International Immunology, Vol. 23, No. 6, pp. 375–384
doi:10.1093/intimm/dxr027
Advance Access publication 6 May 2011

The B lineage transcription factor E2A regulates apoptosis in chronic lymphocytic leukemia (CLL) cells

Lela Kardava[1], Qi Yang[1], Anthony St. Leger[1], Kenneth A. Foon[2], Suzanne Lentzsch[3], Abbe N. Vallejo[4], Christine Milcarek[1] and **Lisa Borghesi[1]**

[1]Department of Immunology, University of Pittsburgh School of Medicine, 200 Lothrop Street, Pittsburgh, PA 15261, USA
[2]Department of Hematological Malignancies, Nevada Cancer Institute, One Breakthrough Way, Las Vegas, NV 89135, USA
[3]Division of Hematology/Oncology, University of Pittsburgh Cancer Institute, 5117 Center Avenue, Pittsburgh, PA 15213, USA
[4]Department of Pediatrics and Immunology, University of Pittsburgh School of Medicine, Children's Hospital of Pittsburgh Rangos Research Center, 3640 Fifth Avenue, Pittsburgh, PA 15261, USA

Correspondence to: L. Borghesi; E-mail: borghesi@pitt.edu

Received 18 February 2011, *accepted* 5 April 2011

Abstract

Chronic lymphocytic leukemia (CLL) is a common malignancy characterized by the accumulation of B lymphocytes with an antigen-experienced activated $CD19^+CD5^+$ clonal phenotype. Clinically, ~50% of cases will behave more aggressively. Here, we investigate the role of the major B-cell transcription factor E2A, a known regulator of B-cell survival and proliferation, to CLL persistence. We show that E2A is elevated at the mRNA and protein levels relative to normal B-cell subsets. E2A silencing in primary CLL cells leads to a significant increase in spontaneous apoptosis in both $CD38^+$ (aggressive) and $CD38^-$ (indolent) cases. Moreover, E2A knockdown synergizes with the immunomodulatory drug lenalidomide to reduce CLL viability. E2A is known to restrain the proliferation of primary B and T lymphocytes at multiple stages of maturation and we report that targeted E2A disruption increases the frequency of Ki-67^+ CLL cells in the absence of effects on *de novo* proliferation. At the molecular level, E2A siRNA-treated CLL cells display reduced expression of key genes associated with survival and cell cycling including p27, p21 and mcl-1, of which the former two are known E2A target genes. Thus, E2A, a key transcription factor associated with the B-cell activation profile, regulates apoptosis in CLL and may contribute to disease pathology.

Keywords: apoptosis, chronic lymphocytic leukemia, E2A, IMiD, lenalidomide

Introduction

Chronic lymphocytic leukemia (CLL), one of the most common forms of adult leukemia in the western world, is associated with the progressive accumulation of malignant clones of apparently mature B-cell origin that express the CD5 antigen. Incapability of undergoing appropriate programmed cell death as well as profound defects in cell cycle regulation play an important role in CLL pathogenesis (1). There are two major CLL disease pathologies: aggressive and indolent. The aggressive disease manifestation is classically associated with the expression of CD38 (2), over-expression of ZAP-70 (3) and unmutated IgV_H gene profile (4, 5). Additional prognostic markers have been described (6–8) including characteristic microRNA signatures (8, 9). Leukemic clones from patients with stable disease usually express mutated rather than unmutated IgV_H genes indicating that the molecular defect(s) is not directly linked to somatic hypermutation processes during B-cell differentiation. Despite the variable phenotypic and mutational status, CLL cells display a common and characteristic gene expression profile that is closer to memory B cells than to naive B cells, $CD5^+$ B cells, germinal center B cells and centroblasts or centrocytes (10). Cells from both the aggressive and indolent clinical categories have an activated and antigen-experienced phenotype (4, 11, 12). Antigen stimulation in the context of co-stimulatory molecules and cytokines and constitutively activated signaling pathways are thought to play an important role in CLL persistence and protection from apoptosis, thereby contributing to leukemic propagation and insensitivity to chemotherapeutics (11, 13–15).

B-cell stimulation through the antigen receptor elicits a host of activation-associated changes including up-regulation of the key B lineage transcription factor E2A (16). E2A is a master transcriptional regulator of B lineage maturation and function. In the absence of E2A, lymphocyte development is

ablated at the progenitor stage in the bone marrow (17, 18). Conditional deletion studies demonstrate a differential requirement for E2A activity in the periphery. Once the peripheral B-cell compartment is formed, E2A is dispensable for the maintenance of mature B cells and plasma cells (19). However, E2A is critical for germinal center B-cell persistence (19) as well as B-cell activation (20), and its down regulation is essential for differentiation of germinal center B cells into plasma cells (21). In peripheral compartments, E2A promotes isotype class switching and somatic hypermutation through the up-regulation of activation-induced deaminase (22, 23). E2A also regulates B lymphocyte proliferation and survival. Work from our group and others have shown that E2A can either restrain or promote cell proliferation, depending on the cell type or developmental stage (24–26). Blockade of E2A activity by the enforced expression of an inhibitory binding partner induces caspase-dependent apoptosis, highlighting a role for E2A in promoting lymphocyte viability (27). In man, disruptions in E2A are associated with cancers of both the B and T lymphocyte lineages. Within the B lineage, lesions in E2A are mechanistically linked to pre-B acute lymphoblastic leukemia and Hodgkin lymphoma as well as cancers of T lineage origin (28, 29). Given the role of E2A in B-cell persistence and activation and the link to cancers of B lineage origin, targeted manipulation of this transcription factor may increase our understanding of disease pathogenesis and improve the efficacy of existing therapies in CLL.

Here, we exploit siRNA knockdown nucleofection to evaluate the contribution of the master transcriptional regulator E2A to CLL persistence. We show that E2A is over-expressed in CLL cells relative to normal B-cell subsets in a manner that is consistent with the activation status of this leukemia. We place these findings in a biological context by showing that loss of E2A leads to increased apoptosis, and the combination of E2A knockdown in the context of the immunomodulatory (IMiD) drug lenalidomide dramatically enhances the pro-apoptotic effects of this agent. We then demonstrate that efficient and specific knockdown of E2A alters the expression of a key set of genes associated with survival.

Methods

Study population

Peripheral blood samples were obtained from 26 B–CLL patients (age range 39–86 years, median age 58 years) as well as eight young (age range 17–51 years) and 10 aged (>80 years) healthy individuals. Blood samples were collected in accordance with the Declaration of Helsinki. Diagnosis of CLL was based on clinical, immunophenotypic and morphologic criteria (30, 31). Patient information including clinical stage and treatment history is depicted in Table 1.

