**IL-10 production by CLL cells is enhanced in the anergic IGHV mutated subset and associates with reduced DNA methylation of the IL10 locus.**

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**Running Title:** IL-10 production varies with anergy and DNA methylation in CLL

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#  Abstract

Chronic lymphocytic leukemias (CLL) with unmutated (U-CLL) or mutated (M-CLL) IGHV have variable features of immunosuppression, possibly influenced by those CLL cells activated to produce IL-10. The two subsets differ in their levels of anergy, defined by low surface IgM levels/signaling capacity, and in their DNA methylation profile, particularly variable in M-CLL. We have now found that levels of IL-10 produced by activated CLL cells were highly variable. Levels were higher in M-CLL than in U-CLL and correlated with anergy. DNA methylation analysis of *IL10* locus revealed two previously uncharacterized ‘variably methylated regions’ (CLL-VMRs1/2) in the gene body, but similarly low methylation in the promoter of both U-CLL and M-CLL. CLL-VMR1/2 methylation was lower in M-CLL than in U-CLL and inversely correlated with IL-10 induction. A functional STAT3 binding site in CLL-VMR2 was confirmed by proximity-ligation and luciferase assays, while inhibition of SYK-mediated STAT3 activation resulted in suppression of *IL10*. The data suggest epigenetic control of IL-10 production. Higher tumor load may compensate the reduced IL-10 production in U-CLL, accounting for clinical immunosuppression in both subsets. The observation that SYK inhibition also suppresses IL-10 provides a potential new rationale for therapeutic targeting and immunological rescue by SYK-inhibitors in CLL.

# Introduction

Chronic lymphocytic leukemia (CLL) is a neoplasm of mature CD5+ B cells and is heterogeneous in many clinical and functional aspects. Much of the heterogeneity associates with the characteristics of the tumor B-cell receptor (BCR). From analysis of the tumor BCR immunoglobulin (Ig) gene heavy-chain variable regions (IGHV), CLL can be divided into two subsets with either unmutated (U-CLL) or mutated IGHV (M-CLL). U-CLL derives from pre-germinal center (GC) CD5+ B cells and has a poorer prognosis while M-CLL derives from post-GC CD5+ B cells and has a good prognosis (1-3).

An important clinical problem is that both U-CLL and M-CLL are associated with significant suppression of the immune system from the early stages of disease (4, 5), and infections are the leading cause of death (6, 7). Immune suppression is multifactorial, but one possibility is that CLL cells themselves produce immunosuppressive factors, such as IL-10, and have features of regulatory B cells (Breg) (5). Breg are a small fraction of the normal B cell population that exert regulatory functions through soluble factors, predominantly IL-10 (8). IL-10 producing Breg (B10) cells can expand significantly during inflammation, autoimmunity and cancer to regulate innate and adaptive immune responses (8-10). Human B10 cells can differentiate from CD5+CD24hiCD27+ antigen-experienced B10 progenitors (B10pro), which are induced to become IL-10-competent following costimulation (8). Both B10 and anergic B-cells arise following prior chronic exposure to antigen (9, 11), and consequently express low levels of surface IgM (sIgM) (8, 10-12), suggesting a potential link between their functions.

CLL cells share multiple phenotypic markers with human B10 cells (13), and are also CD5+CD24hiCD27+ antigen-experienced cells (5, 13). Although they may spontaneously release modest amounts during circulation (14), both U-CLL and M-CLL cells produce significant levels of IL-10 following perturbation by a range of stimuli *in vitro* (5, 13). While the stimulus *in vivo* is unclear, *IL10* transcripts are higher in CLL cells from lymph nodes compared to peripheral blood (15). IL-10 expression in B-cells is induced following STAT3 tyrosine phosphorylation (16), which is activated in the lymph node CLL cells (17). The TLR9 ligand CpG oligonucleotide is a potent tool to reveal the capacity of CLL cells to differentiate into B10 cells, and to mimic STAT3 activation via DOCK8 adaptor and SYK-SRC kinases complex (18).

