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Inactivation of Myc in Murine Two-Hit B lymphomas Causes Dormancy with Elevated Levels of Interleukin 10 Receptor and CD20: Implications for Adjuvant Therapies

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Abstract

Overexpression of c-Myc and inactivation of p53 are hallmarks of human Burkitt's lymphomas. We had previously showed that transduction of murine p53-null bone marrow cells with a Myc-encoding retrovirus is sufficient for B lymphomagenesis. To address the role of Myc in tumor sustenance, we generated lymphomas induced by the Mycestrogen receptor fusion protein (MycER). Engrafted hosts were continuously treated with the ER ligand 4-hydroxytamoxifen (4-OHT) to allow tumor formation. Subsequent inactivation of MycER via 4-OHT deprivation resulted in tumor stasis but only partial regression. At the cellular level, dormant neoplastic lymphocytes withdrew from mitosis and underwent further B-cell differentiation. Concomitantly, they up-regulated genes involved in lymphocyte proliferation and survival, most notably interleukin 10 receptor α (IL10R α) and CD20, the target for antibody therapy with Rituxan. We found that overexpression of IL10R α affords significant proliferative advantages and in 4-OHT-deprived animals correlates with eventual tumor relapse. Both dormant and relapsing tumors maintain IL10R α expression suggesting that they might be sensitive to emerging drugs targeting the IL-10 pathway. Up-regulation of CD20 following Myc inactivation was also observed in immortalized human lymphocytes. Importantly, in this system, Myc^{OFF}CD20^{HIGH} cells were more prone to Rituxan-induced apoptosis than Myc^{ON}CD20^{MED}. Thus, targeting Myc, while moderately effective on its own, shapes the phenotype of dormant neoplastic cells and sensitizes them to adjuvant molecular therapies.

Introduction

Human Burkitt's lymphomas and their murine counterparts plasmacytomas arise from B-cell precursors following translocations juxtaposing the c-Myc proto-oncogene and one of the immunoglobulin enhancers (reviewed in ref. 1). Thus, inactivation of Myc might impair neoplastic growth, and drugs targeting this oncoprotein are being developed (2). However, whether targeting of Myc would afford clinical benefits to lymphoma patients remains unclear. Several strains of transgenic mice bearing inducible or repressible alleles of Myc have been generated to address this issue. Expression of the Myc-estrogen receptor (MycER) fusion in suprabasal compartments of the skin results in estrogen-dependent papillomatosis, a precancerous condition (3). Similarly, expression of a tetracycline-dependent allele of Myc in hematopoietic cells leads to the formation of T lymphomas (4), osteogenic sarcomas (5), and B-cell lymphomas (6). In all these systems, brief inactivation of Myc results in rapid proliferative arrest, terminal differentiation, and apoptosis of tumor cells. However, acquisition

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of secondary mutations can abrogate the reliance of lymphoma cells on continuous Myc expression (7). Similarly, Myc-induced murine breast carcinomas initially respond to Myc-targeting but subsequently accumulate mutations in the Ki-Ras oncogene which render therapy ineffective (8). Moreover, recent work has shown that many neoplastic cells with down-regulated Myc survive in a dormant state while retaining the capacity to form tumors upon Myc reactivation (9,10).

These findings suggest that in the clinical setting anti-Myc therapies will not be curative on their own and will require simultaneous inactivation of other targets. Such adjuvant therapies are likely to be cell type specific, necessitating the investigation of Myc function in neoplastic B cells. The contribution of Myc to B lymphomagenesis has been studied extensively using transgenic mice (reviewed in ref. 11), but only recently have B-cell neoplasms with conditionally active Myc been generated (6). However, it was not clear whether these neoplasms acquire additional oncogenic lesions, in particular, mutations in the p53 pathway characteristic of advanced human B lymphomas. We have previously shown that s.c. coinjection of primary p53-null bone marrow cells (BMC) and Myc retrovirus-producing packaging cells results in the formation of polyclonal, short-latency lymphomas (12). All Myc/p53-null lymphomas possess the B-cell phenotype *in vivo*, although some of them are derived from hematopoietic precursors with the dual B-lymphoid/myeloid potential (13). In this study, we set out to generate neoplasms using the estrogen-dependent allele of Myc and determine the consequences of Myc inactivation in this two-hit system. We were particularly interested in determining whether switching Myc off leads to up-regulation of additional therapeutic targets.