Table 1. Patient characteristics

Patient	Age	Sex	Clinical stage	CD38 (%)[a]	E2A (%)[a]	E2A MFI[b] $CD38^{pos}$:$CD38^{neg}$	Treatment
CLL-1	60	F	Stage I	26	87	n.d.	Observation
CLL-2	57	F	Unknown	18	75	645:638	Unknown
CLL-3	77	M	Stage 0	62	96	713:688	Observation
CLL-4	86	F	Original CLL reconsidered for mantle cell lymphoma	44	83	1137:1124	Observation
CLL-5	49	M	Stage II	14	83	n.d.	Fludarabine/Rituxan/ Bendamustine
CLL-6	78	M	Stage II	27	61	1174:1200	Rituxan/Bendamustine
CLL-7	60	F	Stage IV	0	68	n.d.	Fludarabine/Cytoxan/ Rituxan
CLL-8	63	F	Stage I	13	72	602:594	Observation
CLL-9	51	M	Stage 0	<1	53	n.d.	Observation
CLL-10	53	F	Stage II	12	56	3857:3591	Observation
CLL-11	80	F	Stage I	16	58	n.d.	Observation
CLL-12	64	M	Stage I	18	65	n.d.	Observation
CLL-13	67	F	Stage 0	25	52	n.d.	Observation
CLL-14	48	M	Stage II	42	63	1204:1163	Fludarabine/Cytoxan
CLL-15	50	M	Stage II	35	76	950:947	Observation
CLL-16	39	M	Stage I	45	47	889:890	Observation
CLL-17	50	M	Stage II	21	69	n.d.	Pento/Cytoxan/Rituxan
CLL-18	66	F	Stage IV	33	63	n.d.	Promace CytoBOM
CLL-19	45	M	Stage 0	31	n.d.	n.d.	Observation
CLL-20	57	M	Stage II	34	78	3211:3233	Observation
CLL-21	73	F	Stage II	38	n.d.	n.d.	FCR Lite
CLL-22	54	F	Stage I	36	71	1214:1156	Observation
CLL-23	42	M	Stage I	24	n.d.	n.d.	Observation
CLL-24	66	F	Stage I	48	88	757:731	Observation then FOLFOX
CLL-25	60	F	Stage I	13	56	584:546	Observation
CLL-26	49	M	Stage II	52	97	1760:1709	Fludarabine/Cytoxan

n.d., not done.
[a]Gated on $CD19^{+}CD5^{+}$.
[b]Mean fluorescence intensity (MFI) of E2A expression in $CD38^{+}$ versus $CD38^{-}$ CLL clones within individual donors of $CD38^{+}$ clinical status.

Human peripheral blood buffy coats from healthy donors were from the Pittsburgh Central Blood Bank. Human subjects research was approved by the University of Pittsburgh Institutional Review Board.

Mice

Mice bearing targeted mutations in the *E2A* gene have been described elsewhere (24). Animals were treated humanely in accordance with federal and state government guidelines and the Institutional Animal Care and Use Committee of the University of Pittsburgh.

Isolation and culture of B cells

PBMCs were separated from heparinized venous blood or buffy coats by density gradient centrifugation using lymphocyte separation medium. $CD19^+$ B cells were purified from PBMCs from healthy donors or CLL patients using a negative isolation kit according to manufacturer instructions (Miltenyi Biotec, Auburn, CA, USA). Purified $CD19^+$ B cells were cultured in RPMI-1640 medium with L-glutamine, penicillin (100 U ml^{-1}), streptomycin (100 μg ml^{-1}) and 10% fetal bovine serum, in the presence or absence of anti-CD40 (1 μg ml^{-1}) plus anti-IgM (5 μg ml^{-1}) and incubated for 48 h at 37°C (culture reagents from Fisher Scientific, Waltham, MA, USA).

Surface and intracellular flow cytometry

Surface staining of the cells was performed using the following mAbs from BD Biosciences (San Jose, CA, USA): FITC-conjugated anti-CD24, anti-CD5, APC-conjugated anti-CD19, anti-CD27 and PE-Cy5-conjugated anti-CD38. Intracellular staining for E2A (clone G127-32) was performed as we have described (32). In brief, surface-stained cells were resuspended in 200 μl of Cytofix (BD Biosciences) for 20 min on ice, washed with PBS/5% FCS and permeabilized with 200 μl of PBS/0.2% Tween 20 for 15 min at 37°C, and washed again. Cells were then stained for intracellular E2A and analyzed promptly. Flow cytometric analysis was performed on an LSR II flow cytometer (BD Biosciences) and analyzed with FlowJo software (Tree Star, Ashland, OR).

Viability and proliferation assays

Cell viability was assessed by flow cytometry using Annexin V and DAPI (BD Biosciences) or by MitoTracker Red CMXRos dye (Invitrogen, Carlsbad, CA) following manufacturer's protocol as we have described (24, 32). For cell cycle analysis, cells were re-suspended in 100 μl Cytofix/Cytoperm on ice for 15 min, washed and incubated with 100 μl Cytoperm Plus (both from BD Biosciences). After refixation with Cytofix/Cytoperm, cells were stained with anti-Ki-67 mAb or isotype control per manufacturer's instruction (BD Biosciences). Bromodeoxyuridine (BrdU) and tritiated thymidine (^{3}H-TdR) incorporation assays were performed as we have previously described (32). For BrdU assays, immediately following nucleofection, 2×10^6 CLL cells were cultured in 24-well plates for 24–48 h, and 10 μM BrdU was added during the last 18 h of culture. Cells were then harvested and stained with BrdU antibodies using the BrdU flow kit (BD Bioscience) according to the manufacturer's instructions. For ^{3}H-TdR incorporation assays, 4×10^5 nucleofected CLL cells were cultured in 96-well plates in the presence of 1 μCi per well of isotope (present throughout the entire culture period), and radiolabel incorporation was determined by counting radioactivity after 24 h. As a control for detection of proliferation, total murine splenocytes were seeded at same cell numbers as CLL cells in the presence (positive control) or absence (negative control) of 10 μg ml^{-1} lipopolysaccharide, and wells were pulsed with either BrdU or ^{3}H-TdR for the last 6 h of culture. Murine cultures were harvested in parallel with CLL cultures.

siRNA transfection

Transient transfections of B–CLL cells were performed using the Amaxa (Koln, Germany) nucleofection system according to the manufacturer's instructions. Briefly, 1 to 3×10^6 cells were resuspended in 100 μl of transfection solution, mixed with 3 μg of E2A siRNA or control non-targeting siRNA (Dharmacon, Lafayette, CO, USA; 009384 and 001210, respectively) and nucleofected using program U-15. Cells were rapidly transferred to preheated complete medium (RPMI/10% FCS) and incubated for 24–48 h at 37°C.

Quantitative PCR

Total RNA was isolated from 0.5×10^7 to 1×10^7 cells using the RNEasy kit (Qiagen, Valencia, CA, USA) with on-column DNA digestion as described by the manufacturer. Total RNA was reverse transcribed and cDNA was analyzed by quantitative PCR (QPCR) in duplicate or triplicate wells in the ABI Prism 7900HT. E2A and NFAT levels were measured using TaqMan probe/primer mix (Applied Biosystems, Foster City, CA, USA; hs00413032-m1 and hs00234855-ml, respectively), while all other gene targets were assayed using previously published primers with SYBR Green technology (33–37). Data were normalized to the housekeeping gene 18S rRNA or β-actin by the comparative $\Delta\Delta Ct$ method.

Statistical analysis

Multiple comparisons were performed with either analysis of variance followed by Tukey's HSD post-hoc analysis or, for paired data, the nonparametric Wilcoxon rank sum test. Differences were regarded as significant at $P < 0.05$. Analyses were performed using the JMP version 5.1 statistical software package (SAS Institute, Cary, NC).

