Villagra, Cheng et al. 2009). There currently exists a single clinical trial investigating the efficacy of 5-aza-2'-deoxycytidine in combination with the HDACi valproic acid in previously treated CLL patients, the final results of this trial are still pending(Blum, Liu et al. 2010).

T-cell dysfunction

In healthy individuals B-lymphocytes are critically necessary for the polarization of effective T cell responses (Mosmann and Coffman 1989). However, in CLL an increasingly defective immune synapse enables the malignant B-cell to evade immune detection by inducing T cell anergy as well as improper Th2 polarization (Krackhardt,

Witzens et al. 2002; Scrivener, Goddard et al. 2003; Mellstedt and Choudhury 2006; Horna and Sotomayor 2007). The end result of this immunosupression is a high incidence of severe infections that in the setting of therapeutic interventions, often lead to patient morbidity. (Kay, Rai et al. 2006; Fulci, Chiaretti et al. 2007; Ouillette, Erba et al. 2008; Rassenti, Jain et al. 2008).

Original identification of the Th1 and Th2 T cell subsets established disparate patterns of stimulation underpinned by static and heritable epigenetic changes (Wilson, Rowell et al. 2009). More recently, however, the widely observed plasticity in Th cell differentiation was nailed down to bivalent epigenetic marks that maintained heritability yet provided the flexibility to tailor activation status based upon changing external signals (Araki, Wang et al. 2009). Surprisingly, these bivalent marks were identified on transcription factors previously considered "master regulators" of Th cell differentiation, opening the possibility for diametrically opposed states of activation to alternately persist in a single clonal cell population (Lee, Turner et al. 2009; Wei, Wei et al. 2009).

Immunomodulation in CLL

Advances in disease pathogenesis and cytogenetics have vastly improved our biological understanding and therapeutic arsenal against CLL (Kay, Rai et al. 2006; Fulci, Chiaretti et al. 2007; Ouillette, Erba et al. 2008; Rassenti, Jain et al. 2008). Immunotherapeutic strategies that complement standard therapies, such as monoclonal antibodies, are part of current routine treatment, and next generation therapies are under investigation in CLL patients (Tam, O'Brien et al. 2008). An additional advantage is that, since most patients are of advanced age, an immunotherapeutic approach may be more suitable than aggressive chemotherapeutic regimens (Ramsay, Johnson et al. 2008).

Table 1: Cytokines involved in the development and progression of CLL.

Cytokine	Secretory cell	Receptor	Normal Activities	Abnormal Role in CLL	Anti-CLL immune response	Refrences
IL-2	T-cells (activated)	Yes	Critical lymphocyte signaling molecule for activation of cytotoxic lymphocytes and tumor surveilance	Increases proliferation in activated CLL; absorption of exogenous IL-2 may reduce T-cell activation	Beneficial	(de Totero, Leukemia 1995) (Lantz, J Exp Med 1985) (Fluckiger, Blood 1992) (Mainou Fowler, J Clin Pathol 1995) (Foa, Blood 1985) (Semenzato, Blood 1987) (Callea, Haematologica 1996) (Foa, Blood 1987) (Bartik, Semin Oncol 1998)
IL-12p70	APC	Unknown	Stimulates Th1 proliferation and proinflammatory cytokine generation	Decreased expression leads to a lack of anti-tumor cytolytic T-cells	Beneficial	(Orsini, Cancer Res 2003) (Langenkamp, Nat Immunol 2000) (Trinchieri, Res Immunol 1995) (Macatonia, J Immunol 1995)
IFNα	T-cells (activated)	Yes	Confers resistance to viral infection in uninfected cells	Protects CLL from spontaneous apoptotic death; can therapeutic modulate immune function	Beneficial	(Rubin, Proc Natl Acad Sci 1980) (Nakamura, Nature 1984) (Sen, J Biol Chem 1992) (Goldstein, Cancer Res 1985) (Panayiotidis, Br J Haematol 1994) (Jewell, Br J Haematol 1994) (Chaouchi, Clin Immunol Immunopathol 1994) (McSweeney, Br J Haematol 1993) (Haslop, Br J Haematol 1991) (Jewell, Leuk Lymphoma 1996) (Ostlund, blood 1986) (Totterman, Clin Exp Immunol 1988) (Jewell, Leukemia 1992) (Csanaky, Med Oncol Tumor Pharmacother 1993)
GMCSF	CLL	Yes	Stimulates myelopoiesis, and effects myeloid and B-cell function	Autocrine signaling may sustain CLL cell survival	Beneficial	(Lieschke, N Engl J Med 1992) (Khwaja, Curr Opin Hematol 1994) (Nishijima, Mol Biol Cell 1995) (Ni, Immunol Cell Biol 1992) (Till, Blood 1996) (Harris, J Immunol 2000)
IFNγ	CLL (activation)	Probable	T-cell cytokine responsible for the generation of Th1 cellular immunity	Induction of antiapoptotic molecules in CLL and costimulator of proliferation	Beneficial	(Buschle, J Exp Med 1993) (Rojas, Leukemia 1996) (Zaki, Leuk Res 2000) (Mainou-Fowler, Leuk Lymphoma 1996)
IL-21	CD4 and NKT	Yes	Regulates autoimmunity, Th17 development, and anti-tumor cytotoxicity	Induces apoptosis in CLL cells and enhances cytolytic immune response	Beneficial	(Spolski, Ann Rev Immunol 2008) (Kim, Nat Cell Biol 2005) (Brenne, Blood 2002) (Davis, Clin Cancer Res 2007) (Kotb, Blood 2008) (de Totero, Blood 2007)
IL-1β	CLL	Yes	Proinflammatory, immunostimulant, and chemotactic factor	Autocrine signaling protects CLL from apoptosis; may elicit immune response	Probable	(Auron, Proc Natl Acad Sci 1984) (Dinarello, Blood 1996) (Platsoa, J Immunol 1998) (Morabito, Blood 1987) (Uggla, Blood 1987) (Plate, Hum Immunol 1993) (Jewell, Leuk Lymphoma 1995)
IL-5	CD4	Probable	Hematopoetic growth factor, can induce IgM production in B-cells	Increases spontaneous apoptosis of CLL cells	Probable	(Lopez, J Exp Med 1988) (Sanderson, Blood 1992) (Hayes, Leuk Res 1993) (Carlsson, Leukemia 1989) (Tavernier, DNA 1989) (Mainou-Fowler, Blood 1994) (Mainou-Fowler, J Clin Pathol 1995)
IL-15	MAC and DC	Yes	Microenvironmental factor which supports lymphoid proliferation and survival similar to IL-2	Induces proliferation and inhibits apoptosis in CLL	Probable	(de Totero, Blood 2007) (Trentin, Blood 1996) (Dobbeling, Blood 1998) (Yamada, Blood 1998) (Zambello, Blood 1997) (Fehniger, J Exp Med 2001)
G-CSF	CLL (activation)	Yes	Stimulates myelopoiesis, and effects myeloid and B-cell function	Decreases spontaneous apoptosis	Unknown	(Lieschke, N Engl J Med 1992) (Khwaja, Curr Opin Hematol 1994) (Corcione, Blood 1996) (Corcione, Methods 1997) (Handa, Ann Hematol 2000)
IL-8	CLL	Yes	Neutrophill activation and chemoattraction	Autocrine signaling inhibits CLL apoptosis	Unknown	(Baggiolini, J Clin Invest 1989) (Mukaida, Int J Hematol 2000) (Hermouet, Leuk Lymphoma 2000) (Srivastava, Leuk res 1993) (Francia, Blood 1994) (Molica, Haematologica 1999)
TNFα	CLL	Yes	Proinflammatory cytokine and lymphocyte growth factor	Controversially antiapoptotic autocrine factor capable of inducing CLL expression of IL-1, IL-6, and p55 IL-2 receptor.	Unknown	(Brenner, Br J Haematol 1988) (Gehr, J Immunol 1992) (Cordingley, Lancet 1988) (Foa, Blood 1990) (Trentin, Blood 1994) (Digel, Blood 1989)
IL-7	CLL	Yes	Hematopoetic growth factor regulating lymphoid development, differentiation, and homeostasis	Autocrine antiapoptotic signal in response to microenvironment	Unknown	Frishman, Exp Med 1993) (Plate Hum Immunol 1993) (Digel, Blood 1991) (Long, Proc Natl Acad Sci 1995)
IL-4	CLL and CD4	Yes	Promotes proliferation and differentiation of normal B-cells	Protects CLL from apoptosis; may aid in drug resistance	Negative	(Hovard, J Exp Med 1982) (Banchereau, Res Immunol 1993) (Dancescu, J Exp Med 1992) (Llorente, Eur J Immunol 1996) (Mainou-Fowler, Leuk Lymphoma 1995) (Tangye, Immunol Cell Biol 1997) (Kay, Br J Haematol 2001)
IL-6	CLL and CD4	Yes	B-cell growth and differentiation factor	Supports CLL survival and polarization of Th2 immunity	Negative	(Kishimoto, Blood 1989) (Hsu, Am J Pathol 1992) (Seymour, J Clin Oncol 1995) (Jewell, Leuk Lymphoma 1995) (Mainou-Fowler, J Clin Pathol 1995) (Dancescu, J Exp Med 1992) (Aderka, Blood 1993) (Tangye, Immunol Cell Biol 1997)
IL-10	CLL	Yes	Strong immunosupressant inhibiting Th1-type cytokines; B-cell stimulation, proliferation, and differentiation factor	Autocrine antiapoptotic factor for CLL which also inhibits generation of cellular immune response	Negative	(Fiorentino, J Immunol 1991) (Del Prete, J immunol 1993) (Mosmann, Immunol Today 1991) (Levy J Clin Invest 1994) (Finke, Leukemia 1993) (Sjoberg, Br J Haematol 1996) (Fayad, Blood 2001)
TGFβ	CLL	Yes	Negative autocrine regulator of lymphocyte growth and differentiation	Preferentially eliminates healthy lymphocyte compartments	Negative	(Massague, Annu Rev Cell Biol 1990) (Wallick, J Exp Med 1990) (Kremer, Br J Haematol 1992) (Schena, Leukemia 1992) (Lotz, J Exp Med 1994) (Israels, Leuk Res 1993) (Douglas, Blood 1997) (Lagneaux, Leuk Lymphoma 1998)

Common and immunologically relevant signaling molecules and cytokines are presented. Cytokines are dived into group with either likely beneficial effects, probably beneficial effects, unknown effects, or likely negative effects from the perspective of immunotherapeutic intervention.

More notably, the emergence of novel immunomodulatory compounds has brought to light the importance of immunoregulation and stroma interactions in the management of CLL and has shown promising results, perhaps alleviating the tolerogenic phenotype that

characterizes this clinical malignancy (Chanan-Khan and Porter 2006; Kay, Rai et al. 2006; Horna and Sotomayor 2007; Molica 2007; Rabinovich, Gabrilovich et al. 2007; Wierda and Kipps 2007; Ferrajoli, Lee et al. 2008). On the forefront of these immunomodulatory compounds is lenalidomide, a derivative of thalidomide, which has been shown to repair the T-cell/CLL B-cell immunological synapse and increase natural killer cell and monocyte-mediated antibody-dependant cellular cytotoxicity (Ferrajoli, Lee et al. 2008; Ramsay, Johnson et al. 2008; Wu, Adams et al. 2008). Early clinical trials with lenalidomide have been shown to induce responses in refractory CLL patients (Chanan-Khan, Miller et al. 2006; Ferrajoli, Lee et al. 2008).

CLL cytokine signaling

Development of CLL is inextricably linked to the abrogation of normal cytokine loops in favor of signaling patterns, which promote proliferation or protection from apoptosis. Since the mature B cell is a known intermediary of a healthy immune response, CLL must manage the precarious induction of many pro-inflammatory cytokines through autocrine or juxtacrine mechanisms to obtain antiapoptotic signaling while simultaneously suppressing key cytokines that have the potential to promote anti-tumor cytolytic responses.

While the complex interplay between cytokines involved in CLL may be difficult to understand in its entirety, there are three signaling mediators that, if altered, could potentially drive the generation of an anti-CLL immune response: IL-2, IL-10, and IL-4. IL-2 is a known T-cell stimulatory cytokine that is selectively removed from the CLL microenvironment via upregulation of soluble and membrane bound IL-2 receptor (Table 1) (Foa, Giovarelli et al. 1985; Fluckiger, Rossi et al. 1992; de Totero, Francia di Celle et

al. 1995; Mainou-Fowler, Copplestone et al. 1995). IL-4 polarizes the T-helper compartment toward a Th2 response, simultaneously ensuring adequate CD4+ stimulation and proliferation (which feeds back in the form of CD40 stimulation) while maintaining an ineffective anti-CLL response (Dancescu, Rubio-Trujillo et al. 1992; Banchereau, Bidaud et al. 1993; Mainou-Fowler, Craig et al. 1995). Moreover, IL-4 serves as an antiapoptotic autocrine factor further exacerbating its effects (Howard, Farrar et al. 1982; Llorente, Mitjavila et al. 1990; Tangye and Raison 1997; Kay, Han et al. 2001). IL-10 functions to suppress immune responses and is known to stimulate T-regulatory cell differentiation (Fiorentino, Zlotnik et al. 1991; Mosmann and Moore 1991; Sher, Fiorentino et al. 1991; Del Prete, De Carli et al. 1993; Finke, Ternes et al. 1993). Although IL-10 is actively secreted by CLL cells, its effect on CLL itself is abrogated due to an ineffective intracellular signaling pathway, which ensures that the remaining healthy lymphocyte compartments are disproportionately inhibited (Levy and Brouet 1994; Sjoberg, Aguilar-Santelises et al. 1996; Fayad, Keating et al. 2001).

Epigenetically regulated cancer antigens as immunotherapeutic targets for CLL

Anti-tumor vaccines, also known as active immunotherapies, exemplify some of the newer targeted treatments being investigated in CLL (Dubovsky and McNeel 2007). Ideal antigen candidates would have applicability to a significant percent of patients, have expression restricted to the cancer and high immunogenicity, and would be essential for the survival of the CLL cell, thus restricting immune evasion (Krackhardt, Witzens et al. 2002). One particular class of antigens, the cancer testis antigens (CTAs), has been investigated for these particular characteristics (Dubovsky, Albertini et al. 2007). CTAs have expression restricted to immunologically “privileged” tissues (blood-testis barrier

and lacking surface expression of major histocompatability complex (MHC) molecule) such as germ cells but are often aberrantly expressed in tumor cells (Kowalik, Kurpisz et al. 1989; Scanlan, Simpson et al. 2004). Their expression is thought to be induced by global genome hypomethylation, a well-known characteristic of cancer; it is this aberrant expression that presumably makes them available for immune recognition (De Smet, De Backer et al. 1996). Indeed, recent studies have shown that hypomethylating 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 anti-tumor vaccines (Weber, Salgaller et al. 1994; Grunau, Sanchez et al. 2005; Guo, Hong et al. 2006; Coral, Sigalotti et al. 2007; Dubovsky and McNeel 2007). Concordant results have shown that HDACi can have similar and synergistic effects, presumably due to the increased transcriptional availability of previously epigenetically silenced chromatin (Wischnewski, Pantel et al. 2006; Picard, Bergeron et al. 2007). More importantly, however, HDACi treatment blocks a critical tumor immuno-evasive antigen silencing mechanism enabling the potential generation of a prolonged and effective T-cell response.

In CLL, an immunosuppressive phenotype enables the malignant B cell to evade immune detection (Krackhardt, Witzens et al. 2002; Scrivener, Goddard et al. 2003; Mellstedt and Choudhury 2006; Horna and Sotomayor 2007). Novel strategies have been suggested that may supplant immunosuppression with appropriate cancer-antigen presentation leading to T-cell activation and killing (Khan, Magner et al. 2004; Khan, Magner et al. 2007; Wierda and Kipps 2007). The hallmarks of such strategies hinge upon increasing the APC capacity of the CLL cell, reducing the levels of

immunosuppressive factors and generating a robust response against a non-tolerized cancer-specific antigen (Figure 3). Recent studies have shown increased expression of MHC class I, class II, immunoproteosome subunits, peptide processing machinery, pro-apoptotic molecules such as APO2L/TRAIL, and various costimulatory molecules after treatment with epigenetic modifiers, leading to enhanced cancer-specific immune responses (Borden 2007; Khan, Gregorie et al. 2008; Khan and Tomasi 2008). A role for epigenetic modifiers in the regulation of pro-inflammatory cytokines has also been shown (Sailhamer, Li et al. 2008).

The generation of a therapeutic and CLL-specific immune response is currently under active investigation. Some of the more notable targeted proteins for vaccine approaches include specific idiotype immunoglobulin-derived peptides, survivin, KW-13, fibromodulin, MDM2, and telomerase (Harig, Witzens et al. 2001; Giannopoulos and Schmitt 2006; Kokhaei, Palma et al. 2007). Other strategies have focused on the generation and adoptive transfer of CLL antigen-specific T lymphocytes (Foster, Brenner et al. 2008). Additionally, adenoviral vector gene therapy using CD40-ligand (CD154) has been tested in early-phase clinical trials showing a reduction of disease burden in several patients (Trojan, Schultze et al. 2000; Krackhardt, Witzens et al. 2002; Schmidt, Schag et al. 2003; Mayr, Kofler et al. 2005; Giannopoulos and Schmitt 2006; Wierda and Kipps 2007). These trials have served to reveal one unresolved consequence of CLL: the current antigenic repertoire has likely generated tolerance leading to a weak cytotoxic

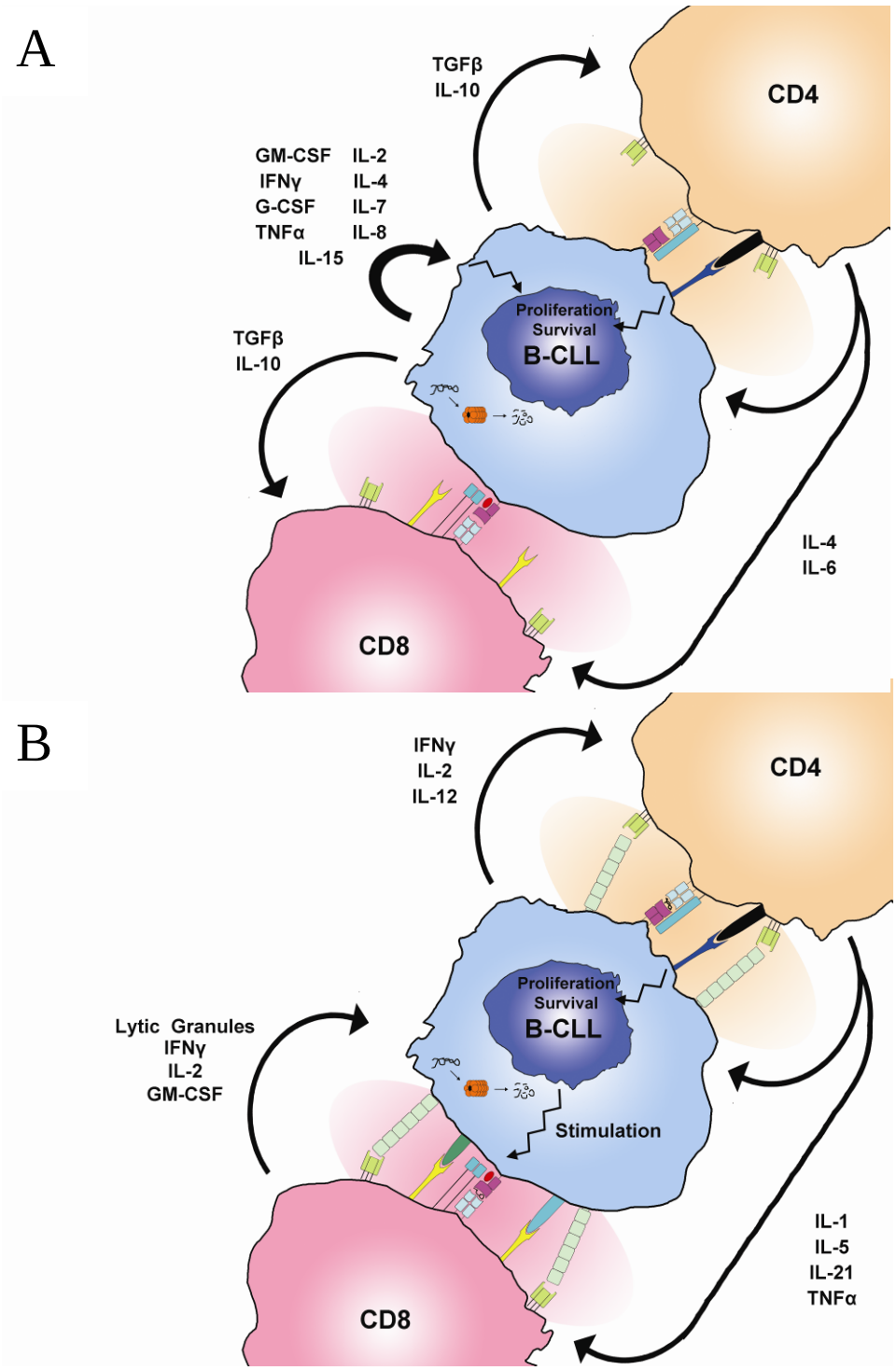

Figure 3: Signaling interactions between CLL cells and T cells. CLL cases vary however the most common signaling interactions are displayed in panel **A**. Panel **B** shows CLL based T cell interactions which may result from an improved immunogenic profile, a goal of many therapeutic interventions currently under development.

response incapable of generating effective tumor cell clearance. Experiments conducted by Guo et al. have shown that treatment of 4T1 tumor-bearing animals with such agents followed by adoptive transfer of P1A CTA-specific cytolytic lymphocytes (CTL) resulted in tumor-specific recognition and eradication(Guo, Hong et al. 2006). These studies were very recently confirmed in a murine orthotopic glioma model using a prominent and highly immunogenic human CTA, NY-ESO-1 (Natsume, Wakabayashi et al. 2008).