Analysis of BCR sIgM levels and signaling function confirms heterogeneity in CLL cells. Circulating cells from both subsets have features of anergy (19, 20), possibly as a consequence of chronic exposure to common (super)antigens in lymphatic tissue (19-21). The degree of anergy is typically different between U-CLL and M-CLL, being more profound in the latter, with additional evidence for intrasubset variation (22). Antigen-driven anergy is a dynamic process and sIgM levels/signaling capacity will recover during circulation *in vivo* following egress from the lymph nodes (22, 23). Anergy will determine cell fate, partly by an increased susceptibility to apoptosis (12, 24), and it is now clear that a lower grade of anergy accompanies more rapid progression in CLL (25).

However, clinical behavior is also influenced by the DNA methylation of the transformed cell (26). DNA methylation is involved in controlling cellular differentiation and cell type specification during hematopoietic development (27, 28). GC maturation of normal B cells involves widespread epigenetic programming, including targeted changes to the DNA methylation of genes important for immune function (29-31). In CLL, the methylation profile of U-CLL and M-CLL is clearly different, likely reflecting the maturation of the cell of origin (29, 30), and is stable over the course of disease (30, 32). Overall methylation levels can be deduced through analysis of a small set of genes including *IL10* (31). We have recently found that the methylation levels inversely correlate with lower sIgM levels/signaling in CLL (25). Although IgM levels/signaling in CLL cells are also influenced by reversibility during circulation (22, 23), the data suggest a connection between IgM levels/signaling and *IL10* gene methylation, although the possible epigenetic control on IL10 has not been investigated in normal and tumor B-cells.

In this study, we investigated the link between anergy and the ability of CLL cells to produce IL-10. We found that induced production of IL-10 is variable in CLL and that the variability is closely associated with grade of anergy and with differential *IL10* gene methylation.

# Material and Methods

## Patients, IgM expression and signaling capacity

The study was performed from peripheral blood mononuclear cells (PBMCs) of CLL patients collected at the Cancer Sciences Unit in Southampton (CSU cohort, Supplementary\_Table\_S1a) and, where specified, at the German Cancer Research Center/National Center for Tumor Diseases (DKFZ cohort, Supplementary\_Table\_S1b). Samples were collected prior to treatment from patients with CLL diagnosis according to the IWCLL/NCI 2008 criteria. The study was approved by the Institutional Review Boards at the University of Southampton (REC: H228/02/t) and at the Heidelberg University Hospital. All patients provided written informed consent.

PBMCs were obtained using density gradient centrifugation and cryopreserved as previously described (22). Prior to each assay, PBMCs were thawed, immediately washed in RPMI1640 medium (supplemented with 10% fetal calf serum, 2mM glutamine and 1% penicillin/streptomycin) and allowed to recover for 1 hour at 37°C in complete RPMI1640 medium before use (22-25). *IGHV* usage, mutational status, expression of sIgM and signaling capacity by intracellular Ca2+ mobilization assay were determined as described previously (22, 25). Our previous cut-offs of 50 and 5% were used to distinguish patients with high/low sIgM expression (MFI) or signaling capacity (iCa2+ mobilization %), respectively (22, 25).

## Flow cytometric assessment of IL-10 production

PBMCs (2x107 cells/ml) were re-suspended in complete RPMI1640 medium, cultured in a 96-well flat-bottom plate (250 μl/well) and stimulated with CpG (ODN 2006, 7.5µg/mL, Source Bioscience) for 24 hours. PMA (50ng/mL), ionomycin (0.001mM; Sigma-Aldrich) and brefeldin-A (PIB; BD Biosciences) were subsequently added for 5 hours before washing in FACS buffer (1% bovine serum albumin, 4mM EDTA, and 0.15 mM NaN3 in PBS) and staining with anti-CD5-PerCP/Cy5.5 (UCHT2; Biolegend) and anti-CD19-Pacific Blue (HIB19; Biolegend) on ice for 30 minutes protected from light. Cells were washed, fixed and permeabilised by the BD Cytofix/CytopermTM Fixation/Permeabilisation Kit before staining with anti-IL-10-PE (JES3-19F1; BD Biosciences) or isotype control (BD Biosciences). Cells were washed, re-suspended in FACS buffer and 2.5x104 lymphocytes were acquired on a FACS Canto II. Analysis and determination of percentages of IL-10+ CD19+CD5+ CLL cells were performed with FACSDiva software (BD Biosciences).

