The mTORC1 Inhibitor Everolimus Prevents and Treats Eμ-Myc Lymphoma by Restoring Oncogene-Induced Senescence

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INTRODUCTION

Deregulated expression of the MYC proto-oncogene is one of the most ubiquitous aberrations in human cancer. In up to 15% of cancers, chromosome translocation or gene amplification results in inappropriate expression of MYC. In a further 50% of cases, MYC overexpression results from a variety of mechanisms including enhanced translation, increased protein stability, or disordered signaling upstream of MYC (1). MYC is a bHLH-LZ transcription factor. In most instances, it acts by binding E-boxes and recruiting transcriptional coactivators to regulatory promoter elements in target genes, but MYC also binds MIZ1 to represses gene transcription at a small subset of targets. Despite evidence from preclinical models that inactivating MYC translates into therapeutic benefits, it has proven difficult to target MYC pharmacologically as it lacks a simple enzymatic function that mediates its activity (2). However, oncogenic MYC gives rise to cellular transformation through an aberrant transcriptional program, and it is known that up to one-third of MYC target genes are regulators of energy metabolism and cell growth (3, 4).

The signal transduction molecule mTOR is also a critical mediator of cell growth. In the mTOR complex 1 (mTORC1) multiprotein complex, mTOR associates with GβL, raptor, PRAS40, and deptor to promote nutrient- and growth factor–dependent signaling (5). However, unlike MYC, mTORC1 is readily amenable to allosteric inhibition by rapamycin and analogues including everolimus (also known as RAD001). The Eμ-Myc transgenic mouse is a preclinical model that has been used extensively to understand the sequelae of MYC deregulation (6). The transgene mimics the human t(8;14) (q24;q32) that is characteristic of Burkitt lymphoma and juxtaposes MYC to the immunoglobulin heavy chain enhancer (IG) leading to tissue-specific deregulation of MYC expression. Expression of the Eμ-Myc transgene initially results in a premalignant phenotype notable for abnormal B-cell development (7). The premalignant phase comprises 2 stages. First, there is polyclonal B-cell expansion with accumulation of undifferentiated B cells in hemopoietic organs (7, 8). During this phase, B cells at equivalent stages of development are larger than their counterparts in control mice and exhibit increased protein synthesis, indicating that the failure of B cells from Eμ-Myc mice to differentiate is accompanied by deregulated cell growth (9). Subsequently, mice enter a phase characterized by more rapid proliferation and turnover of B-cell precursors, increased hemophagocytic activity, and relative normalization of peripheral blood counts (8, 10).

During the premalignant phase, unconstrained expression of MYC is counterbalanced by activation of the ARF/p53...
network and compensatory changes in Bcl2 family members resulting in cell-cycle arrest and cell death. Genetic deletion of Arf, p53, or Bim and overexpression of Bcl2 accelerates lymphomagenesis in Eμ-MyC mice (11–14). Furthermore, p53 mutation or biallelic deletion of Arf coincides with outgrowth of monor oligoclonal malignant disease in half to two-thirds of spontaneously arising lymphomas showing that counter-regulatory measures must be disabled for malignant transformation (12).

Anticancer strategies that target processes driven by the cell growth component of the MYC transcriptome can be therapeutically beneficial. Blocking mTORC1 signal transduction through cotransfection of TSC2 reduced colony formation driven by MYC (15), and crossing mice heterozygous for ribosomal proteins with Eμ-MyC mice to restore ribosome biogenesis and protein synthesis levels to those of normal B cells increased the latency of Eμ-MyC lymphomas (16). Moreover, interventions to decrease transcription of the ribosomal RNA genes have therapeutic efficacy in established Eμ-MyC lymphoma (17).

We hypothesized that administration of everolimus to Eμ-MyC mice would restore B-cell differentiation and delay lymphoma onset. In fact, everolimus specifically rescued B-cell development and conferred near-complete protection from malignant transformation concomitant with enhanced senescence and clearance of prelymphomatous B cells. In addition, everolimus afforded significant control over malignant disease in a manner that corresponded to senescence induction and the presence of a functional p53 response. These data reveal that mTORC1 is necessary for MYC to bypass tumor suppression through induction of cellular senescence.