Chapter Two:

Treatment of chronic lymphocytic leukemia with a hypomethylating agent induces expression of NXF2, an immunogenic cancer testis antigen

Abstract

Critical to success of active immunotherapy against cancer is the identification of immunologically recognized cancer-specific proteins with low tolerogenic potential. Cancer testis antigens (CTAs) 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). Using a two-pronged approach we evaluated the immunogenicity of 29 CTAs in 22 patients with CLL and correlated these results to RT-PCR data from CLL cell lines and patient cells. 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 which 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 CTA expression and effective antigen presentation in malignant B-cells.

Results

An IgG response specific to NXF2, a known cancer-testis antigen, was identified in CLL.

In order to identify CTAs which may be recognized by the T-cell repertoire in the

Table 2: Cancer-testis antigen panel

#	Antigen	GenBank ID	Sense	Antisense
1	MAGE-A1	NM_004988	CACCTCCTCCTCCTCTCCTC	TCTCCAGCATTTCTCGCCTTT
2	SSX-2	BC007343	GGTGCTCAAATACCAGAGAAG	GGTGCTCAAATACCAGAGAAG
3	NY-ESO-1	AJ003149	GCTTCAGGGCTGAATGGAT	AAAAACACGGGCAGAAAGC
4	GAGE-7	NM_021123	GCCTAGACCAAGGCGCTAT	CCTTCTTCAGGCGTTTTCAC
5	SSX-4	U90841	CGAAACCACAGGAATCAGGT	CTCAGGGTCGCTGATCTCTT
6	NXF-2	BC015020	TGAAACCCTGCAAGGAAAAC	GCACTGAGGGAGTCCACAAT
7	TPX-1	BC022011	AGAGGACCGCAAAACCAGTA	TTCCTTGTTGGTACGGGGTA
8	XAGE-1	BC009538	GTATCCGAGTCCCAGAAGCA	GATTTATCCCCGGTGTTTGA
9	LAGE-1	BC002833	TTCTGCGCAGGATGGAAG	AAAAACACGGGCAGAAAGC
10	PAGE-1	BC010897	TCCAGAGGAAGAGGAGATGG	CTTAGCACGCTCCGGATTAG
11	MAGE-E1	BC081566	AGAGCATCACAGCCCTCATT	TCAGGTGGATCCCAAACTTC
12	SPANXC	BC054023	AATGGACAAACAATCCAGTGC	CATGAATTCCTCCTCCTCCA
13	ADAM2	BC064547	TGCACCCCAGAACCATAAGT	CTCTCCTGCTTCCAGCTTTG
14	TSP50	BC037775	TGCACCCCAGAACCATAAGT	CTCTCCTGCTTCCAGCTTTG
15	NY-SAR-35	BC034320	AAAGCGAAGGGGAGGAATAG	GGGCAGGATATGTCCATTTG
16	FATE-1	BC022064	GAAATGTCCCTGGCAGAAGA	AATGGAAACGTATGCCTTGG
17	PAGE-5	BC009230	CCCAATCCTCAGAAAGAGGAA	TTCACCTGCTTCCAGCACTT
18	LIP-1	BC023635	CAGACCGTTAAGCTCCTTGC	GGTGGCATATTCTCCACCTC
19	SPA17	BC032457	ACGCGAGATTCTGAGAGAGC	CAGCTTGGATTTTGACAGCA
20	MAGE-A8	BE387798	AGAAGTGGACCCCTTTGTCC	GGATCACTATTGGGCACCTG
21	MAGE-B1	BE897525	TGCTGCAGCTGTGTCATGTA	TGGCCACTAGGGTTGTCTTC
22	MAGE-B2	BC026071	CTTCAAGCTCTCCTGCTGCT	GGAAGTGCTCCCTGAACCTT
23	MAGE-A4	BC017723	GCAAGTATCGAGCCAAGGAG	TCCCAGATTTCCTCCTCAGA
24	SSX-1	BC001003	AGAAGCAAGGCCTTTGATGA	GCTTCTTGGGCATGATCTTC
25	GAGE-2	BC069397	GCCTAGACCAAGACGCTACG	CCTTCTTCAGGCGTTTTCAC
26	GAGE-4	BC069470	GCCTAGACCAAGGCGCTAT	CCTTCTTCAGGCGTTTTCAC
27	MAGE-A3	BC016803	GCAAAGCTTCCAGTTCCTTG	AAATGTTGGGTGAGCAGCTT
28	MAD-CT-1	NM_002762	AGGTGTACAGGCAGCAGTTG	TCTGCATGTTCTCTTCCTGGT
29	MAD-CT-2	AK097414	ACCCTGAGACAACCACTTCG	ACCTGTCTGCCTTCTTGTGC

The names and GenBank identifiers for each CTA chosen for analysis are shown. In addition, the 5' and 3' primers used for the gene-specific RT-PCR amplifications are shown.

context of CLL we used high-throughput phage immunoblot, a technique we have previously described (Dubovsky, Albertini et al. 2007; Dubovsky and McNeel 2007). In brief, lambda-phage encoding 29 known CTAs (Table 2) were spotted robotically in duplicate onto a bacterial lawn and overlaid with nitrocellulose membranes impregnated

with IPGT to induce protein expression. These membranes were then probed with sera from 22 patients with CLL (Table 3).

Table 3: Clinical characteristics of CLL patients

Number	Sex	Age	Stage	FISH/cytogenetics	Treatment
1	M	60	I	trisomy 12	+
2	M	67	I	normal	+
3	M	56	0	del 13q14	-
4	M	63	0	normal	-
5	M	69	0	del 13q14	-
6	M	63	I	del 13q14	-
7	M	70	I	del 13q14	-
8	M	55	I	normal	-
9	M	61	I	del 13q14	-
10	F	70	I	del 13q14	+
11	M	37	I	del 13q14	+
12	F	77	0	ND	-
13	M	66	I	trisomy 12	-
14	F	76	IV	trisomy 12	+
15	M	47	0/I	normal	-
16	M	57	0/I	normal	-
17	F	50	0	del 17p13	-
18	M	69	IV	trisomy 12; del 13q14	-
19	F	67	II	del 13q14; del 17p13	-
20	M	71	I	normal	-
21	M	68	IV	del 13q14; del 17p13	+
22	F	53	I	normal	+

Serum and cells from CLL patients was used to identify immunologically recognized and inducible CTAs. Patient Characteristics, Rai stage, cytogenetics, and prior treatments are shown. "del" indicates a deletion of the indicated chromosomal arm or gene.

Immunoreactive plaques specific for NXF2 were identified in CLL patient 9 (Figure 4A). This immunoreactivity was also found in serum collected from the same patient both three and nine months later, corroborating our initial data (Figures 4B and 4C). 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 which confirmed a robust IgG response in patient 9 at both available timepoints (Figure 5A). Using subisotype specific ELISA this NXF2 reactivity was found to be predominantly composed of subisotype IgG_1 (Figure 5B). As further confirmation of

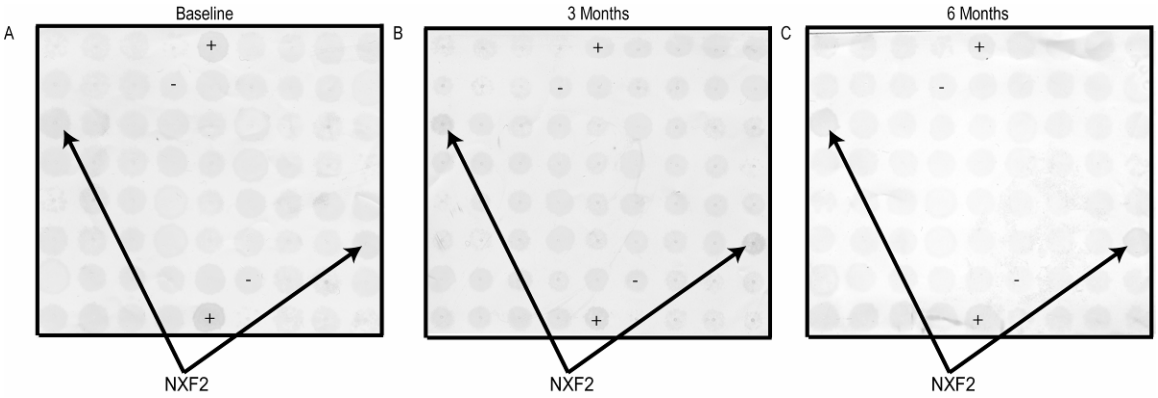

Figure 4: High Throughput Phage Immunoblot indicates an IgG response to NXF2
Patient number 9 had a detectable response to NXF2 (**panel A**). Three months later on a return visit for further observation the same patient displayed reactivity to NXF2 once again, confirming the first occurrence (**panel B**). Reactivity to NXF2 was also seen at a six month observation point for patient 9 (**panel C**). Immunoreactive spots are determined by visually comparing them with the negative "-" and positive control "+" spots on each filter. Of the 22 patients screened NXF2 was the only reactive CTA discovered.

reactivity a Western blot was performed using serum from a subset of patients (CLL 9, 1, 13, 2, 22, and 10) probing NXF2 transiently transfected 293T cell lysates which demonstrated that the IgG response was specific for NXF2 (Figure 5C).

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 Table 2 and used this primer panel to profile CTA expression in a variety of CLL cells with or without 1μM 5A2 treatment using RT-PCR. The CLL cell lines MEC1, MEC2, and WaC3 were kindly

provided to us by Dr. John Byrd at the Ohio State University Medical Center and are further described in (Wendel-Hansen, Sallstrom et al. 1994; Stacchini, Aragno et al. 1999). Using these cell lines we identified multiple genes which were either induced de

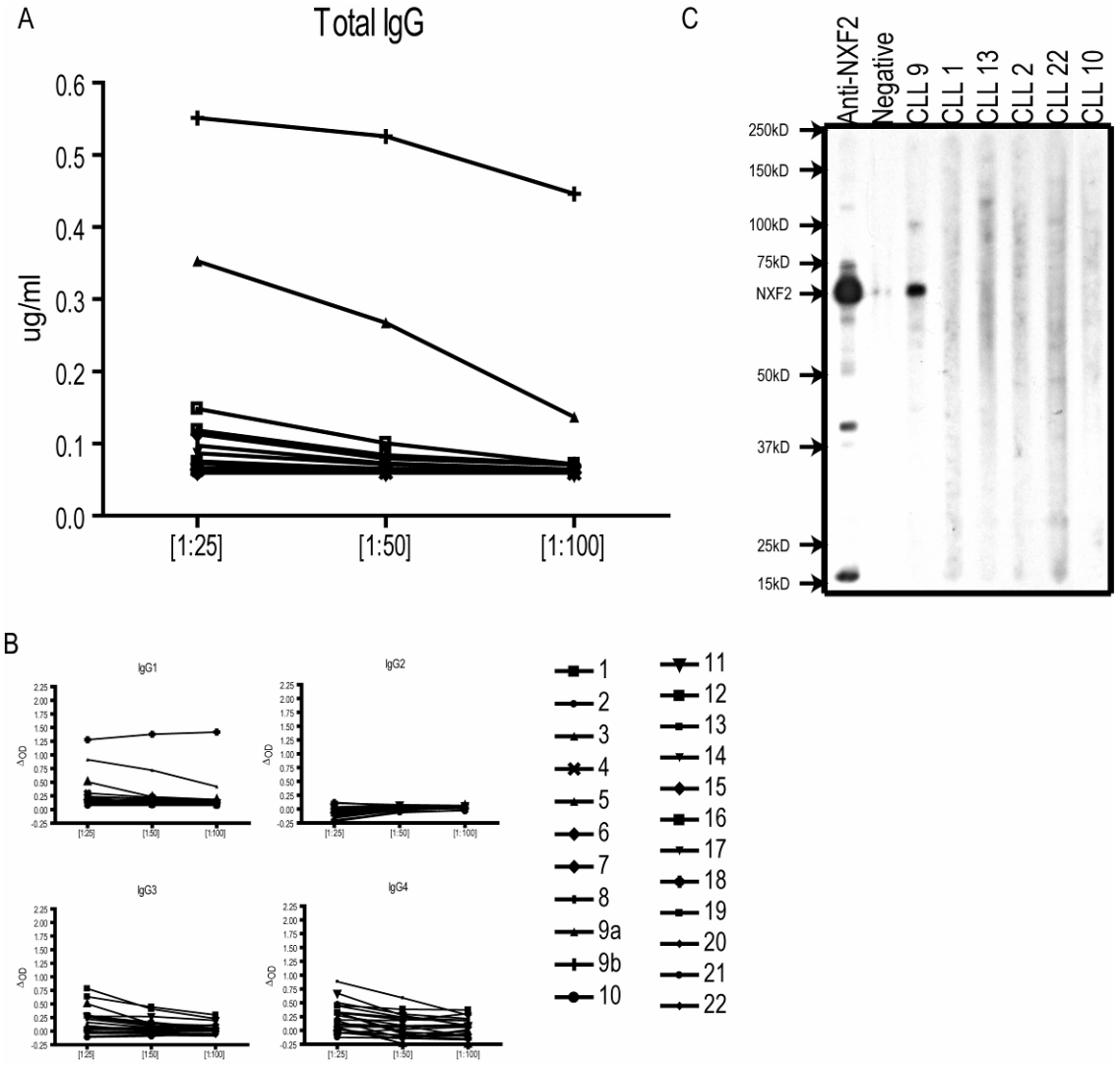

Figure 5: ELISA and Western Blot results confirm IgG response to NXF2 and identify major subisotype

Sera from patients with CLL were subjected to ELISA using GST tagged NXF2 purified protein (**panel A**). The NXF2-specific antibody response is primarily of the IgG_1 subisotype (**panel B**). To eliminate the possibility of potential GST-specific reactivity serum from a subset of patients, including the reactive patient #9, were used to probe NXF2 transient transfection Lysates (positive = anti-NXF2 polyclonal antibody) (negative = no loaded protein) (**Panel C**). Patient 9 donated serum on two separate occasions delineated by 9a and 9b in all ELISA data.

novo, were significantly upregulated, or were constitutively upregulated (Figure 7A). Of the 29 CTAs tested expression of NY-SAR-35, XAGE-1, GAGE-2, 7, and 4, LAGE-1, NXF2, NY-ESO-1, and SSX2 was induced de novo after treatment with 5A2. Expression

of MAGE-A1, A3, A4, and B2, SSX1, and 4, TPX-1, and FATE-1 was significantly upregulated by treatment with 1μM 5A2. Additionally, we found that mRNA levels of LIP1, MAD-CT-1 and 2, SPA17, MAGE-B1 and 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

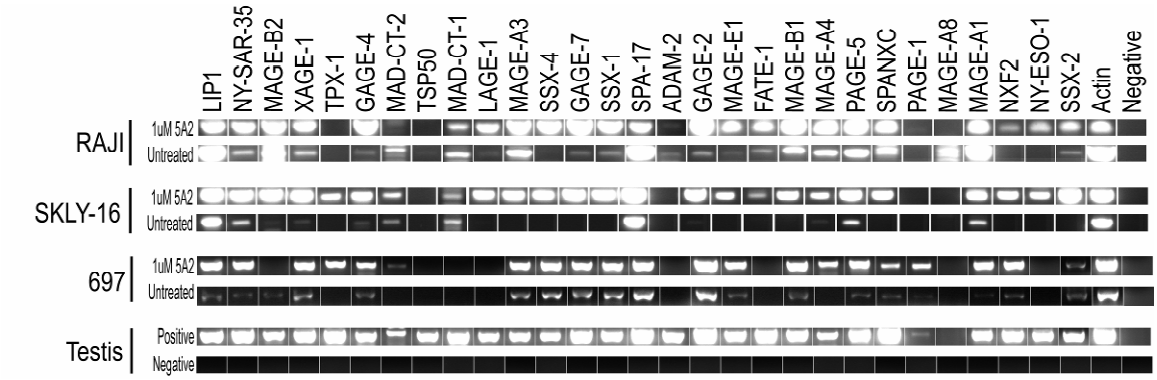

Figure 6: Analysis of B-Cell leukemia confirms inducibility of certain CTAs. As additional confirmation of inducibility the B-Cell leukemia lines RAJI, SKLY-16, and 697 were analyzed for CTA expression pre and post treatment with 1μM 5A2. CTA inducibility was found to be similar to that of CLL.

total RNA from purified CLL cells (>90% purity) (Figure 7B). We found that CTA expression was extremely similar to that of the cell lines. 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 (Figure 6). In this screening the only consistently

inducible CTA was NXF2, although other CTAs were inducible in particular cell lines.

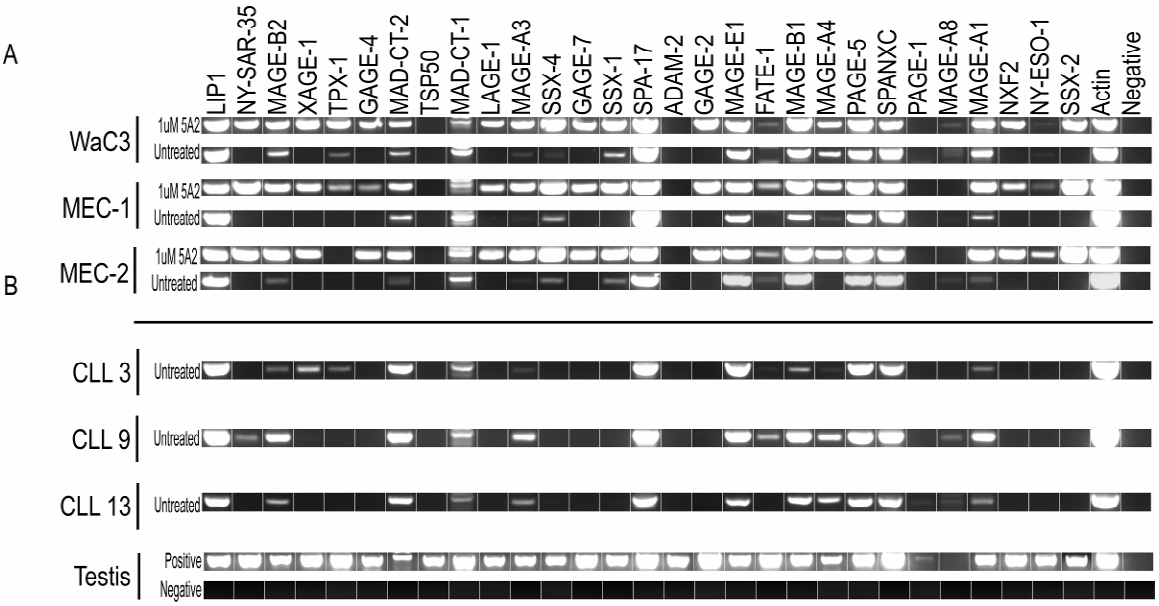

Figure 7: RT-PCR reveals novel constitutively expressed, inducible, and upregulated CTAs in CLL.
Three cell lines, MEC1, MEC2, and WaC3 were analyzed via transcript-specific RT-PCR for mRNA expression of CTAs pre and post treatment with 1μM 5A2 (**panel A**). To ensure that CTA expression patterns in the CLL cell lines are representative CLL cells from patients 3, 9, and 13 were analyzed for basal CTA expression (**panel B**). Testis cDNA was analyzed as a positive control as well as a no-RNA transcript negative control, confirming the identity of the amplified products.

Of the three classes of leukemia CTAs (inducible de novo, upregulated, and constitutively expressed) currently the most attractive class from the prospective of CLL immunotherapy is those CTAs which 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.

While NXF2 has demonstrated 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(Loriot, Boon et al. 2003). Consequently, we evaluated the Oncomine micro-array database, which contains data from over 20,000 human microarray studies for mRNA expression of NXF2 in normal tissues. In a panel of 40 normal tissues NXF2 was only significantly upregulated in the testis, as expected (Figure 8).