Materials and Methods

Mice

p53-null and wild type C57BL6/J mice were obtained from The Jackson Laboratory (Bar Harbor, ME). BMC harvest and infection and production of BMC-derived neoplasms have been described previously (12). For 4-hydroxytamoxifen (4-OHT) treatment, the hormone powder (H6278, Sigma, St. Louis, MO) was dispersed, via sonication, in corn oil (Sigma) at the concentration of 10 mg/mL. The administration of 4-OHT was done via daily i.p. injections at the dose of 1 mg per mouse.

Analyses of tumor specimens

Histologic staining and flow cytometric analyses were done as described previously (12). TUNEL staining was done using the *In situ* Cell Death Detection Kit/TMR red (Roche Diagnostics, Indianapolis, IN) per manufacturer's recommendations. For Ki-67 staining, paraffin-embedded 4- μ m tissue sections were deparaffinized in xylene and rehydrated in graded ethyl alcohol. Endogenous peroxidase was quenched by placing slides in 3% hydrogen peroxide (H1009, Sigma) for 15 minutes. Ki-67 antigen unmasking was accomplished by heating the sections in Antigen Unmasking Solution (H3300, Vector Laboratories, Burlingame, CA) in a microwave oven. Nonspecific staining was blocked by applying 5% normal rabbit serum for 20 minutes at room temperature. The slides were incubated with 100 μ L diluted (1:40) primary antibody (TEC-3 Ki-67, DAKO Corp., Carpinteria, CA) for 2 hours at room temperature in a humidified chamber. Biotinylated rabbit anti-rat secondary antibody was applied for 60 minutes at room temperature followed by incubation with the appropriate reagents from the avidin-biotin complex method Kit (PK-4004, Vector Laboratories) for 45 minutes. The slides were rinsed and stained with Dako DAB Chromagen (K3465, DAKO) and counterstained with Hematoxylin QS (H3404, Vector Laboratories) for 20 seconds. Green fluorescent protein (GFP)-expressing neoplastic cells were also detected using fluorescent microscopy. For flow cytometric analysis of BMC, cell suspensions were washed once with

PBS, and RBC were hypotonically lysed by resuspending cell pellets in 9 mL of distilled water. 20 seconds later 1 mL of 10× PBS was added, and cells were washed again before analysis. The analysis of tumor clonality was done using the detection of DJ-recombination events as described previously (12).

Generation of and infection with the MycER retrovirus

The MycER transgene was excised from the pBabePuro/MycER vector (14) and subcloned into the *EcoRI* site of MIGR1. MIGR1-MycER and the control MIGR1 retroviral DNAs were transfected into GP+E86 packaging cells using the LipofectAMINE Plus Reagent (Invitrogen, Carlsbad, CA). The GFP-positive single cell clones were obtained by fluorescence-activated cell sorting (FACS) as described in (13). Ten microliters of conditioned medium from each clone were used to infect 1×10^5 NIH3T3 cells cultured in 6-well plates. Percentages of GFP-positive NIH3T3 cells were determined 48 hours later using flow cytometry. Cell clones yielding >2% of GFP-positive NIH3T3 cells were pooled together and used for BMC transduction *in vivo*.

Generation of and infection with the interleukin 10 receptor α -encoding retrovirus

To generate the MIGR1-mIL10R α retrovirus, total RNA was isolated using TriReagent (Sigma) from Myc3-mBCL cells and the cDNA was prepared using the SuperScript First-Strand Synthesis System for reverse transcription-PCR (RT-PCR; Invitrogen). The coding sequence of mIL10R α was amplified using the Advantage HF2 PCR kit (Clontech, Palo Alto, CA) and the following primers: 5V-GCCGGAGGCGTAAAGGCCGGCTCCAG-3' (sense) and 5'-GGTACAGGGAGGGGAGCAGGCATGGCTG-3' (antisense). IL10R α cDNA was initially cloned into the PCR-II vector using the TOPO TA Cloning Kit (Invitrogen) and after sequence verification was inserted into the MIGR1 retrovirus using *Bam*HI and *Xho*I restriction sites. For *in vitro* infections with the IL10R α retrovirus, BOSC23 packaging cells were transfected with either MIGR1 or MIGR1-IL10R α using Lipofect-AMINE 2000 (Invitrogen). Conditioned media were harvested and added to Myc3-mBCL cells for 12 hours. Three days post infection, GFP-positive cells were obtained using FACS.

Culturing of B-lymphoid cells *in vitro*

MycER-expressing tumor cells were cultured on monolayers of γ -irradiated S17 cells as described in ref. (13). P493-6 cells (15) were cultured in RPMI 1640 with or without doxycycline (10 ng/mL). When indicated, P493-6 cells were treated with Rituxan (Genentech, Inc., South San Francisco, CA) at the final concentration of 20 μ g/mL. For analytic purposes (flow cytometry), Rituxan was conjugated with FITC at the University of Pennsylvania Cell Center Services Facility.

