Post-transcriptional upregulation of p53 by reactive oxygen species in chronic lymphocytic leukemia

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ABSTRACT

Chronic lymphocytic leukemia (CLL) cells multiply and become more resistant to immunochemotherapy in 'proliferation centers' within tissues, whereas apoptosis occurs in the periphery. Various models recapitulate these microenvironments in vitro, such as stimulation with CD154 and IL-4. Using this system, we observed a 30-40 fold induction of wild-type p53 protein in 50 distinct human CLL specimens tested, without the induction of either cell cycle arrest or apoptosis. In contrast, the mRNA levels for p53 did not increase, indicating that its elevation occurred posttranscriptionally. Mechanistic investigations revealed that under the conditions studied p53 was phosphorylated on residues associated with p53 activation and increased half-life. However, p53 protein induced in this manner could transcriptionally activate only a subset of target genes. The addition of a DNA damaging agent further upregulated p53 protein levels which led to apoptosis. p53 induction relied on the increase in intracellular reactive oxygen species (ROS) observed after CD154 and IL-4 stimulation. We propose that chronic oxidative stress is a characteristic of the microenvironment in B cell 'proliferation centers' in CLL that are capable of elevating the basal expression of p53, but to levels below the threshold needed to induce arrest or apoptosis. Our findings suggest that reactivation of the full transcriptional activities of p53 in proliferating CLL cells may offer a possible therapeutic strategy.
INTRODUCTION

Chronic Lymphocytic Leukemia (CLL) is a B-cell malignancy that is highly sensitive to microenvironmental signaling. Depending on the presence or absence of these signals, proliferation or apoptosis occur, respectively (1). These responses usually take place in different anatomical compartments. Proliferation centers within lymph nodes, bone marrow and spleen provide specialized microenvironments that foster survival and cell division, whereas outside these centers CLL cells may undergo apoptosis (2-7).

Mesenchymal marrow stromal cells (MSCs)(7-9) and monocyte-derived nurse-like cells (NLCs) (6, 10, 11) within lymph nodes secrete potent chemotactic signals that facilitate tissue homing and retention of circulating CLL cells (12). Once resident, CLL cells engage in complex bidirectional cellular and molecular interactions with the stromal cells, which secrete chemokines such as CCL3 and CCL4 to attract accessory CD3+ T-cells and monocytes and create a more supportive microenvironment (13, 14). The T-cells recruited are largely CD4+/CD154(CD40L)+ and play a crucial role in CLL cell activation mediated in part via the TNF superfamily member CD40(14). In conjunction with T cell-derived cytokines such as interleukin (IL)-4 and IL-8, CD40 ligation enhances CLL survival, proliferation and resistance to regular immune-chemotherapy (15-19).

When cultured under regular conditions in vitro, peripheral blood CLL cells usually undergo spontaneous apoptosis. However, this can be suppressed by coculturing CLL cells with stromal and blood-derived nurse-like cells (2, 6, 20). Furthermore, stimulating CLL cells in vitro either via engagement of BCR, TLR, CD40 or cytokine receptors also induces proliferation (16, 21-23). Therefore, these
interactions could be considered to reflect those within a proliferation center, and such
culture systems may allow the study of CLL cells in a microenvironment that mimics
that in which they normally thrive in vivo. For instance, using a combination of CD40
signaling and IL4 stimulation, we have previously demonstrated that the increased
apoptotic resistance of stimulated CLL cells is dependent on transcriptional up-
regulation of alternative BCL2 family members including BCL2A1 and BCL-XL (24,
25).

The loss of function of p53 (also known as TP53 and TRP53) is a key
tumorigenic event associated with deregulation of cell cycle, genomic instability and
resistance to chemotherapy (26). Almost all cancer cells escape the control by p53,
either by mutating p53 itself, seen in ~50% of all human cancers, or by disrupting
critical upstream and downstream signals of the pathway (27). The frequency of p53
mutations in CLL is relatively low, generally under 10% at diagnosis (28). This
increases with disease progression following treatment and is associated with a poor
prognosis and resistance to chemotherapy (29).