RESULTS

mTORC1 Is Required for Tumor Initiation

To determine whether mTORC1 activity was necessary for tumor initiation by MYC, we randomized 4-week-old Eμ-MyC mice with no overt evidence of malignancy to receive everolimus or the equivalent volume of a placebo (Supplementary Table S1). Mice underwent weekly lymph node palpation for the duration of the study in addition to peripheral blood monitoring after 2, 4, and 8 weeks of treatment. As expected, placebo-treated mice developed fatal pre-B or B-cell leukemia/lymphoma (Fig. 1A and B) with a median lymphoma-free survival of 73 days (Fig. 1A). Overall, mTORC1 inhibition protected strongly against malignant transformation with only 4 of 33 everolimus-treated mice developing leukemia/lymphoma after more than 150 days of therapy (Fig. 1A and B). The biology of tumors in everolimus-treated mice was also distinct. Tumors arising in placebo-treated mice were approximately evenly distributed between B-cell [B220+/surface IgM (slgM)+/surface IgD (slgD)+] and pre-B cell [B220+/slgM+/slgD− (46.2%)] tumors as expected from previous studies (18). In contrast, all tumors in everolimus-treated mice had the pre-B immunophenotype (Supplementary Fig. S1A and S1B). Therefore everolimus prevents Eμ-MyC lymphoma and treatment failure selects for lymphomas with a pre-B phenotype.

Everolimus Restores Normal B-Cell Development

Because there is an expanded polyclonal B-cell population in Eμ-MyC mice, we examined whether tumor prevention by everolimus was associated with reversal of this phenotype. Immunophenotyping indicated that everolimus reduced the percentage of circulating B cells at the immature B (B220+/slgM+) and pre-B cell (B220+/slgM−) stages compared with placebo (Supplementary Fig. S2A and S2B). Furthermore, absolute numbers of circulating B cells, and particularly undifferentiated B cells, were reduced by mTORC1 inhibition to levels equivalent to those in wild-type mice (Supplementary Fig. S2C–S2E). These data indicate that mTORC1 inhibition rescued aberrant B-cell differentiation in Eμ-MyC mice.

To thoroughly investigate the effects of everolimus on B-cell development, we next took cohorts of 4-week-old Eμ-MyC mice and analyzed them after 2 weeks of therapy. We observed that the spleen weight was restored to wild-type levels in association with a 50% reduction in splenic B-cell numbers (Fig. 2A and B). Purified B220+ splenocytes in everolimus-treated mice also had similar morphologic characteristics to differentiated cells observed in wild-type spleens.
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with more condensed nuclear chromatin and greater cytoplasmic pallor than B220+ splenocytes from placebo-treated mice (Fig. 2C). The mean cell volume (MCV) of B220+ cells from everolimus-treated mice was significantly reduced in the spleen [placebo 264.9 ± 6.1 femtoliters (fl) vs. everolimus 230.8 ± 5.3 fl (P = 0.002)]. Furthermore, analysis of B220+ B cells showed reduced percentages of the less differentiated slgM−/slgD− and slgM+/slgD− populations (Fig. 2D).

In the bone marrow, although overall cellularity was not significantly reduced by everolimus therapy (Supplementary Fig. S3A), there was more than 50% reduction in the percentage of B220+ lymphocytes from the bone marrow of everolimus-treated Eμ-Myc mice were also smaller than those from control mice [MCV placebo 288.4 ± 2.4 fl vs. everolimus 266.9 ± 3.9 fl (P = 0.002)]. Histology revealed preserved trilineage hemopoiesis after everolimus therapy with loss of the expanded population of B lymphoblasts that remained apparent in the marrow of placebo-treated mice (Supplementary Fig. S3C). Immunophenotyping showed reduced proportions of both B220+/slgM− and B220+/slgM+ B cells (Supplementary Fig. S3D). These findings show selective reduction of B lymphocytes in the bone marrow after everolimus therapy in the absence of nonspecific myelosuppression.

Figure 2. B-cell precursors in the spleen of Eμ-Myc mice are reduced by mTORC1 inhibition. Six- to 7-week-old wild-type littermate control mice (WT; n = 5), Eμ-Myc mice treated with placebo (P) for 2 weeks (n = 5), and Eμ-Myc mice treated with everolimus (E) for 2 weeks (n = 7) were analyzed. A, average spleen weight (P vs. E, P = 0.001). B, percent B220+ splenocytes (P vs. E, P = 0.001). C, May Grunwald–Giemsa–stained cytospin preparations of unsorted splenocytes (top) and purified B220+ cells (bottom) in a representative mouse from each group. D, Fluorescence-activated cell sorting (FACS) plots showing surface expression of IgM and IgD in B220+– gated splenocytes for a representative mouse from each group. Error bars represent the SEM. P values were generated using a Student unpaired 2-tailed t-test, *, P < 0.05.
To directly compare the effects of mTORC1 inhibition on B-cell populations from mice with wild-type levels of MYC expression versus transgenic levels in Eμ-Myc mice, we also administered everolimus to wild-type mice. As in Eμ-Myc mice, we did not observe myelosuppression in wild-type mice after everolimus therapy (Supplementary Fig. S4A–S4E). However, unlike Eμ-Myc mice, B-cell numbers in the spleen and bone marrow of wild-type mice were unchanged by everolimus treatment, showing the heightened sensitivity of B cells with oncogenic expression of MYC to mTORC1 inhibition (Supplementary Fig. S5A and S5B). Taken altogether, these results suggest that everolimus prevented tumor initiation through preferential elimination of tumor-susceptible undifferentiated B-cell populations from the spleen and bone marrow of Eμ-Myc mice.