## Assessment of IL-10 secretion and transcript expression

CLL cells were negatively selected from PBMCs using the B-CLL Isolation Kit (Miltenyi Biotec). Isolated cells (2x107 cells/ml) were cultured as above in the presence or absence of CpG for 24 hours. Cells and supernatant were subsequently separated and collected for RNA isolation and cytokine secretion, respectively.

Cytokine levels were measured in the supernatants using the Luminex multiplex bead array assay kit (Cytokine human 10-plex panel; Life Technologies) and a Luminex 100 instrument.

*For IL10* transcript measurement (CSU cohort), total RNA was isolated from cells using the RNeasy Mini-Kit (QIAGEN) and cDNA was generated using oligo(dT) primers and M-MLV reverse transcriptase (Promega). Quantitative PCR was performed using TaqMan primers for *IL10* (Hs99999035-m1; Applied Biosystems) and human beta-2-microglobulin endogenous control (4333766T; Applied Biosystems), on a 7500 Real-Time PCR System (Applied Biosystems). A standard curve was generated for each gene from untreated HUT78 cells (33). The average *IL10* cDNA concentration was determined using the standard curve method and made relative to *B2M*. For the DKFZ cohort, RNA was reverse-transcribed using Superscript II oligo(dT) (Invitrogen) and analyzed using the Universal ProbeLibrary System and a LightCycler-480 Real-Time PCR instrument (Roche). *IL10* gene expression was measured relative to HPRT.

Cell viability using our conditions was >85% in all samples analyzed and did not correlate with IL10 production.

## Assessment of STAT3 activity

PBMCs were cultured in the presence or absence of CpG for 5 hours as above. Incubation with SYK inhibitor R406 (Selleck Chemicals) or PYK2 inhibitor AG-17 (Merck Millipore) was performed for 1 hour prior to CpG stimulation (18). Following stimulation, cells were collected for protein extraction and RNA isolation (33).

For immunoblotting, proteins were separated on 10% polyacrylamide gels, transferred to nitrocellulose membranes (GE Healthcare), probed for phosphorylated STAT3 (Y705), total STAT3 (Cell Signalling Technology) and actin (Sigma) as a loading control and subsequently stained with HRP-linked secondary antibodies (Dako). Following enhancement by chemiluminescence reagents (Thermo Scientific), proteins were visualized using the ChemiDoc-It imaging system (UVP). Band intensities were quantified using ImageJ and normalized to actin.

## DNA methylation analysis

DNA methylation analysis was performed by MassARRAY using standard procedures (Agena Biosciences). Briefly, DNA was isolated using the Qiagen DNeasy blood and tissue kit followed by bisulfite-conversion using the EZ DNA Methylation Kit (Zymo Research). Bisulfite PCR primers were used to cover relevant regions of the *IL10* locus (primer sequences and positions are listed in Supplementary\_Table\_S2). Infinium 450K genome-wide DNA methylation data on 122 CLL cases were generated previously (31). Data are available at the European Genome-phenome Archive (EGA, accession EGAS00001000534). Raw data were normalized using the BMIQ method without background subtraction using the RnBeads software (34, 35). For ranking of *IL10* among all genes analyzed by 450K arrays, we considered all annotated genes (20,622) and ranked them by their most differentially methylated CpG (459,625 CpGs interrogated).

## Luciferase reporter Assay

Dual luciferase reporter assays were performed as described previously (36). Luciferase reporters were cloned into pGL4.23 vectors (Promega) or PCpGfree-promoter-lucia (Invivogen) for methylation studies. *In vitro* methylation was carried out using M.SssI CpG methyltransferase (Thermo Fisher Scientific) according to the manufacturer’s instructions. Reporter constructs were validated by Sanger sequencing (GATC Biotech). Site-directed mutagenesis was carried out with QuikChange II mutagenesis kit (Agilent Technologies). HEK293T cells were transfected with TransIT-LT1 transfection reagent (Mirus Bio, Madison, WI). Raji and MEC-1 cell lines were transfected with an Amaxa Nucleofector II device using kit V (Lonza, Verviers, Belgium). Data were normalized to co-transfected luciferase reporter vectors (pRL-*Renilla*, Promega) for pGL4-based reporters and pGL3-CMV-firefly luciferase for pCpGfree-lucia reporters. Overexpressed transcription factors (TFs) were cloned in Gateway pDest-11-FLAG-CMV vectors (Invitrogen) and co-transfected together with reporter plasmids. Primer and oligo sequences are available in Supplementary\_Table\_S3.