Results

E2A protein is expressed at high levels in CLL cells

Perturbations in E2A expression are associated with B lineage cancers. The profile of E2A expression in B-cell subsets from healthy individuals versus CLL patients has not been examined, and we analyzed E2A at both the protein and RNA levels. Fig. 1(A) depicts the phenotypic resolution of mature B cells ($CD19^+CD24^+CD38^+CD27^-$), memory B cells ($CD19^+CD24^+CD38^{lo/-}CD27^+$) and the rare $CD19^+CD5^+$ B-cell subset from healthy donors as compared with the $CD19^+CD5^+$ population characteristic of CLL. We first examined E2A protein levels in young healthy donors (<51 years) by intracellular staining and flow cytometry. Figure 1(B) (left column) shows a representative E2A protein profile of each

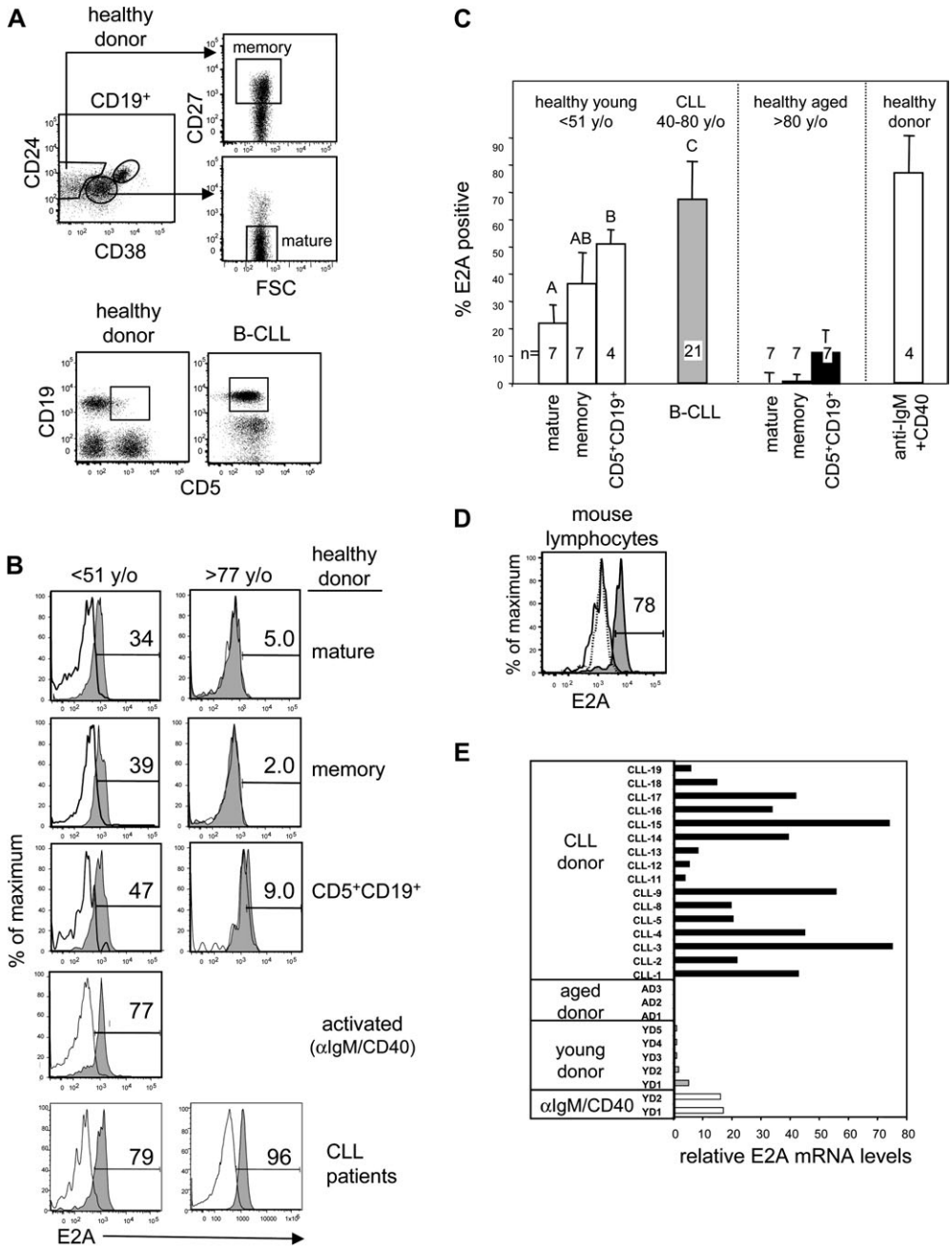

Fig. 1. CLL cells have increased E2A protein and mRNA relative to normal B-cell subsets. (A) Phenotypic gating of normal B-cell subsets or CLL. $CD19^+$ peripheral blood lymphocytes from healthy donors purified by negative selection with magnetic separation were stained with antibodies to CD24, CD38 and CD27 to distinguish mature and memory subsets (upper panels) or the $CD19^+CD5^+$ compartment in normal donors versus CLL patients (CLL-3 is shown) (lower panels). (B) $CD19^+$ PBMCs from healthy donors or CLL patients (CLL-15 left; CLL-3 right) stained with antibodies to detect surface phenotype were then fixed and permeabilized and incubated with antibodies to intracellular E2A (shaded histogram) or the isotype control (open histogram). Donor age range is indicated. Activated $CD19^+$ cells were stimulated with anti-CD40/anti-IgM for 48 h before analysis. (C) Comprehensive analysis of E2A protein expression across individuals. The number of independent donors is indicated in the figure (*n*). The data represent mean ± SD of patients. The letters A, B and C indicate statistical significance as determined in an analysis of variance followed by Tukey–Kramer HSD post-hoc analysis, $P < 0.05$. Donors were CLL-1, -2, -3, -5, -6, -7, -8, -9, -10, -11, -12, -13, -14, -15, -17, -18, -20, -22, -24, -25 and -26. (D) The specificity of the anti-E2A antibody (recognizes both mouse and human E2A) was confirmed by examining intracellular E2A in bone marrow B lineage progenitors from wild-type mice (shaded histogram) versus mice bearing a targeted mutation in the *E2A* gene (open histogram). Nonspecific staining of wild-type cells with the isotype control is also shown (dotted line). (E) E2A mRNA levels were examined in normal $CD19^+$ peripheral B lymphocytes, anti-CD40/anti-IgM-stimulated $CD19^+$ cells or CLL cells from the indicated donors using real-time QPCR. Data were normalized to 18S and analyzed relative to expression in healthy B cells from young donors, which was arbitrarily set to 1.

phenotypic subset, while Fig. 1(C) presents a cumulative analysis across multiple donors. E2A protein expression in mature, memory and $CD5^+CD19^+$ B-cell subsets from the peripheral blood of young donors was 23 ± 6.5% (n = 7), 38 ± 11.0% (n = 7) and 52.9 ± 4.6% (n = 4), respectively. The anti-E2A mAb recognizes both human and mouse E2A, enabling us to confirm the sensitivity and integrity of this detection method by using knockout mice. While E2A was detectable in 78% of bone marrow precursors from wild-type mice, this signal was reduced to background levels in mice bearing a targeted mutation in the *E2A* gene, indicating the specificity of staining (Fig. 1D). Our finding that E2A is present at high levels in human B lineage progenitors in the bone marrow (32) and at low levels in peripheral B-cell subsets parallels data in mice, indicating a conserved pattern of expression across species.

CLL is a disease of elderly individuals (median age 65 years) and analysis of B-cell subsets from aged healthy donors (>80 years) reveals that E2A is virtually undetectable in mature, memory and $CD5^+CD19^+$ B-cell subsets: 0.7 ± 1.7% (n = 7), 1.1 ± 2.8% (n = 7) and 12 ± 8.0% (n = 4), respectively (Fig. 1B, right column, and Fig. 1(C), left). In marked contrast, CLL cells express high levels of E2A protein in a manner not characteristic of normal resting peripheral B-cell subsets of any age. Across 21 individuals, 71 ± 14% of CLL cells were $E2A^+$ (Fig. 1B and C); donor age range 40–80 years. E2A expression is known to be upregulated following B-cell activation (38) and approximately 68–77% of $CD19^+$ B cells are $E2A^+$ following anti-CD40/anti-IgM stimulation (Fig. 1B, left column, and data not shown). Consistent with this finding, CLL cells exhibit increased expression of CD23, CD25 and CD71 as well as other markers characteristic of antigen-experienced cells (39), suggesting the possibility that chronic antigen stimulation may contribute to the persistent elevation of E2A expression.