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). Since 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 IL-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 figure 9, we saw a marked increase in FATE-1, MAGE-A4, and MAGE-A8 transcription and a de-novo induction of

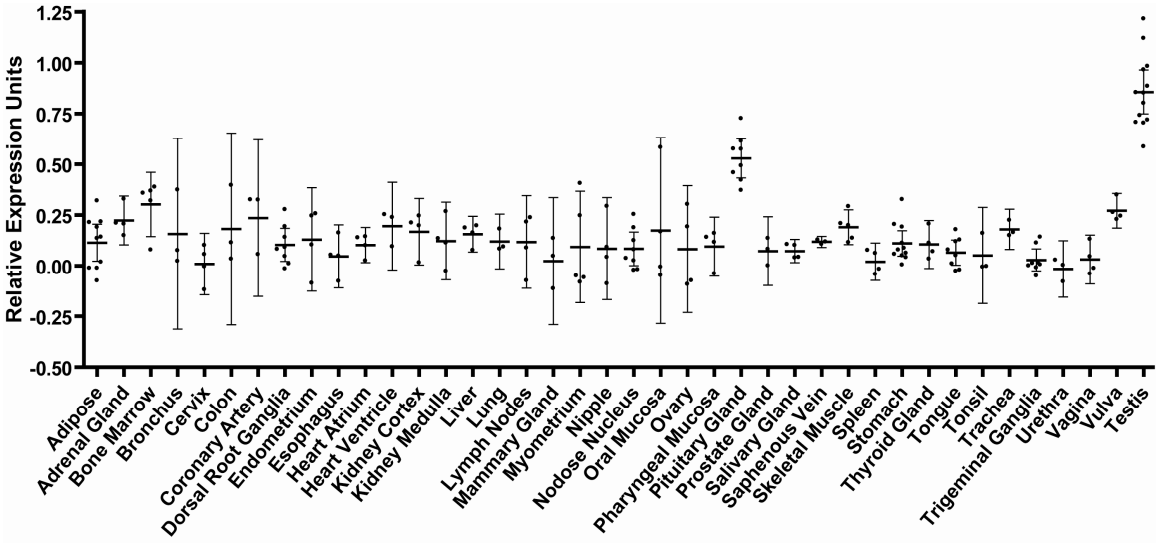

Figure 8: NXF2 expression is primarily restricted to the testis among normal tissues.
The Oncomine database, containing cancer gene expression profiles from over 20,000 microarrays (Rhodes, Kalyana-Sundaram et al. 2007) (available at www.oncomine.org), was queried for the presence of NXF2 mRNA among different normal tissue sets. Shown are the relative expression units of NXF2 among 40 normal tissues including testis with mean and standard error indicated (n ranges from 3 to 13).

XAGE, GAGE-4, 2, and 7, SSX-1 and 2, PAGE-1, and NXF2 transcripts. These data served to confirm our initial antigen identification and to demonstrate NXF2 inducibility in primary cells.

Immunogenic CTA expression can be modulated for a possible immunotherapeutic approach.

Since 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 1μM 5A2 for 72 hours and then washed and cultured with serum-supplemented media only for up to 20 days. Cells were harvested at various timepoints after washout and assessed for NXF2 mRNA by qRT-PCR. As shown in Figure 10A, in the MEC2 cell

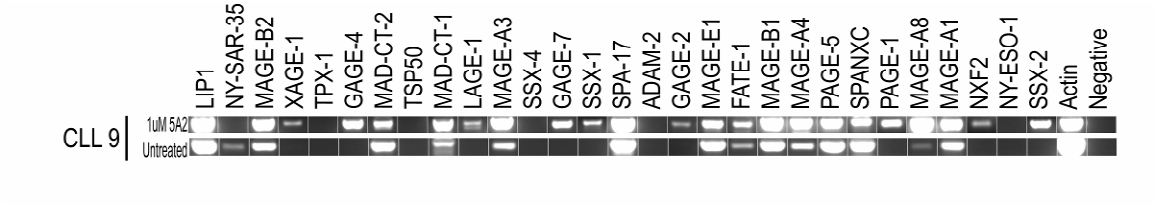

Figure 9: NXF2 is among a variety of inducible CTAs in primary human CLL treated with 5-aza-2'-deoxycytidine.
CLL cells cultured with IL-4 atop an irradiated CD40L expressing murine fibroblast stromal layer were analyzed via transcript-specific RT-PCR for mRNA expression of CTAs with and without 1μM 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 previous figure).

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 MEC1 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 upregulated 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 which 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 (Figure 10B). Moreover, we found that both SSX1 and

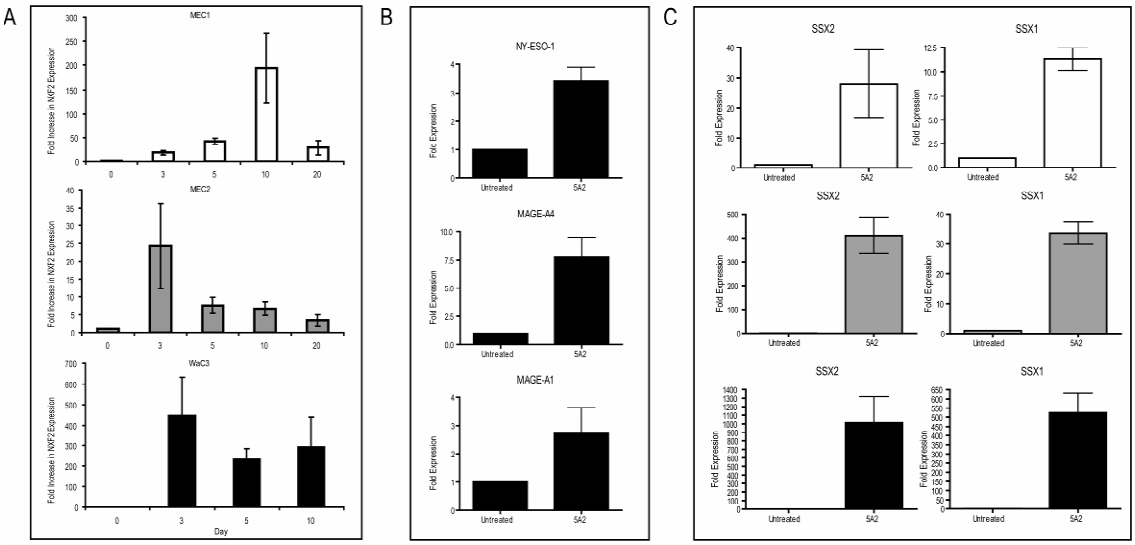

Figure 10: NXF2 expression in CLL cell lines can be modulated by treatment with the DNA methylation inhibitor 5-aza-2'-deoxycytidine.
CLL cell lines (*white bars* = MEC1, *grey bars* = MEC2, *black bars* = WaC3) were cultured for 72hr in the presence of 1μM 5A2. *Panel A:* Cells were washed and then 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 qRT-PCR. *Panel B*: RNA from the WaC3 cell line was subjected to qRT-PCR specific for NY-ESO-1, MAGE-A4, and MAGE-A1. *Panel C*: RNA from all CLL lines was subjected to qRT-PCR for SSX2 and SSX1 indicating upregulation 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 $2^{-\Delta\Delta CT}$ method(Pfaffl 2001). The data shown is the mean and standard deviation of three independent qRT-PCR experiments.

SSX2 were highly upregulated in all three cell lines after 5A2 treatment (Figure 10C). 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 (APC) 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 Figure 11A we

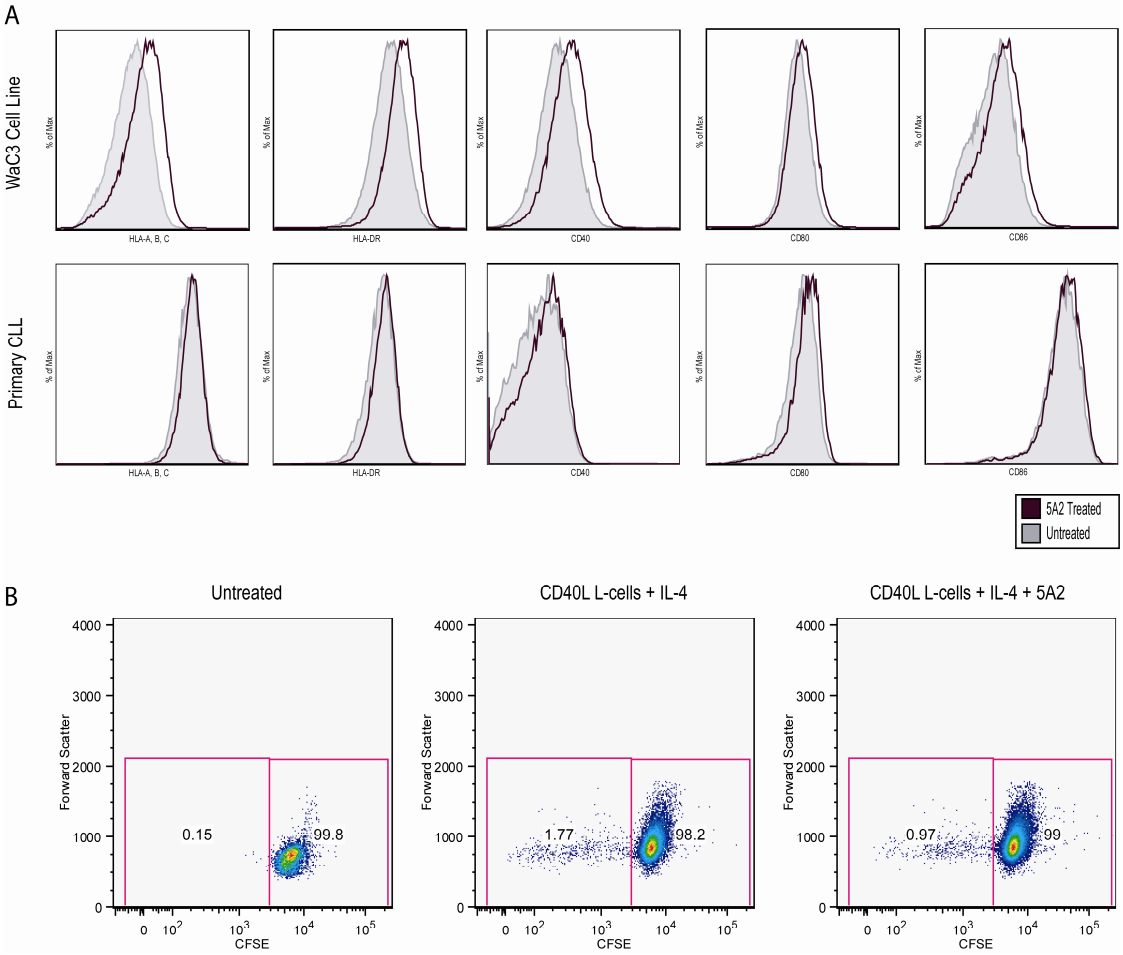

Figure 11: Demethylation restores effective antigen presentation characteristics in a CLL cells.

The representative CLL cell line MEC1, or human primary CLL cells cultured using IL-4 and an irradiated CD40L transfected fibroblast feeder layer, were cultured for 72hr in the presence of 1μM 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 APC costimulatory molecules (CD80, CD86, and CD40) as compared to basal levels under an identical culture system without 5A2 (untreated) (**panel A**). The 72 hour proliferative capacity (and likewise 5A2 uptake capacity) is demonstrated in CFSE labeled primary human CLL cells in a separate experiment using only serum supplemented RPMI (untreated), the CD40L fibroblast stromal layer + IL-4, or the CD40L fibroblast stromal layer + IL-4 + 1μM 5A2 (**panel B**). Data shows that 5A2 treated cells (black histograms) upregulate expression of all five molecules when compared to 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.

found that both MHC class I and class II molecules were upregulated after treatment. Furthermore, the costimulatory molecules CD40, CD80, and CD86 were also upregulated. 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 IL-4. As an independent CFSE staining experiment demonstrated 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 (Figure 11B). These data suggest that treatment with 5A2 may aid in effective cancer-testis antigen 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 have used similar high-throughput antibody screening methods to identify antigens in both prostate cancer and melanoma (Dubovsky, Albertini et al. 2007; Dubovsky and McNeel 2007). 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 which require access to small biopsy or post surgical resection specimens for mRNA or protein analysis. Our study has taken full advantage of this characteristic to complement 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 (Hoeppner, Dubovsky et al. 2006). 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 heterogenic levels of global CpG methylation(Melki, Vincent et al. 2000; Melki and Clark 2002; Rush, Raval et al. 2004). 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(Plass, Byrd et al. 2007).

Despite these hurdles we have managed to identify NXF2 as an immunogenic CTA in a CLL patient. We have 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 immunoediting, the repeated evasion of immune recognition(Dunn, Bruce et al. 2002; Dhodapkar, Krasovsky et al. 2003; Dunn, Old et al. 2004). Although, it is still unclear as to the exact antigen milieu which shapes CLL since 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 since they persist years after antigen stimulation. It is postulated that these antigens already have a proven capacity to eliminate CLL clones, but their non obligate 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 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 (2.1g/dl compared to a 1.5g/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, since a specific IgG response requires CD4+ 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 which occur in CLL upon treatment with such inhibitors(Wheeler and Gordon 1996; Jacob, Pound et al. 1998; Willimott, Baou et al. 2007). 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 the specific fraction of CLL cells who 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(Wang, McCarrey et al. 2001; Loriot, Boon et al. 2003). Fortunately, Loriot et. al. had the foresight to attempt modulation of this gene using 5-aza-2'-deoxycytidine at the time of discovery, a result which has shaped our current investigation. Although little attention has been paid to its antigenicity a few groups have investigated its function as

an mRNA exporter, interactions with cytoplasmic motor proteins, and role in male infertility(Takano, Miki et al. 2007). It has been hypothesized that NXF2 may have multiple roles critical to the meiotic process(Wang and Pan 2007). Conceivably, these roles would have wide reaching benefits to the pre-malignant B-cell including the independent segregation of undesired chromosomes leading to anuploidy and the recombination of genetic material leading to translocations and deletions(Simpson, Caballero et al. 2005). Moreover, Wang and co-workers 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 IgG_1 which is consistent with the generation of a Th2 immune response which does not necessitate CD8+ 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 IL-4(Rossmann, Lewin et al. 2002). This effect will need to be overcome in order for T-Cell based vaccines to be truly effective. However, emerging evidence implicates a CD4+ T-Cell response in significant, rapid, and antigen specific cytotoxic responses in CLL and CTA (NY-ESO-1) vaccine trials(Chu, Deforce et al. 2002; Hunder, Wallen et al. 2008).

We have shown here that NXF2 mRNA can be upregulated for extended periods of time after treatment with the methylation inhibitor 5-aza-2'-deoxycytidine. In prior studies we and others have found that transient treatment with 5A2 was able to induce

prolonged and cancer restricted expression of CTAs and based on our results it appears 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 (DNMT1) combined with an inability to upregulate expression of this critical gene post-treatment. However, in our experience mRNA expression of DNMT1 mRNA seems unchanged in both normal and cancer cells lending evidence to the contrary(Dubovsky and McNeel 2007). Nevertheless, this characteristic will likely be essential to the generation of an effective immune response since 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 which 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 and 2, SPA17, MAGE-B1 and 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, A3, A4, and B2, SSX1 and 4, 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(Jager, Gnjatic et al. 2000; Brichard and Lejeune 2008). 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(Krackhardt, Witzens et al. 2002; Horna and Sotomayor 2007; Wierda and Kipps 2007). Wierda and Kipps recently reviewed this topic putting together multiple

strategies which have shown promising results. In the current study we 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 semi-professional APC(Coral, Sigalotti et al. 1999). 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 which 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 5-aza-2'-deoxycytidine, followed by adoptive transfer of P1A CTA-specific cytolytic lymphocytes (CTL), resulted in tumor-specific recognition and eradication(Guo, Hong et al. 2006).

In the current study we have 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 which are both immunogenic and specific for CLL. We identified IgG specific for one novel CLL cancer-testis antigen NXF2 and confirmed this response by ELISA and western blot. In addition, we confirmed that treatment of CLL with 5-aza-2'-deoxycytidine can induce expression of NXF2 for weeks post treatment. Treatment also increases levels of MHC and costimulatory molecules necessary for antigen presentation. In addition, RT-PCR results identified other promising antigens which 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 vaccinate-induce strategy.

Materials and methods

Subject Populations

Sera and peripheral blood mononuclear cells (PBMCs) were obtained from 22 patients with CLL (16 males and 6 females, mean age 62 years, range 37-77 years). 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 (Table 3). Rai stage for the 22 patients was: 5 patients = 0, 13 patients = I, 1 patient = II, 3 patients = IV. All subjects gave written institutional review board (IRB)-approved informed consent for their blood products to be used for immunological 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 have previously reported the construction of a panel of lambda phage encoding 29 cancer-testis antigens(Dubovsky, Albertini et al. 2007). Analysis of this panel was conducted similarly to what we have previously reported (Dubovsky, Albertini et al. 2007; Dubovsky and McNeel 2007). In brief, XL-1 blue MRF *E. coli* were grown overnight, collected by centrifugation, resuspended in 10mM $MgSO_4$, and poured in top agarose (LB broth/10mM $MgSO_4$/0.2% maltose/0.7% agarose) over LB agar in Omniwell plates (Nunc, Rochester, NY). Phage encoding individual (9,000 pfu) CTA's were then

spotted in replicates onto multiple bacterial agar lawns using a liquid handling robot (Biomek FX, Beckman, Fullerton, CA). Spotted plates were allowed to sit undisturbed for 15 minutes and then overlaid with nitrocellulose membranes impregnated with 10mM IPTG. Plates were incubated overnight at 37°C. The next day, filters were washed, blocked with TBS (50mM Tris pH 7.2, 100mM NaCL) + 1%BSA (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 anti-human IgG detection antibody (Sigma, St. Louis, MO). The filters were washed again and then developed with 0.3mg/ml nitro-blue tetrazolium chloride (NBT) + 0.15mg/ml 5-bromo-4-chloro-3'-indolyphosphate p-toluidine salt (BCIP). 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 beta-galactosidase) control plaques.

Enzyme-Linked Immunosorbent Assay (ELISA)

Ninety-six-well high binding plates (Corning, Corning, NY) were coated with purified Glutathione-S-Transferase linked NXF2 protein (Novus Biologicals, Littleton, CO) at 2ug/ml or purified human IgG (Sigma) titrations starting at 2ug/ml in 50mM sodium carbonate buffer (pH 9.6) overnight at 4°C. After blocking for 2 hours at room temperature with phosphate buffered saline (PBS)/1% bovine serum albumin (BSA) 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 anti-human IgG HRP antibody (GE-

biosciences, Piscataway, NJ) was added at 1:1000 in blocking buffer. Alternatively, for the IgG subisotype ELISAs, biotinylated anti-IgG_1, -IgG_2, -IgG_3, or -IgG_4 (Sigma) were used followed by HRP-labeled streptavidin (GE-biosciences). After a three-wash step reactivity was measured using TMB substrate (KPL, Gaithersburg, MD) according to manufacturer instructions.

Western Blot

Lysates from NXF2 transiently transfected 293T cells were mixed 1:2 with 2X sodium dodecyl sulfate (SDS) Laemmli's loading buffer (0.04M Tris, pH 6.8, 12% glycerol, 1.25% SDS, 3% β-mercaptoethanol, 0.06% bromophenol blue) and boiled for 5 minutes 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, Boston, MA) and autoradiography film.

Primary Cell Culture

CLL cells were isolated from peripheral blood by density gradient centrifugation, washed, and resuspended at $3X10^6$ in RPMI 1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal calf serum and 20ng/ml recombinant human IL-4 (RDI, Concord, MA). Isolated CLL cells (>95% purity) were cultured in $25cm^2$ tissue culture flasks atop an adherent bed of irradiated (30Gy) CD40L (CD154) expressing murine fibroblast L-cells. Cultures were maintained in the presence or absence of 1μM 5A2 for 72 hours and were subsequently assayed via RT-PCR or flow cytometry.

Reverse Transcriptase-PCR (RT-PCR)

Total RNA was prepared from centrifugally pelleted cell cultures (RNeasy mini columns and RNAse free DNAse, Qiagen, Valencia, CA) or was commercially obtained (BioChain, Hayward, CA). RT-PCR reactions were conducted using the Qiagen one-step RT-PCR kit (Qiagen) with transcript-specific primers (Table 2) and total RNA from various B-Cell leukemia and CLL cell lines as templates (generous gifs 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 confirmed by comparison with DNA size markers (GelPilot 1Kb Plus Ladder, Qiagen).

Quantitative Reverse Transcriptase-PCR (qRT-PCR)

Cell lines were cultured in the presence or absence of 5-aza-2'deoxycytidine (5A2) at 1μM. After 72 hours of culture, cells were washed multiple times with PBS and re-cultured in medium without 5A2 for up to 20 days. Total RNA obtained from these cultured cell lines (RNeasy mini columns, Qiagen) was analyzed for β-actin and NXF2 RNA by qRT-PCR using a manufacturer's standard protocol (iScript RT-PCR with SYBR green, BioRad, Hercules, CA) and NXF2, MAGE-A3, MAGE-A4, SSX1, SSX-2, or β-actin-specific gene primers (Table 2, and β-actin-5' TCATGAAGTGTGACGTTGACATCCGT, β-actin-3' CTTAGAAGCATTTGCGGTGCACGATG). Fluorescent amplicon signatures and cycle of transmittance values (CT) were obtained using a BioRad MyCycler and its associated software, MyIQ v1.0 (BioRad). Fold changes in NXF2 expression relative to actin were calculated according to the $2^{-\Delta\Delta CT}$ method previously described(Pfaffl 2001). The

reported fold change in gene expression was determined from two independent qRT-PCR measurements performed at different times.