**MYC Expression Is Maintained in the Face of mTORC1 Inhibition**

To confirm the molecular inhibition of mTORC1 signaling in everolimus-treated mice, we monitored RPS6 phosphorylation. We observed an increase in phosphorylated RPS6 in extracts from placebo-treated B cells compared with wild-type controls, corroborating the established positive correlation between MYC levels and mTORC1 activity (refs. 15, 19; Fig. 3A). In addition, phosphorylated RPS6 was reduced 24 hours after the last dose of everolimus, confirming continued robust inhibition of mTORC1 in the target cell population at trough drug levels. Given that rapamycin has been shown to regulate expression of MYC at a posttranscriptional level (20, 21), we assayed expression of MYC protein and the MYC transcriptional target genes *ODC1* (B) and *UBTF* (C) in B220+ cells from the bone marrow and spleen of WT (*n* = 3), placebo-treated transgenic (*n* = 5), and everolimus-treated transgenic mice (*n* = 7). mRNA expression levels were corrected for the expression of ubiquitin B and normalized to expression in WT controls. Data shown is the average ± SEM. *ODC1*, ornithine decarboxylase 1; *UBTF*, upstream binding transcription factor.

**Everolimus Has Single-Agent Activity Against Eμ-Myc Lymphoma**

Although short-term dosing with rapamycin lacked efficacy in treating established Eμ-Myc tumors (19, 22), chronic regular administration of everolimus has not been assessed as a
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Figure 4. Everolimus-induced tumor regression and resistance in established Eμ-Myc lymphoma. A and B, syngeneic mice were injected with Eμ-Myc lymphoma cells (tumor #299). Dosing with placebo (P) or everolimus (E) commenced on day 14 with the onset of overt malignancy (n = 5 mice/group). Surviving mice were bled 14, 24, and 38 days after tail vein injection of tumor cells. A, survival curves. Median survival was 25 days for placebo and 53 days for everolimus (P = 0.003). B, leukocyte counts of mice 14, 24, and 38 days after tumor injection. C and D, syngeneic mice were injected with EN Eμ-Myc lymphoma cells (tumor #299) or equal-passage cells previously exposed to everolimus (EE). Dosing with placebo or everolimus started 72 hours after injection (n = 10 mice/group). C, leukocyte counts 10 days after transplantation (EN tumor: placebo vs. everolimus, P < 0.001, EE tumor: placebo vs. everolimus, P = 0.07). Error bars represent the SEM. P values were generated using a Student unpaired 2-tailed t test, *, P < 0.05, NS, not statistically significant. D, survival curves. For EN tumor, the median survival was 22 days for placebo and 33 days for everolimus. For EE tumor, the median survival was 20 days for placebo and 22 days for everolimus. EE, everolimus exposed; EN, everolimus naive.

Everolimus Activity Does Not Correlate with Apoptosis

Because widespread apoptosis in response to chemoradiotherapy is a feature of Eμ-Myc lymphoma, we suspected that......
everolimus treatment might also trigger apoptosis to affect tumor regression. Accordingly, mice with overt lymphoma were analyzed after a single dose of everolimus for evidence of apoptosis over a 24-hour time period. Progressive diminution in leukocyte counts of treated mice occurred (Fig. 5A) and corresponded with a G1 cell-cycle arrest in involved lymph nodes (Fig. 5B). However, the increased sub-G1 DNA characteristic of apoptosis was minimal (Fig. 5B). To exclude the possibility of delayed apoptosis, we also carried out continuous daily dosing: Disease regression occurred, followed by stabilization between day 2 and 7 of therapy and then relapse by day 11 (Supplementary Fig. S8A). As seen at the shorter time points, disease response during ongoing everolimus administration was also associated with G1 arrest, but again, without marked increases in sub-G1 DNA (Supplementary Fig. S8B).