Here, we show that p53 protein is induced up to 30-40 fold post-
transcriptionally in CLL B cells following conditions that mimic those within the
lymph node. This induced p53 is phosphorylated and capable of some but not all of its
transcriptional activation functions. Moreover, we show that p53 protein up-
regulation depends on a concurrent and substantial increase in intracellular reactive
oxygen species (ROS) and the resulting oxidative DNA damage. We propose a model
in which basal levels of functional p53 are normally elevated due to a constitutive
oxidative DNA damage in stimulated CLL cells, which would be more resistant to
p53 due to the presence of anti-apoptotic and pro-survival signals, such as those
provided by BCL2-related proteins and p53 itself.
MATERIALS AND METHODS

Patient samples. Peripheral blood samples were obtained from CLL patients attending clinics at the Leicester Royal Infirmary (Leicester, UK) following informed consent and approval from the local Research Ethics Committee and in accordance with the Declaration of Helsinki. All patients, diagnosed according to IWCLL-NCI 2008 guidelines (30), were treatment free for at least 6 months and had a cell count >50 x 10^9/l. Peripheral blood mononuclear cells (PBMCs) were separated from whole blood by density centrifugation. Heparinized whole blood was diluted 1:1 with PBS and gently layered onto 15ml Ficoll (Histopaque 1077, Sigma-Aldrich, Poole, UK) prior to centrifugation at 400g for 30 minutes. The mononuclear cell layer was removed from the interphase, washed and resuspended in RPMI-1640 medium (Life Technologies, Paisley, UK) supplemented with 10% fetal bovine serum (Lonza, Slough, UK), L-Glutamax (2mmol/l), penicillin (50U/ml) and streptomycin (50mg/ml). The isolated mononuclear cells had a CLL cell purity of >90% in all cases, as determined by flow cytometry.

Cell Culture. Primary CLL cells were cultured alone or co-cultured with 35Gy irradiated mouse fibroblast L-cells (NTL) or human CD40 ligand (CD154) expressing mouse fibroblast L-cells seeded at 80-90% confluence, together with 10ng/ml rhIL-4 (CD154/IL-4, R&D systems, Abingdon, UK), as described (25). Alternatively, they were supplemented with 10ng/ml His-tagged CD154, 1μg/ml anti-Histidine monoclonal antibody crosslinking enhancer and 10ng/ml IL-4 (all from R&D
systems). BCR stimulation was performed with 20 μg/mL goat F(ab’)2 anti–human IgM (Sigma-Aldrich) for 48 hours. Stimulation via TLR was performed with 1 μM CpG-oligodeoxynucleotide (CpG-ODN) DSP30 (Sequence: 5’-TCGTCGCTGTCTCCGCTTCTTCTTGCC-3’) (TIB MOLBIOL, Berlin, Germany) and 10 ng/ml rhIL-2 (R&D systems).

**Immunoblotting.** Cells were lysed for 20 minutes on ice in RIPA lysis buffer (10mM Tris pH 7.4, 150mM NaCl, 1% TritonX-100, 0.1% sodium deoxycholate, 0.1% SDS, 5mM EDTA) supplemented with protease and phosphatase inhibitors and centrifuged for 15 minutes. Protein concentration was determined using the Bradford protein assay (Thermo Scientific, Loughborough, UK). Equal amounts of protein lysates were separated on 12% polyacrylamide gels and transferred to nitrocellulose membranes (Hybond-C, Amersham, Little Chalfont, UK). Membranes were probed with primary antibodies (Table S3) and detected after incubation with HRP-linked secondary antibodies (Thermo Scientific) with enhanced chemiluminescence reagents (Thermo Scientific).