Flow Cytometry

Flow cytometric analysis of CLL cell lines was performed using fluorochrome-labeled monoclonal antibodies (mAbs; anti-HLA-A,B,C, -HLA-DR, -CD40, -CD80, -CD86, -CD19, and –CD20, Becton Dickinson, San Jose, CA and eBiosciences, San Diego, CA) and the vitality die 4',6-diamidino-2-phenylindole (DAPI, Sigma). For CFDA-SE (CFSE) staining cells were resuspended in 0.5μM CFSE (Invitrogen) in RPMI medium for 15 minutes at 37°C followed by a wash and an additional 30 minute incubation in serum supplemented medium prior to resuspension in culture medium. Data was acquired on an LSRII cytometer (Beckman Coulter), and analyzed with FlowJo software (Tree Star, Ashland, OR).

Chapter Three:

Restoring the Functional Immunogenicity of Chronic Lymphocytic Leukemia Using Epigenetic Modifiers

Abstract

Chronic lymphocytic leukemia (CLL) is a malignancy arising from immune cells (B-lymphocytes) endowed with intrinsic antigen-presenting capabilities. Such a function however is lost during malignant transformation and CLL cells are well known for their inability to process and present antigens to the T-cell arm of the immune system. Instead, malignant CLL cells elicit a vast array of immune regulatory mechanisms conducive to T-cell dysfunction and immunosupression. Previously, we have shown that treatment of CLL cells with the demethylating agent 5-aza-2'-deoxycytidine unleashed target antigen expression. Here we show for the first time that combining two epigenetic modifiers, 5-aza-2'-deoxycytidine and the histone deacetylase inhibitor LAQ824 effectively restores the immunogenicity of CLL cell lines as well as primary cells obtained from CLL patients. Indeed, such a combination induces the expression of novel and highly antigenic cancer testis antigens (CTAs) and co-stimulatory molecules. These changes facilitate the formation of robust supramolecular activation complexes (SMAC) between CLL cells and responder T-cells leading to intracellular signaling, lytic granule mobilization, and polarization of functional and relevant T-cell responses. This cascade of T-cell activating events triggered by CLL cells with restored APC function, points to

combined epigenetic modifier treatment as a potential immunotherapeutic strategy for CLL patients.

Results

Epigenetic modifiers synergistically induce CTA expression in CLL cells.

Our prior studies in CLL indicate that 5A2 treatment alone is capable of inducing the expression of highly antigenic CTAs(Dubovsky, McNeel et al. 2009). Given this prior knowledge we wanted to examine the combined effect of 5A2 and histone deacetylase inhibitors in this regard.

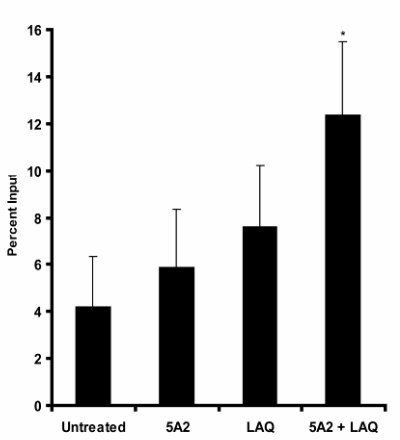

Figure 12: 5A2 and LAQ treatment of a CLL cell line induces histone acetylation changes at the chromatin level. Chromatin immunoprecipitation experiments using acetylated Histone–H4 specific antibody carried out with MEC1 CLL cells untreated, treated with 1uM 5A2, 25nM LAQ, or both 1uM 5A2 and 25nM LAQ were quantified via Q-PCR and normalized to input controls. Primers specific for a known distal regulatory region of the IFN-γ gene were used as a marker for effective rearrangement of chromatin acetylation patterns. Reactions were repeated in triplicate, error bars display standard deviation and significance ($p<0.05$) above the untreated condition is indicated by an asterisk.

Prior to initiating experimentation, we first wanted to confirm that our epigenetic modifiers were capable of altering chromatin structure in a CLL cell line. Previous studies have documented acetylation changes at conserved interferon gamma distal regulatory elements (Shnyreva, Weaver et al. 2004; Schoenborn and Wilson 2007). To

confirm the activity of 5A2, LAQ, and 5A2/LAQ in MEC1 cells, we profiled the acetylation changes occurring at the regulatory CNS+18 locus of the IFNG gene. As expected, our results demonstrated increased H4 acetylation in treated MEC1 cells indicating that both drugs were capable altering chromatin structure in a CLL cell line (Figure 12).

We therefore compared the mRNA expression pattern of 29 known CTAs between our CLL cell lines WaC3, MEC1, and MEC2 and primary CLL samples by RT-PCR. As shown in Figure 13A there is diverse low-level expression of multiple CTAs including SPANXC, MAD-CT-1 and -2, LIP1, SPA-17, and many members of the MAGE CTA family. Additionally, mRNA expression of SSX-1, -2, and -4, NY-SAR-35, GAGE-2, -4, and -7, XAGE-1, NXF2, LAGE-1, NY-ESO-1, TPX-1, FATE-1, ADAM-2, and TSP50 was extremely low or nonexistent in the majority of CLL samples and the CLL cell lines. The observed CTA expression profile indicated that interpatient expression patterns are somewhat variable. Nevertheless, our CLL cell lines accurately mirror the CTA expression trends seen in ten random patients with primary CLL making them valuable tools for in-vitro studies.

We next sought to improve upon basal expression patterns by treating our CLL cell lines with 5A2, LAQ, or a combination treatment of both 5A2 and LAQ (Figure 13B). mRNA expression data from these studies indicated that while both 5A2 and LAQ were capable of inducing the expression of varied CTAs only the combination treatment was capable of eliciting robust expression amongst virtually all of the CTAs tested including SSX-1, -2, and -4, NY-SAR-35, GAGE-2, and -7, XAGE-1, NXF2, LAGE-1, NY-ESO-1, TPX-1, FATE-1, ADAM-2, and TSP50, antigens which were nonexistent

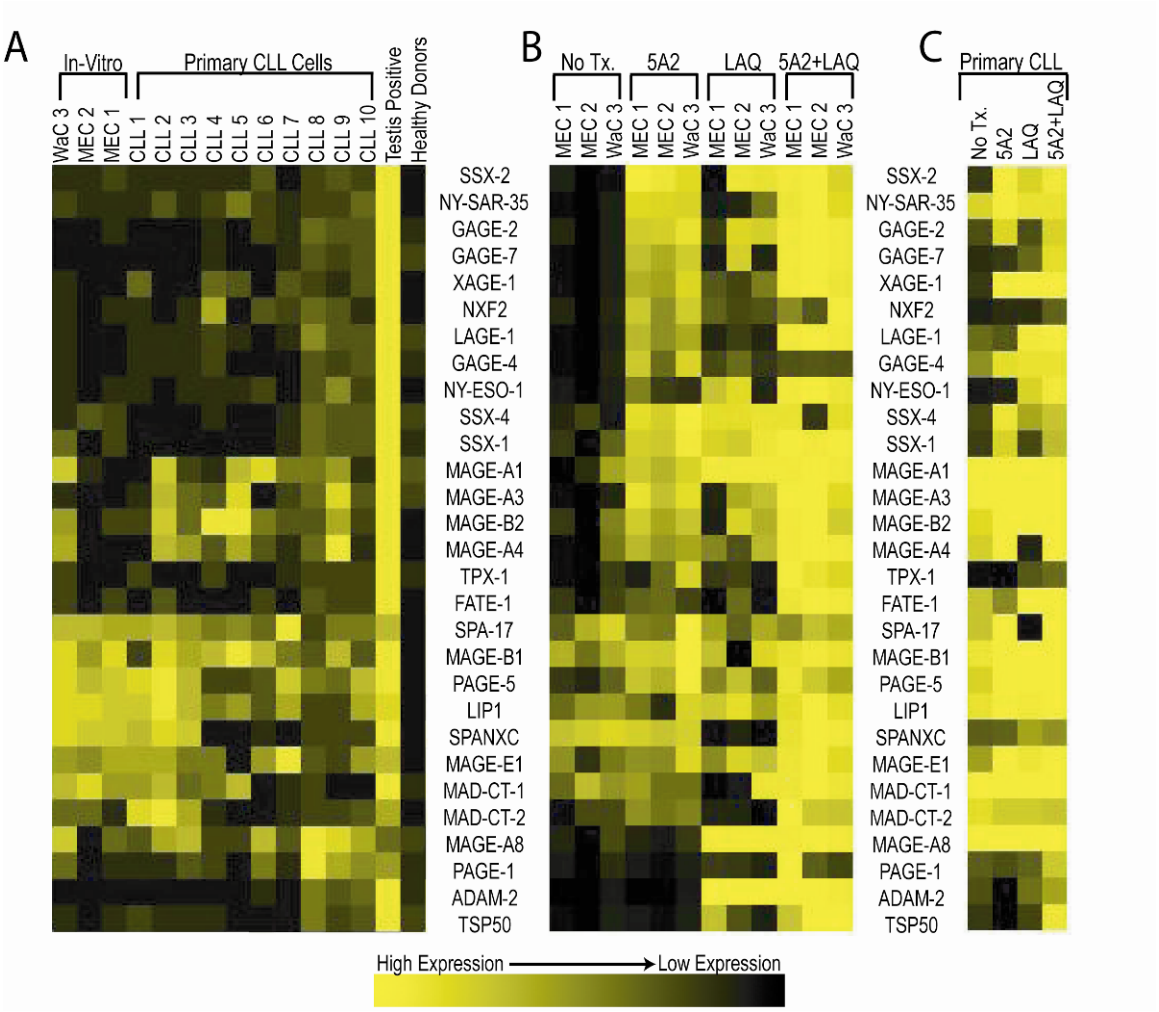

Figure 13: Cancer-testis antigen expression is significantly increased in CLL after treatment with combined epigenetic modifiers. RT-PCR results using transcript-specific primers for each of 29 known CTAs shows that our CLL cell lines mimic basal expression when compared to primary CLL (**Panel A**). Normal Human testis cDNA serves as a positive control and a mixture of PBMC RNA from 10 healthy blood donors serves as the negative control. A progressive increase in CTA expression can be seen in all cell lines after treatment with either 1uM 5-aza-2'deoxycytidine (5A2) or 25nM LAQ824 (LAQ) with the greatest levels achieved after combined treatment with both inhibitors (**Panel B**). Similar results were achieved utilizing primary CLL samples cultured on CD40L expressing murine fibroblasts and treated with the same concentrations of 5A2 and LAQ (**Panel C**). Experiments conducted on cell lines were repeated three times and experiments conducted on patient samples were repeated at least two times with similar results.

prior to treatment. While these results indicate a cooperative nature between the two

drugs we also observed synergistic activity with respect to certain CTAs, namely NY-ESO-1, FATE-1, TPX-1, PAGE-1, and TSP-50.

Given that primary CLL cells do not proliferate ex-vivo and thus do not incorporate 5A2 into their DNA it has previously been challenging to study the demethylating effects of nucleoside analogs outside of in-vivo or cell culture systems. To circumvent this challenge we utilize a CD40L expressing murine fibroblast feeder cell line which, in conjunction with IL-4, induces limited proliferation in some human CLL primary cells in-vitro. Using this system we characterized the epigenetic changes to the CTA expression pattern in primary CLL cells. As shown in Figure 13C, we saw a marked increase in CTA transcription with 5A2 or LAQ alone, although maximal effects were only observed with the combination treatment. These data confirm our initial hypothesis by demonstrating robust CTA inducibility in primary cells using epigenetic modifiers.

Epigenetic modifiers modulate the costimulatory profile and cytokine signaling of B-CLL.

Prior studies indicate that treatment of specific solid tumors with HDACi can improve the costimulatory phenotype and can increase tumor immunogenicity(Khan, Magner et al. 2004; Tomasi, Magner et al. 2006; Khan, Magner et al. 2007; Khan, Gregorie et al. 2008; Khan and Tomasi 2008). These data along with our prior studies using 5A2 on CLL led us to investigate the possibility that 5A2 and LAQ may potentiate an improved costimulatory phenotype in CLL. Our flow cytometry profiling experiments revealed modest increases in the CLL cell line surface expression of CD86, CD80, HLA-DR, CD40, and MHC class I (HLA-A,B,C) after treatment with 1μM 5A2 and 25nM LAQ (Figure 14A). In two additional repeat experiments this modest upregulation was consistent. Given that CLL cells are inherently derived from APCs, it is possible that

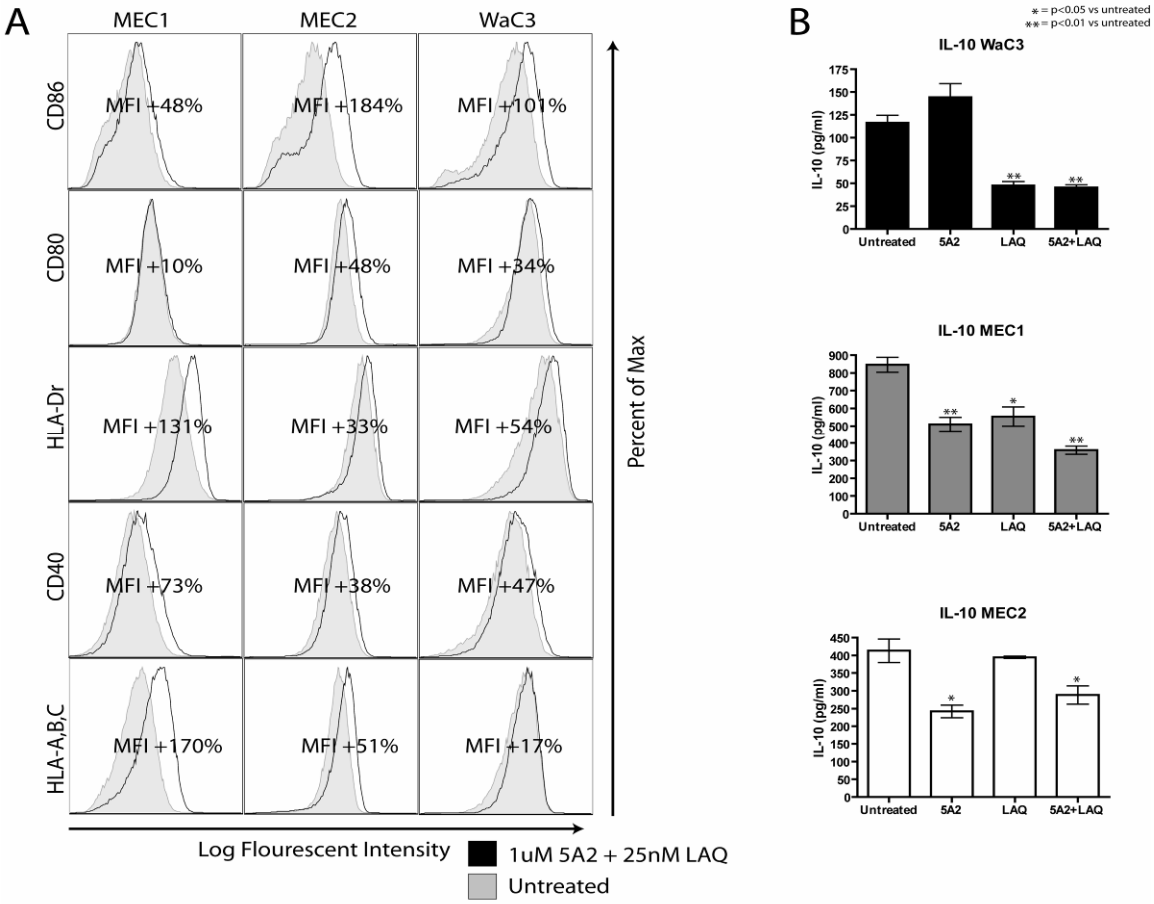

Figure 14: Immunophenotype can be enhanced and secretion of IL-10, an immunosuppressive cytokine, abrogated by treating CLL cells with 5A2 and LAQ. FACS analysis of three CLL cell lines (MEC1, MEC2, and WaC3) after treatment with 1uM 5A2 and 25nM LAQ reveals a moderate increase in costimulatory molecule surface expression when compared to untreated controls (grey histograms) (**Panel A**) mean fluorescent intensity (MFI) shifts are depicted for each histogram. Cytokine analysis by CBA showed significantly lower levels of IL-10 secretion in all CLL cell lines after 5A2+LAQ treatment (**Panel B**). These data suggest that there may be changes to the functional immunogenicity of CLL cells after treatment with epigenetic modifiers.

even slight increases in the costimulatory phenotype may tilt the balance towards the formation of an anti-CLL immune response.

Along with costimulatory molecules B-cells also provide critical immunostimulatory and immunosuppressive cytokine signals. B-CLL cells in particular, secrete high levels of immunosuppressive factors which inhibit the generation of anti-CLL T-cell responses. One such potent immunosuppressive factor is IL-10(Sjoberg, Aguilar-Santelises et al. 1996). In light of this we decided to investigate the IL-10 secretion by CLL cell lines after treatment with 5A2 and LAQ. Results from our cytokine analysis indicated that 5A2 and LAQ had the potential to independently inhibit IL-10 secretion in particular cell lines, however the combination of both inhibitors was essential to obtain significant inhibition in all cell lines, indicating that combination 5A2+LAQ therapy may release CLL-induced T-cell suppression (Figure 14B).

DNA demethylation and histone acetylation cooperate to increase the potency of the CLL cell – T-cell interaction.

Our results thus far point to improved antigen expression, costimulatory phenotype, and cytokine signaling which have been postulated to directly correlate with the formation of a healthy immunological synapse, or SMAC. To further investigate the changes in the resulting immune synapse we utilized superantigen (sAg) stimulation via staphylococcal enterotoxins A and B to induce a TCR mediated response in healthy allogenic donor CD8 or CD4 purified T-cells. As APCs we utilized 5A2 and LAQ treated CLL cell lines or alternatively healthy allogenic B-cells as a control. Our confocal imaging data suggested that the untreated CLL cells rarely formed robust interaction complexes with either CD8 or CD4 T-cells. In sharp contrast, after treatment with 5A2, LAQ, or both, SMAC complexes were readily abundant, robust, symmetrical, and concave, indicative of more effective and prolonged APC signaling (Figure 15A and

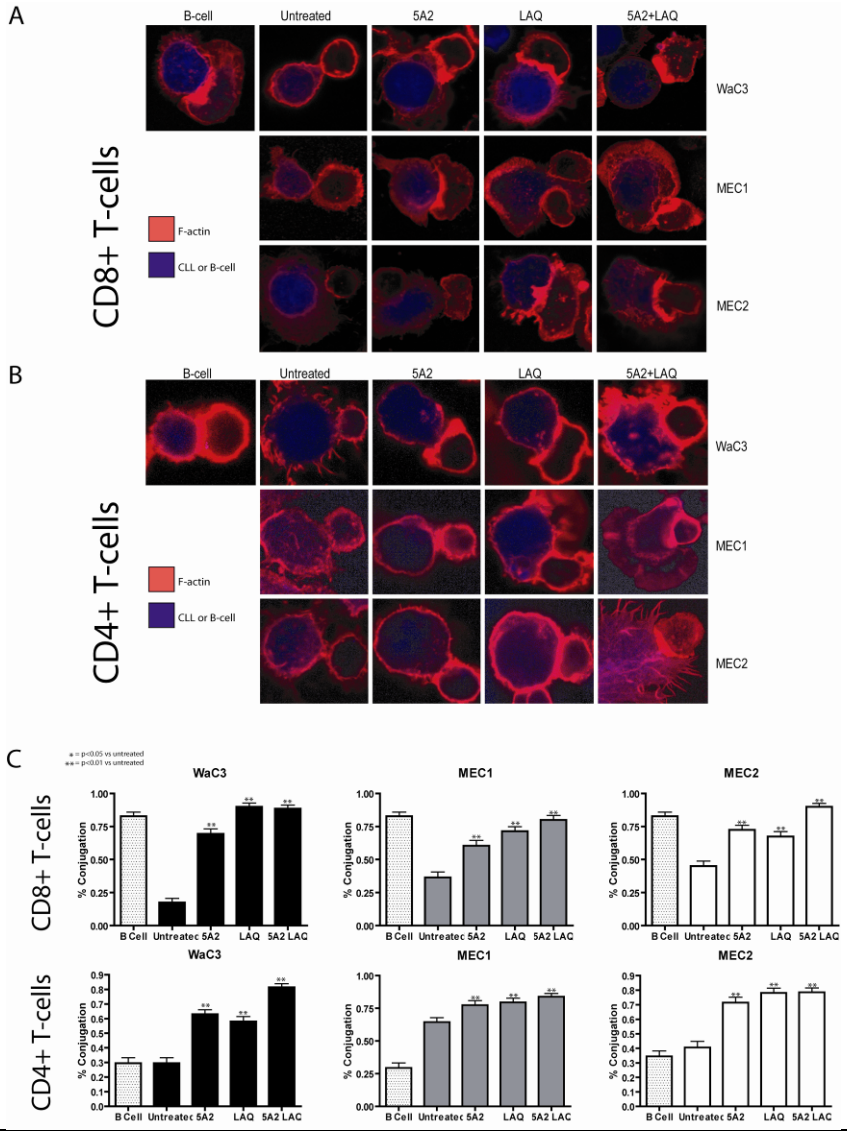

Figure 15: Demethylation and histone acetylation improves the quality of the immune-synapse between T-cells and CLL cells. Purified healthy donor CD8 T-cells (**Panel A**) or CD4 T-cells (**Panel B**) were allowed to conjugate with sAg pulsed CLL cell lines which were treated with 1uM 5A2, 25nM LAQ, both or neither drug (or allogenic B-cells) (blue stained) and were stained for actin polymerization (red) using phalloidin rhodamine. Representative confocal images are presented of T-cell/APC interactions for each of the three CLL cell lines under each of the treatment conditions. Quantification of the conjugation events was conducted by scoring polymerization as either 0 = dysfunctional, unorganized, and unpolarized synapse, 0.5 = somewhat organized, somewhat polarized, or 1.0 = fully formed, concave, and polarized synapse; data is presented with standard error of 100 individual events (**Panel C**). Data indicated both an increased quantity and increased quality of immune synapses occurring between the allogenic T-cells and the CLL cells after treatment with epigenetic modifiers imparting some functional significance to the observed expression differences.