We then used isogenic tumor lines with constitutive BCL2 expression to examine whether functional apoptotic machinery was required for everolimus sensitivity. Everolimus treatment conferred a significant survival benefit over placebo in these tumor lines (median survival 13.5 days for placebo and 19.0 days for everolimus, P = 0.001; Fig. 5C). Importantly, the survival benefit of everolimus was maintained with enforced BCL2 expression (median survival 11.5 days for placebo and 16.0 days for everolimus, P < 0.001; Fig. 5D), suggesting that functional apoptotic networks are dispensable for everolimus activity. Thus, everolimus administration did not elicit an apoptotic response in Eμ-Myc lymphoma.

**Everolimus Induces Cellular Senescence**

Analysis of tumor morphology to characterize responses to everolimus more thoroughly revealed the presence of a mixed inflammatory cell infiltrate in involved lymph nodes that was particularly prominent after 2, 4, and 7 days of therapy (Fig. 6A) coinciding with tumor regression and disease stabilization (Supplementary Fig. S8A) and occurring in the absence of histopathologic changes in apoptosis. Given that...
cellular senescence has a prominent inflammatory component in in vivo tumor models (23), we investigated whether induction of senescence might account for everolimus activity. Everolimus treatment was associated with robust acquisition of senescence-associated β-galactosidase (SA-β-gal) activity in tumors after 4 and 7 days of treatment that was lost upon disease relapse at day 11, indicating that they no longer retain the capacity to undergo senescence (Fig. 6A and Supplementary Fig. S9A). Furthermore, immunostaining to identify granulocytes and macrophages using the markers Gr1 and F4/80, respectively, confirmed an increase in infiltrating innate immune cells capable of tumor clearance from day 2 forward (Fig. 6A and Supplementary Fig. S9B and S9C). Interrogation of tumor samples by Western blot analysis of samples obtained from everolimus-treated mice showed p53/ARF induction in the context of persistent inhibition of RPS6 phosphorylation (Fig. 6B). p21 levels were also upregulated, its expression coinciding with p53 serine-15 phosphorylation but preceding maximal p53 stabilization, thus possibly activated by low levels of active p53 in this setting. Consistent with a senescence response, activation of the senescence regulatory kinase p38 mitogen-activated protein kinase (p38MAPK) occurred after 4 days of everolimus treatment (24). We also observed an increase in H3K9 trimethylation (H3K9me3), a chromatin marker of transcriptional silencing mechanistically linked to cellular senescence, likely through its role in directing the silencing of E2F target genes (refs. 25, 26; Fig. 6B). Thus, treatment of Eμ-Myc lymphoma with everolimus was characterized by cell-cycle arrest, SA-β-gal staining, an innate immune response, and expression of tumor suppressor and senescence-associated genes consistent with oncogene-induced senescence as a mechanism for tumor clearance.

We hypothesized that a senescence mechanism was also operative during lymphoma prevention by everolimus in premalignant Eμ-Myc mice. Therefore we treated 4-week-old mice with everolimus and analyzed them on day 4. In everolimus-treated mice, morphologic analysis showed selective clearance of lymphoblasts known to be responsible for expansion of the splenic red pulp in transgenic mice (18), and this finding was associated with acquisition of SA-β-gal activity (Fig. 6C and Supplementary Fig. S10A). We also observed a gene expression profile, including increased expression of transcripts encoding the extracellular signaling molecules ICAM1, IGFBP7, and interleukin (IL)-6 that is reflective of a senescence response in B220⁺ but not B220⁻ cell populations in bone marrow isolated from mice treated for 4 days with everolimus (Supplementary Fig. S10B). Overall, these data in the prevention model corroborate those in the established Eμ-Myc tumor model and provide further evidence that activity of mTORC1 is required for avoidance of MYC-induced senescence in B lymphocytes.