**Immunofluorescence microscopy.** Cells were resuspended in serum-free RPMI-1640 media with 100nM MitoTracker Red CM-H2XRos (see below) in the dark, then washed thrice with PBS. And allowed to attach to adhesion microscope slides (Superfrost Plus glass slides, Thermo Fisher Scientific), maintained within hydrophobic circles marked out using PAP marker (Sigma-Aldrich) prior to addition of cells. 50-100μl of cell suspension was added to the slide, placed in a humidified chamber and kept at 4°C for 1 hour. Cells were fixed with 4% paraformaldehyde, permeabilized with 1% Triton X-100 in PBS and blocked with 3% BSA, 0.1%
Tween-20 in PBS for 30 minutes. Cells were stained overnight with anti-p53 antibody (DO1, GeneTex, Irvine, USA) followed by DyLight488-conjugated secondary antibody (Thermo Scientific) for 1 hour in the dark. After nuclear staining with DAPI (Life Technologies), samples were mounted with Fluoromount aqueous mounting media (Sigma-Aldrich). Slides were photographed using a Nikon TE300 microscope and the image analyzed using Volocity 3D software (Perkin Elmer, Waltham, MA, USA).

**Flow cytometry.** For surface staining, cells were incubated with anti-CD19-PE conjugated antibody at predetermined optimal concentration. For intracellular staining, cells were fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100 (Sigma-Aldrich) and 1% BSA (Sigma-Aldrich) in PBS. Cells were stained with primary antibody followed by DyLight Fluor-conjugated secondary antibodies (Thermo Scientific). Data were acquired on a FACSCanto II and analyzed using FACSDiva version 6.0 (Beckton Dickinson, San Jose, CA, USA). Lymphocytes were gated on the basis of their forward and side light scatter and protein expression was analyzed in CD19+ cells. Cells with fluorescence intensity greater than that of the FITC-conjugated IgG control were considered positive. See Table S4 for antibodies used.

**Cell cycle analysis.** Cells were fixed and permeabilized using ice cold 70% Ethanol and incubated at -20°C for at least 30 minutes. Cells were then washed once in PBS and resuspended in 500μl of freshly prepared PI buffer (50μg/ml Propidium Iodide (Sigma-Aldrich) and 10μg/ml RNase A (ThermoScientific) in PBS). Cells were
incubated at 37°C for 30 minutes and analyzed with a FACSCanto II and FACSDiva (Beckton Dickinson, USA).

**Cell proliferation and viability analysis.** Cell proliferation was assessed using CellTrace™ Cell Proliferation Kits (Invitrogen), following manufacturer’s instruction. Briefly, cells were washed with PBS and resuspended with PBS containing 5μM CSFE. Cells were incubated for 10 minutes at 37°C in the dark, then five times volume of culture medium was added and incubated for 5 minutes. After removing free dye solution, cells were cultured with pre-warmed RPMI-1640 complete medium for at least 10 minutes. CellTrace™ CSFE reagent staining in cells was detected with FACSCanto II (BD biosciences) and data was analyzed with FlowJo software (TreeStar, Ashland, OR). Cell proliferation was also determined by analysis of Ki67 expression by flow cytometry as described previously using anti-Ki67-FITC conjugated antibody (Abcam, Cambridge, UK). To assess viability, isolated mononuclear cells were harvested and assayed using the CellTiter-Glo Luminescent Cell Viability Assay (Promega, Southampton, UK). 100μl of cell suspension from a single well of a 6-well plate were plated in triplicate into 96-well plates. Cells were incubated with 50μl CellTitre-Glo reagent for 10 minutes and luminescence measured using a Tecan Infinite M200 Pro plate reader (Tecan, Männedorf, Switzerland).

**Gene expression analysis.** RNA was extracted from pellets of 1 x 10⁷ CLL cells (isolated from peripheral blood or after 72h co-culture) using TRIzol (Invitrogen, Paisley, UK). The aqueous phase containing RNA was separated after adding 1-bromo-3-chloro-propane and the RNA precipitated using isopropanol. After washing
with ethanol, the RNA pellet was resuspended in sterile RNase-free water. RNA quality was assessed by determining the $A_{260}/A_{280}$ ratio using a Nanodrop spectrophotometer (Thermo Scientific) and analysed on an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). cDNA was synthesised using the SuperScript III First-Strand Synthesis system (Invitrogen). Gene expression was measured using an ABI Prism 7000 sequence detector (Applied Biosystems, Foster City, USA). Expression values were normalized to β-actin and TATA box-binding protein (TBP). For primer sequences, see Table S5. A custom StellArray™ qPCR array (Lonza) was designed to analyze the expression of 28 p53 target genes and 4 housekeeping genes. This array was a 384-well plate containing wet-lab validated primer sets and the reactions were performed using a Lightcycler 480 Real Time PCR machine (Roche, Sussex, UK).