15B). To quantify these data we manually counted and scored 100 synaptic events for each experiment (Figure 15C) according to the methodology developed by Ramsay et. al. and demonstrated in Figure 16(Ramsay, Johnson et al. 2008). Data showed a significant shift towards increasingly numerous and robust interaction zones after treatment, with

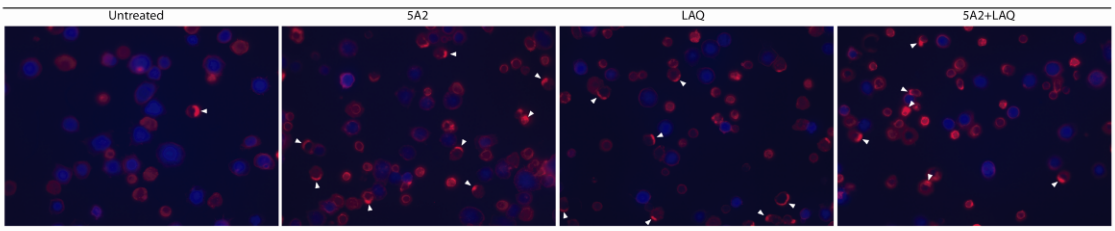

Figure 16: Microscopic identification and of immune synapse between CLL cell lines and allogenic T cells. Purified healthy donor CD8 T-cells were allowed to conjugate with sAg pulsed CLL cell lines which were treated with 1uM 5A2, 25nM LAQ, both or neither drug (blue stained) and were stained for actin polymerization (red) using phalloidin rhodamine. White arrows indicate actin polarized T cells. Scoring of synapse events involves counting only T cells which directly interact with adjacent blue-stained APCs and have not been sheared from their interacting partner by the process of plating. Interactions are scored with either a "0" indicating little to no actin polymerization, "0.5" indicating slight polarization, and"1.0" indicating strong polarization. Interactions are counted until 100 events are recorded.

optimal actin polarization observed with combination 5A2+LAQ treatment.

To further confirm our microscopy data, we conducted flow cytometric sAg-induced conjugation assays between healthy allogenic CD8 or CD4 T-cells and our CLL cell lines. As depicted in an example flow plot (Figure 17A) the percentage of TCR specific conjugated cells was determined by subtracting the conjugates forming in an unstimulated sample from those forming in the sAg stimulated sample. Data obtained in this manner indicated that robust TCR mediated conjugation occurred with greater frequency in both CD8 and CD4 conjugates after treatment with 5A2 or LAQ. In all cases maximal conjugation was observable after combined therapy (Figure 17B).

Epigenetic alteration can restore T-cell signaling capacity and lytic granule mobilization in the context of CLL.

To this point our data has shown that epigenetic modifiers such as 5A2 and LAQ could enhance CLL cell – T-cell SMAC formation. However, we also sought to demonstrate that intracellular signaling, a key component of a functional APC – T-cell interaction, was improved. It has been well demonstrated that mitochondrial localization at the synaptic interface is directly associated with CRAC mediated calcium entry, an essential downstream signal required for T-cell activation(Oakes 2007; Quintana, Schwindling et al. 2007). To determine if mitochondria were localizing along the SMAC complex in our experiments we performed immunofluorescent confocal microscopy of CLL cell – allogenic T-cell conjugates after treatment with our epigenetic modifiers (Figure 18A and 18B). Our observations led us to conclude that mitochondrial localization was deficient in untreated CLL cell conjugates, but was qualitatively restored by treatment with 5A2 plus LAQ in both CD4 and CD8 T-cell conjugations.

Primed CD8 T-cells utilize TCR mediated signaling to mobilize and eventually release lytic granules containing perforin, granzyme-B, and interferon gamma. By immunofluorescent staining of perforin we found that appropriate SMAC F-actin polarization, mitochondrial localization, and lytic granule mobilization was improved in the CD8 T-cell conjugates after CLL cells had been treated with both epigenetic modifiers (Figure 18A). These data serve to confirm our prior results and indicate a definitive signaling enhancement.

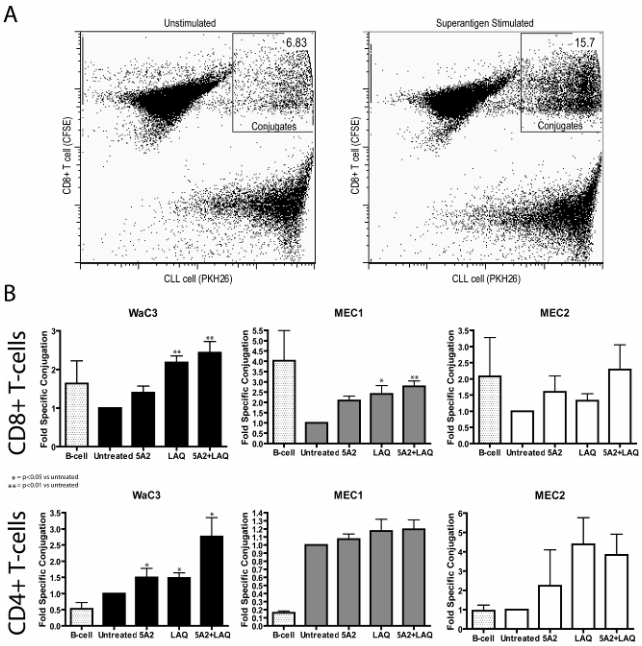

Figure 17: Epigenetic modification of CLL cells improves TCR-induced conjugation with both CD4 and CD8 T-cells. FACS based conjugation assays were conducted between healthy allogenic T-cells and sAg stimulated CLL cell lines (or healthy allogenic B-cells) after treatment with 5A2, LAQ, both or neither. TCR-specific conjugation was ascertained by subtracting the sAg induced conjugation level from the basal allogenic conjugation level (example depicted in **Panel A**) data was then normalized to the untreated condition. Treatment revealed reproducible and significant increases in TCR-specific conjugation levels between CLL cells after treatment with both inhibitors (**Panel B**). These data supplement prior analyses and confirm the amplification of TCR-specific synapses between CLL cells and both CD4 and CD8 T-cells.

Epigenetically altered CLL APC signaling results in T-cells with Th1 polarization, increased proliferative capacity, and lytic activity.

The functional significance of an improved CLL APC is, in part, determined by the polarization of the responding T-cells. To characterize this polarization we sorted conjugates formed between CD4+ T-cells and CLL cells and restimulated them with PHA following a 48 hour rest period. The cytokine profile was then measured using cytokine bead array. Our data suggest that, the balance between Th1 and Th2 signaling ([Th1]:[Th2]) was improved (increasingly Th1) after treatment with both inhibitors

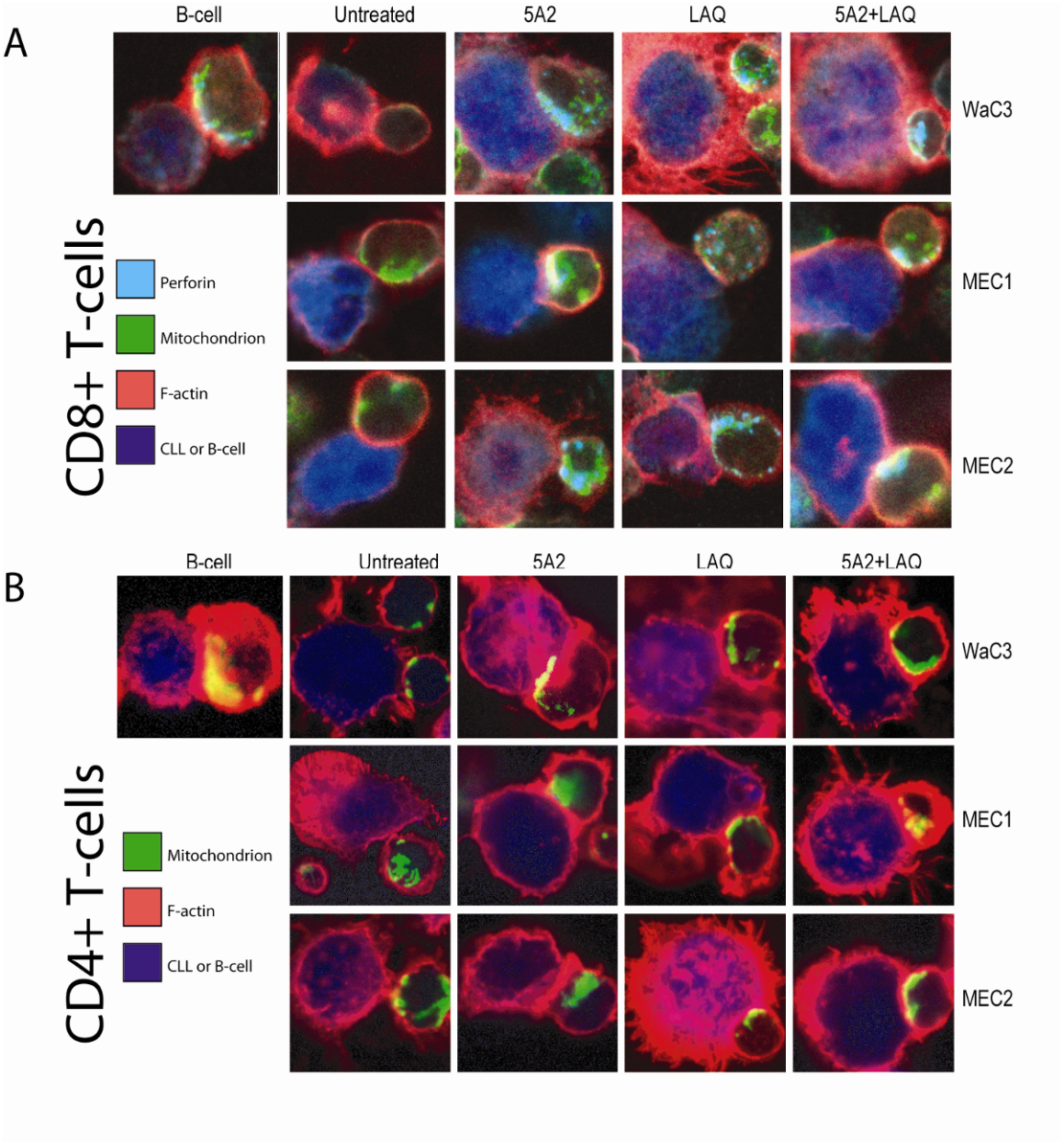

Figure 18: Epigenetic modifiers improve the functionality of the immune-synapse between T-cells and CLL cells as indicated by recruitment of mitochondrion and perforin to the immune synapse. Purified CD8 T-cells (Panel A) or CD4 T-cells (Panel B) were allowed to conjugate for 30 minutes with sAg stimulated CLL cell lines which were treated with 1uM 5A2, 25nM LAQ, both or neither drug (or allogenic B-cells) (blue stained) and were stained for F-actin polymerization (red) using phalloidin rhodamine, mitochondrion (green), and perforin (cyan) (CD8 only). Representative confocal images are presented of T-cell/APC interactions for each of the three CLL cell lines under each of the treatment conditions. Data indicated increased functionality of immune synapses occurring between the allogenic T-cells and the CLL cell lines after treatment with epigenetic modifiers.

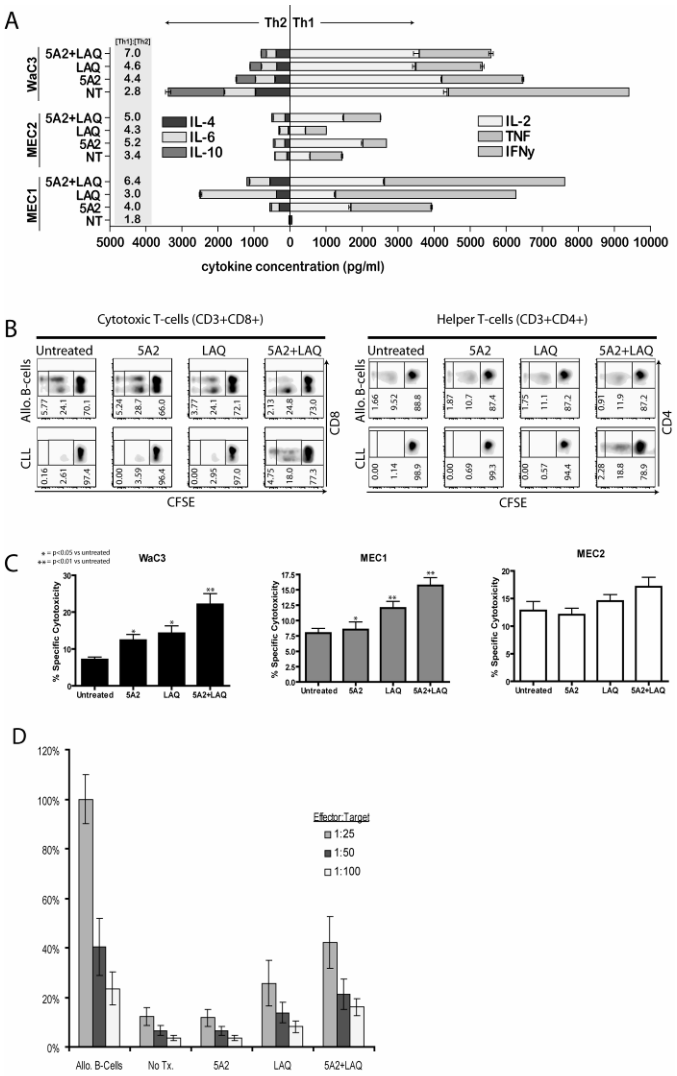

Figure 19: CLL cells treated with 5A2 and LAQ recover the functional capacity to induce Th1 cytokine responses, proliferation, and TCR-specific cytotoxicity in responder lymphocytes. Cytokine analysis of sorted 5A2 or LAQ treated CLL cell – T-cell conjugates indicated polarization towards a Th1 type cytokine profile as depicted by the [Th1]:[Th2] ratio and the relative change to IL-10, IL-6, and IL-4 concentration versus IL-2, TNF, and IFNγ concentrations (**Panel A**). In a MLR lymphocytes of both the CD4 and CD8 lineage were induced to proliferate only after CLL target cells had been treated with 5A2 and LAQ, while healthy allogenic B-cells were capable of inducing proliferation regardless of treatment (**Panel B**). Additionally, we found increased TCR-specific cytotoxicity by FACS based analysis of T-cell/dead-CLL conjugates after treating the CLL cells with epigenetic modifiers and stimulating with sAg (Effector:Target = 10:1), as control non-sAg based conjugation between T-cells and dead-CLL cells was subtracted from the percentage displayed (**Panel C**). . Cytotoxicity assays using untreated, 1μM 5A2 treated, 25nM LAQ treated, or 5A2+LAQ treated primary CLL cells with healthy allogenic CD8 T-cells show higher cytolytic function, approaching that of a healthy allogenic reaction (**Panel D**).

(Figure 19A). For the MEC1 cell line, treatment completely restored cytokine signaling, while for the WaC3 cell line treatment constricted Th2 signaling predominantly decreasing IL-10, IL-4, IL-6 and IFNγ. These data serve to highlight clinically observed differences in cytokine signaling amongst CLL patients as well as a potential methodology to restore therapeutic T-cell polarization.

Proliferative self-renewal of antigen specific T-cells is a requirement in the context of sustaining any potential anti-tumor effect. To determine the effects of 5A2 and LAQ have on the proliferation of T-cells stimulated with drug-treated MEC1 CLL cells we CFSE stained healthy donor allogenic CD8 and CD4 T-cells and subjected them to mixed lymphocyte reactions. As shown in Figure 19B a normal allogenic response between healthy B-lymphocytes and healthy allogenic T-lymphocytes (both CD8 and CD4) induces approximately 30% proliferation. On the other hand, only CLL cells which had been treated with a combination of 5A2 and LAQ were capable of stimulating appreciable proliferation (22.7% for CD8 and 21.1% for CD4). This proliferation data served as evidence that the combined 5A2+LAQ treatment could potentiate significant functional improvements when compared to either drug alone.

As a final indicator of proper APC function of CLL cells we wanted to examine the TCR mediated lytic ability of CD8 cytotoxic T-cells. To investigate this we utilized sAg mediated killing and analyzed the results by flow cytometry, quantifying the percentage of T-cells specifically conjugating with dead CLL cells in a sAg specific (or TCR specific) manner(Vitale, Zamai et al. 1991; Morgan, Labno et al. 2001). Results from this analysis showed improved lytic function of allogenic CD8 T-cells after being stimulated with 5A2+LAQ treated CLL cells (Figure 19C). Significance ($P<0.01$) was

achieved in the WaC3 and MEC1 cell lines, correlating with and corroborating prior evidence of APC functionality.

5-aza-2'-deoxycytidine and LAQ824 enhance allogenic CD8 cytotoxicity against early stage primary CLL cells.

It is well established that CLL cells are deficient in their ability to stimulate T-cell responses, even in allogenic MLR (Figure 19B). It has also been established that the level of dysfunction is directly correlated to increased RAI staging(Ramsay, Johnson et al. 2008). To ensure that our in-vitro results hold true in primary CLL, we conducted cytotoxicity assays using allogenic CD8 T-cells and 5A2 or LAQ treated primary CLL cells. We found that when primary purified CLL cells were treated with 5A2 and LAQ, there was a significant increase in allogenic cytotoxic potential above that seen in either drug treatment alone (Figure 19D). These results indicate that combined 5A2+LAQ therapy may increase the APC potential of primary CLL.

Discussion

Our studies unveil a previously unknown effect of 5A2 and LAQ on the immunobiology of B-CLL. As anticipated, our results show a therapeutic improvement in antigenic protein expression, costimulatory potential, cytokine signaling, SMAC synapse formation, and T-cell stimulation. These changes reinforce the three signals of APC function: antigenic peptide (signal 1), costimulation (signal 2), and cytokine stimulation (signal 3), resulting in functional changes which may benefit current immunotherapeutic approaches for CLL.

One caveat to our studies is that the majority of mechanistic experimentation has been carried out in CLL cell lines. In our experience, the treatment of primary CLL cells

in-vitro with proliferation-dependent epigenetic modifiers such as 5A2 can be quite tedious, requiring the addition of external cytokines, growth factors, and feeder cells, making primary cells inappropriate for large scale reproducible molecular assays. To avoid such pitfalls, our approach focused on validating the CLL cell lines as appropriate epigenetic surrogates of primary CLL cells and utilizing them for the short term in-vitro treatments necessary to foster an enhanced understanding of the mechanisms behind CLL cell epigenetic dysfunction. Subsequently we confirm increased immunogenicity and epigenetic upregulation of potential target antigens using primary CLL cells to ensure the accuracy of our final conclusions. In addition to our independent validation, other investigators have found these cell lines ideal for studying CLL epigenetics(Plass, Byrd et al. 2007; Chen, Raval et al. 2009).

We postulate that the B-CLL cells are capable of directly presenting cancer antigens to the immune system, however it is likely that cross-presentation of CLL antigens via dendritic cells (DCs) is also occurring. In our experience, treatment of DCs with epigenetic modifiers consistently inhibited the production of IL-10. Such an effect was accompanied by an increased expression of co-stimulatory molecules and enhanced production of pro-inflammatory mediators. In addition, HDACi-treated APCs were capable of effectively priming naïve antigen-specific CD4 T-cells and restoring the responsiveness of anergic CD4 T-cells isolated from tumor bearing mice (Wang et. al. unpublished observations). These studies led us to unveil a novel role for HDAC11, in particular, as a transcriptional repressor of IL-10 in murine and human APCs, providing one potential molecular mechanism which we plan to specifically examine in the setting of CLL(Villagra, Cheng et al. 2009).

Our studies did not directly focus on the direct cytotoxic effect of HDAC and DNMT inhibitors; instead we focused our efforts on the epigenetic changes which result in improved immunological function. In preliminary in-vitro testing we established a working concentration of 1μM for 5A2 and 25nM for LAQ which demonstrated negligible direct cytotoxicity over the 72 hour treatment, as measured by Annexin V and propidium iodide staining, yet yielded sustained epigenetic effect, as measured by CTA upregulation or histone acetylation. Although we cannot rule out the possibility that autophagy may occur(Fink and Cookson 2005), we have demonstrated quantifiable epigenetic changes which could conceivably be independently responsible for the demonstrated functional modifications. In addition, although our studies do not directly focus on treating T-cell and instead focused on the polarization of T-cells by treated CLL APCs , preliminary data from our laboratory suggest that these drugs do not negatively affect the proliferation or IFNγ production capacity of primary CD4 and CD8 T-cells . Future ongoing studies in our laboratory will focus on the direct effects of epigenetic modifiers on the T-cells themselves (Dubovsky et. al. unpublished observations).