Tumor Response to Everolimus Requires an Operative Senescence Response and a Functional p53 Pathway

A robust temporal relationship was shown between loss of response to everolimus and intratumoral selection for cells incapable of undergoing cellular senescence (Fig. 6A and Supplementary Fig. S9, compare day 4 and 7 with day 11). In murine models, p53 is widely regarded as a key mediator of senescence, and in Eμ-Myc lymphoma, p53 mutation is a well-characterized secondary genetic alteration (12). Therefore we examined whether everolimus resistance was associated with loss of p53 function. Given that etoposide sensitivity is a known indicator of p53 function (27, 28), we challenged everolimus-resistant tumors with etoposide. Whereas mice transplanted with EN tumors showed improved survival with etoposide treatment, those with EE tumors displayed markedly compromised etoposide sensitivity (Fig. 7A and B). To genetically interrogate the requirement for p53 function in everolimus responsiveness, tumors derived from Eμ-Myc mice with either genetic deletion of p53 or characterized by spontaneous p53 mutation were transplanted and mice were monitored for survival. The average survival advantage conferred by everolimus over placebo was 1.1-fold for lymphomas with homozygous deletion or mutation of p53 (Fig. 7C and D, Supplementary Fig. S11) compared with 1.7-fold for the panel of p53–wild-type lymphomas we screened initially (Fig. 4A and Supplementary Fig. S6A and S6B). Thus, the effectiveness of everolimus therapy was diminished in Eμ-Myc lymphomas in which p53 was deleted or p53 signaling was dysfunctional.

DISCUSSION

Rapamycin and rapamycin analogues are potent and selective inhibitors of mTORC1 with on-target activity at low nanomolar concentrations and no off-target kinase inhibition at levels less than 1 nmol/L (29). Everolimus improves clinical outcomes and is approved for use in the treatment of metastatic renal cell carcinoma (30) and subependymal giant cell astrocytomas associated with tuberous sclerosis (31). mTORC1 inhibitors are currently being assessed in clinical trials in a variety of other human cancers. Therefore mTORC1 inhibitor drugs serve both as tools that allow us to address important biologic questions about mTORC1 loss of function and as validated cancer therapeutics.

MYC transcriptionally regulates several components of the mTOR pathway, and a positive relationship exists between expression of MYC and mTORC1 activity. We found that mTORC1 activity was increased in premalignant B cells isolated from Eμ-Myc mice, and we have shown that mTORC1 activity in this model can be safely and effectively inhibited by once-daily dosing with everolimus. Our results indicate that therapeutic intervention to inhibit mTORC1 during the premalignant phase acts as a powerful barrier to the acquisition of additional genetic hits that facilitate malignant transformation. Transcripts that encode MYC have a complex 5' untranslated region (UTR) rendering MYC vulnerable to posttranscriptional inhibition by mTORC1 inhibition, and posttranscriptional modification of MYC expression can influence MYC-driven phenotypes under some experimental conditions (20, 21). However, in this study, continued expression and transcriptional activity of MYC was observed in B lymphocytes from transgenic mice treated with everolimus. These data are consistent with a model in which everolimus does not mediate its effects by reducing MYC function but rather acts via a parallel pathway or downstream of MYC to determine the cellular response to oncogenic MYC expression.

We found that everolimus improved the survival of mice transplanted with spontaneously arising Eμ-Myc lymphomas...
Figure 6. mTORC1 is required to prevent cellular senescence. A and B, syngeneic mice were injected with Eμ-Myc lymphoma cells (tumor #299), treated once daily with everolimus (E) after the development of overt malignancy, and sacrificed after 0, 2, 4, 7, and 11 days of therapy (n = 4 mice per group). A, sections from lymph nodes of untreated mice and mice sacrificed 2, 4, 7, and 11 days after chronic daily dosing with everolimus were stained with hematoxylin and eosin (H&E), SA-β-gal, anti-Gr1, and anti-F4/80. B, expression of phospho-(P-S15 p53) and total p53, p21, p19ARF, phospho-(P-p38MAPK) and total p38 MAPK, phospho-(P-RPS6), and total RPS6 and H3K9me3 in tumor lysates by Western blotting. Actin was used as a loading control. Full-length blots are presented in Supplementary Fig. S12. C, Four-week-old Eμ-Myc mice were treated with daily placebo (n = 4) or everolimus (n = 4) and sacrificed after 4 days. Splenic sections were stained with H&E (top), or SA-β-gal (bottom).
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Figure 7. Everolimus resistance is associated with loss of functional p53. A and B, syngeneic mice were injected with EN Eμ-Myc lymphoma cells (tumor #299) or equal-passage cells previously exposed to everolimus (EE). After the development of overt malignancy on day 14, mice were treated with placebo or etoposide (25 mg/kg intraperitoneally) on 3 consecutive days (n = 7 mice per group). Surviving mice were relabeled on day 26. A, leukocyte counts of mice before therapy with etoposide at 14 days (D14) after tail vein injection (TVI) and 14 days after etoposide (D28, P < 0.001 for EN vs. EE tumors treated with etoposide at D28), *P < 0.05. B, survival curves. Median survival durations were 24 days and 25 days for placebo-treated EE and EN tumors, respectively, and 35 days and 54.5 days for etoposide-treated EE and EN tumors (P = 0.03 for EN vs. EE tumors treated with etoposide). C and D, syngeneic mice were injected with lymphoma cells (tumor #3391 or tumor #3239) derived from spontaneously arising tumors in Eμ-Myc;p53−/− mice. Dosing with placebo or everolimus was started 72 hours after injection. C, leukocyte counts of mice measured 10 days after commencement of therapy, (top, placebo vs. everolimus; P = 0.26). D, survival curves for everolimus-treated and placebo-treated mice (bottom). Median survival was 17 days in the placebo group and 18 days in the everolimus group (n = 10 mice per group). Error bars represent the SEM. P values were generated using a Student unpaired 2-tailed t test, *P < 0.05.