**Whole transcriptome RNA sequencing.** CLL cells isolated from the peripheral blood and after 72 hours co-culture with CD154/IL4 from 6 patients were used. CD19+ selection was performed using labeled magnetic beads (Miltenyi Biotec, Bisley, UK). RNA was extracted from the CD19+ cells as described above. RNA quality was assessed using a bioanalyser and samples with a RNA integrity number (RIN score) >8.0 out of a possible 10, were selected for RNA-seq. Ribosomal RNA sequences were depleted using RiboMinus rRNA depletion kits (Life Technologies). RNA whole transcriptome sequencing (RNA-seq) was performed by the genomics facility at the MRC Toxicology Unit, Leicester using an Applied Biosystems Solid 4 (Life Technologies) system. Whole genome alignment was done using TopHat software (Centre for Computational Biology, Johns Hopkins University, USA) using the aligner Bowtie, as well as Novoalign (Novocraft Technologies Sdn Bhd,
Malaysia). Transcript expression analysis was done using Cufflinks (CCB, Johns Hopkins University, USA).

**Phosphatase treatment.** Protein extracts were obtained from CLL cell pellets and protein concentration determined as described above. 50μg of lysate was treated with 200U Lambda Protein Phosphatase (Sigma-Aldrich) in 1X Lambda Protein Phosphatase buffer, with a final concentration of 50mM Tris (pH 7.5), 0.1mM Na₂EDTA, 5mM dithiothreitol, 2mM MnCl₂ and protease inhibitor cocktail. The reaction was incubated at 30°C for 1 hour and terminated by adding SDS-loading buffer.

**Cyclohexamide pulse chase assay.** CLL cells were co-cultured with CD154 expressing mouse L cells as described above for 72hr, or co-cultured for 48hr and treated further with 1μM Doxorubicin for 16 hours. Cells were then treated with 20μg/ml cyclohexamide and collected at the indicated times, fixed and subjected to intracellular staining or lysed and subjected to immunoblotting.

**Analysis of Reactive Oxygen Species (ROS).** Intracellular ROS were quantified using the fluorescent probe CM-H₂DCFDA (DCF, Life Technologies, Paisley, UK). About 1x10⁵ freshly isolated or co-cultured mononuclear cells were incubated with 1μM DCF for 1hr at 37°C in the dark. Cells were then washed twice, resuspended in phosphate buffered saline and fluorescence analyzed using FACSCanto II (Beckton Dickson). Alternatively, Dihydrorhodamine 123 (ThermoFisher Scientific), an uncharged and non-fluorescent ROS indicator that can passively diffuse across membranes, where it is oxidized to the fluorescent rhodamine 123, or MitoTracker
Red CM-H2XRos (ThermoFisher Scientific), a reduced, nonfluorescent dye that enters mitochondria fluoresces upon oxidation, were used following manufacturer’s protocols.

**Measurement of Oxidative Damage to DNA.** Analysis of oxidatively damaged purine DNA base lesion levels was done using the enzyme-modified comet assay protocol, as described previously (31). After lysis, slides were washed once with distilled water and immersed in three changes of enzyme digestion buffer (40mM HEPES, 0.1M KCl, 0.5mM EDTA and 0.2mg/ml bovine serum albumin (pH 8.0)), for 5 minutes each time at room temperature. Formamidopyrimidine DNA glycosylase (Fpg, Sigma-Aldrich) was added to the gel (50μl/gel) at 1/1000 dilution; gels were covered with a coverslip and incubated in a humidified chamber at 37°C for 30 minutes. The coverslips were removed, and the slides subject to electrophoresis at 30 V (0.7 V/cm) and 300 mA for 20 minutes in a horizontal electrophoresis tank containing ice-cold alkaline electrophoresis buffer (300 mM NaOH, 1 mM disodium EDTA, pH ≥ 13). Slides were neutralized with 0.4 M Tris–HCl (pH 7.5) for 20 min, washed with double-distilled water and dried. DNA staining was carried out after rehydration with 2.5μg/ml propidium iodide for 20 minutes, washed and then dried. Comets were visualized using a fluorescence microscope and analyzed with the Komet Analysis software version 5.5 (Andor Bioimaging, Nottingham, UK). A total of 100 comets were analyzed per sample. DNA damage was expressed as the percentage of DNA in the comet tails.
RESULTS