## STAT3 proximity ligation assay

Proximity ligation assay was carried out based on published protocols (37). Briefly, FLAG-tagged STAT3 was expressed in HEK293T cells and nuclear protein was harvested after 48 hours. Oligonucleotide-coupled anti-FLAG-antibody (Clone M2, Sigma-Aldrich) was generated using Thunderlink oligonucleotide conjugation kit (Innova Biosciences) with a 5’-amino oligo. Proximity ligation probes (200 pM), conjugated antibody, and proteins were incubated at room temperature for 2 hours followed by 1 hour of ligation at 16°C. Ligation efficiency was analyzed by real-time PCR using the LightCycler 480 system and human Universal ProbeLibrary hydrolysis probes (Roche). Oligo sequences are available in Supplementary\_Table\_S3.

## Statistical analysis

Continuous variables were compared by Mann-Whitney non-parametric test for independent samples. For statistical correlation between two variables, the non-parametric Spearman’s rank test was used. All statistical tests were 2-sided. Statistical significance was defined as *P*-value <0.05. Analyses were performed with GraphPad Prism 6 software (La Jolla, CA). The JASPAR database was used to obtain the consensus STAT3 motif and to perform TF motif prediction (38). The Interspecies sequence conservation track and Basewise conservation by PhyloP for 100 vertebrate species was obtained from the UCSC Genome Browser (<https://genome.ucsc.edu>). STAT3 sequence conservation data are from the Vertebrate Multiz Alignment (39).

# Results

## Human CLL cells have preferential and variable capacity to produce IL-10

While it is known that CLL cells can produce IL-10 (13), less is known about how the production relates to other cytokines. Spontaneous and CpG-induced cytokine secretion was measured in the supernatants of CLL tumor B-cells purified from the peripheral blood of 16 patients patients at CSU (Supplementary\_Table\_S1a), including 8 U-CLL and 8 M-CLL. Purity was ≥99.1% CD19+CD5+ cells in all samples. Cytokine secretion was measured after 24 hours of culture. Supernatant from unstimulated CLL cells contained undetectable or trace amounts of IL-10, measurable in 6/16 patients (range 0-110 pg/ml, median 0, mean 36pg/ml), while other cytokines were either lower or undetectable in the matched samples. Following stimulation, IL-10 levels increased significantly in the supernatant of all samples, with concentrations varying widely from 232 to 65000 pg/ml (mean 7765pg/ml) (Figure\_1 and Supplementary\_Table\_S4). Stimulation also increased IL-6, IL-8 and TNF-α levels in some samples, while the other cytokines failed to be detected in the majority (Supplementary\_Table\_S4). Sample-matched cytokine analysis demonstrated that IL-10 levels were significantly higher than any other cytokine, with the exception of 2/16 cases (M-CLL 281 and U-CLL 501), where TNF-α levels were higher than or similar to IL-10 (Supplementary\_Table\_S4).

The evidence that CLL cells secreted IL-10 following stimulation, confirmed that CLL cells have basal features very similar to B10pro cells (13). These results prompted us to look for features associated with capacity to produce IL-10 in CLL.

## The variable capacity of CLL cells to become IL-10+ associates with anergy

Percentages of IL-10+ CLL cells were determined by flow cytometry following CpG stimulation and PIB treatment as previously published (13). Variable percentages of IL-10+ CLL cells became evident in the CLL population of all patients (Figure\_2A and Supplementary\_Table\_S5). Since there was significant correlation between intracellular levels and secretion in the initial 16 CSU patients (r=0.61, *P=*0.01), we used the intracellular assay to extend our analysis to 27 patients patients at CSU (Supplementary\_Tables S1a and S5). Analysis of the extended cohort confirmed capacity of the CLL cells to produce variable amounts of IL-10 (range 1.1-34.4% of all CLL cells). Importantly, the percentage of tumor cells producing IL-10 was significantly higher in M-CLL than in U-CLL patients (Figure\_2B).