that were wild type for p53. Tumor regression in response to mTORC1 inhibition was not associated with apoptosis. Furthermore, everolimus sensitivity persisted in tumors with enforced expression of BCL2. In keeping with our findings, everolimus did not induce apoptosis of B-cell acute lymphoblastic leukemia cells in xenograft experiments (32). It is known that the apoptotic response to rapamycin in Eμ-Myc lymphoma can be heightened by interventions that activate signaling upstream of mTORC1, such as expression of miR-34a and loss of PTEN, or loss of TSC2 (19, 22, 33). Notably, in our studies, we did not hyperactivate AKT and observed cellular senescence rather than apoptotic cell death after mTORC1 inhibition. Thus, mTORC1 signal intensity may determine whether tumor cells undergo apoptosis or senescence in response to mTORC1 inhibition.

Oncogene-induced senescence is thought to function as a safeguard that premalignant cells must circumvent to undergo malignant transformation. Accordingly, as malignant potential evolves, the risk of dysfunction or inactiva-
increased histone H3K9 trimethylation (H3K9me3), G1 cell-cycle arrest, activation of p38MAPK, and markers of tumor inflammation. Indeed, many regard the sustained and irreversible cessation of proliferation as a fundamental characteristic of senescence. Of all the senescence indicators present in our study, perhaps the best testament to the irreversibility of the everolimus effect is the long-term protection it affords prelymphomatous mice from malignant transformation.

The importance of oncogene-induced senescence in Eμ-Myc lymphomas has been highlighted by recent articles showing that senescence abrogation through genetic deletion of the histone methyltransferase SUV39h1 greatly reduced the tumor latency of Eμ-Myc lymphomas (26) and that senescence induction by genetic deletion of CDK2 delayed lymphomagenesis in Eμ-Myc mice (35). Our work critically extends these observations by showing that the route to malignant transformation through suppressed senescence can be selectively targeted pharmacologically to realize biologically significant improvements in survival.

The TGF-β pathway has been linked to senescence induced by MYC. Van Riggelen and colleagues (36) reported that senescence occurring in T-cell lymphomas after MYC inactivation requires TGF-β signaling and that the MIZ1-mediated effects of MYC negatively regulate senescence in response to TGF-β. Complex interplay has also been observed between the tumor and the host immune system during senescence. In a mouse model of T-cell ALL, the senescence and clearance of malignant cells after tetracycline-mediated suppression of MYC expression was impaired in the absence of CD4+ T cells (37). Reimann and colleagues (26) identified 2 pathways to MYC-induced senescence in Eμ-Myc lymphomas: a relatively weak cell-autonomous pathway and a stronger non-cell-autonomous pathway that required secretion of TGF-β by activated macrophages in the tumor stroma. The senescence response was dependent on SUV39h1 activity as monitored by the repressive chromatin mark, H3K9me3. Our studies show that macrophage recruitment and H3K9me3 are features of the senescence response induced by everolimus. In addition, we did not observe markers of senescence after treatment of Eμ-Myc lymphoma cell lines with everolimus in vitro (data not shown), suggesting that nonmalignant immune cells in the tumor stroma make a significant contribution to the senescence triggered by mTORC1 inhibition in this model.