CD154/IL4 stimulates wild type p53 expression in CLL cells in the absence of arrest or death. In order to study CLL cells in an environment reflecting that of the proliferation centers, we cultured cells from 50 CLL patients with wild-type p53 (no chromosomal abnormalities in the 17p13 or p53 mutations, Table S1) in the presence of CD154 and IL4. As previously reported (16, 17), cells cultured under these conditions showed increased proliferation, as measured by Ki67 expression (Figure S1A), cell cycle analysis (Figure S1B) and CSFE (Figure S1C), in the absence of significant cell death (Figure S1B). These cells remained viable for prolonged periods of time (Figure S1D). As shown in Figure 1A, CLL B cells showed a 30-40 fold increase in the levels of p53 protein under these conditions. Of note, the predominant p53 band on the immunoblots was of higher molecular weight than that of the unstimulated cells, suggesting the presence of post-translational modifications (see below). Also, up-regulation of p53 protein was observed regardless of the stimulation protocol used (co-culture with mouse L-cells expressing CD154 in combination with IL-4 or incubation with anti-IgM or CpG-ODN in combination with IL-2). The detection of p53 protein under these conditions was unanticipated, since such stimulation results in proliferation, decreased apoptosis and enhanced resistance to chemotherapeutic drugs (17, 24, 25).

The induced p53 protein appeared to be the full-length form from immunoblotting with antibodies that recognize either the C or the N terminus (Table S1 and Figure S1E). Using flow cytometry, we observed that the percentage of cells induced to express p53 was variable (from 25 to 85% after 6 days) but increased with time after stimulation (Figure 1B). There was no obvious correlation between levels
of p53 and proliferation, since the percentages of Ki67 positive cells expressing p53 or not were similar (Figure 1C). Of note, p53 induction was consistently observed in all cases of CLL irrespective of cytogenetic abnormalities or IGHV mutational status. These results together suggest that CLL cells in proliferation centers may have high basal levels of p53 in the absence of the usual cell fate responses subsequent to its expression.

**p53 induced in stimulated CLL cells is transcriptionally active.** We next studied the functional capacity of p53 in stimulated CLL cells. Immunofluorescence microscopy showed that p53 was expressed in the nucleus, thus ruling out a loss of activity due to a cytoplasmic localization (Figure S2A). We observed by Western blot that the induction of p53 correlated with the up-regulation of p53 targets MDM2 and SCO2, but not of TIGAR (Figure 2A), suggesting that although p53 had intact transactivation capabilities, not all target genes were equally induced. Consistent with this hypothesis, a custom qRT-PCR array revealed selective up-regulation of 26 p53 target genes (Figure 2B). Of note, pro-apoptotic p53 target genes such as Puma, Noxa and Bax (32-34) were not upregulated under these conditions, whereas pro-survival genes (such as DDR1 (35-37) and Cox2 (38, 39)) were. Also, despite the increase in protein levels, p53 mRNA was not increased (see below). RNA-Seq experiments were also performed on six CLL cases pre and post stimulation. Expression of three p53 target genes upregulated in the RNASeq experiments was confirmed by qRT-PCR: ICAM1, NR4A2, SOCS1 were all induced three days after CD154/IL-4 stimulation (Figure 2C). However, another p53 target, CCL5, showed no increase, which further supports the idea that p53 was not able to transactivate all target genes. As previously described (17, 25), CD154/IL-4 stimulation of CLL cells also showed an induction of
the pro-survival BCL2 family proteins BCL2A1, BCLXL and MCL1, together with a decrease in BCL2 expression (Figure 2D). In addition, the pro-apoptotic protein BID was induced, whilst BIM was down-regulated following stimulation.