It has been suggested that the survival of CLL hinges upon the balance between receiving lymphocyte activation, survival, and proliferation signals while maintaining immunosupression of the remaining healthy lymphocyte compartments(Tangye and Raison 1997; Rossmann, Lewin et al. 2002). This model implies that a given CLL clone may preserve this balance in a different manner explaining the heterogeneity we see in the disease. Our results elucidate some of these subtle differences and provide a potential mechanism for tilting the balance towards immune stimulation. This balance is exemplified in our costimulatory phenotyping experiments (Figure 14A) which

demonstrated that slight to moderate improvements in the surface expression of costimulatory and MHC molecules can facilitate the changes seen in the SMAC complex. Our studies confirm the current understanding that slight alterations to APC molecular machinery enable CLL cells to facilitate their own survival while circumventing or diverting T-cell stimulation. It is also possible that the epigenetic modifiers alter functional affinity and avidity of certain costimulatory molecules.

Our studies focused on drug treatment and its effects on B-CLL immunology. However, in the clinical setting it is unlikely that these inhibitors will be so exclusive in their actions. In prior studies we have demonstrated that the epigenetic effects of 5A2 do preferentially act on malignant cells, potentially due to deregulation of the chromatin packaging machinery such as DNA methyltransferase I (DNMT1) or brother of the regulator of imprinted sites (BORIS)(Klenova, Morse et al. 2002; Loukinov, Pugacheva et al. 2002; Vatolin, Abdullaev et al. 2005; Dubovsky and McNeel 2007). Our previous efforts also indicate a potential for drug treatment to elicit long term molecular changes specifically in cancer cells, allowing for temporal separation of drug treatment and effector reaction. Additional evidence comes in the form of studies conducted by Guo et. al. demonstrating elimination of lung metastases by CTA specific cytolytic T-lymphocytes in a murine mammary carcinoma model after treatment with 5A2(Guo, Hong et al. 2006).

Altogether, our results suggest that epigenetic modifiers which release chromatin constriction via DNA demethylation and histone acetylation may effectively restore the functional immunogenicity of chronic lymphocytic leukemia by inducing the expression of novel and highly antigenic tumor targets, increasing costimulatory potential, repairing

defective SMAC formation, and rectifying cytokine stimulation. These effects could aid in the generation of more robust TCR mediated cytolytic responses in primary CLL.

Materials and Methods

Subject Populations

Sera and peripheral blood mononuclear cells (PBMCs) were obtained from patients with CLL. All subjects gave written institutional review board (IRB)-approved informed consent for their blood products to be used for research. Blood was collected at the H. Lee Moffitt Cancer Center (Tampa, FL). PBMCs were stored in 1ml aliquots at -140°C and sera were stored in aliquots at -80°C until used.

Cell Culture and Drug Treatments

Unless otherwise stated cells were cultured in-vitro at 37°C and 5%CO2 using RPMI1640 medium supplemented with 10% fetal calf serum and antibiotics. The MEC1, MEC2, and WaC3 cell lines were kindly provided by Dr. John Byrd at Ohio State University and were previously characterized in the following references(Wendel-Hansen, Sallstrom et al. 1994; Stacchini, Aragno et al. 1999). Drug treatments were carried out on the CLL cells (separate from T-cells) for 72hr in complete medium using either 1uM 5-aza-2'-deoxycytidine (5A2), 25nM LAQ824 (LAQ), 1uM5A2 + 25nM LAQ, or neither. At the conclusion of treatment cells were washed twice using pre-warmed serum-free RPMI1640 and subjected to the various assays. For treatments involving primary CLL cells, cultures of purified B-CLL cells (>95% purity) were maintained in 6 well plates atop irradiated (30Gy) monolayers of CD40L expressing murine fibroblasts (a kind gift from Dr. John Gordon at the University of Birmingham) in

the presence of 500U/ml recombinant human IL-4 (Research Diagnostic Inc., Concord, MA)

Reverse Transcriptase-PCR (RT-PCR)

Total RNA was prepared from pelleted cells (RNeasy mini columns and RNAse free DNAse, Qiagen, Valencia, CA) or was commercially obtained (BioChain, Hayward, CA). RT-PCR reactions were conducted using the Qiagen one-step RT-PCR kit (Qiagen) with transcript-specific primers and total RNA from CLL cell lines as templates. RT-PCR amplification reactions were resolved on 2% agarose gels and the size of the amplified transcript confirmed by comparison with a standard DNA ladder (GelPilot 1Kb Plus Ladder, Qiagen). Heatmap depictions were created using Image Quant 5.1 software (Molecular Dynamics) and the Heatmap Builder tool kindly provided by the Quertermous lab at Stanford.

Cell Conjugation Assays

Healthy or malignant B-cells were stained with CellTracker Blue (PKH26 for flow cytometric analysis) following the manufacturer's instructions and pulsed with or without 2ug/ml of a cocktail of staphylococcal superantigens (SEA and SEB; Toxin Technologies; Sarasota, FL) for 30min at 37°C. B-cells were then centrifuged (200g for 5min) with 5 times the number of T cells stained with mitotracker deep-red (Invitrogen, Carlsbad, CA) (or CFSE for flow cytometric analysis) and were incubated at 37°C for 10min unless otherwise stated, then plated onto poly-L-lysine slides and fixed using 3.7% formaldehyde in PBS for 15min. Additional antibody staining was conducted using 1:50 anti-perforin (Pierce, Rockford, IL) followed by 1:125 anti-mouse FITC (Sigma). Microscopic acquisition and analysis of immune synapses was conducted using a Leica

upright fluorescent confocal microscope with the associated software according to the methods developed by Ramsay et. al.(Ramsay, Johnson et al. 2008). Controls which lacked sufficient conjugation to acquire microscopic images (controls without SEA/SEB, without APCs, or without T-cells) were investigated and documented in low-resolution wide-field images showing no significant conjugation, unless otherwise stated. All microscopy experiments were repeated three independent times unless otherwise stated.

Flow Cytometry Immunophenotyping

Flow cytometric analysis of cultured cells was performed using fluorochrome-labeled monoclonal antibodies (mAbs; anti-HLA-A,B,C, -HLA-DR, -CD40, -CD80, -CD86, -CD19, and –CD20, Becton Dickinson, San Jose, CA and eBiosciences, San Diego, CA) and the viability dye 4',6-diamidino-2-phenylindole (DAPI, Sigma). For CFDA-SE (CFSE) staining cells were resuspended in 0.5μM CFSE (Invitrogen) in RPMI medium for 15 minutes at 37°C followed by a wash and additional 30 minute incubation in serum supplemented medium prior to resuspension in culture medium. For conjugation based T-cell phenotype experiments cells were allowed to conjugate according to the methods described in cell conjugation assays and conjugated cells were stained for anti-CD20 and anti-CD4. Conjugation events were sorted using a FACS Aria cell sorter and separate populations were assayed for cytokine production using CBA array. Cytokine bead array (CBA) (Becton Dickinson) was conducted according to the manufacturers published protocol using cellular supernatant from three replicate experiments. PKH26 staining was carried out and data was acquired on an LSRII cytometer (Beckman Coulter), and analyzed with FlowJo software (Tree Star, Ashland, OR). For analysis

Mixed Lymphocyte Proliferation

CLL cells or healthy B-cells were incubated in serum supplemented RPMI1640 (Invitrogen) with allogenic, ficoll density gradient separated, PBMCs from a healthy donor which had been stained 30 minutes prior with 0.5μM CFSE at a 1:10 effector:target (E:T) ratio. Cultures were maintained at 37°C for 60 hours and were subsequently subjected to flow cytometric analysis. Viability dye was used to gate out dead cells, CD4 and CD8 cells were stained using spectrally separate fluorophores and were individually investigated for CFSE dilution resulting from proliferation.

FACS Based Cytotoxicity Assay

Healthy allogenic T-cells were stained with CFSE as previously described. CLL cells were pulsed with SEA and SEB superantigens at 2ug/ml for 2 hours and were centrifugally collected and incubated with T-cells at a 1:10 E:T ratio for 45 minutes at 37°C in serum-free RPMI1640. Cells were then carefully chilled to 4°C, centrifuged, and resuspended in FACS buffer containing the viability dye propidium iodide. FACS analysis was immediately conducted and the percentage of T-cells conjugated with dead cells was tracked and compared to a T-cell only control. Cytotoxicity analyses were conducted in triplicate and experiments were repeated at least twice.

Lactate Dehydrogenase (LDH) Cytotoxicity Assay

Allogenic CD8 T-cells (or B-cells) were separated from healthy donor ficoll density purified PBMCs using magnetic negative selection on an MACS LS magnetic column (Miltenyi Biotec, Auburn, CA) using the manufacturer specified protocol. CD8 T-cells and purified primary CLL B-cells (>95%) were washed twice with 37°C RPMI1640 without phenol red/1%FCS. Cells were then incubated together at 1:100,

1:50, and 1:25 E:T ratios in RPMI1640 without phenol red/1%FCS in 96 well round bottom plates for 4 hours at 37°C/5%CO2. Cytotoxicity was measured using the cytotox96 non-radioactive LDH assay kit according to the manufacturers published protocol (Promega, Madison, WI). Control wells corresponding to minimum cytotoxicity (targets alone), maximum cytotoxicity (Triton X100 lysed targets), and culture medium alone were used to calculate experimental sample percent lysis.

Statistical Analysis

Unless otherwise stated, all continuous data conducted in replicates were analyzed using a two tailed Students T test. P values are displayed on the graph to indicate the level of significance. Error bars presented on graphical representations of data indicate standard deviation unless otherwise stated.

Chromatin Immunoprecipitation (ChIP)

After 72 hours treatment with 5-aza, LAQ, 5'-aza + LAQ or DMSO control (no treatment) cells were crosslinked with 1% formaldehyde and incubate with rotation at room temperature. After 10 minutes, reactions were stopped by the addition of glycine to a final concentration of 0.125M. Cells were washed twice with ice cold PBS and resuspended in ice cold TX-100/NP40 buffer (10mM Tris pH 8.1, 10mM EDTA, 0.5M EGTA, 0.25% TX-100, 0.5% NP40, 1mM PMSF, 0.5x Protease inhibitors) at a density of $4x10^6$ cells/ml. Cells were re-suspended in 10ml ice cold Salt-wash buffer (10mM Tris pH 8.1, 1mM EDTA, 0.5M EGTA, 200mM NaCl,1mM PMSF, 0.5x Protease inhibitors) and incubated for 10 minutes at 4 ^oC. Cells were lysed by adding sonication buffer (10mM Tris pH8.1, 1mM EDTA, 0.5M EGTA, 1% SDS, 1mM PMSF, 1x Protease inhibitors) at a cell density of $1x10^6$ cells/30µl. Lysate was sonicated using a water bath

sonicator (Diagenode). Chromatin immunoprecipitation was carried out for 16 hours at 4 ^{o}C using 4.5x10^{6} cells and 5µg of specific antibody (anti acetyl histone-H4)(Upstate) or 5 µg of rabbit IgG (Upstate). Immunoprecipitated chromatin was captured by incubation with pre-blocked protein A/G beads (Santa Cruz) for 4hrs at 4^{o}C then washed sequentially with low salt wash (20mM Tris pH8.1, 2mM EDTA, 150mM NaCl, 0.1% SDS, 1% tritonX 100), high salt wash (20mM Tris pH8.1, 2mM EDTA, 500mM NaCl, 0.1% SDS, 1% tritonX 100) and LiCl wash (10mM Tris pH8.1, 250 mM LiCl, 1% NP-40, 1% sodium deoxycholic acid, 1mM EDTA). DNA was eluted with elution buffer (10mM Tris pH8, 1% SDS, 1mM EDTA) and crosslinks were reversed by incubating with 312 mM NaCl at 65^{o}C for 4 hours. The immunoprecipitated DNA was treated with RNase (Ambion) for 30 minutes at 37^{o}C and proteinase K (Roche) for 1 hour at 45^{o}C. The DNA was purified with Qiagen PCR spin columns per manufacturer's instructions. Purified DNA was analyzed by quantitative PCR using primers to the CNS+18 region of the human IFNγ locus (FWD 5'-GACTGGGTGAGGGAGATTG-3', REV 5'-GGGAGTGACAGGTAGGGAGA-3') using an annealing temperature of 55°C. Data was analyzed by calculating enrichment for acetylation versus IgG using percent input of each sample.

Chapter Four:

Molecular, epigenetic, and phenotypic repolarization of T lymphocytes from chronic lymphocytic leukemia patients using 5-aza-2'-deoxycytidine.

Abstract

T cell immune dysfunction has an important role in the profound immunosupression that characterizes chronic lymphocytic leukemia (CLL). Improper polarization of T cells has been proposed as one of the mechanism involved. Mounting data implicates chromatin regulation, namely promoter methylation, in the plasticity of naïve human T cells. Recent in-vitro evidence indicates that this plasticity may be phenotypically altered by using methylation inhibitors which are approved for clinical use in certain types of cancer. These results beg the question: can the ineffective polarization of T lymphocytes in the context of CLL be effectively modulated using methylation inhibitors in a sustainable therapeutic fashion? To answer this question our laboratory has studied the effects of 5-aza-2'-deoxycytidine (5A2) in helper and cytotoxic T lymphocytes from healthy donors and CLL patients in well characterized molecular and epigenetic signaling pathways involved in effective polarization. Moreover, we sought to investigate the consequences of methylation inhibitor treatment on lymphocyte survival, activation intensity, and naïve cell polarization. Our data indicates that 5A2 treatment can repolarize Th2 cells to effectively secrete interferon gamma, signal via T-bet, and achieve demethylation of critical Th1 specific promoters. Moreover, we demonstrate that 5A2 can force Th1 polarization of naïve T cells despite a strong IL-4 stimuli and a lack of IL-12.

In conclusion our data seeks to define a modality in which improper or ineffective T cell polarization can be altered by 5AZA and could be incorporated in future therapeutic interventions.

Results

T lymphocytes display phenotypic Th1 repolarization after treatment with 5A2

To understand the activity of DNA methyltransferase inhibitors (DNMTi) on T helper cell polarization we examined the effects of a single dose of 5A2 (0.3μM, 1μM, 3μM, 10μM, and 30μM) on fresh CD4 T lymphocytes obtained from healthy blood donors. Intracellular staining revealed an increase in IFNγ (Th1 cytokine) positive cells, ranging from 10-13% at baseline to 39% in the 30μM dosage (Figure 20A). To identify any potential detriment to CD8 T lymphocyte responses we examined IFNγ production in CD8 T cells from healthy donors and observed a similar increase which directly correlated with drug treatment (Figure 21).

To more specifically address alterations to Th2 cells we conducted phosflow analysis of pSTAT6 levels in CD4 T cells from healthy donors. Since pSTAT6 levels in Th2 cells quickly rise following activation via CD3ζ and CD28 we conducted a timecourse analysis spanning 15 to 75 minutes post activation(Chapoval, Dasgupta et al. 2010). Interestingly, pSTAT6 was found to decrease with drug treatment indicating that Th2 signaling patterns were abrogated by the DNMTi (Figure 20B).

To better understand the effects of 5A2 on Th1 and Th2 cell populations in-vitro polarized T cells were subjected to 5A2 treatment and the secreted levels of IFNγ were measured by CBA (Figure 23A and Figure 22). The results confirmed that IFNγ

production in Th2 cells increased in a dose dependent manner with 5A2. It was also noted that Th1 cells produced more IFNγ, however this was less pronounced.

In addition we tested the resulting polarity of 5A2 treated naïve cells after stimulation under strong Th1 or Th2 polarizing conditions using intracellular staining of

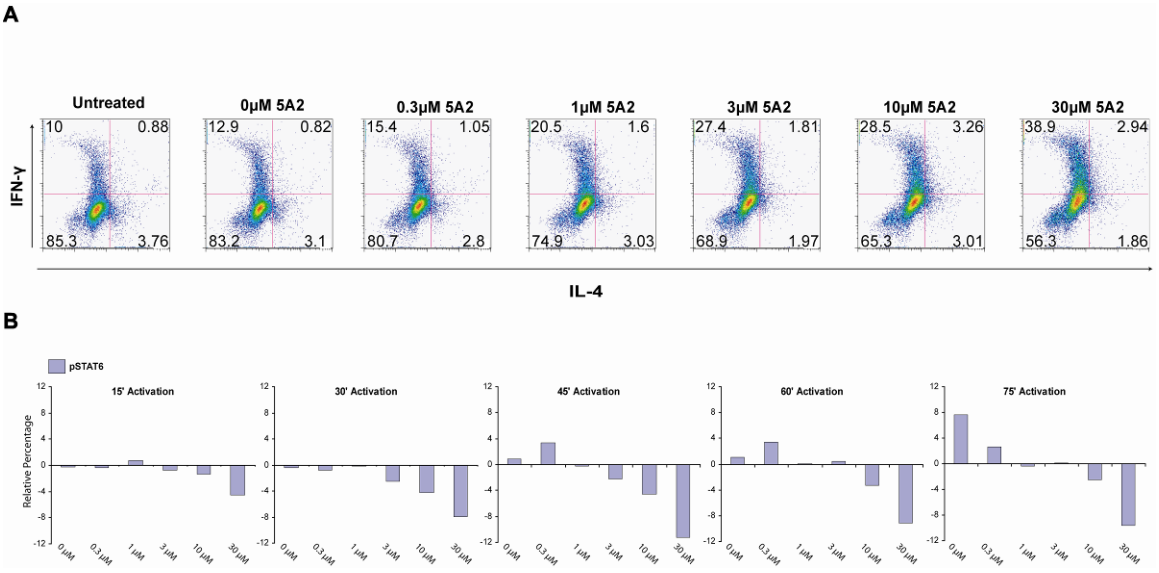

Figure 20: Intracellular staining reveals 5A2 induced IFNγ and reduced pSTAT6. Magnetically isolated CD4+ T cells from healthy donors were treated with 5A2 (30, 10, 3, 1, 0.3, and 0μM) or left untreated and stimulated in-vitro prior to FACS analysis. **A)** Intracellular levels of IFNγ and IL-4 after 12 hours of PMA/ionomycin stimulation. **B)** T cells were assayed for intracellular pSTAT6 at various timepoints after stimulation with αCD3 and αCD28 (15, 30, 45, 60, and 75 minutes) all percentage data were normalized to an untreated and unstimulated sample. All experiments were repeated at least three times with various healthy

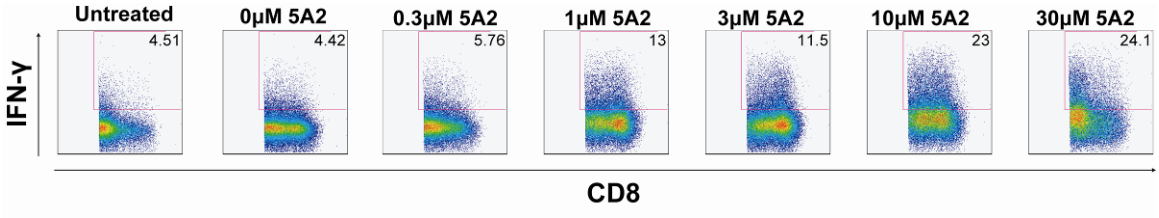

Figure 21: 5A2 increases IFNγ response in CD8 T cells from a healthy donor. Purified and PMA/ionomycin stimulated CD3+ T cells were treated with the indicated concentration of 5A2 and assayed via flow cytometry for CD8α and intracellular IFNγ. Cells were gated on CD8+ and the gated population represents IFNγ positive.

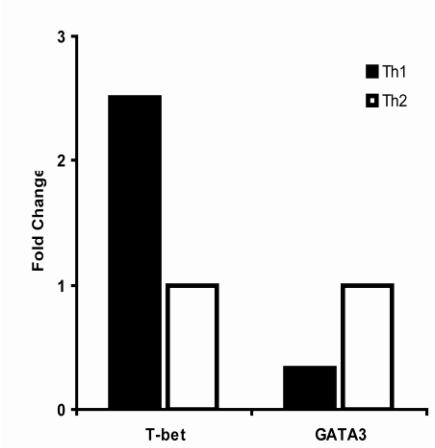

Figure 22: In-vitro polarized Th1 and Th2 T cells have divergent expression of T-bet and GATA3 by qRT-PCR. mRNA analysis of 5 week in-vitro polarized Th1 and Th2 cultures reveals that Th1 cells express relatively high levels of T-bet meanwhile Th2 cultures express relatively high levels of GATA3 indicating polarity.

IFNγ and IL4. As expected, in the control sample Th2 polarizing conditions resulted in exclusively IL4 secreting cells, however 5A2 was capable of eliminating the IL4 response and inducing the secretion of IFNγ in a dose dependent manner (Figure 23B). Our data also confirmed that Th1 polarizing conditions resulted in exclusive IFNγ production which was further exacerbated by 5A2 treatment.