We compared IL-10 production with initial sIgM levels and signaling capacity to analyze associations with anergy. We observed that the CLL cases with low sIgM produced significantly more IL-10 than those with high sIgM (Figure\_2C) and that that the IgM non-signalers produced significantly more IL-10 than signalers (Figure\_2D). Similar conclusions could be obtained if signaling was measured as a quantitative variable and a strong inverse linear correlation was documented between intracellular Ca2+ mobilization following anti-IgM stimulation and IL-10 production following CpG and PIB stimulation (r=-0.74, *P*<0.0001, Supplementary\_Figure\_S1).

## Induced IL10 transcript levels associate with IL-10 secretion and percentage of IL-10+ cells

In order to assess linkage between mRNA and protein levels of IL-10, transcripts were quantified from purified CLL cells after 24-hour culture with or without CpG. Without CpG stimuation, *IL10* transcript levels were very low but measurable in all M-CLL, while they were undetectable in U-CLL (Figure\_3A and Supplementary\_Table\_S6). With CpG stimulation, *IL10* transcript levels increased in all 15 U-CLL and M-CLL (Figure\_3A and Supplementary\_Table\_S6). The induced levels were significantly higher in M-CLL than U-CLL (*P*=0.02), and associated with percentage of IL-10+ cells (Figure 3B) and IL-10 secretion (r=0.73, P=0.003).

## DNA methylation of the IL10 locus inversely associates with IgM signaling capacity

The linear correlation between induced transcript and protein levels suggested transcriptional control of IL-10 secretion. Hence, we explored possible mechanisms that regulate IL-10 transcript levels in CLL. As global DNA methylation has been shown to be different between M-CLL and U-CLL (29, 30, 32), we first looked for associations between *IL10* gene methylation and transcription.

Initial profiling by quantitative MassARRAY of the entire *IL10* locus in 36 CLL at DKFZ (Supplementary\_Table\_S1b) revealed that the core promoter region was largely unmethylated in both U-CLL and M-CLL (Figure\_4A). However, methylation differences were detected immediately downstream of the promoter within the first intron (approximately +200 to +500 bp) and in the gene body (approximately +1300 to +1800 bp). We termed these two regions as CLL variably methylated regions (CLL-VMRs) 1 and 2, respectively. Both CLL-VMR1 and CLL-VMR2 displayed lower methylation in M-CLL than in U-CLL (Figure\_4A). An analysis of published Illumina 450K array data (30), that includes the methylation probes interrogating the *IL10* upstream enhancer, promoter and CLL-VMR1 (but does not include CLL-VMR2) in an expanded set of 122 patients at DKFZ confirmed that methylation differences were specifically found in the gene body (Supplementary\_Figure\_S2A). In absence of stimulation, modest *IL10* transcript levels were detectable in M-CLL but not in U-CLL. Transcript levels inversely associated with the methylation status of CLL-VMR1 or CLL-VMR2, but not of the promoter (Supplementary\_Figures\_S2B). This suggested that the capacity for IL-10 production may relate to the epigenetic state of the CLL-VMRs.

Analysis of the CSU cohort revealed that methylation of CLL-VMR1 or CLL-VMR2 also correlated with IL-10 protein expression following CpG stimulation (Figure\_4B-C). Consistently, the relative degree of *IL10* gene methylation associated inversely with IGHV mutational status and sIgM signaling capacity (Figures 4D-G). These results suggest a possible functional link of CLL-VMR1/2 methylation with IL-10 production and with the grade of anergy.

## Functional analysis of variably-methylated regions in the IL10 gene

CLL-VMRs 1 and 2 display histone H3 lysine 27 acetylation (H3K27ac) in B cells (GM12878 cells), indicating they may contain functional DNA elements interacting with transcription factors (TFs) (Figure\_4A) (40). We chose to further investigate CLL-VMR2, as it contains high inter-species sequence conservation and predicted TF binding (Figure\_4A). *In silico* TF motif analysis predicted a binding site for STAT3. This site is widely conserved among mammalian species (Figure\_5A). To validate our hypothesis that STAT3 interacts with CLL-VMR2, we performed proximity ligation assays of the CLL-VMR2 sequence with STAT3 protein. We found that STAT3 showed specific binding to CLL-VMR2 in a manner similar to the consensus STAT3 motif (Figure\_5B). To determine if CLL-VMR2 shows STAT3-dependent enhancer activity, we transfected a luciferase enhancer reporter plasmid containing the CLL-VMR2 sequence (600 bp) together with a minimal promoter in combination with a STAT3-expressing plasmid carrying a phosphomimetic S727E modification. We found that CLL-VMR2 shows STAT3-dependent enhancer activity in all cell lines tested (Figure\_5C). Furthermore, CLL-VMR2 functions regardless of its orientation (a general feature of enhancers) and mutation of the STAT3 motif abrogates activity. Importantly, methylation of the CLL-VMR2 sequence resulted in complete loss of STAT3-dependent CLL-VMR2 enhancer activity (Figure\_5D).