**p53 is post-translationally modified and stabilized in stimulated CLL cells.** Our results show that a functional p53 is induced following CD154/IL4 stimulation in CLL B cells. PCR array, QRT-PCR and RNA Seq experiments showed that p53 RNA transcripts were not upregulated (see Figures 2B and C), suggesting a transcription-independent mechanism of p53 protein induction. To study this, we first investigated the post-translational modifications of p53 in stimulated cells. Treatment with phosphatase led to the disappearance of the top band on a p53 Western blot (Figure S2B), which suggests that the predominant form of p53 may be phosphorylated. Indeed, using phosphor-specific antibodies we observed that p53 was phosphorylated at serines 15, 20, 46, 392 and threonine 81 (Figure 3A), all of which are markers of p53 activation (40-43). Phosphorylation of Ser15, which plays an important role in p53 stabilization through the disruption of p53-MDM2 interaction (41), progressively increased with time after stimulation (Figure 3B). These results suggest that p53 had acquired the necessary post-translational modifications to allow dissociation from MDM2 and thus prevent proteolytic degradation and stabilizing protein levels, which is the main mechanism of p53 up-regulation (44).

To confirm whether this is the case, we assessed the half-life of p53 in CD154/IL4 stimulated CLL cells. p53 is normally a short-lived protein that is continuously cycled within the cell with a half-life of 5-20 minutes (45, 46). As shown in Figures 3C and D, stimulation of CLL cells increased p53 stability, with 50% of cells still expressing p53 after 2 hours (compared to <30 minutes in
unstimulated cells, and >7 hours in DNA damaged cells). These data provide a mechanism to explain the elevation in p53 protein levels after stimulation.

**Induction of p53 after stimulation is mediated by an elevation in intracellular reactive oxygen species (ROS).** While looking for a possible trigger of p53 induction in stimulated CLL cells, we observed a progressive and elevation in the levels of intracellular ROS, up to a 50-fold increase above basal levels (Figures 4A, S2A and S2C), which was higher in cells with unmutated than with mutated IGHV (Figure 4B). We reasoned that this increase in ROS could explain the induction of p53 after stimulation through the activation of DNA damage pathways. As shown in Figure 4C, levels of oxidative damage to DNA increased over 8-fold in stimulated cells, as analyzed by a modified Comet assay, comparable to that induced by the positive control (H$_2$O$_2$ treatment). This was supported by a Western blot that showed an increase after stimulation of phosphorylated histone 2AX, a commonly used marker of DNA damage (47) (Figure 4D). Consistent with these results, the induction of p53 protein was suppressed when different antioxidants were used to block the increase in ROS (Figure 4E). This confirms the hypothesis that an elevated level of oxidative stress is responsible for p53 induction after CD154/IL4 stimulation. Importantly, ROS levels correlated with cell viability (Figure 4F), and when ROS were blocked by antioxidants, viability (Figure 4F) and p53 protein (Figure 4G) reverted to levels similar to those of unstimulated cells. This indicates a causal relationship between ROS, p53 and survival in stimulated CLL cells.

Importantly, p53 in stimulated cells was still responsive to chemotherapeutic drugs, as shown by a further increase in cells expressing p53 after doxorubicin treatment (see Figures 3D and 4D). When these levels of p53 were achieved, cell
viability was reduced (Figure 4H). Taken together, these data show that a wild type p53 is induced and activated in stimulated CLL cells due to an increase in basal intracellular ROS levels that induce DNA damage, but to levels below a threshold sufficient to induce apoptosis in these conditions. However, this threshold can be reached with further DNA damage.