Microarray analysis

Total RNAs from MycER tumors 118 and 119 (Myc^{ON}) and 120 and 121 (Myc^{OFF}) were used. cRNAs were synthesized using *in vitro* transcription with biotinylated CTP and UTP. Labeled probes were hybridized to the U74v2 gene chip (Affymetrix, Inc., Santa Clara, CA) using the PENN Microarray Facility's standard protocol (<http://www.med.upenn.edu/microarr/Data%20Analysis/Affymetrix/methods.htm>). Affymetrix MAS 5 probe set signals and presence/absence flags were calculated. The local pooled error test for differential expression as implemented in S+ArrayAnalyzer v 1.1 (Insightful Corp., Seattle, WA) was applied with 1% Bonferonni multiple testing correction to median IQR normalized MAS 5 signal values. The resulting list of 686 genes was imported into GeneSpring v 6.1 (Silicon Genetics, San Carlos CA), filtered for Presence (per Affymetrix MAS5 analysis) in two of two samples in one or more conditions (Myc^{ON} or Myc^{OFF}), and then filtered for fold change (>2.5-fold). This analysis yielded 352 genes; 184 of them were expressed at higher levels in Myc^{ON} tumors and

168 in Myc^{OFF} tumors. The lists were compared with the Gene Ontology Biological Process category list using the EASE v 2.0 software (NIH, NIAID; ref. 16) and Bonferroni correction.

Flow cytometric analyses

To detect expression of surface markers, standard techniques were applied. Staining with antibodies was done on ice for 30 to 45 minutes. For B220 staining, a PE-conjugated anti-mouse B220 antibody was used (PharMingen, San Diego, CA). For mIL10R α staining, a biotin-conjugated anti-mouse IL10R α antibody was used (PharMingen). For human CD20 staining, either Rituxan or a FITC-conjugated anti-hCD20 antibody were used (MHCD2001, Caltag, Burlingame, CA).

Real-time PCR

Total RNAs were isolated using TRI Reagent (Sigma) and treated with a TURBO DNA-free kit (Ambion, Inc., Austin, TX). cDNAs were prepared using SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen). Amplification reactions were done using Titanium Taq DNA polymerase (BD Biosciences, Palo Alto, CA), SYBRgreen I (Roche Diagnostics) in LightCycler (Roche Diagnostics). The following cycling variables were commonly used: 1 second at 95°C for denaturation and 10 to 47 seconds at 68°C to 72°C for annealing and extension for the total of 30 cycles. Primer sequence information is available upon request.

Cell death, proliferation, and accumulation assays

P493-6 cells (2×10^4 cells per well) were cultured in media with 10% tetracycline-free fetal bovine serum (BD Biosciences) in the presence or absence of doxycycline (10 ng/mL). Silencing of Myc was confirmed using immunoblotting with an anti-c-Myc antibody (Santa Cruz Biotechnology, Santa Cruz, CA). For apoptosis induction, after 12 hours of incubation with doxycycline, Rituxan (20 μ g/mL) was added for additional 12 hours. Apoptotic cells were detected using staining with Annexin V (17). Annexin V was used in either PE- or APC-conjugated form (BD Biosciences) or as a fusion with GFP. To measure the extent of cell lysis, release of lactate dehydrogenase (LDH) was quantitated in aliquots of culture supernatant from duplicate wells using a colorimetric cytotoxicity detection kit (Roche Diagnostics) and a plate reader set at the wavelength of 492 nm. Cell proliferation was assessed using ³H-thymidine incorporation. Cell accumulation was quantitated in a colorimetric assay using the WST-1 reagent (Roche Diagnostics) and a plate reader set at the wavelength of 450 nm.

Results

Deactivation of Myc in murine B-cell lymphomas results in tumor dormancy and eventual relapse

The MycER-encoding retrovirus was generated as described in Materials and Methods. To generate MycER/p53-null neoplasms, BMC from p53-null animals were transduced *in vivo* per our protocol (12). Hosts engrafted with transduced cells received daily injections of 4-OHT as detailed in Materials and Methods and developed neoplasms after 4 to 5 weeks. Two independently derived neoplasms (MycER1 and MycER2) were chosen for tumor load experiments and were found to behave in a similar fashion.

In the first experiment, 5×10^6 MycER cells were injected into the tail veins of nonirradiated mice, and the spread and survival of lymphoma cells were monitored using GFP fluorescence. In animals continuously treated with 4-OHT, by day 14, MycER lymphoblasts comprised the majority of cells in the bone marrow (Fig. 1A, B, and C, *left*), as evidenced by both flow cytometry and fluorescent microscopy. They were also efficient in colonizing lymph nodes and visceral organs, especially the liver and the spleen (data not shown). In contrast, on day 14 bone marrows of 4-OHT-deprived animals contained only solitary GFP-positive cells (Fig.