Examination of E2A mRNA expression by QPCR mirrored the protein data. We quantified E2A mRNA in B cells from 16 CLL patients, 5 young normal donors, 3 aged normal donors as well as anti-CD40/anti-IgM-activated B cells from 4 young donors and found that E2A transcript is highly expressed in CLL cells, moderately expressed in activated B cells, is expressed at low levels in primary peripheral B cells from both young and aged normal donors (Fig. 1E). Thus, our data reveal interesting differences in E2A expression in peripheral blood subsets from CLL patients versus normal aged-matched donors, a finding that extends previous studies in which *CLL* gene expression was compared with tonsillar subsets or cord blood (10, 40). CD38 expression is linked to cellular activation and disease course but no relationship was detectable between the expression of E2A and CD38 in CLL cells (data not shown). Taken together, these data demonstrate that E2A is expressed at heightened levels in CLL cells relative to normal peripheral blood B-cell subsets at both the mRNA and the protein levels, in a manner consistent with cellular activation.

Efficient knockdown of E2A in primary CLL cells

E2A is a major regulator of B-cell survival and proliferation, and we aimed to establish the mechanistic contribution of this transcription factor to CLL pathology. We designed a knockdown approach using siRNA delivered by nucleofection, a strategy that has been recently used with success in primary CLL (41, 42). We nucleofected primary CLL cells with E2A siRNA or control siRNA, and 24 h later measured E2A protein and transcript levels. As shown in Fig. 2(A) intracellular E2A was reduced from 82 to 2%, indicating the efficacy of knockdown at the protein level. In an analysis across cells from multiple donors, intracellular staining was reduced from 71.6 ± 8.7% to 7.6 ± 3.7% (n = 8), a 90% reduction in expression. Similar knockdown results were obtained 48 h after transfection (data not shown). Likewise, E2A mRNA levels were reduced 80–90% as assessed by reverse transcription (RT)–QPCR (Fig. 2B and data not shown). No gross differences in viability were detectable between mock (non-transfected) versus control siRNA transfected treatments as assessed using paired samples from five individual donors (67 ± 12% and 70 ± 14%, respectively, $P > 0.05$, CLL-6, -7, -9, -11 and -12; and Fig. 3B). The targeted knockdown was specific for E2A because nucleofection with E2A siRNA had no detectable effect on

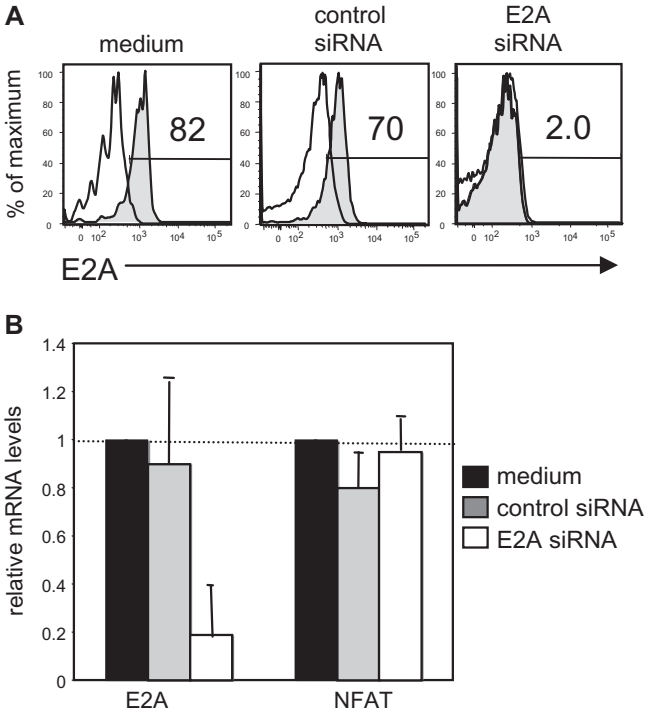

Fig. 2. Targeted knockdown of E2A in CLL using siRNA. CLL cells were transfected with medium alone, control siRNA or E2A-specific siRNA using Amaxa Nucleofection. (A) E2A protein levels were measured in CLL cells 24 h after transfection as in Fig. 1. Cells were stained with antibodies to intracellular E2A (shaded histogram) or the isotype control (open histogram). The results are representative of eight independent experiments (CLL-6 is depicted). (B) E2A mRNA levels were quantified in CLL cells 24 h after transfection using real-time PCR. Data were normalized to 18S and analyzed relative to expression in mock-transfected CLL cells (defined as 1.0, dotted line). As a control for siRNA specificity, levels of NFAT were also examined. The results represent the average of three to four independent experiments using donors CLL-3, -4, -6 and -7.