Having documented a functional interaction of STAT3 with CLL-VMR2, we investigated the consequence of STAT3 inhibition on IL-10 production in 7 CLL samples producing variable amounts of IL-10 (U-CLL 635, 668, 695 and M-CLL 348, 482, 604, 684, Supplementary tables S1a and S5). Following stimulation, STAT3 phosphorylation (pSTAT3) was induced in all U-CLL and M-CLL (Figure 6A). Levels were similar between samples and irrespective of IL-10 production. Specific inhibition of the TLR9-mediated STAT3 phosphorylation pathway was performed in the two M-CLL (482 and 684) producing the highest levels of IL-10 and one U-CLL sample (635), using SYK inhibitor R406 or PYK2 inhibitor AG-17 (18). In all cases inhibition of STAT3 phosphorylation associated with suppression of *IL10* transcript induction (Figure 6B). These data show that STAT3 activity can be induced in all CLLs and is required for IL-10 induction following CpG stimulation. Differential IL-10 induction between U-CLL and M-CLL despite equal STAT3 phosphorylation among CLLs advocates that methylation of CLL-VMR2 may play a role in controlling STAT3-dependent IL-10 expression.

# Discussion

Immunosuppression is a key clinical feature accompanying CLL course from the early stages and we have hypothesized that this may be a consequence of the regulatory activity of CLL cells (5). CLL cells are known to have competence to produce IL-10, which is more evident in M-CLL (5, 13). We have now observed that the variable capacity to produce IL-10 is associated with the nature of the cell of origin, and particularly with the associated degree of anergy. We also found a clear link between IL-10 production and methylation status of the *IL10* locus, including a region with a STAT3 biding site, suggesting epigenetic programming of B10 function in CLL.

IL-10 has different immunoregulatory functions by acting on innate and cognate immunity, including regulation of cytotoxic and helper T cells (41). IL10 activity on T-cells is more difficult to investigate because autologous CLL T-cells are functionally impaired *in vivo* (42).

The IL-10 producing B10 cells differentiate from B10pro typically within the CD5+ B-cell population (8, 43-45). The phenotype of circulating CLL cells is remarkably similar to normal B10pro (5, 13). Our study now adds that pro-inflammatory cytokines were not increased following TLR9 stimulation, while IL-10 was the most highly produced cytokine and we focused on it for its known function in B10 cells (8).

A number of studies have indicated that serum levels of IL-10 in CLL and lymphomas, can be of viral or human origin and can predict prognosis (46-50). However the serum amounts in CLL may likely reflect increases in the viral IL-10 (5). In fact, human IL-10 can hardly be detected (46-50), and, like in our cohort, circulating (human) IL-10+ CLL cells are very hard to identify (14). However, analysis of the public gene expression profile database has revealed that *IL10* is higher in the CLL cells from lymph node than from those in peripheral blood (15), and serum levels are not expected to be indicative of the cytokine profiles within CLL tissue sites (17).

A recently proposed ‘natural’ stimulus for CLL and Breg to produce IL-10 in lymph node is BAFF (51). BAFF receptors require SYK for signal transduction (52). Although CpG is unlikely operating *in vivo* for CLL activation, the incidental observation that TLR9 also require SYK to transduce STAT3 mediated signals was interesting (18). SYK is emerging as a key therapeutic target which is under investigation in clinical trials with different compounds in CLL (53). In the Eµ-Tcl1 mouse CLL model, IL-10 production is inhibited by the SYK inhibitor BAY 61-3606 (54). Our result in human CLL that R406 suppresses induction of IL-10 may set a new rationale to therapeutic targeting of CLL with SYK inhibitors, in antithesis to chemotherapy- or PI3K-inhibitors-based regimens that aim to reduce tumor burden while having a negative impact on immune function (55, 56).