1A, *middle*, B, C, *right*). However, by day 19, MycER lymphoblasts started to colonize bone marrow even in untreated mice (Fig. 1A, *right*). Consequently, the animals appeared moribund and had to be sacrificed. On postmortem examination, numerous GFP-positive cells were detectable in lymph nodes and viscera (data not shown).

To obtain a better measurement of tumor growth kinetics, 8×10^6 primary tumor cells were also injected s.c. into flanks of syngeneic C57B6/J mice that continued to receive 4-OHT. Palpable tumors were allowed to form, and two experimental groups were established on day 8. Mice in the first group continued to receive 4-OHT (Myc^{ON}), rapidly developed very large tumors, and had to be sacrificed (Fig. 2A, *solid lines*). In the second group (Myc^{OFF}), 4-OHT treatment was discontinued (Fig. 2A, *dotted lines*). Differences in tumor volumes between 4-OHT-treated and untreated animals became apparent as early as after 24 to 48 hours. However, in animals taken off 4-OHT, tumors did not undergo complete regression; instead tumor volumes stabilized until approximately day 21.

To determine the cellular mechanisms of tumor stasis, TUNEL, H&E, and Ki-67 stainings of Myc^{ON} and Myc^{OFF} tumor sections were done. We observed that upon inactivation of Myc, rates of cell death actually decreased (data not shown), consistent with the well-established role of Myc in apoptosis (reviewed in ref. 18). However, rates of cell proliferation were also lower, as reflected in mitotic indices on H&E-stained sections (Fig. 2B). On average, Myc^{OFF} tumors contained 10 times fewer mitotic cells than Myc^{ON} tumors (Fig. 2B, *inset*). Furthermore, unlike their Myc^{ON} counterparts, Myc^{OFF} cells were also positive for CD45R (B220 antigen; Fig. 2D), an evidence of their commitment to the B-cell lineage (19). Finally, we stained the samples with the monoclonal antibody Ki-67 raised against the proliferation-associated antigen that is expressed in all active phases of the cell cycle (G₁-M) but is absent in G₀ cells (20). Whereas there was a significant decrease in the number of Ki-67-positive cells in Myc^{OFF} cells (Fig. 2C), Ki-67-positive cells were not completely eliminated. Given very low mitotic indices, they were presumably arrested in G₁ or G₂ phases of the cell cycle, suggesting that Myc^{OFF} tumors have the potential to relapse. Indeed, at about day 21 post-implantation, all lymphomas in 4-OHT-deprived mice resumed progressive growth, defined as the doubling of tumor volume in ≤ 2 days.

Tumor relapse is not due to clonal selection or 4-hydroxytamoxifen-independent Myc activity

We wished to determine whether tumors are relapsing because of the adaptation of Myc^{OFF} cells to MycER-independent growth. Alternatively, tumor relapse could be brought about by mutations in additional oncogenes or 4-OHT-independent MycER activity. To distinguish among these possibilities, we first assessed the clonality on relapsing tumors. This assessment was done by analyzing rearrangements of D and J regions of the immunoglobulin heavy chain loci. D-J junctions were amplified using PCR and the specificity of resultant fragments was confirmed using Southern blotting (21). As described in our prior publication (12), all original Myc/p53-null lymphomas are polyclonal, as evidenced by the abundance of individual recombination products (Fig. 3A, *first two and last lanes*). In this respect, they closely resemble patently polyclonal bone marrow populations (Fig. 3A, *penultimate lane*). When relapsing tumors were analyzed, we found that their genetic complexity equals that of the original neoplasms (Fig. 3A, compare specimens A/B and C/D). This suggested that no clonal selection takes place during the period of tumor stasis and that tumor relapse is not due to mutations in additional oncogenes or MycER itself.

It was still possible that tumors relapse because they no longer require 4-OHT for the activity of MycER. To rule out this possibility, we retreated mice bearing Myc^{OFF} tumors on the verge of relapse with 4-OHT. Readministration of 4-OHT immediately resulted in exponential growth (Fig. 3B) suggesting that Myc^{OFF} tumors are still highly sensitive to 4-OHT.