With respect to other forms of oncogene-induced senescence, there is a growing body of evidence to support the contention that phosphoinositide 3-kinase (PI3K)/AKT/mTOR signaling is inhibitory to senescence triggered by deregulation of the RAS pathway. In the inherited condition neurofibromatosis type 1, inactivating mutations of the NFI gene lead to RAS activation; within benign neurofibromas from these patients, generation of a negative feedback loop that downregulates PI3K/AKT signaling triggers senescence (38). A more recent study that used a mouse model of pancreatic cancer showed that RAS-induced senescence was suppressed by activation of the PI3K pathway via PTEN deletion and that loss of PTEN accelerated tumorogenesis in a gene dosage-dependent manner. Rapamycin administration rescued senescence, suggesting that signaling through mTORC1 was necessary to restrain RAS-induced senescence in premalignant lesions in the pancreas (39). Likewise, in human melanocytes, a short hairpin RNA (shRNA) that reduced expression of PTEN prevented senescence provoked by the oncoprotein BRAF<sup>V600E</sup> (40). Our study is the first to show that mTORC1 inhibitors can exert their anticancer activity by provoking senescence induced by the MYC oncogene, suggesting that inhibition of senescence by PI3K/AKT/mTOR signaling may occur in oncogene-induced senescence other than that due to oncogenic RAS signaling. Thus, mTORC1 inhibition can prevent or delay the onset of malignancy in other cancer-prone mice (41–46). Whether cellular senescence occurs in other mouse models in which cancer is prevented by mTORC1 inhibitors is unclear.

Growing understanding of the role senescence plays in cancer has spurred interest in the idea of harnessing senescence induction for therapeutic benefit. Our study serves as proof-of-principle that targeted therapy can bring about tumor regression by activating senescence. At the same time, our data illustrate some potential pitfalls of this approach. In established lymphoma, the response to everolimus was not sustained because of strong selective pressure favoring preexisting senescence-defective tumor subpopulations. Thus, future strategies will need to anticipate and avoid outgrowth of evolved clones with intrinsic drug resistance due to failure to senesce if we are to leverage such therapies for maximal clinical gain.

Consensus is lacking in the literature about whether a functional p53 pathway is required for the anticancer activity of mTORC1 inhibitors. Studies in myeloma (47), breast (48), and ovarian (49) cancer cells <i>in vitro</i> and in ovarian cancer xenografts (50) suggest that tumors dependent on AKT signaling for survival respond to mTORC1 inhibition irrespective of p53 status. In contrast, Beuvink and colleagues (51) showed that RNA interference knockdown of p53 abolished synergistic killing of A549 lung cancer cell lines by RAD001 and cisplatin, and Wendel and colleagues (22) showed p53-dependent resistance to rapamycin in Eμ-Myc;PTEN<sup>−/−</sup> lymphomas. Given the clinical implications of these findings, we made it a priority to establish the p53 dependence of the everolimus response in Eμ-Myc lymphomas.

In the current study, we found that Eμ-Myc lymphomas generated on the background of p53 genetic loss of function display intrinsic everolimus resistance (Fig. 7C and D), showing that a therapeutic response to everolimus requires functional p53. Consistent with this finding, resistance to everolimus coincided with the outgrowth of resistant clones that were defective for the p53 pathway. Surprisingly, although etoposide sensitivity is a reliable indicator of intact p53 function, sequencing of p53 exons did not identify any somatic mutations to account for the loss of etoposide sensitivity that tracked with everolimus resistance (data not shown). Thus, loss of p53 function is likely to be mediated through mechanisms other than mutations in the coding region of p53 as previously reported in malignant disease (52). Interestingly, when we treated Eμ-Myc mice with CX-5461, a small-molecule inhibitor of Pol I transcription and the ribosomal RNA synthesis pathway that is under the direct control of mTOR, animal survival was significantly improved in a p53-dependent manner. Likewise, sequencing of p53 exons in CX-5461-resistant clones failed to uncover the expected p53 mutations, suggesting that, in this model, drug pressure on a functional p53 pathway in response to inhibition of growth and translation is borne out via molecular lesions other than in p53 itself (data not shown; ref. 17). Greater understanding of the factors that mediate everolimus resistance may be of universal benefit by identifying ways to improve the clinical
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performance of mTORC1 inhibitors through the use of rational drug combinations. One potential approach to combat the outgrowth of resistant clones is the use of everolimus in combination with drugs that are known to have p53-independent cytotoxicity, such as vorinostat (53). Although the survival advantage conferred by wild-type p53 over deleted or mutated p53 was robust overall (an average of 12.8 compared with 2.5 days; see Fig. 4A, Supplementary Fig. S6 and Supplementary Fig. 7C and D, and Supplementary Fig. S11, respectively), the variability in the observed everolimus response among the p53 wild-type tumors is also of interest. These findings suggest that additional factors, such as cooperating genetic lesions that affect disease aggression or influence interaction with host stromal cells, have a role to play in determining the relative everolimus sensitivity of those tumors with wild-type p53.