**DISCUSSION**

Unlike solid tumors, p53 mutations are uncommon in hematological malignancies, occurring in only a fraction of cases at diagnosis in most subtypes of disease. The retention of wild-type p53 suggests that p53 activation might be a useful therapeutic strategy. However, B-cells may have an increased threshold to the pro-apoptotic and anti-proliferative actions of wild type p53. For example, some diffuse large B-cell lymphoma express high levels of wild-type p53 in the nucleus, along with the p53 target gene p21(48); in one series, 107/474 (22.6%) of cases expressed wild-type p53 to variable levels in the absence of p53 mutation(49). Similarly, infection of normal human resting B-cells with Epstein-Barr virus results in high-level expression of p53 protein with no induction of apoptosis (50) and the viral protein EBNA5 interferes with normal p53 protein transactivating functions by binding directly to the p53/MDM2 complex (50). Our results indicate that CLL cells have similarly an increased “tolerance” of the presence of wild type p53 and that this protects them from the induction of apoptosis or cell cycle arrest following CD154/IL4 stimulation.

The p53 expressed in CLL cells was full length, wild type, located to the nucleus and could be normally phosphorylated in key residues, which lead to the
expected increase in half-life that is necessary for the protein to accumulate. However, despite of all these normal features, p53 was unable to transactivate certain key target genes in stimulated CLL cells, especially those involved in cell death, and induce the usual cellular responses, such as apoptosis or arrest. Instead, there was an activation of pro-survival pathways.

The general determinants of cell fate after p53 induction are still not clear. One hypothesis is that the decision to undergo apoptosis, arrest or any of the usual p53 responses is made by a conjunction of factors that modulate p53 functions, including protein and ROS levels (51), oxygen availability (52) and a specific pattern of post-translational modifications of p53 (40, 43). In the case of stimulated CLL cells, microenvironmental factors could activate signals that tip the balance away from the apoptotic response of p53 and favor a pro-survival one. Which factors are determinant in this effect remains to be elucidated. Some may be related to the induction of key antiapoptotic BCL2 family members, which could allow the cell to take advantage of the pro-survival effects of p53 (53). Overall reduction in pro-apoptotic BCL2-related proteins and increase in anti-apoptotic proteins in CLL cells could also play a role in providing a higher threshold for cell death. Our hypothesis is that the increased levels of p53 in stimulated CLL cells are not sufficient to induce key target genes in pro-apoptotic pathways and that this is only achieved at higher levels of p53 expression. This would be consistent with the fact that induction of apoptosis by p53 depends, among other factors, on the protein levels triggered by the stress stimuli (51, 54).

In summary, stimulated CLL cells accumulate functional p53 due to constitutive oxidative stress and this does not lead to apoptotic or arrest responses. This could provide new avenues to define specific therapeutic strategies that would
only affect stimulated B cells. Finding a way to reduce the tolerance of these cells to the presence of p53 would selectively kill them without having to use additional DNA damaging agents.

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REFERENCES


FIGURE LEGENDS

Figure 1. p53 is induced in stimulated, proliferating CLL cells. (A) Western blot showing induction of p53 in primary CLL cells freshly extracted from the patient (C), or cultured with NTL, CD154/IL4, Anti IgM or CpG/IL2 (see protocols for details) for the specified days. (B) Levels of expression of p53 or Ki67 in primary CLL cells from 20 patients (see details on Table S1) cultured in the presence of CD154/IL4 for the specified days, as measured by FACS. (C) Representative FACS plot of primary CLL cells cultured in the presence of CD154/IL4 for 3 days, double stained with Ki67 and p53 antibodies.

Figure 2. p53 is able to transactivate a subset of its target genes in stimulated CLL cells. (A) Representative Western blot of primary CLL cells cultured in the presence of CD154/IL4 for the specified days showing expression of p53 and some of its target genes. (B) mRNA expression of p53 and some of its target genes in primary CLL cells cultured in the presence of CD154/IL4 for 3 days, as measured by a custom StellArray™ qPCR array. Results represent mean and standard deviation and error
bars represent maximum and minimum values of the 12 primary cells analyzed. (C) mRNA expression of p53 and some of its target genes in primary CLL cells cultured in the presence of CD154/IL4 for 3 days, as measured by qRT-PCR. Results represent mean and standard deviation and error bars represent maximum and minimum values of the 12 primary cells analyzed. (D) Representative Western blot of primary CLL cells cultured in the presence of CD154/IL4 for the specified days showing expression of Bcl2 family members.