Furthermore, the escaping tumors were distinct from the original neoplasms in that there were modest rates of cell proliferation but almost no apoptosis (data not shown). Lastly, when cultured *in vitro*, Myc^{OFF} cells were still positive for CD45R, but treatment with 4-OHT resulted in efficient CD45R down-regulation (Fig. 3C). We thus concluded that tumor relapse is due to the efficient adaptation of Myc^{OFF} cells to MycER-independent growth and not to the leakiness of the MycER system. This scenario is fully consistent with our observation that in tumor production experiments only 4-OHT-treated animals bearing transduced bone marrow cells develop neoplasms (data not shown).

Inactivation of Myc results in up-regulation of the interleukin 10 receptor which promotes neoplastic growth

To identify genes that might be responsible for Myc-independent tumor growth and eventual relapse, we performed microarray analysis of gene expression profiles in Myc^{ON} versus Myc^{OFF} lymphomas. We then searched the list of differentially expressed genes for B-cell growth factors or receptors thereof, reasoning that up-regulation of such genes might result in increased cell proliferation and/or survival in the absence of functional Myc. The only such gene corresponded to the α subunit of the IL10R. IL-10 has been known to stimulate proliferation of normal B cells *in vitro* (22), but its *in vivo* effects on both normal and neoplastic B cells are poorly characterized. Recently, it has been suggested that high levels of IL-10 signaling are in fact incompatible with B lymphomagenesis (23).

To determine the significance of IL10R α regulation in Myc-induced lymphomas, we first validated the microarray data using real-time RT-PCR (see Materials and Methods). We observed that the IL10R α mRNA was indeed up-regulated 8- to 10-fold upon inactivation of MycER whereas mRNAs encoding actin and the β subunit of the IL10R β were unaffected (Fig. 4A, *first two columns*; data not shown). Also unaffected were IL10R α levels in 4-OHT-treated Myc, not MycER, -induced tumors (data not shown). This suggested that the effects of 4-OHT are mediated by MycER and not the endogenous ER. We also stained the corresponding tumor samples with an antibody against IL10R α . The IL10R α protein levels were several fold higher in tumor cells from 4-OHT-deprived animals (Fig. 4B). These cells were then briefly cultured *in vitro* where they maintained elevated levels of IL10R α (Fig. 4C, *left*). However, upon restimulation with 4-OHT, levels of IL10R α decreased again (Fig. 4C, *right*). This reversible regulation in briskly growing cells suggested that up-regulation of the *il10ra* gene in 4-OHT-deprived cells is not merely a marker of growth arrest.

To determine whether the expression levels of IL10R α are rate limiting, we have generated a retrovirus coexpressing murine IL10R α and GFP (see Materials and Methods). This virus was then used to infect Myc3-mBCL cells (13) in which IL10R α levels were as low as in Myc^{ON} cells (Fig. 4D, *left*). Upon infection and FACS for GFP-positive cells, transduced lymphoma cells expressed elevated levels of IL10R α that were comparable to that observed in Myc^{OFF} cells (Fig. 4D, *right*). Following sorting for GFP, these cells formed tumors that were on average 4-fold larger than those formed by vector-transduced cells (Fig. 4E). We also observed a strong selection for high levels of IL10R α *in vivo*, when IL10R α ^{HIGH} and IL10R α ^{LOW} cell were admixed before engraftment (data not shown). The Myc^{OFF}IL10R^{HIGH} phenotype of dormant tumors suggests that to be clinically effective, Myc-targeting might have to be combined with an anti-IL-10 therapy. Such a therapy could target either IL10R α itself or its downstream effectors (see Discussion).

Inactivation of Myc causes the emergence of well-differentiated CD20-positive neoplasms: implications for Rituxan therapy

To determine whether neoplasms with inactivated Myc express additional therapeutic targets, we analyzed all the genes that were expressed differentially in Myc^{ON} versus Myc^{OFF} samples.

Statistical analysis (see Materials and Methods) yielded 352 mRNAs that were present in either Myc^{ON} or Myc^{OFF} tumors and whose expression levels differed by >2.5-fold; 184 genes were expressed at higher levels in Myc^{ON} cells and 168 in Myc^{OFF} cells. The two lists were run against the Gene Ontology database using the EASE software package (ref. 16; <http://david.niaid.nih.gov/david/ease.htm>), to determine what categories of genes are primarily affected by Myc.

Predictably, for Myc-activated mRNAs, the highest EASE score was assigned to genes falling into the Gene Ontology category “Mitotic cell cycle/Proliferation.” Thirty-eight of Myc target genes belonged to this category, and 15 of them exhibited >4-fold down-regulation when Myc was switched off (Table 1). Some of them represented known targets such as Dp1, the component of the E2F multimeric transcription factor (24,25). Interestingly, the list of Myc-repressed genes bore the strongest similarity to the Gene Ontology category “Defense/Immune Response” containing numerous B-cell differentiation markers such as MHC class II molecules and immunoglobulin heavy and light chains. In all, 34 Myc-repressed genes were represented by this category, and 18 of them were up-regulated >4-fold upon Myc inactivation (Table 1). This was consistent with the observation that Myc overexpression renders B cells nonimmunogenic (26).