The association between the B10pro cell nature of CLL cells and the anergic status is a novel finding. Anergy has been defined as a state of lethargy which follows an initial response to antigen in the absence of cognate T-cell help (12). The functional signature of anergy is high basal intracellular Ca2+ levels, constitutive activation of ERK1/2 and SHIP-1, NF-ATc1 nuclear translocation with low sIgM levels/signaling capacity (12). The circulating CLL cells summarize these features, particularly prominent in the M-CLL subset (19, 20, 22, 24). We now find that levels of induced IL-10 inversely correlate with IgM levels and signaling capacity. This points to the capacity of cells to produce IL-10 as a new feature of anergy at least in CLL. Normal human regulatory B10 cells also have lower surface IgM with maintained levels of sIgD (8, 43, 57).

Our analyses of the *IL10* locus methylation profile suggests that differential epigenetic programming may underlie the degree of B10 differentiation capacity in CLL. The distinct clinical behavior and origin of the M-CLL and U-CLL subsets now appear to be reflected by broad differences in the epigenomes of U-CLL and M-CLL (29-32). Our genome-wide analysis of DNA methylation reveals that *IL10* ranks within the top 1% of the most highly differentially methylated genes (146 out of 20,622 genes) between U-CLL and M-CLL, revealing that hypomethylation of the *IL10* gene body is a marked feature of M-CLL (30, 31). In concordance with the vast majority of global methylation differences between CLL subtypes occurring outside of promoters (29, 31), here we find that differences in the *IL10* locus are located at discrete regions within the gene body, not involving the promoter. Non-promoter regions, such as enhancers, have now been appreciated to provide an essential function in the regulation of gene expression (40).

Here we find that variable methylation of *IL10* occurs within the *IL10* gene body and a region variably-methylated between U-CLL and M-CLL, CLL-VMR2, contains a functional STAT3 binding site. We observe that IL-10 inducibility via CpG stimulation appears to require STAT3 activity, but that STAT3 phosphorylation does not differ between the U-CLL and M-CLL subsets, advocating that the differential response to CpG stimulation may depend on the ability of pSTAT3 to interact with the *IL10* locus. IL-10 expression is known to be activated by STAT3 interacting with the *IL10* promoter in normal or CLL B cells and in T cells (16, 58, 59). STAT3 also interacts with an upstream enhancer (-12 kb) and other STATs interact with an enhancer element within intron 4 (+3110–3272 bp) (58, 60). However, these regions are all equally unmethylated among CLLs, thus in an epigenetic state likely permissive for STAT3 binding. We show that STAT3 will bind and activate CLL-VMR2 only when the region is not methylated, suggesting a role for variable methylation of CLL-VMR2 controlling the observed inverse correlation with *IL10* expression. Together these data point to this novel gene body region as being potentially involved in the differential expression of IL-10 between U-CLL and M-CLL and raises the possibility that the epigenetic configuration of the *IL10* locus at the CLL-VMR2 STAT3-binding site plays an important role in controlling IL-10 production in CLL.

In conclusion, our study shows that CLL cells appear to be B10pro cells, poised to produce IL-10, possibly in tissue sites. M-CLL cases have a higher capacity to respond than U-CLL, reflected by differential epigenetic programming of the *IL10* gene between the two subsets. There is a clear link between capacity to produce IL-10 and anergy. However, due to the high burden of disease in CLL, even low levels of production of IL-10 by individual CLL cells could lead to suppressive effects on immune status.

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# Conflict-of-interest-disclosure

The authors declare no competing financial interests.

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# Figure legends

**Figure 1. Immunoregulatory cytokine profile of CLL cells.** Purified CLL cells from the patient cohort at CSU (n=16) were cultured with CpG (7.5µg/ml) for 24 hours. Secreted cytokine concentrations in the culture supernatant were determined using the Luminex multiplex bead array assay. Horizontal lines indicate mean values for each cytokine. All cytokines were significantly lower than IL-10 secretion (TNF-α, *P*=0.002; all other cytokines, *P*<0.0001).