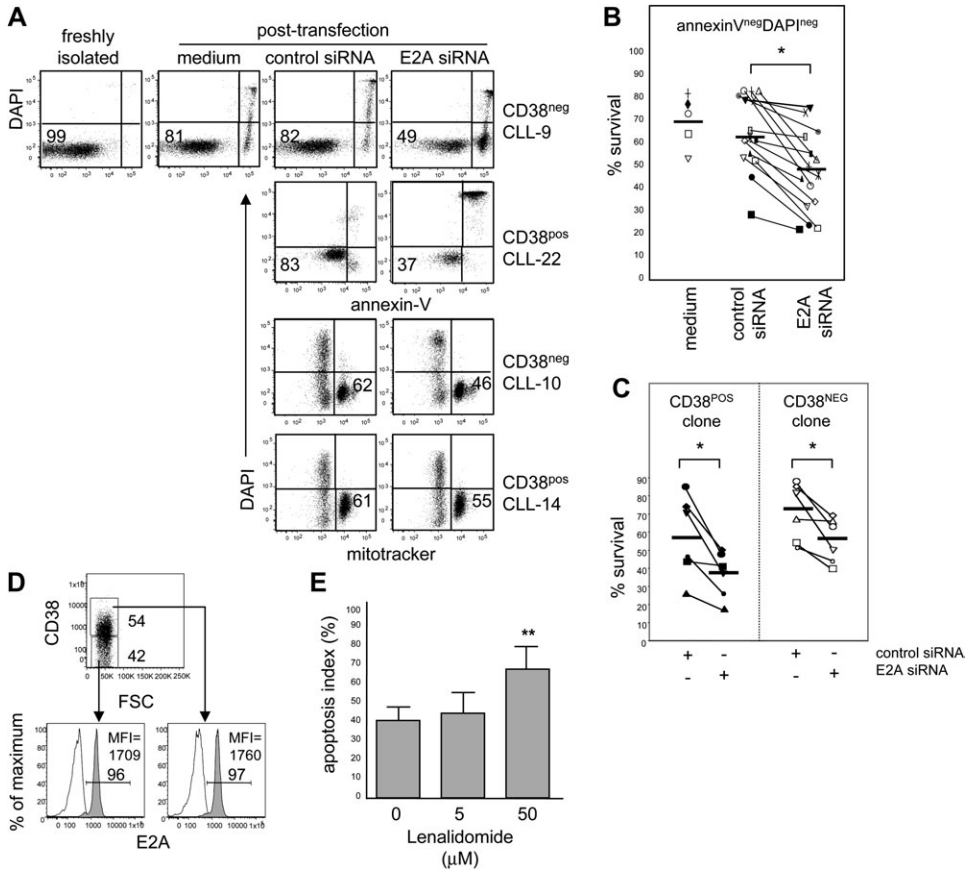

Fig. 3. E2A inhibition promotes spontaneous apoptosis of CLL cells. CLL cells were mock-transfected (no siRNA) or transfected with control siRNA or E2A siRNA, and 48 h later cells were stained with either annexinV/DAPI or mitotracker/DAPI. Representative staining of individual donors is shown in (A), while an analysis across multiple patients is summarized in (B), $*P < 0.05$ in a non-parametric Wilcoxon signed rank test for paired samples. The individual CLL donors in Fig. 3(A) are named by each panel; Fig. 3(B) depicts data from CLL-4, -7, -8, -9, -10, -11, -12, -13, -14, -15, -16, -17, -19, -20, -21 and -22. In (C), $CD38^-$ and $CD38^+$ CLL clones within individual patients diagnosed were examined: CLL-12, -16, -20, -21, -22 and 23. (D) The magnitude of E2A expression in $CD38^-$ versus $CD38^+$ CLL clones within an individual donor is depicted for CLL-26; additional patients are detailed in Table 1. Intracellular E2A (shaded histogram), isotype control (open histogram), MFI = mean fluorescence intensity. (E) CLL cells transfected with E2A siRNA or control siRNA were cultured in the presence or absence of the indicated concentration of lenalidomide or vehicle (DMSO) for 48 h after which apoptosis was assessed by Annexin V staining. The data are presented as the mean apoptosis index using the formula [100 − (% live cells E2A siRNA)/(% live cells control siRNA) × 100] across three independent CLL donors (CLL-22, -23 and -24).

another transcription factor, NFAT, which is constitutively expressed in CLL cells (Fig. 2B) (43).

E2A knockdown increases CLL cell apoptosis

E proteins are required for the persistence of B cells in a developmental stage-specific manner (26, 32). We examined the survival of leukemic cells from 12 CLL cases 48 h after nucleofection with E2A siRNA versus control siRNA or treated with medium alone. For this work, we assessed spontaneous apoptosis using Annexin V/DAPI or mitotracker staining, and identical results were observed with both methods. Representative staining profiles are depicted in Fig. 3(A) and an analysis across multiple patients in shown in Fig. 3(B). Both approaches revealed the frequency of viable CLL cells (Annexin V^-DAPI^- or mitotracker$^+DAPI^-$) was significantly reduced from 63 ± 16% to 44 ± 16% ($n = 14$) in control siRNA transfected versus E2A siRNA-transfected cells, respectively ($P < 0.05$); the survival of mock-transfected cells was 69 ± 12% ($n = 5$) comparable to previous reports (44). Increased sensitivity to spontaneous apoptosis was detectable in patients with $CD38^+$ or $CD38^-$ CLL status (Fig. 3B and data not shown) as well as from the $CD38^+$ and $CD38^-$ clones within individual patients (Fig. 3C; $n = 6$ independent donors). Consistent with these findings, E2A was expressed at comparable levels as assessed by mean fluorescence intensity in $CD38^+$ and $CD38^-$ clones within nine individual donors with $CD38^+$ CLL status (Fig. 3D, representative profile, and Table 1).

Despite the availability of chemotherapeutic drugs including alkylating agents (chlorambucil, cyclophosphamide), purine analogs (fludarabine) or monoclonal antibodies (rituximab), patients with CLL are not cured and additional therapeutic options are important (45). The IMiD agent lenalidomide has high clinical activity in CLL and other B cell malignancies; it appears to be nontoxic at low doses and requires combination with other synergistic agents for optimal effect (46). IMiD anticancer agents can cause apoptosis or growth arrest in a cancer cell-type-specific manner

(47, 48); however, the precise mechanisms of action remain unclear. We determined if the effect of E2A down regulation on CLL apoptosis can be sensitized by the treatment with lenalidomide. CLL cells transfected with E2A siRNA or control siRNA were exposed to two different concentrations of lenalidomide (5 or 50 μM) for 48 h. Consistent with previous findings (47), lenalidomide alone failed to induce apoptosis of primary CLL cells (Fig. 3E). By contrast, the combined effects of lenalidomide treatment and E2A knockdown on CLL apoptosis were significantly increased relative to the effect of E2A knockdown alone ($P < 0.05$). As shown in Fig. 3(E), after 48 h of combined treatment, the viability of CLL cells was significantly inhibited in a dose-dependent manner. The mean apoptosis index was 39 ± 3% after E2A knockdown alone and 66 ± 21% at 50 μM lenalidomide plus E2A knockdown ($n = 3$ donors). These results indicate that E2A knockdown synergizes with lenalidomide to reduce CLL viability. This effect appeared to be specific to lenalidomide as E2A siRNA knockdown in the context of a different IMiD derivative, actimid, had no significant effect on CLL apoptosis (data not shown).

E2A knockdown alters the relative frequency of Ki-67$^+$ CLL cells but does not promote de novo proliferation

Despite features of cellular activation, circulating CLL cells are arrested at the $G_{0/}$early G_1 stage and do not undergo proliferation (1, 39). We and others have recently shown that E2A inhibits lymphocyte proliferation (26, 32) and we examined a similar role for E2A in primary CLL cells. Interestingly, E2A down regulation was sufficient to alter the cell cycle profile of CLL as assessed using the Ki-67 proliferation antigen. Figure 4(A) shows a representative flow cytometry profile for individual patients, while Fig. 4(B) summarizes the data across multiple donors. The percentage of Ki-67$^+$ cells was increased 5-fold from 5.3 ± 3.4% versus 24.2 ± 12.6% ($n = 7$) in control versus E2A-targeted CLL samples, respectively ($P < 0.05$ in a paired sample analysis) (Fig. 4B, left side) Stimulation with anti-CD40 plus