Also notable was the fact that the second highest gene on that list (14-fold up-regulation in Myc^{OFF} cells) was the B-lymphocyte surface antigen CD20 (also called MS4A1; ref. 27) whose human orthologue is the target for Rituxan (IDEC-C2B8), a humanized monoclonal antibody approved by Food and Drug Administration for the treatment of B-cell lymphoma (28). Insofar as responses to Rituxan are dictated by levels of CD20, its up-regulation in Myc^{OFF} cells could render them more sensitive to this antibody-based therapy. CD20 mRNA was indeed up-regulated 10- to 20-fold upon inactivation of MycER, whereas the *Dp1* gene was down-regulated (real-time PCR data; Fig. 2A, *four middle columns*). CD20 levels were unaffected by 4-OHT in tumors not expressing MycER (data not shown).

We were hindered in the analysis of the murine CD20 protein levels by the lack of a commercially available antibody. Whereas anti-mouse CD20 antibodies have been described in the literature (29), they were not made available for this study. We thus used another experimental system, human B-lymphocytes immortalized by the EBV EBNA1 protein and conditionally transformed by doxycycline-repressible c-Myc (15). We confirmed, using Western blotting, that exposure of these P493-6 cells to doxycycline for 24 or 48 hours results in strong suppression of c-Myc levels (Fig. 5A) and a sharp decrease in cell proliferation (ref. 15; data not shown). This was reminiscent of the effects of 4-OHT withdrawal in our B-lymphoma model. We thus analyzed CD20 levels using flow cytometry and fluorescently labeled Rituxan. When grown in doxycycline-free medium, P493-6 cells express intermediate levels of CD20. Upon addition of doxycycline and ensuing silencing of Myc, levels of CD20 increased 3- to 4-fold (Fig. 3B). This result suggested that repression of CD20 levels by Myc is a common feature of B-lymphocytes from different species and organs (bone marrow and peripheral blood).

To determine whether Rituxan induces cell death in P493-6 cells, we treated them with this antibody for 12 hours and measured cell death by staining with fluorescently labeled Annexin V. As evidenced by flow cytometric profiles in Fig. 5C, pretreatment with Rituxan significantly increased Annexin V binding, which is indicative of impending apoptosis (17). To corroborate this finding, cell lysis was quantitated by measuring the release of LDH (see Materials and Methods). Consistent with the Annexin V data, pretreatment with Rituxan increased LDH release as well (data not shown).

We then set out to determine whether Myc^{OFF}/CD20^{HIGH} P493-6 cells are more sensitive to Rituxan-mediated apoptosis than Myc^{ON}/CD20^{MED} cells. To this end, both cell populations were either left untreated or treated with Rituxan, and cell death was measured by calculating median intensities of Annexin V staining. After subtracting the background (staining induced by control antibody, dotted line), we observed a 3-fold increase in Annexin V levels after Myc was turned off (Fig. 5D). No effect of Myc on background staining was apparent. To measure accumulation of viable cells, WST assay was also done. We observed that both down-regulation of Myc and the treatment with Rituxan significantly reduced cell outgrowth, but the strongest reduction occurred when these two treatments were combined (data not shown). Thus, down-regulation of Myc indeed sensitizes B lymphocytes to Rituxan-mediated apoptosis *in vitro* and might synergize with this drug *in vivo*.

Discussion

The apparent genetic complexity of tumor cells (30) suggests that truly “smart” drugs must target molecules actively contributing to tumor sustenance. Still, to achieve sustained therapeutic effects, even the “smartest” drugs might have to be used in combinations, much like highly active anti-HIV retroviral therapy (HAART) relies on simultaneous delivery of protease inhibitors and nucleoside analogues (31). Herein, we show, for the first time, that inactivation of Myc in a two-hit mouse tumor model results in immediate cessation of cell proliferation but no complete regression (which was readily observed in one-hit models; refs. 5,6). Following 4-OHT withdrawal, MycER/p53-null neoplasms enter a state of dormancy, with very low mitotic indices. At the same time, dormant Myc^{OFF} neoplasms contain much fewer apoptotic cells than their Myc^{ON} counterparts. Predictably, 4-OHT withdrawal improves tumor cell survival, and after a lag period, all lymphomas resume progressive growth. Apparently, this relapse is not caused by 4-OHT-independent Myc activity, because readministration of 4-OHT immediately boosts neoplastic growth *in vivo* and readily reverses changes in gene expression *in vitro*. Most importantly, we found no evidence of clonal selection for additional mutations described in other model systems (7,8). Thus, we concluded that Myc-initiated changes in cell physiology might activate proliferation and survival pathways that circumvent the requirement for Myc.