T cells from CLL patients alter polarization towards Th1 in response to 5A2

It has been previously demonstrated that T cells from CLL patients generally proliferate poorly after in-vitro stimulation with low IL2 (20U/ml) and secrete high levels of IL4, and relatively low levels of IFNγ(Scrivener, Kaminski et al. 2001; Frydecka, Kosmaczewska et al. 2004). Given our previous data we wanted to test the repolarizing effects of 5A2 on these cells. We started by examining the IFNγ secreted by T cells isolated from four CLL patients using CBA. Our results matched the characteristic dose-dependent increase in IFNγ revealed earlier in healthy donor T cells (Figure 24A). These results were further confirmed using intraceullular staining to examine the levels of IFNγ

produced in response to stimulation (Figure 24B). In addition, we found that the percentage of IL4 positive T cells decreased under 5A2 treatment, indicating that a high percentage of Th2 polarized cells were no longer responding to in-vitro stimulation via IL4.

As mentioned earlier, the proliferation of T cells from CLL patients after in-vitro stimulation is characteristically poor, thus we were curious if 5A2 treatment could alleviate this anergic condition. Using MTX proliferation assays we found that CD4 T cell proliferation directly correlated with 5A2 dose resulting in a 500% increase in proliferation compared to untreated samples (Figure 24C).

Th2 cells treated with 5A2 induce constitutive pSTAT1 signaling and express T-bet

The phenotypic alterations we have identified thus far must derive from a specific set of molecular signaling networks. T-bet is considered the master regulator of Th1 phenotype and it is controlled by activated STAT1 signaling. We decided to investigate the protein expression levels of T-bet and pSTAT1 in healthy donor and CLL CD4 T lymphocytes by western blot. As depicted in Figure 25A T-bet in healthy donor CD4 T cells is expressed at a basal level, however treatment with 5A2 increases this expression. This correlates with the increased phosphorylation of Tyr 701 on STAT1. Similar results were observed in T cells obtained from a CLL patient; however both T-bet and pSTAT1 were not identified at a basal level (Figure 25B).

Our prior experiments revealed phenotypic repolarization was restricted to the Th2 cells, thus we wanted to confirm that molecular alterations in purified populations of Th1 and Th2 were restricted to the Th2 population. Using western blot analysis on polarized T cells we identified an increase in pSTAT1 Tyr 701 in Th2 cells when treated

with 5A2. Notably, pSTAT1 levels were maintained at a higher basal level in Th1 cells

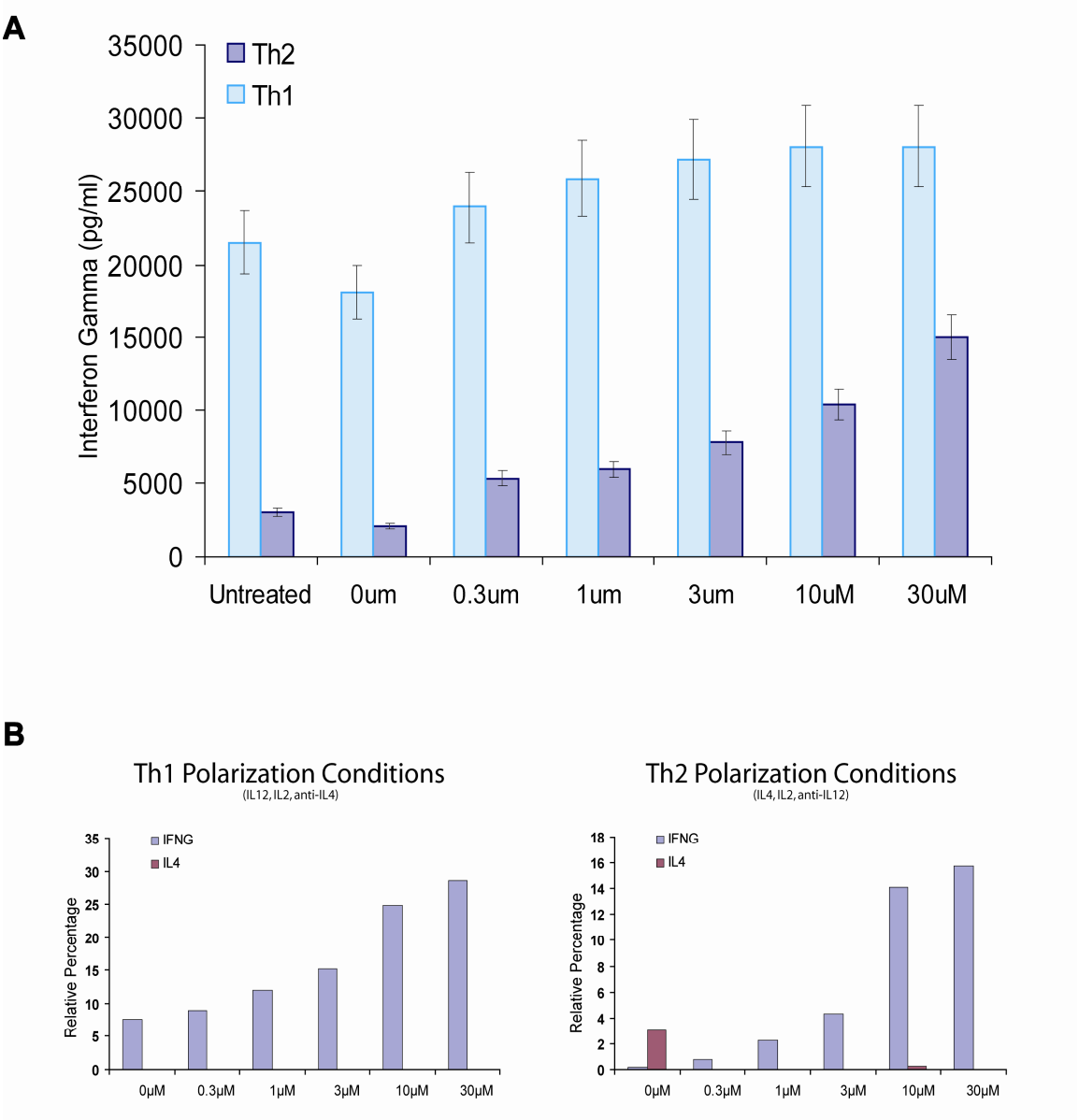

Figure 23: Th2 cytokine polarization is inhibited by 5A2 treatment. A) IFNγ released by In-vitro polarized and 5A2 treated Th1 and Th2 T cells subjected to 24 hours of stimulation with αCD3 and αCD28 was assayed via CBA. Error bars represent the standard deviation of four replicate samples. **B)** Intracellular staining of IL4 and IFNγ in magnetically isolated 5A2 treated naïve CD4 T cells from a healthy blood donor subjected to stimulation with αCD3 and αCD28 along with strong Th1 or Th2 stimulation (indicated under title).

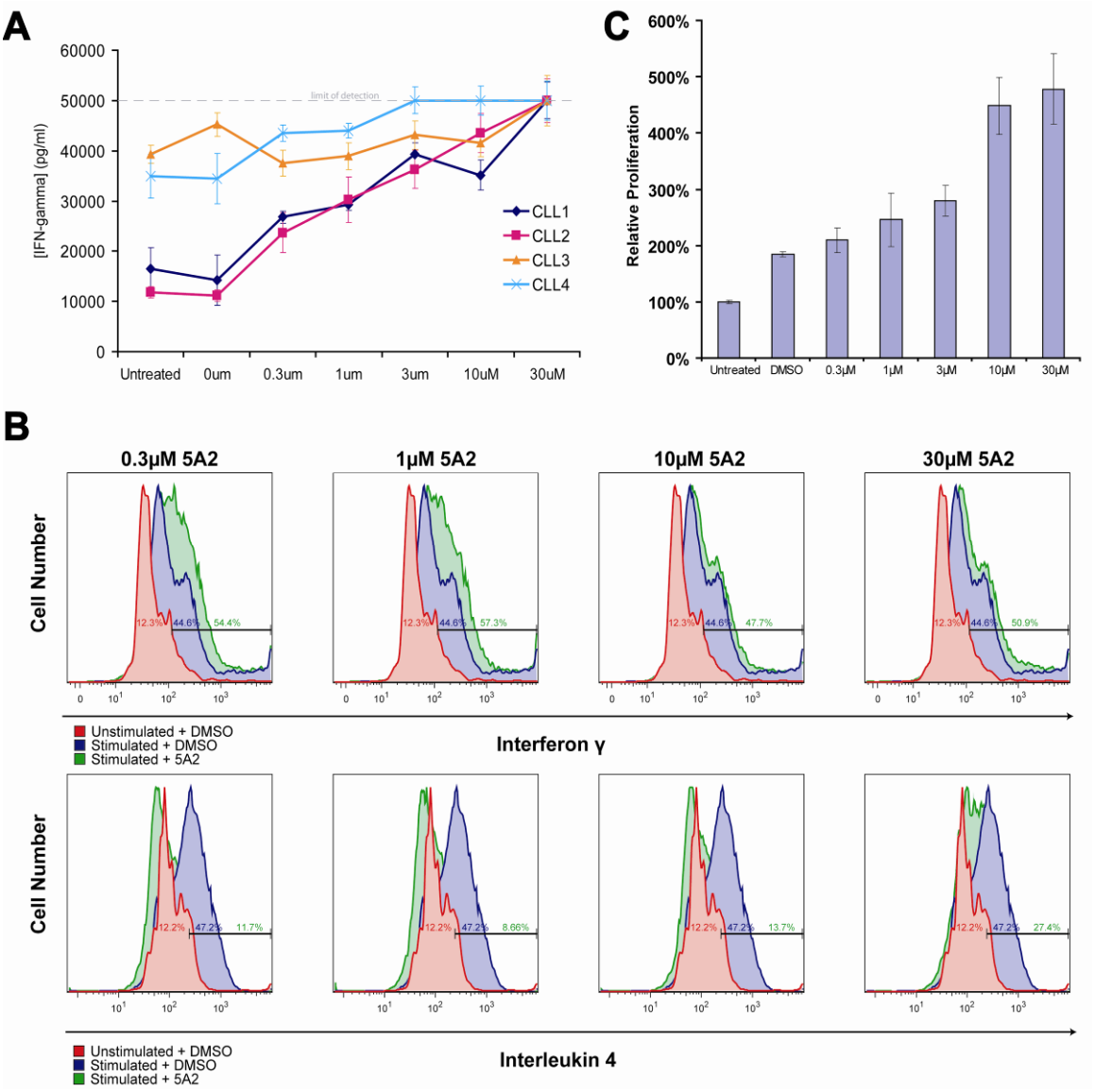

Figure 24: T cells from CLL patients are phenotypically repolarized by 5A2. CD4 T cells were magnetically isolated from CLL patients, immediately subjected to 5A2 treatments, and assayed for alteration to phenotype. **A)** T cells secretion of IFNγ was measured using CBA. Data represents the mean and standard deviation of triplicate samples of four independent CLL patients. **B)** T cells from a CLL patient were assayed via FACS analysis for intracellular levels of IFNγ (*top panel*) and IL4 (*bottom panel*) in response to 5A2 treatment and PMA/ionomycin stimulation. For comparison histograms representing an unstimulated (*red*) and stimulated yet untreated (*blue*) sample are provided along with the relative percentage of positive cells for each histogram. **C)** Proliferation of T cells from CLL patients in response to αCD28 and αCD3 along with 20U/ml IL2 was interrogated using an MTX assay. Data are normalized to the untreated sample and the graph represents triplicate well from four independent CLL patients with error bars representing standard deviation.

potentially rendering any alteration of pSTAT1 levels by 5A2 less evident (Figure 25C and D.

5A2 treatment of T cells stimulates a well characterized IFNγ autocrine loop

Our data identified a link between T-bet, pSTAT1, and 5A2 treatment of CLL and Th2 T cells. We wanted to know if the demethylating agent was eliciting the expression and secretion of IFNγ thus inducing a well defined STAT1α mediated autocrine feedback

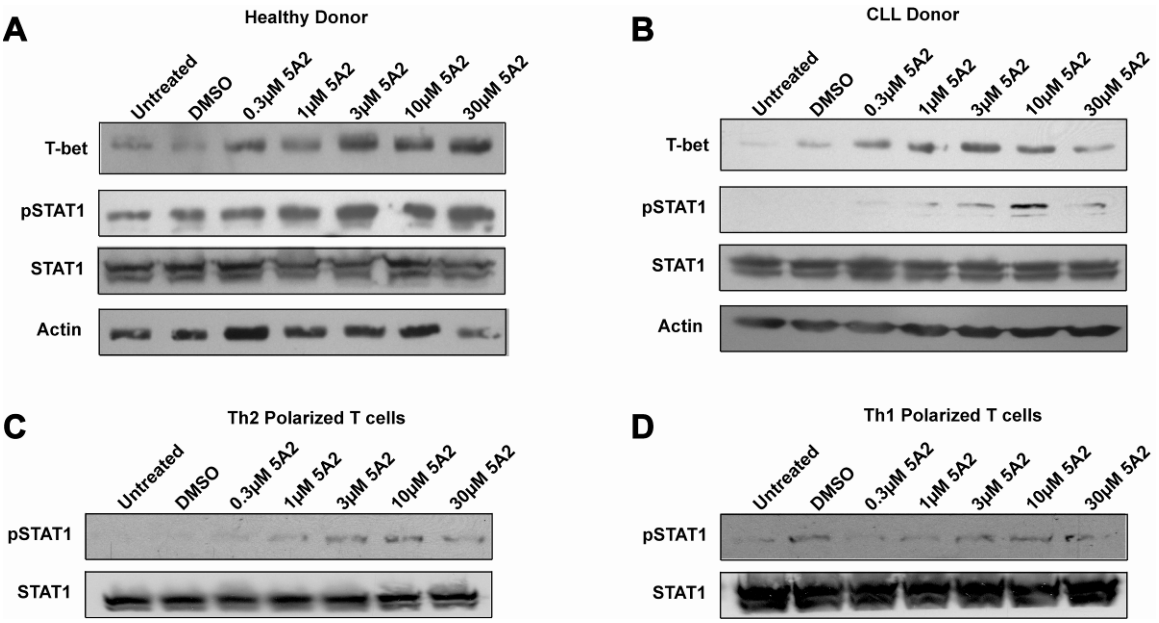

Figure 25: 5A2 induces pSTAT1 and T-bet signaling in T cells. Magnetically purified 5A2 treated CD4 T cells from a healthy blood donor **A)** or a CLL patient **B)** were assayed via western blot for total cellular levels of pSTAT1 ant T-bet protein. In-vitro polarized **C)** Th2 and **D)** Th 1 T cells were assayed via western blot for pSTAT1. For comparison β-Actin and total STAT1 levels were also included. Western blots are representative of three independent experiments.

loop. To explore this possibility we decided to abrogate this autocrine loop using anti-IFNγ. Our experiments revealed that soluble IFNγ was directly linked to the activation of STAT1α in response to 5A2, confirming our hypothesis (Figure 26A). To confirm that the IFNγ and T-bet were upregulated at the transcriptional level we conducted qRT-PCR.

Our examination of mRNA levels indicated that both T-bet and IFNγ mRNA were increased after 5A2 treatment (Figure 26B).

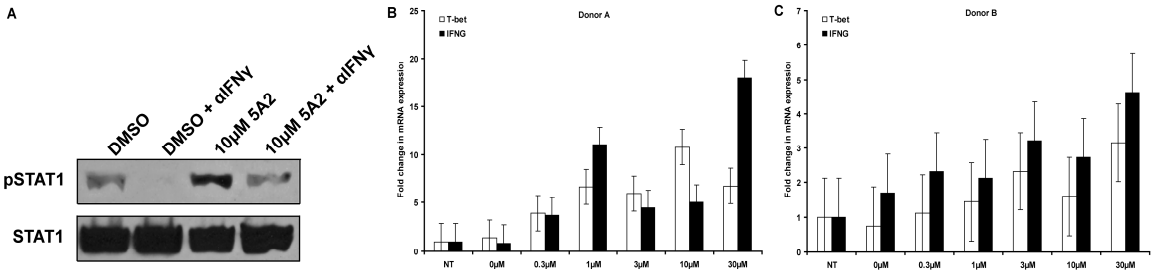

Figure 26: pSTAT1 signaling is induced by autocrine IFNγ. A) Magnetically isolated CD4 T cells from healthy donors were treated with 5A2 and αIFNγ as indicated and subjected to western blot for pSTAT1 and total STAT 1. **B and C)** Total RNA obtained from magnetically isolated CD4 T cells from healthy donors were subjected to T-bet and IFNG transcript specific qRT-PCR. Error bars indicate two times the standard deviation of the mean of three replicate experiments.

5A2 treatment of CLL T cells specifically induces demethylation of the IFNG promoter

In order to link the phenotypic changes with the molecular alterations we observed it was necessary to provide a mechanism for 5A2 induced IFNγ expression. The IFNG locus spans a 110kb region on human chromosome 12. Using methylation specific PCR and bisulfite sequencing we identified a region comprised of 200bp proximal to exon I of IFNG which was demethylated by 5A2 (Figure 27A, B, and C). Our results reveal the methylation percentage of two independent CpG sites was decreased in T cell populations from CLL patients after treatment with 5A2. Although, using Methyl-ChIP, we tested other regions of the IFNG locus, including the Conserved noncoding sequence (CNS)-56kb, -54kb, -34kb, Intron I, +18-20kb, +24kb, +46kb, and +55kb, we could only identify significant alterations in methylation within the promoter

region (data not shown). Notably, the basal methylation level of untreated CLL cells resembled the highly methylated state seen in purified Th2 T cells.

In mouse studies it has been shown that the CNS-6kb region was divergently

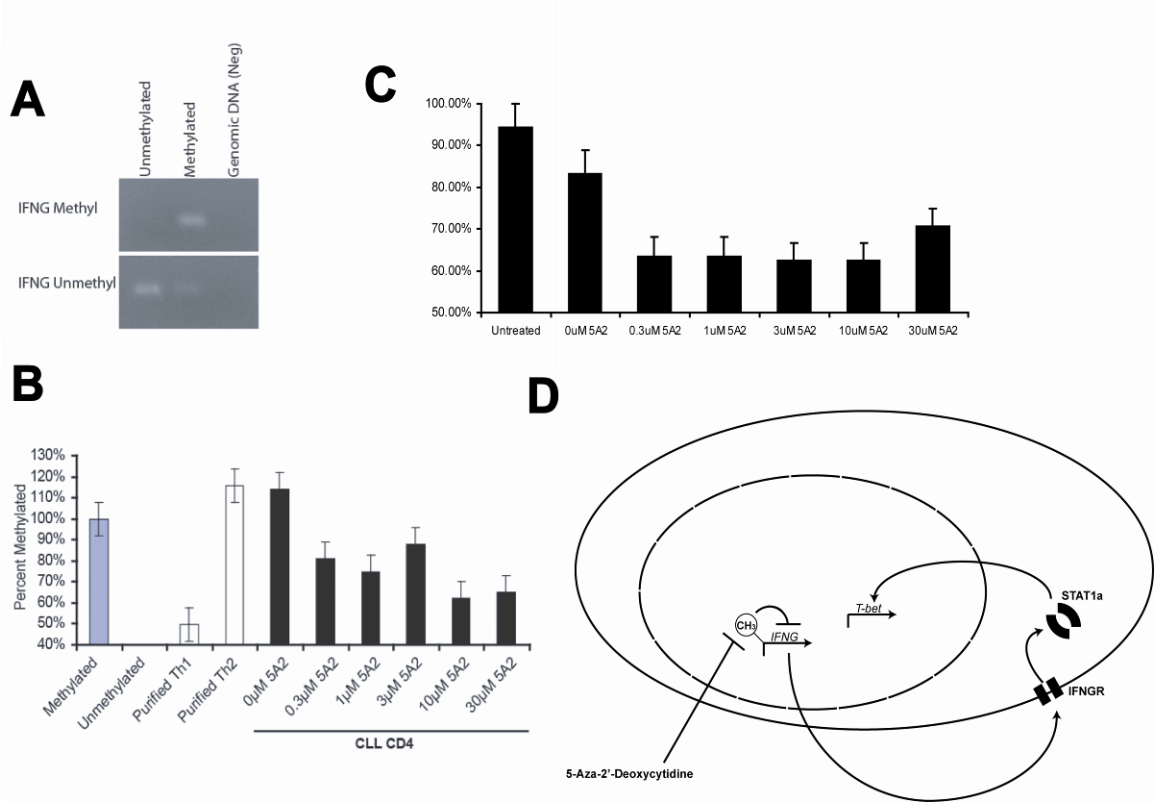

Figure 27: 5A2 specifically demethylates the IFNG promoter. A) Synthetically methylated, unmethylated, and gemomic DNA were assayed using methylation specific PCR primers demonstrating transcript specificity. **B)** MSP was carried out on bisulfite treated DNA obtained from Th1, Th2, and CD4 T cells obtained from CLL patients treated with various concentrations of 5A2 in addition to synthetically methylated and unmethylated DNA samples. **C)** Bisulfite sequencing of the promoter region of the IFNG locus confirms MSP results. For methylation analysis the relative level of methylation for three independent experiments is depicted along with error representing the standard deviation. Note that the graphical range is abbreviated for graph clarity. **D)** A graphical depiction of our proposed signaling model in which 5A2 induces ectopic demethylation, expression, and secretion of IFNγ resulting in autocrine IFNGR signaling via pSTAT1α a known T-bet transcription factor resulting in T-bet expression.

methylated in Th1 and Th2 cells. Additionally, it has been postulated that 5A2 treatment demethylates this region resulting in transcription of the IFNG gene in human T cells.