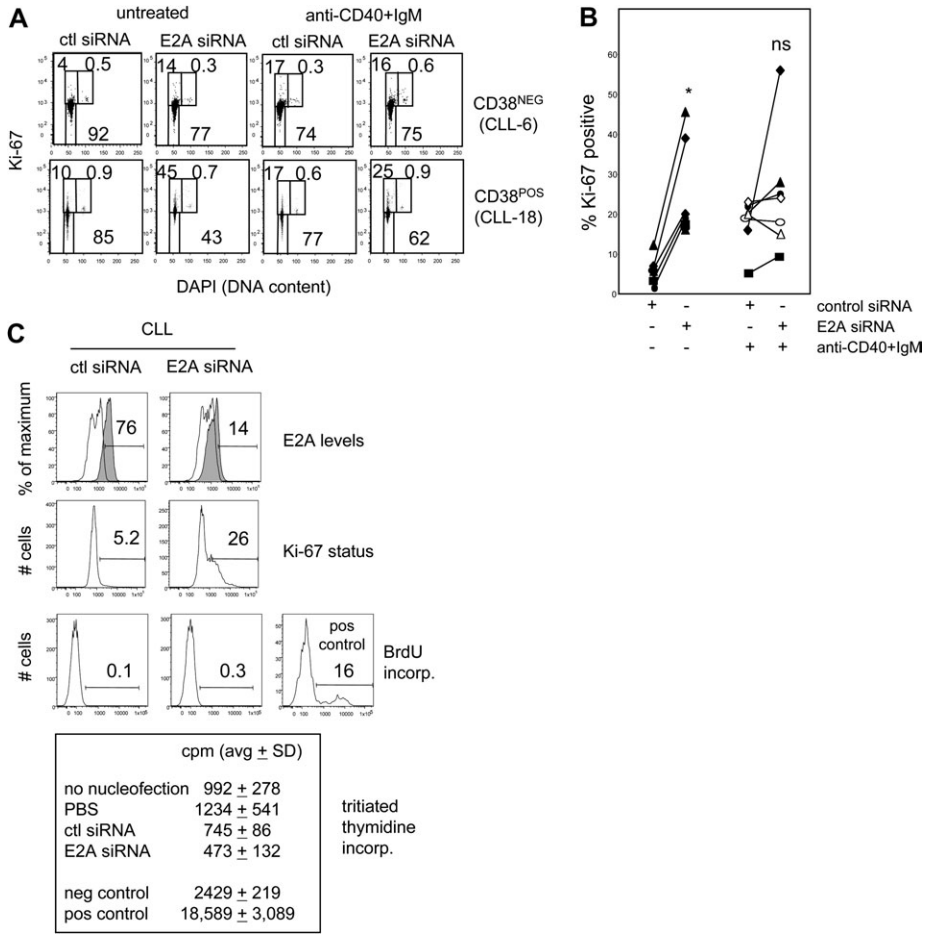

Fig. 4. E2A knockdown alters cell cycle distribution. CLL cells were mock-transfected (no siRNA) or transfected with control siRNA or E2A siRNA, cultured without or with anti-IgM + anti-CD40 antibodies and 48 h later, cells were stained with intracellular Ki-67 and DAPI and analyzed immediately by flow cytometry. Representative staining profiles of two independent donors (CLL-6 and CLL-18) are shown in (A). DAPI and Ki-67 staining are used to visualize the G_0 compartment (lower left quadrant), G_1 compartment (upper left quadrant) and S/G_2/M compartment (upper right quadrant). Dot plots in (B) represent data across multiple independent CD38$^+$ (CLL-14, -16, -18 and -22) and CD38$^-$ (CLL-6, -13 and -17) donors. In (C), CLL cells transfected as above were cultured for 48 h during which BrdU was added for the last 18 h of culture, or ^{3}H-TdR was added for the last 24 h as detailed in Methods. Data from CLL-7 is shown.

anti-IgM increased the frequency of Ki-67$^+$ cells in control-transfected populations but did not further elevate Ki-67 expression in E2A-targeted subsets, relative to their unstimulated counterparts (Fig. 4B, right side).

Analysis of cell cycle distribution based on DNA content demonstrated that after knockdown of E2A, CLL cells accumulate in G_1 but rarely are found in later stages of cell cycle (Fig. 4A). Therefore, we directly measured active DNA synthesis at the clonal level by BrdU and ^{3}H-TdR incorporation following E2A down regulation. Figure 4(C) shows that despite increased Ki-67 expression, there is no evidence of induction of *de novo* proliferation in E2A down regulated CLL cells as assessed by either method. Our results suggest that E2A regulates the transition from G_0 to G_1 phase of cell cycle by increasing the relative frequency of Ki-67$^+$ cells within the total CLL population but has no effect on the proliferation of individual CLL clones. These findings are consistent with observations that Ki67$^+$ CLL cells can accumulate in the G_1 phase without entering other phases of the cell cycle (11).

Targeted knockdown of E2A in CLL disrupts the expression of key genes associated with survival and cell cycling

We further analyzed the effect of E2A down regulation on the expression of key genes associated with CLL cell survival or cell cycling. QPCR analyses revealed that E2A knockdown did not affect expression of the major regulators of apoptosis bcl-2 or bax, but mcl-1 levels were significantly decreased in all cases studied regardless of CD38 status (Fig. 5). Furthermore, E2A knockdown altered levels of key cyclin-dependent kinase inhibitors. In particular, the expression of p27, which is up-regulated in CLL and is involved in cell cycle arrest (49, 50) as well as impaired apoptosis (51), was markedly reduced after E2A knockdown. Moreover, p21, a known target of E2A (25, 26, 32) and a downstream target of the ATM-p53 pathway that is thought to play an important role in CLL resistance to chemotherapy (52) was also down regulated in CLL cells after E2A inhibition.

Taken together, these results suggest that E2A may enhance CLL cell survival and resistance to chemotherapy, at least in part, by potentiating the effects of mcl-1, p21 and p27.

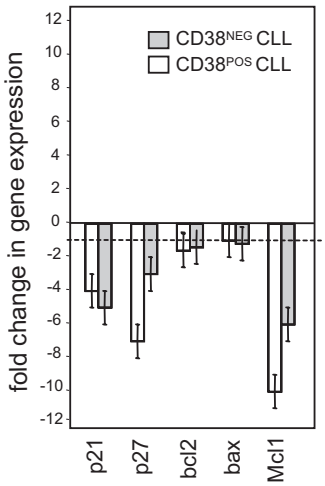

Fig. 5. Fold changes in quantitative expression of the indicated cell cycle and apoptosis-related genes in CLL cells after E2A knockdown. Data from CD38$^+$ donors CLL-4, -14, -15 and -16 and CD38$^-$ donors CLL-6, -7 and -10 are shown. Relative expression was measured by RT–QPCR, normalized to the actin housekeeping gene. Genes were considered as up-regulated or down-regulated if the differential expression between control and E2A siRNA-transfected cells was at least 2-fold in all experiments. The data are presented as the average ± SD across three independent experiments.

Discussion

While CLL patients exhibit variable disease courses, a unifying property of CLL cells whether aggressive or indolent is an activated phenotype. Our data indicate that E2A mRNA and protein are elevated in CLL relative to normal B-cell subsets in a manner characteristic of antigen-activated cells. Here, we used siRNA knockdown to specifically silence the B lineage transcription factor E2A, a known regulator of B lymphocyte survival, proliferation and activation. We show that efficient disruption of E2A expression promotes apoptosis of primary leukemic cells, which is further sensitized by treatment with the IMiD agent lenalidomide. Loss of E2A also alters Ki-67 expression without promoting *de novo* proliferation and deregulates the expression of cell cycle and apoptosis components. Together, our findings provide insight into the transcriptional mechanisms of CLL persistence.

E2A is a major transcription factor that controls B lineage development and function. E2A expression patterns are best detailed in the mouse but several recent studies have formally begun to establish E2A levels in man. Like mouse, E2A protein increases during the earliest stages of human B lineage progression in the bone marrow (32) and is detectable at low levels in mature and memory B-cell subsets from healthy donors. In striking contrast, E2A is expressed at high levels in CLL cells, to a degree that is only comparable with that of B-cell receptor/anti-CD40 stimulation of normal B cells. These observations provide additional support for the idea that at least some CLL cases represent the transformation of an activated B cell (12). Since E2A expression is strongly associated with lymphocyte activation (53, 54), a characteristic feature of CLL (11, 39, 55), it is somewhat perplexing that E2A has not been readily flagged in *CLL* gene expression profiling studies (10, 40, 56). These array studies focused on B-cell subsets from cord blood and tonsil, two subsets for which E2A levels have not been explicitly characterized. Moreover, E2A expression is clearly age-dependent (57), and a direct comparison of CLL versus B-cell subsets from age-matched individuals may reveal previously unappreciated differences. Indeed, our data show striking properties of E2A with normal aging. E2A expression dramatically decreases with age (>60 years) at the levels of both mRNA expression (57) and protein yet remains highly expressed in age-matched CLL patients. Since CLL cells appear to exhibit an activated phenotype, we hypothesized that disruption of the cellular activation status might provide the opportunity for improved therapy.