To identify such pathways, we analyzed the list of genes differentially expressed in Myc^{OFF} versus Myc^{ON} cells. No transcriptional changes in well-recognized survival pathways (Akt, nuclear factor- κ B, etc.) were apparent (data not shown). However, we observed pronounced up-regulation of the α subunit of the IL10R. To the best of our knowledge, this is the first demonstration that Myc controls IL10R α levels. This is significant given the emerging role of IL-10 in B-lymphomagenesis. IL-10 has been known to stimulate proliferation of normal B cells (22) *in vitro*, but its *in vivo* effects are more complex. For instance, IL-10 can exert strong antiangiogenic and sometimes overt antineoplastic effects (23,32). Herein, we show that in the murine system the IL-10 pathway strongly enhances B lymphomagenesis. IL-10 is known to signal predominantly through signal transducers and activators of transcription 3 (STAT3; reviewed in ref. 33), which is constitutively phosphorylated on Tyr⁷⁰⁵ in a variety of human neoplasms (34) including Burkitt's lymphoma (35). In turn, STAT3 activates expression of several antiapoptotic genes including Bcl-2, Bcl-xl, survivin, and Mcl1 (36). Provocatively, per our microarray data, Mcl1 was up-regulated in Myc^{OFF} tumors which have significantly lower levels of cell death compared with Myc^{ON} tumors (data not shown). Thus, targeting of the IL-10 pathway using soluble IL10R α (37) or a dominant-negative STAT3 (38) could block expansion of lymphoma cells that have survived an anti-Myc therapy.

With regard to additional therapeutic targets, Myc down-modulation also causes up-regulation of the CD20 antigen. Importantly, a humanized monoclonal antibody against CD20 (IDEC-C2B8, Rituxan) has proven an effective anti-lymphoma drug in murine systems (39) and

human patients (28). However, in humans, the response rates do not exceed 50% and the average response lasts only 1 year (40). The mechanisms of resistance to Rituxan are poorly understood. One suggested mechanism invokes CD20 levels that may vary between individual cells over a log range (41). The molecular basis for such a heterogeneity remains undeciphered and no specific inhibitors of CD20 expression have been identified. Our data suggest that overexpression of Myc might interfere with CD20 expression and by inference, limit sensitivity to Rituxan. Indeed, using as a model P493-6 cells, we showed that lowering Myc levels synergizes with Rituxan treatment *in vitro*. While we cannot rule out the possibility that this occurs in a CD20-independent manner, elevation of CD20 levels should allow more efficient binding of Rituxan and presumably more robust downstream signaling.

Furthermore, future immunotherapies could use neutralizing antibodies against additional Myc-repressed B-cell differentiation markers identified by us (Mb1, lymphotoxin B, etc.) and by others (24-26). Such antibodies might be particularly effective in eliminating neoplastic cells that have survived anti-Myc therapies. As the latter are being developed and tested (2), these hypotheses could be addressed in pre-clinical and eventually clinical settings.

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Figure 1.

Effects of 4-OHT on colonization of bone marrow by MycER tumor cells. A, flow cytometric analysis of bone marrows of mice injected i.v. with MycER cells and maintained with or without 4-OHT. Day 14 and day 19 are days after injection. Analysis of bone marrow cells using bright field (B) and fluorescent microscopy (C). Panels in both columns depict same microscopic fields.

Figure 2.

Effects of 4-OHT on subcutaneous growth of MycER tumor cells. **A**, tumor volumes versus days after engraftment of individual neoplasms from 4-OHT-treated (*solid lines*) and untreated (*dotted lines*) animals. **B**, histologic staining of representative specimens from (**A**). *Inset*, mitotic indices in Myc^{ON} and Myc^{OFF} tumors on day 12. **B**, Ki-67 staining of the same specimens. Ki-67-positive nuclei appear in brown, all nuclei are counterstained in blue. **D**, flow cytometric analysis of B220 expression on the surface of cells from Myc^{ON} and Myc^{OFF} tumors. Gray plots are samples stained with a control antibody.

Figure 3.