Our studies confirmed the notion that Th1 and Th2 cells differ in their respective methylation levels within the CNS-6kb region using bisulfite sequencing, however after detailed analysis using bisulfite sequencing we could not confirm demethylation in T cells after treatment with 5A2 indicating that this region is not responsible for the phenotypic and molecular changes in T cell polarity that we have identified (Figure 28A and B). Figure 27D shows our proposed schema for the activation of T-bet using 5A2 based on the evidence at hand.

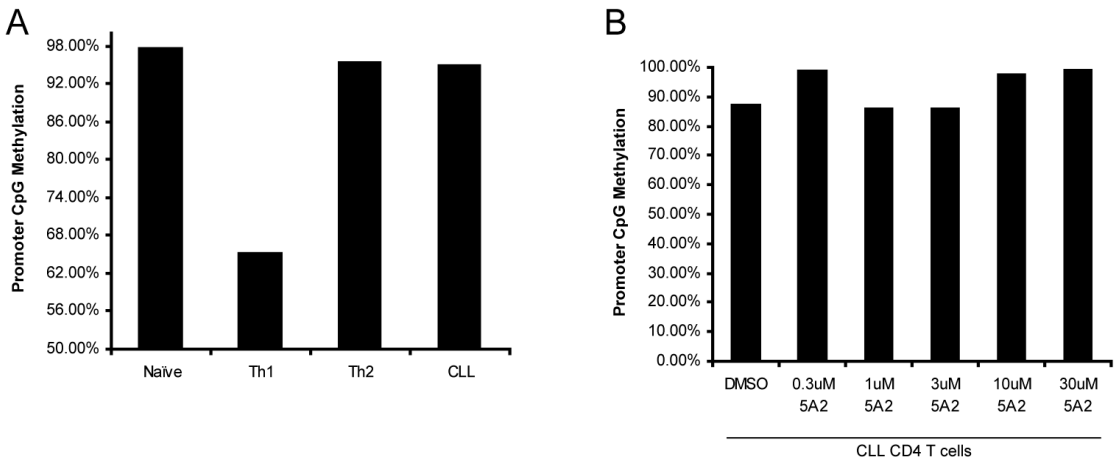

Figure 28: 5A2 does not induce demethylation of the CNS-6kb region of the IFNG locus. A) Bisulfite sequencing analysis of the CNS-6kb region of the IFNG gene revealed divergent patterns of methylation between Naïve, Th1, Th2, and CLL T cells however **B)** no change was identified in CLL T cells after treatment with various concentrations of 5A2. Note that the graphical range is abbreviated for graph clarity.

Discussion

The heritable epigenetic alterations which underpin T cell polarity can also become chromatin-based patterns which are altered in disease states. Our studies have shed light on a potential mechanism by which therapeutic application of demethylating agents such as 5A2 can induce the signaling patterns of Th1 phenotype in what would generally be considered Th2 T cells. We have also demonstrated this in a disease state,

CLL, for which Th2 polarization regularly results in the inability to adequately deal with external pathogens. As of yet it is uncertain how long these molecular alterations can persist in the absence of continued 5A2 treatment. In unpublished studies we have identified phenotypic changes which continue for weeks post treatment and our ongoing studies will work to identify any temporal limitations to these chromatin modifications. It is conceivable, that even temporary alteration of T cell phenotype may have an impact in the T cell imunosuppression seen in patients with CLL.

With the general goal of chemotherapeutic effect many of the clinical trials to date have used concentrations of 5A2 which are detrimental to the healthy lymphoid compartment. We too have occasionally witnessed a reversal of immunotherapeutic effects at higher doses of 5A2 (30μM or higher). A recent clinical trial has been conducted using a relatively low dose 5A2 in CLL patients. While 5A2 alone demonstrated little cytotoxic effect on CLL cells the 10mg/m^2 dosage was well tolerated; unfortunately however, the effects to T lymphocytes were not studied(Blum, Liu et al. 2010). In a similar Phase I study of T-cell lymphomas 5A2 at 10mg/m^2 reduced global DNA methylation in T lymphocytes by 2.5-6%, lending credence to the idea that sub-chemotherapeutic levels of demethylating agents may alter the methylation of the T cell compartment(Stewart, Issa et al. 2009).

Currently, there is scant evidence regarding the regulation of IFNG in human T lymphocytes, however regulation in mice has been comprehensively studied by CB Wilson(Schoenborn, Dorschner et al. 2007). A single manuscript has previously identified the epigenetic repolarization of human tumor infiltrating lymphocyte clones using 5A2, however the scope of this study lacked a detailed analysis of the mechanism

and molecular phenotype(Janson, Marits et al. 2008). In prior published studies we identified a number of demethylation-induced immunotherapeutic effects which in total lead to an increased antigen presenting phenotype in CLL cells, a result which complements our current findings(Dubovsky, Wang et al. 2010).

We have identified molecular signatures which parallel the increased IFNγ response. The molecular signals recapitulate an expected Th1 T cell including T-bet, pSTAT1, and decreased pSTAT6. To our knowledge we are the first to identify these molecular signature alterations in response to demethylating agents. In addition, we have identified a region of the IFNG locus which is actively demethylated by 5A2 and potentially induces expression in T cells. In previous reports using human T cells methylation of the CNS-6kb region of the IFNG locus was shown to mirror T cell phenotype. Our studies clearly demonstrate that demethylation of the CNS-6kb region is not necessary for IFNG expression and a Th1 phenotype. It remains unclear why the CNS-6kb region was unchanged by 5A2 however it is possible that this region may be progressively demethylated as a Th1 clone expands, further reinforcing Th1 polarity.

Our findings generate provocative questions regarding the efficacy of new immunotherapeutic strategies based around 5A2. Numerous studies have identified the induced expression of various cancer germline antigens in both solid and hematologic malignancy, including CLL(Dubovsky, Villagra et al. 2010). A strategy which incorporated the restoration of T cell cytolytic capacity with the expression of potent immunotherapeutic antigens specifically within tumor cells would likely be attractive.

Materials and Methods

Subject Populations

Sera and peripheral blood mononuclear cells (PBMCs) were obtained from patients with CLL. All subjects gave written institutional review board (IRB)-approved informed consent for their blood products to be used for research. Blood was collected at the H. Lee Moffitt Cancer Center (Tampa, FL). PBMCs were stored in 1ml aliquots at -140°C and sera were stored in aliquots at -80°C until used.

Cell Culture, Drug Treatments, and T cell Polarization

Unless otherwise stated cells were cultured in-vitro at 37°C and 5%CO2 using RPMI1640 medium supplemented with 10% fetal calf serum and antibiotics. Drug treatments were carried out on plate-bound anti-CD3, soluble anti-CD28, and IL-2 (20U/ml) stimulated magnetically isolated T cells (>95% purity) for 72hr in complete medium using the indicated concentration of 5-aza-2'-deoxycytidine (5A2) (dissolved at 10mM in DMSO). At the conclusion of treatment cells were washed twice using pre-warmed serum-free RPMI1640 and subjected to the various assays (Supplementary Figure 1). Th1 and Th2 polarized T cells were obtained from magnetically purified naïve human CD4 T cells by weekly stimulation with plate-bound anti-CD3, soluble anti-CD28, and IL-2 (20U/ml) in the presence of IL-12 (10ng/ml) and anti-IL-4 (1:100) for Th1 or IL-4 (5ng/ml) and anti-IL-12 (1:100). Medium was replaced twice weekly and stimulation was repeated for four weeks upon which a sample was collected and tested via CBA array and qRT-PCR for polarity (Figure 29).

Reverse Transcriptase-PCR (RT-PCR)

Total RNA was prepared from pelleted cells (RNeasy mini columns and RNAse free DNAse, Qiagen, Valencia, CA). RT-PCR and qRT-PCR reactions were conducted using the Qiagen one-step RT-PCR kit (Qiagen) or the iScript SYBR green RT-PCR kit

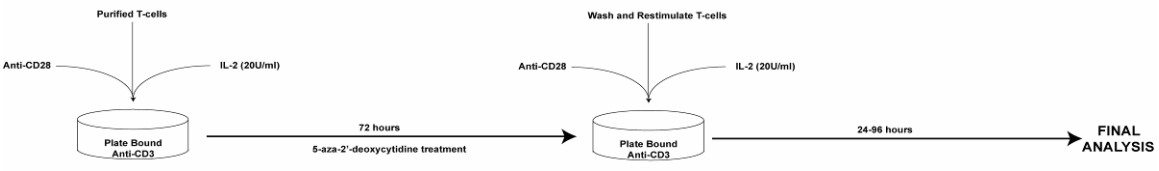

Figure 29: Schema for in-vitro experimental analysis of T cells. Purified CD4 or CD8 T cells are stimulated using plate bound αCD3, soluble αCD28 and 20U/ml IL2 for 72 hours in the presence of the indicated concentration of 5A2. Cells are then washed and restimulated using the same methodology prior to analysis.

(BioRad, Hercules, CA) with transcript-specific primers (T-bet: 5'TGACCCAGATGATTGTGCTC, 3'ATCTCCCCCAAGGAATTGAC) (GATA3: 5'AAGGCAGGGAGTGTGTGAAC, 3'TGGATGCCTTCCTTCTTCAT) (IFNG: TTCAGATGTAGCGGATAATGGA, 3'TCAGCCATCACTTGGATGAG) and 200ng of total RNA. RT-PCR amplification reactions were resolved on 2% agarose gels and the size of the amplified transcript confirmed by comparison with a standard DNA ladder (GelPilot 1Kb Plus Ladder, Qiagen). qRT-PCR experiments were analyzed using the MyiQ software package. After confirming a single melt curve peak CT values for Actin were compared to CT values for the transcript of interest using the $2^{-\Delta\Delta CT}$ method.

Flow Cytometry and Cytokine Bead Array

Flow cytometric analysis was performed using fluorochrome-labeled monoclonal antibodies (mAbs; anti-CD3, -CD4, -CD8, -IL-4, -IFNγ, and anti-pSTAT6, Becton Dickinson, San Jose, CA and eBiosciences, San Diego, CA) and the viability dye 4',6-diamidino-2-phenylindole (DAPI, Sigma). Intracellular staining of IL-4, IFNγ, and pSTAT6 was conducted according the appropriate manufacturer protocol (Becton Dickinson). Cytokine bead array (CBA) (Becton Dickinson) was conducted according to the manufacturers published protocol using cellular supernatant from three replicate

experiments. Flow cytometric data was analyzed with FlowJo software (Tree Star, Ashland, OR) on a minimum of 30,000 collected events.

Methylation Analysis

For both methylation specific PCR (MSP) and bisulfite sequencing purified DNA (Qiagen DNA miniprep kit, Qiagen) was bisulfite treated according to the manufacturer protocol using the Epitect Bisulfite treatment kit (Qiagen). MSP was conducted using transcript specific primers designed to specifically recognize either methylated or unmethylated CpG motifs (IFNG-methyl 5'AAGAGTTAATATTTTATTAGGGCGA, 3'TAAACTCCTTAAATCCTTTAACGAT) (IFNG-unmethyl 5'TGAAGAGTTAATATTTTATTAGGGTGA, 3'TAAACTCCTTAAATCCTTTAACAAT). Touchdown PCR was utilized to generate PCR products which were subjected to gel electrophoresis, stained with ethidium bromide and densitometrically analyzed.

Bisulfite sequencing was conducted using primers indifferent to methylation status (IFNG-Promoter 5'TAGAATGGTATAGGTGGGTATAATGG, 3' ATAACAACCAAAAAAACCCAAAAC) (CNS-6kb 5'TGAGTAAAGGTTTAGGGTATTTTTT, 3'ACTCACTACAAACTCTACCTCCC). PCR amplicons were cloned into plasmid vectors using a T-A cloning kit (Qiagen) and transformed bacterial colonies were directly sequenced using vector primers. A minimum of 12 bacterial colonies were sequenced for each sample. Sequencing results were used to calculate methylation status of CpG motifs and average methylation stats was depicted in graphical form.

Western Blot Analysis

Western blotting experiments were conducted using conventional methodology previously described(Dubovsky, McNeel et al. 2009). Blotting was conducted using pSTAT1 Y701 (Cell Signaling Technologies, Danvers, MA), T-bet (eBiosciences), STAT1, and β-Actin (Santa Cruz Biotechnology, Santa Cruz, CA) specific antibodies.

Chapter Five:

Scientific significance and future directions

Antigen specific immunotherapy for CLL

Vaccines in general have had wide-ranging success and, in fact, are the only means by which the human race has achieved eradication of a disease. Unfortunately, however, vaccines are generally only effective when administered prior to challenge.

Active immunotherapeutic strategies are hardly a novel concept in solid tumors. For the past two decades immunotherapeutic vaccines have been tested, primarily in melanoma and renal cell carcinoma, which tend to be slightly more immunogenic in nature. While many of these vaccine strategies and antigens have been ineffective in fast growing solid tumors there are a few select strategies which persist, among these are cancer testis antigen based cellular vaccines.

In hematologic malignancies, namely CLL, antigen specific vaccines have not been studied in such a fervent manner. Instead, much attention was initially devoted to the development of idiotype-specific vaccines which would elicit an immune response against the clonal CLL antigen receptor. While this was a meritous concept, few trials have demonstrated success and, like antigen-specific vaccines for solid tumors, only a few permutations of this idea persist. Nowadays, immunotherapeutics for CLL have followed the general trend initiated by the success of anti-CD20 monoclonal antibody therapy; however there exists little curative potential with conventional antibody therapy.

With the new era of epigenetics the novel concept of vaccinate-induce has the potential to recapitulate the twentieth century success of anti-viral vaccines.

Our studies represent the first comprehensive identification of inducible antigens in CLL. While true identification of circulating T cells capable of responding to CLL antigens is extremely difficult in CLL we were able to identify isotype-switched antibody responses. The generation of an IgG response towards any antigen requires isotype switching, a phenomenon which occurs in response to helper T-cell antigen recognition. Thus a robust IgG response to a given cancer-specific antigen can be an extremely informative event indicating the presence of a functional T-cell repertoire specific to the antigen; data which becomes vastly more important when you consider that CLL-induced antigen-specific T-cell inhibition tends to eliminate the majority of these clones. One could envision that the most potent antigens should have no detectable immune responses. Although this may also be true, it is now understood that cancer progression is marked by repeated immune-evasive events, leading to the epigenetic suppression of many highly antigenic proteins. Immune-evasion conceivably leads to the generation of an antibody milieu specific to potent tumor antigens. Thus finding a single response towards an antigen which is not expressed by the current tumor potentially indicates 1) epigenetic suppression of the antigen and 2) antigen expression leads to an immune response capable of eliminating a particular CLL clonal population.

In future studies it will be necessary to assess the efficacy of various inducible-antigen based immunotherapeutic strategies. Amongst the most promising is a novel strategy which utilizes patient T cells transfected with chimeric antigen receptors capable of responding to surface antigens on CLL cells. Since the chimeric receptor is composed

of an antibody-based antigen recognition regions and T cell derived signaling the possibilities are not limited to MHC presented peptide antigens. One conceivable permutation of this therapeutic tool would be to derive chimeric antigen receptors which recognize an epigenetically inducible surface antigen on CLL. Conceivably, this antigen could even be an epigenetically induced carbohydrate molecule; a concept which is still in its virgin stage.

Alleviating B cell dysfunction in CLL

In healthy individuals B cells play a critical role in promoting the adaptive immune response via presentation of antigens to the T lymphocyte wing of the immune system. This antigen presentation normally provides costimulatory and cytokine signals which direct T cell polarization, proliferation, and effector function as well as inducing proliferation, isotype switching, and plasma cell differentiation of the B cell. Since CLL patients progressively loose their healthy B cell compartment due to competition with the malignant clone these necessary functions of B cell immunity are lost. In addition, the loss of normal B lymphocytes truncates the available naïve B cell pool, limiting the ability to respond to new pathogens.

Clinically, this dysfunction poses a huge obstacle to effectively treating the disease. Currently, the majority of approved therapeutic interventions, including anti-CD20 monoclonal antibody therapy and chemotherapeutic agents such as fludarabine, have the capacity to kill both healthy and malignant B cells exacerbating the immunosupression. In light of this, there is an obvious need for novel therapeutics which are significantly more specific in their cytotoxic efforts. While many of the epigenetic modifiers, including 5-aza-2'-deoxycytidine and LAQ, were initially developed as

cytotoxic chemotherapeutic agents, their effect at sub-chemotherapeutic levels may lead to a more profound clinical advantage.

Our studies demonstrated that using low doses of both hypomethylating and histone deacetylation agents resulted in an alteration of immune phenotype amongst CLL cells. Importantly, we were able to effectively alter the antigen presentation signals such that Th1 T cell responses were enhanced, immune synapse signaling was improved, immunosuppressive cytokines were reduced, and CLL specific cytotoxicity was achievable. Since our studies were focused on the malignant arm of the B cell repertoire it remains to be seen whether or not healthy B cells derived from the bone marrow will recapitulate healthy antibody responses alleviating the other half of B cell base immunosupression. These studies will be most effectively carried out in animal model systems, a task which our lab actively seeks to carry out.

In B-CLL the stromal environment has drastic impact on the survival, proliferation, signaling, and localization of tumor cells further complicating the transition from benchtop to bedside. There currently exists a single mouse model which adequately recapitulates CLL. Developed by Dr. Carlo Croce this mouse has the TCL1 oncogene under the transcriptional control of the antibody immunoglobulin μ promoter. Recent molecular evidence suggests that the μTCL1 mouse model accurately mimics the stromal environment of CLL, inducing T-cell dysfunction and ignorance identical to that found in human CLL patients. However, the unfortunate consequence of conventional murine models for immunotherapeutic research is that the antigens derived have variable conserved levels of peptide sequence, epigenetic control, cell specificity, and expression. In the past this has led to conclusions which were not translatable to the human system.

An alternative approach would be to utilize the new genetic stock of IL-2R common γ chain deficient NOG (NOD-SCID/IL-2Rγnull) mice for the implantation of human tissue. These mice have demonstrated superior engraftment of human cells attributable to their lack of functional NK, B-, and T-lymphocytes. In our preliminary work we have successfully engrafted B-CLL along with T-cells from patients within our PBMC bank enabling us to conduct preclinical studies, including immunotherapeutic strategies. Another benefit is the ability to utilize human antigens as opposed to murine homologues of varying antigenicity as would be necessary in other spontaneous tumor models. Although this research is still in its infant stages, this new model provides a necessary tool for preclinical testing of immunotherapeutic strategies which incorporate the alteration of B cell immunogenicity.

Alleviating T cell dysfunction in CLL

While more indirect, the immunosuppressive effect CLL has on the T cell armament is no less profound. In healthy individuals professional antigen presenting cells such as B cells are critically necessary for the appropriate licensing of CD4 helper and CD8 cytotoxic responses. Although the complex interactions which take place between T cells and B cells in-vivo are still being studied, there are a few variables which have proven critical for effective T cell polarization. First and foremost is adequate synapse formation. Research indicates that synapse formation consisting of long lasting TCR engagement followed by costimulatory signals derived from interactions involving CD28 and stabilized by engagement of the ICAM1/LFA scaffolding promote memory cell formation, polarization, and proliferation of the recently mature lymphocyte clone.

Interestingly, our research focusing on relieving B cell immunosuppressive had the unintended consequences of improving the dynamics of naïve T cell activation and polarization via interaction with CLL APCs. Our data demonstrated that T cells interacting with epigenetically modified CLL cells were of Th1 polarity. Evidence indicates that this leads to a cytotoxic response capable of eliminating intracellular pathogens such as virus infection or potentially even CLL cells.

While the proper polarization of naïve T cells responding to new pathogens is important, it does not repair the damage which has already accumulated within the T cell repertoire of a CLL patient. It is postulated that the T cell repertoire still contains the receptor diversity necessary to respond to numerous external pathogens and perhaps even CLL. However, as a result of the CLL improper Th2 polarization, anergy, and the inability to form new antibody responses renders T cells powerless against the majority of challenges. Our studies successfully demonstrated a methodology which could be utilized to regain Th1 functionality amongst T helper cells in CLL. Moreover, we demonstrated that newly formed T cell responses could be therapeutically modulated as well. All in all we have identified what may become a useful therapeutic tool for CLL.

What remains unclear is whether or not a revitalized T cell compartment will, without further intervention, be capable of eliminating CLL cells. We have started to investigate this hypothesis; however using conventional methodology it is difficult to isolate adequate CD4 and CD8 T cells along with CLL cells from a single blood donor. Moreover, in-vitro culture of T cells from CLL patients requires supra-physiologic concentrations of IL2. For these reasons it would be ideal to directly test this hypothesis in an in-vivo tumor model. A model such as the NOG/SCID model described earlier

would be ideal. In preliminary studies we have identified a methodology which allows for selective engraftment of the CD3 T cell compartment allowing for experimental control animals to be generated alongside fully engrafted littermates.

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