Here, we targeted a key transcriptional regulator associated with the activation status of CLL. We show that the transcription factor E2A is over-expressed at the transcript and protein levels in CLL cells relative to age-matched normal

B-cell subsets. E2A silencing in CLL promotes apoptosis, which is sensitized by the treatment with IMiD agent lenalidomide. E2A knockdown also alters cell cycle status and is associated with reduced levels of the anti-apoptotic gene mcl-1 and cell cycle inhibitors p21, p27. Although bcl2 is a hallmark anti-apoptotic gene expressed in CLL, the pro-survival effects of E2A appear to be bcl2 independent as bcl2 transcript was not altered following E2A disruption (Fig. 5). As such, our observation that expression of mcl-1, a bcl2 family member associated with CLL drug resistance (7), is responsive to E2A levels merit further investigation. We speculate that inhibition of E2A may facilitate transition from G_0 to G_1 phase of the cell cycle and therefore increase the sensitivity to apoptotic cell death. Finally, our study suggests that aberrant over-expression of E2A plays an important role in CLL cell apoptosis resistance. We anticipate that the specific and efficient siRNA targeting of primary CLL cells as described here can readily be used for the development of new therapeutic strategies aiming to alleviate apoptosis resistance in CLL.

Funding

National Institutes of Health (AI079047, AR054529 to L.B.; CA086433 to C.M.; AG023379 to A.N.V.); National Science Foundation MCB-0842725 to C.M. Elsa U. Pardee Foundation to L.B.; University of Pittsburgh Cancer and Aging Program (P20-CA103730) to A.N.V.; Mario Limieux Foundation to K.A.F.

Acknowledgements

We gratefully acknowledge Dewayne Falkner for indispensable assistance with experiments. Early discussions with Tony Brickner were very helpful. Russ Salter and Patrizia Fuschiotti kindly shared equipment and reagents. We thank Patricia Schaefer and Anne Morris for sample coordination and honest broker efforts. Author contributions: L.K. performed all experiments except 1D (Q.Y.), 4C (L.B.) and a small portion of 1C (L.B.). L.B. performed statistics. A.St.L. performed experiments that informed this work. K.A.F. and S.L. provided CLL samples; A.N.V. and C.M. gave advice. L.K. and L.B. wrote the paper.

References

1 Damle, R. N., Calissano, C. and Chiorazzi, N. 2010. Chronic lymphocytic leukaemia: a disease of activated monoclonal B cells. *Best Pract. Res. Clin. Haematol.* 23:33.
2 Del Poeta, G., Maurillo, L., Venditti, A. *et al.* 2001. Clinical significance of CD38 expression in chronic lymphocytic leukemia. *Blood* 98:2633.
3 Rassenti, L. Z., Huynh, L., Toy, T. L. *et al.* 2004. ZAP-70 compared with immunoglobulin heavy-chain gene mutation status as a predictor of disease progression in chronic lymphocytic leukemia. *N. Engl. J. Med.* 351:893.
4 Chiorazzi, N. and Ferrarini, M. 2011. Cellular origin(s) of chronic lymphocytic leukemia: cautionary notes and additional considerations and possibilities. *Blood* 117:1781.
5 Oscier, D. G., Gardiner, A. C., Mould, S. J. *et al.* 2002. Multivariate analysis of prognostic factors in CLL: clinical stage, IGVH gene mutational status, and loss or mutation of the p53 gene are independent prognostic factors. *Blood* 100:1177.
6 Li, F. J., Ding, S., Pan, J. *et al.* 2008. FCRL2 expression predicts IGHV mutation status and clinical progression in chronic lymphocytic leukemia. *Blood* 112:179.
7 Pepper, C., Lin, T. T., Pratt, G. *et al.* 2008. Mcl-1 expression has *in vitro* and *in vivo* significance in chronic lymphocytic leukemia and is associated with other poor prognostic markers. *Blood* 112:3807.
8 Van Bockstaele, F., Verhasselt, B. and Philippe, J. 2009. Prognostic markers in chronic lymphocytic leukemia: a comprehensive review. *Blood Rev.* 23:25.
9 Moreno, C. and Montserrat, E. 2008. New prognostic markers in chronic lymphocytic leukemia. *Blood Rev.* 22:211.
10 Klein, U., Tu, Y., Stolovitzky, G. A. *et al.* 2001. Gene expression profiling of B cell chronic lymphocytic leukemia reveals a homogeneous phenotype related to memory B cells. *J. Exp. Med.* 194:1625.
11 Chiorazzi, N., Rai, K. R. and Ferrarini, M. 2005. Chronic lymphocytic leukemia. *N. Engl. J. Med.* 352:804.
12 Damle, R. N., Ghiotto, F., Valetto, A. *et al.* 2002. B-cell chronic lymphocytic leukemia cells express a surface membrane phenotype of activated, antigen-experienced B lymphocytes. *Blood* 99:4087.
13 Guarini, A., Chiaretti, S., Tavolaro, S. *et al.* 2008. BCR ligation induced by IgM stimulation results in gene expression and functional changes only in IgV H unmutated chronic lymphocytic leukemia (CLL) cells. *Blood* 112:782.
14 Pleyer, L., Egle, A., Hartmann, T. N. and Greil, R. 2009. Molecular and cellular mechanisms of CLL: novel therapeutic approaches. *Nat. Rev. Clin. Oncol.* 6:405.
15 Rosati, E., Sabatini, R., Rampino, G. *et al.* 2009. Constitutively activated Notch signaling is involved in survival and apoptosis resistance of B-CLL cells. *Blood* 113:856.
16 Santos, P. and Borghesi, L. 2011. Molecular resolution of the B cell landscape. *Curr. Opin. Immunol.* 23:163.
17 Bain, G., Maandag, E. C., Izon, D. J. *et al.* 1994. E2A proteins are required for proper B cell development and initiation of immunoglobulin gene rearrangements. *Cell* 79:885.
18 Zhuang, Y., Soriano, P. and Weintraub, H. 1994. The helix-loop-helix gene E2A is required for B cell formation. *Cell* 79:875.
19 Kwon, K., Hutter, C., Sun, Q. *et al.* 2008. Instructive role of the transcription factor E2A in early B lymphopoiesis and germinal center B cell development. *Immunity* 28:751.
20 Quong, M. W., Martensson, A., Langerak, A. W., Rivera, R. R., Nemazee, D. and Murre, C. 2004. Receptor editing and marginal zone B cell development are regulated by the helix-loop-helix protein, E2A. *J. Exp. Med.* 199:1101.
21 Hauser, J., Verma-Gaur, J., Wallenius, A. and Grundstrom, T. 2009. Initiation of antigen receptor-dependent differentiation into plasma cells by calmodulin inhibition of E2A. *J. Immunol.* 183:1179.
22 Hauser, J., Sveshnikova, N., Wallenius, A., Baradaran, S., Saarikettu, J. and Grundstrom, T. 2008. B-cell receptor activation inhibits AID expression through calmodulin inhibition of E-proteins. *Proc. Natl Acad. Sci. USA* 105:1267.
23 Sayegh, C. E., Quong, M. W., Agata, Y. and Murre, C. 2003. E-proteins directly regulate expression of activation-induced deaminase in mature B cells. *Nat. Immunol.* 4:586.
24 Borghesi, L., Aites, J., Nelson, S., Lefterov, P., James, P. and Gerstein, R. 2005. E47 is required for V(D)J recombinase activity in common lymphoid progenitors. *J. Exp. Med.* 202:1669.
25 Prabhu, S., Ignatova, A., Park, S. T. and Sun, X. H. 1997. Regulation of the expression of cyclin-dependent kinase inhibitor p21 by E2A and Id proteins. *Mol. Cell. Biol.* 17:5888.
26 Yang, Q., Esplin, B. L. and Borghesi, L. 2011. E47 regulates hematopoietic stem cell proliferation and energetics but not myeloid lineage restriction. *Blood* 117:3529.
27 Kee, B. L. 2005. Id3 induces growth arrest and caspase-2-dependent apoptosis in B lymphocyte progenitors. *J. Immunol.* 175:4518.
28 Mathas, S., Janz, M., Hummel, F. *et al.* 2006. Intrinsic inhibition of transcription factor E2A by HLH proteins ABF-1 and Id2 mediates reprogramming of neoplastic B cells in Hodgkin lymphoma. *Nat. Immunol.* 7:207.
29 Mullighan, C. G., Goorha, S., Radtke, I. *et al.* 2007. Genome-wide analysis of genetic alterations in acute lymphoblastic leukaemia. *Nature* 446:758.
30 Abbott, B. L. 2006. Chronic lymphocytic leukemia: recent advances in diagnosis and treatment. *Oncologist* 11:21.
31 Craig, F. E. and Foon, K. A. 2008. Flow cytometric immunophenotyping for hematologic neoplasms. *Blood* 111:3941.