**Figure 2. Production of IL-10 by CLL is associated with an anergic phenotype.** PBMCs from CLL patients at CSU were cultured at 2 x 107/ml and stimulated with CpG (7.5µg/ml). Following 24 hours stimulation, PMA, ionomycin and brefeldin A was added for 5 hours before IL-10 production by CD19+CD5+ cells was detected by flow cytometry. (A) Two representative plots showing IL-10 production by the CLL population (case 482, M-CLL and case 668 U-CLL).The percentage of IL-10 competent CLL cells amongst the patient cohort was divided by (B) mutational status, (C) sIgM expression and (D) anti-IgM calcium signaling capacity. Circles represent M-CLL, squares represent U-CLL. Horizontal lines indicate mean values. The statistical significance of difference was analyzed using the Mann-Whitney test.

**Figure 3. *IL10* transcript levels by CLL are associated with IGHV status and correlate with IL-10 protein production.** (A) *IL10* transcript was quantified by qPCR after culture with no stimulation or with CpG (7.5µg/ml) for 24 hours of purified CLL cells (n=15, CSU cohort). Horizontal lines indicate mean values. The statistical differences between M-CLL and U-CLL were analyzed using the Mann-Whitney test. (B) Correlation between *IL10* transcript levels and percentage of IL-10+ CLL cells determined by intracellular flow cytometry. Linear regression and Spearman correlation are shown. Circles represent M-CLL, squares represent U-CLL.

**Figure 4. Methylation of the *IL10* gene locus and the association with IL-10 production and anergy**. (A) DNA methylation profiling of the *IL10* gene locus by MassARRAY in 36 CLL cases from the DKFZ cohort (M-CLL=26, U-CLL=10). Analyzed CpGs are indicated as black lines at the bottom of the panel and positions are relative to the *IL10* transcriptional start site. The CpGs used to calculate the mean methylation of the promoter and CLL-VMR1 and CLL-VMR2 are indicated. H3K27ac data were generated from GM12878 cells and were obtained from the UCSC Genome Browser and produced by the ENCODE consortium. The sequence cloned into luciferase vectors for functional analysis of CLL-VMR2 is indicated. (B-C) CLL cells from the CSU cohort were assessed for *IL10* gene methylation by MassARRAY. Correlation between the degree of methylation in (B) CLL-VMR1 and (D) CLL-VMR2 of the *IL10* gene and IL-10 protein production following CpG stimulation. IL-10 protein production was measured by intracellular flow cytometry. Linear regression and Spearman correlation shown. (D-G) DNA methylation of CLL-VMR1 and CLL-VMR2 in U-CLL versus M-CLL (D-E) and in sIgM signalers versus non-signalers (F-G). Circles represent M-CLL, squares represent U-CLL.

**Figure 5. Functional analysis of CLL-VMR2 reveals STAT3-dependent activity**. (A) Alignment of the consensus STAT3 motif with the predicted STAT3 binding site in CLL-VMR2 in 7 mammalian species. (B) Proximity ligation assays evaluating the binding of STAT3 protein to the putative STAT3 binding site within CLL-VMR2. Error bars represent the standard deviation of n=3 experimental replicates. (C) Luciferase enhancer assays evaluating the activity of CLL-VMR2 in combination with STAT3 expression in cell lines. Luciferase activity was further evaluated in reverse orientation and following mutation of the STAT3 binding site. A reporter containing consensus STAT3 binding sites was used as a positive control. Error bars represent the standard deviation of n=3 experimental replicates for all luciferase assays. (D) The CLL-VMR2 sequence was cloned into a CpG-free enhancer plasmid and luciferase activity was evaluated following *in vitro* methylation of the CLL-VMR2 sequence. \*indicates P<0.05 in all panels.

**Figure 6. STAT3 activity and *IL10* induction.** (A) PBMCs from CLL patients were cultured at 2 x 107/ml in the presence or absence of CpG (7.5µg/ml) for 5hrs. STAT3 activity was assessed by immunoblotting, probing for phosphorylated STAT3 (Y705). Actin was used as a loading control. Band intensities were quantified using ImageJ and normalized to actin. The percentage of IL-10+ CLL cells was determined by intracellular flow cytometry. (B) PBMCs from CLL patients (n=3, 2 M-CLL samples 482 and 684, and 1 U-CLL sample 635) were cultured in the presence or absence of varying concentrations of R406 (SYK inhibitor) or AG-17 (PYK2 inhibitor) for 1 hour before stimulation with CpG. A representative blot (482) with inhibition of STAT3 phosphorylation is shown as an example. *IL10* transcript was quantified by qPCR after culture (n=3). Bars indicate mean values + SEM.