Molecular characterization of relapsing MycER tumors. *A*, analysis of tumor clonality. PCR amplification and detection of D-J junctions by Southern blotting were done as described in Materials and Methods. *A* and *B*, two original MycER tumors; *C* and *D*, two relapsing tumors. Genomic DNAs from rodent fibroblasts and pro-B cells without D-J rearrangements were used as negative controls. Genomic DNAs from total bone marrow and a previously characterized Myc-induced polyclonal lymphoma were used as positive controls. *B*, tumor volumes versus days after engraftment of individual neoplasms from 4-OHT–deprived (*dotted lines*) and retreated (*solid lines*) animals. *C*, flow cytometric analysis of B220 expression on the surface of cultured Myc^{OFF} cells. Cells were maintained in 4-OHT–free (*left*) or 4-OHT–containing media. Gray plots are samples stained with a control antibody.

Figure 4.

The effects of IL10 signaling on growth of murine B-cell lymphomas *in vivo*. A, real-time RT-PCR analyses of mRNAs corresponding to indicated genes. All reactions were done in triplicates, and at least two independent tumors of each type were analyzed. B-D, flow cytometric analysis of IL10Rα expression on the surface of Myc-transformed cells. Myc^{ON} and Myc^{OFF} refer to primary tumors or cultured Myc^{OFF} cells (“in vitro”). “off>on” denotes Myc^{OFF} cells reexposed to 4-OHT. Myc3-mBCL cells were infected with either empty vector (MIGR1, left) or the IL10Rα-encoding retrovirus (MIGR1/IL10Rα, right). Gray plots are samples stained with a control antibody. E, tumor weights corresponding to neoplasms derived from Myc3-mBCL cells transduced with the empty vector or the IL10Rα-encoding retrovirus.

Figure 5.

CD20 expression and sensitivity to Rituxan of B-lymphoid cells conditionally transformed by Myc. *A*, Western blotting done on P493-6 cells, untreated or treated with doxycycline for 24 or 48 hours. Migration of bands corresponding to c-Myc and β -actin is indicated (*arrows*). *B*, flow cytometric analysis of CD20 expression on the surface of the cells from (*A*). *Bottom*, cells were treated with doxycycline for 24 hours. *C*, flow cytometric analyses of Myc^{ON} P493-6 cells using fluorescently labeled Annexin V. P493-6 cells (*top*) were pretreated with a control antibody. Cells (*bottom*) were pretreated with Rituxan as described in Materials and Methods. Gray plots are samples stained with a control antibody. *D*, median intensities of Annexin V

staining in P493-6 cells maintained with and without doxycyclin (Myc^{OFF} and Myc^{ON}, respectively). Cells were also pretreated with a control antibody or Rituxan as in (C).

Table 1

Groups of Myc-regulated genes with strongest similarities to known gene ontology categories

Myc-activated genes with the best EASE score (1.3×10^{-12}): gene ontology category “Mitotic cell cycle/Proliferation” (top 15 of 38)		Fold change
ect2 oncogene		6.4
cyclin B1		6.0
topoisomerase (DNA) II α		5.3
polo-like kinase (<i>Drosophila</i>)		5.0
proliferating cell nuclear antigen		5.0
Geminin		4.9
CDC28 protein kinase regulatory subunit 2		4.7
ribonucleotide reductase M1		4.6
antigen identified by monoclonal antibody Ki 67		4.6
cell division cycle 2 homologue A (<i>Schizosaccharomyce pombe</i>)		4.5
breast cancer 2		4.4
karyopherin (importin) α 2		4.2
budding uninhibited by benzimidazoles 1 homologue (<i>Saccharomyces cerevisiae</i>)		4.2
cyclin B2		4.0
serine/threonine kinase 6		4.0
Myc-repressed genes with the best EASE score (6×10^{-14}): gene ontology category “Defense/Immune response” (top 18 of 34)		Fold change
histocompatibility 2, class II, locus <i>Mb1</i>		20.4
<i>CD20</i> ; membrane-spanning four domains, subfamily A, member 1		14.0
lymphotoxin B		13.6
CD1d1 antigen		9.7
histocompatibility 2, K region		8.7
CD2 antigen		7.8
histocompatibility 2, class II antigen A, α		7.6
guanylate nucleotide binding protein 3		7.1
IFN-activated gene 205		6.8
immunoglobulin heavy chain 4 (<i>serum IgG1</i>)		6.6
histocompatibility 2, class II antigen A, β 1		5.9
Ia-associated invariant chain		5.5
protein tyrosine phosphatase, receptor type, C		4.8
histocompatibility 2, class II antigen E β		4.3
IFN-activated gene 203		4.2
histocompatibility 2, class II, locus <i>DMA</i>		4.1
immunoglobulin κ chain variable 8 (V8)		4.1
histocompatibility 2, T region locus 23		4.1