32 Yang, Q., Kardava, L., St Leger, A. *et al.* 2008. E47 controls the developmental integrity and cell cycle quiescence of multipotential hematopoietic progenitors. *J. Immunol.* 181:5885.
33 Jamshidi-Parsian, A., Dong, Y., Zheng, X., Zhou, H. S., Zacharias, W. and McMasters, K. M. 2005. Gene expression profiling of E2F-1-induced apoptosis. *Gene* 344:67.
34 Lindberg, D., Akerstrom, G. and Westin, G. 2008. Evaluation of CDKN2C/p18, CDKN1B/p27 and CDKN2B/p15 mRNA expression, and CpG methylation status in sporadic and MEN1-associated pancreatic endocrine tumours. *Clin. Endocrinol. (Oxf.)* 68:271.
35 McCord, A. M., Resto-Ruiz, S. I. and Anderson, B. E. 2006. Autocrine role for interleukin-8 in Bartonella henselae-induced angiogenesis. *Infect. Immun.* 74:5185.
36 Petre, C. E., Sin, S. H. and Dittmer, D. P. 2007. Functional p53 signaling in Kaposi's sarcoma-associated herpesvirus lymphomas: implications for therapy. *J. Virol.* 81:1912.
37 Saramaki, A., Banwell, C. M., Campbell, M. J. and Carlberg, C. 2006. Regulation of the human p21(waf1/cip1) gene promoter via multiple binding sites for p53 and the vitamin D3 receptor. *Nucleic Acids Res.* 34:543.
38 Frasca, D., Nguyen, D., Riley, R. L. and Blomberg, B. B. 2003. Decreased E12 and/or E47 transcription factor activity in the bone marrow as well as in the spleen of aged mice. *J. Immunol.* 170:719.
39 Damle, R. N., Temburni, S., Calissano, C. *et al.* 2007. CD38 expression labels an activated subset within chronic lymphocytic leukemia clones enriched in proliferating B cells. *Blood* 110:3352.
40 Kuppers, R., Klein, U., Schwering, I. *et al.* 2003. Identification of Hodgkin and Reed-Sternberg cell-specific genes by gene expression profiling. *J. Clin. Invest.* 111:529.
41 Gandhirajan, R. K., Staib, P. A., Minke, K. *et al.* 2010. Small molecule inhibitors of Wnt/beta-catenin/lef-1 signaling induces apoptosis in chronic lymphocytic leukemia cells *in vitro* and *in vivo*. *Neoplasia* 12:326.
42 Vogler, M., Butterworth, M., Majid, A. *et al.* 2009. Concurrent up-regulation of BCL-XL and BCL2A1 induces approximately 1000-fold resistance to ABT-737 in chronic lymphocytic leukemia. *Blood* 113:4403.
43 Schuh, K., Avots, A., Tony, H. P., Serfling, E. and Kneitz, C. 1996. Nuclear NF-ATp is a hallmark of unstimulated B cells from B-CLL patients. *Leuk Lymphoma* 23:583.
44 Seiffert, M., Stilgenbauer, S., Dohner, H. and Lichter, P. 2007. Efficient nucleofection of primary human B cells and B-CLL cells induces apoptosis, which depends on the microenvironment and on the structure of transfected nucleic acids. *Leukemia* 21:1977.
45 Wierda, W. G., Chiorazzi, N., Dearden, C. *et al.* 2010. Chronic lymphocytic leukemia: new concepts for future therapy. *Clin. Lymphoma Myeloma Leuk.* 10:369.
46 Chen, C. I., Bergsagel, P. L., Paul, H. *et al.* 2011. Single-Agent lenalidomide in the treatment of previously Untreated chronic lymphocytic leukemia. *J. Clin. Oncol.* 29:1175.
47 Chanan-Khan, A. and Porter, C. W. 2006. Immunomodulating drugs for chronic lymphocytic leukaemia. *Lancet Oncol.* 7:480.
48 Hideshima, T., Chauhan, D., Shima, Y. *et al.* 2000. Thalidomide and its analogs overcome drug resistance of human multiple myeloma cells to conventional therapy. *Blood* 96:2943.
49 Bogner, C., Schneller, F., Hipp, S., Ringshausen, I., Peschel, C. and Decker, T. 2003. Cycling B-CLL cells are highly susceptible to inhibition of the proteasome: involvement of p27, early D-type cyclins, Bax, and caspase-dependent and -independent pathways. *Exp. Hematol.* 31:218.
50 Vrhovac, R., Delmer, A., Tang, R., Marie, J. P., Zittoun, R. and Ajchenbaum-Cymbalista, F. 1998. Prognostic significance of the cell cycle inhibitor p27Kip1 in chronic B-cell lymphocytic leukemia. *Blood* 91:4694.
51 Sanhes, L., Tang, R., Delmer, A., DeCaprio, J. A. and Ajchenbaum-Cymbalista, F. 2003. Fludarabine-induced apoptosis of B chronic lymphocytic leukemia cells includes early cleavage of p27kip1 by caspases. *Leukemia* 17:1104.
52 Johnson, G. G., Sherrington, P. D., Carter, A. *et al.* 2009. A novel type of p53 pathway dysfunction in chronic lymphocytic leukemia resulting from two interacting single nucleotide polymorphisms within the p21 gene. *Cancer Res.* 69:5210.
53 Frasca, D., Van der Put, E., Riley, R. L. and Blomberg, B. B. 2004. Reduced Ig class switch in aged mice correlates with decreased E47 and activation-induced cytidine deaminase. *J. Immunol.* 172:2155.
54 Quong, M. W., Harris, D. P., Swain, S. L. and Murre, C. 1999. E2A activity is induced during B-cell activation to promote immunoglobulin class switch recombination. *EMBO J.* 18:6307.
55 Quiroga, M. P., Balakrishnan, K., Kurtova, A. V. *et al.* 2009. B-cell antigen receptor signaling enhances chronic lymphocytic leukemia cell migration and survival: specific targeting with a novel spleen tyrosine kinase inhibitor, R406. *Blood* 114:1029.
56 Zheng, Z., Venkatapathy, S., Rao, G. and Harrington, C. A. 2002. Expression profiling of B cell chronic lymphocytic leukemia suggests deficient CD1-mediated immunity, polarized cytokine response, altered adhesion and increased intracellular protein transport and processing of leukemic cells. *Leukemia* 16:2429.
57 Frasca, D., Landin, A. M., Lechner, S. C. *et al.* 2008. Aging down-regulates the transcription factor E2A, activation-induced cytidine deaminase, and Ig class switch in human B cells. *J. Immunol.* 180:5283.