**Figure 3. Phosphorylation and stabilization of p53 in stimulated p53 cells.** (A) Percentage of primary CLL cells positive for the expression of total (T) or phosphorylated p53, as measured by FACS using specific antibodies against phosphorylated serines (S15, S20, S46 and S392) or threonine (T81). Cells were cultured in the presence of CD154/IL4 for 3 days. Results represent mean of 8 primary cells analyzed and error bars represent standard deviation. (B) Percentage of primary CLL cells positive for the expression of p53 phosphorylated at serine 15, as measured by FACS. Cells from 20 patients were cultured in the presence of CD154/IL4 for the indicated amount of days. (C) Representative western blot of primary CLL cells cultured in the presence of CD154/IL4 for 3 days and then treated with cyclohexamide to stop protein synthesis for the indicated hours. Cells were treated with DMSO to provide a negative control (C). (D) Levels of remaining p53 protein in primary unstimulated CLL cells (Control), cultured in the presence of CD154/IL4 for 3 days or the same treated with 1μM doxorubicin for 24 hours, as measured by intracellular flow cytometry. Graph show mean values of 5 primary cells (2 for controls) and error bars show standard deviation. Horizontal line marks p53 expression in 50% of cells.
**Figure 4. ROS induces oxidative damage, proliferation and p53 expression in stimulated CLL cells.** (A) Fold changes in Mean Fluorescence Intensity (MFI) of 12 primary CLL cells cultured in the presence of CD154/IL4 for the specified amount of days and then stained with DCF (to detect ROS), as measured by FACS. Graph show mean values of MFI and error bars show standard deviation. Statistics between 0 and 3 days: p<0.0001 (paired t-tests). (B) ROS levels in 11 Vh mutated and 4 unmutated primary CLL cells fresh from the patient or cultured in NTL or CD154/IL4 for 3 days, as measured by DCF staining and analyzed by FACS. Statistics: * P=0.046. (C) Comet assay of primary CLL cells from 8 patients cultured in the presence of CD154/IL4 for 3 days. Positive control are cells treated with 50 μM H2O2 for 30 minutes on ice right before analysis. Experiments were performed in the absence or presence of fpg to determine specific damage due to oxidation (the difference in tail DNA percentage between the two conditions). Mean percentage of tail DNA is plotted. Error bars represent standard deviation. Experiments were performed twice in triplicates. (D) Representative Western blot of primary CLL cells cultured in the presence of CD154/IL4 for 3 days and treated with DMSO or 1μM doxorubicin for 24 hours, showing expression of p53 or phosphorylated H2AX. (E) Representative Western blot showing expression of p53 in primary CLL cells lysed right after being extracted from the patient (C) or cultured in the presence of CD154/IL4 for 3 days. Cells were incubated with 1mM NAC or 1mM GSH for 16 hours. (F) Top: Changes in intracellular ROS levels (plotted as fold changes in MFI of DCF-stained primary CLL cells, compared to control cells) in cells cultured in NTL or CD154/IL4 for 3 days and incubated with 1mM NAC or 1mM GSH for 24 hours. (Statistics, unpaired t-tests: Control NTL to CD154/IL4, CD154/IL4 Control to NAC and CD154/IL4
Control to GSH all p=0.0001) Bottom: Cell viability expressed in fold change in luminescence in the same cells (compared to plastic), as measured by a CellTiter-Glo assay. Graph show mean of 15 samples and error bars show standard deviation. (Statistics, unpaired t-tests. Control NTL to CD154/IL4: p=0.02; CD154/IL4 Control to NAC: p=0.025; CD154/IL4 Control to GSH: p=0.03) (G) Percentage of cells expressing p53 in primary CLL cells cultured in the presence of CD154/IL4 for 3 days and treated further with 1 mM NAC or 1 µM doxorubicin (Dox) for 24 hours, as measured by FACS analysis. Results represent mean and standard deviation and error bars represent maximum and minimum values of the 6 primary cells analyzed. Statistics (paired t-tests): *** p≤0.0006 (H) Percentage of viable cells in primary CLL cells treated with doxorubicin as described above for up to 2 days, as measured by a CellTiter-Glo assay.