Everolimus is currently undergoing testing in clinical trials in mantle cell lymphoma and diffuse large B-cell lymphoma. MYC translocations and p53 mutation/deletion are known to occur in both of these tumor types (54, 55). Furthermore, a common criterion for patient inclusion in such clinical trials is failed therapy with standard first-line treatment regimens that incorporate multiagent chemotherapy, and it is this particular cohort that may be enriched for patients with tumors that have lost functional p53—and/or have a rearrangement of MYC. Our findings are of immediate clinical relevance as they suggest that MYC rearrangement and p53 status may constitute predictive biomarkers for response to everolimus in B-cell lymphomas.

METHODS

Experimental Animals

Ever-Myc C57BL/6 transgenic mice were generated as described previously (6). Six- to 8-week-old C57BL/6J male mice were used as recipient syngeneic mice for tumor transplantation studies. Donor C57BL/6 transgenic mice were generated as described above. Lymphadenopathy was assessed by weekly palpation, in addition to exhibiting resistance to etoposide (17, 56).

Blood Sampling

Seventy-five to 100 μL of blood was obtained from the retro-orbital sinus. Leukocyte counts were measured using an Advia 120 automated hematology analyzer (Bayer Diagnostics).

B-Cell Isolation

Cells suspended at 10⁶ per 100 μL were incubated with biotinylated rat anti-mouse B220 antibody (BD Pharmingen) followed by washing and resuspension in 80 μL of magnetic-activated cell sorting buffer per 10⁵ cells. Twenty microliters of goat anti-rat IgG microbeads (Miltenyi Biotec) was added to each sample, and the cells were incubated for 15 minutes. Cells were labeled with streptavidin-conjugated phycoerythrin (PE; BD Pharmingen) and resuspended in buffer before magnetic separation with the autoMACS (Miltenyi Biotec) POSSEL program. Cells were deemed to be of adequate purity if more than 90% were B220⁺.

Immunophenotyping

Single-cell suspensions were labeled with APC-conjugated rat anti-mouse B220 (CD45R, eBioscience), fluorescein isothiocyanate (FITC)-conjugated rat anti-mouse IgM and PE-conjugated rat anti-mouse IgD (BD Pharmingen), or APC-conjugated rat anti-mouse B220, FITC-conjugated rat anti-mouse CD24 (BD Pharmingen), and PE-conjugated rat anti-mouse CD43 (BD Pharmingen), washed, and then resuspended in buffer containing 2 μmol/L FluoroGold (hydroxystilbamidine, Molecular Probes, Invitrogen) before data collection on an LSR-II flow cytometer and analysis using FCS Express software. Immunophenotyping was used to stage B cells developmentally based on the model of Hardy and colleagues (57) as adapted by Iritani and Eisenman (9).

Western Blotting

Equal amounts (20–50 μg) of protein lysates were separated by SDS-PAGE as described previously (21). Separated proteins were transferred to Immobilon-P membranes (Millipore) and probed with antibodies before detection by enhanced chemiluminescence and autoradiography. Antibodies used were c-MYC (9402), RPS6 5G10 (2217), P-RPS6 S240/244 (2215), P-p53 S15 (9284), p38MAPK (9212), P-p38MAPK (9211; all were obtained from Cell Signaling Technology), actin C4 (69100, MP Biomedicals), p19 ARF 5-C3-1 (sc-32748), p21 C-19, (sc-397; both obtained from Santa Cruz Biotechnology), p53 (NCL-p53-505, Novocastra, Leica Microsystems), and H3K9me3 (ab8898, Abcam). Equal amounts (20–50 μg) of protein lysates were separated by SDS-PAGE as described previously (21). Separated proteins were transferred to Immobilon-P membranes (Millipore) and probed with antibodies before detection by enhanced chemiluminescence and autoradiography. Antibodies used were c-MYC (9402), RPS6 5G10 (2217), P-RPS6 S240/244 (2215), P-p53 S15 (9284), p38MAPK (9212), P-p38MAPK (9211; all were obtained from Cell Signaling Technology), actin C4 (69100, MP Biomedicals), p19 ARF 5-C3-1 (sc-32748), p21 C-19, (sc-397; both obtained from Santa Cruz Biotechnology), p53 (NCL-p53-505, Novocastra, Leica Microsystems), and H3K9me3 (ab8898, Abcam).

Quantitative Real-Time PCR

RNA was isolated by direct cell lysis using TRIzol reagent according to the manufacturer’s instructions (Invitrogen). Equal starting amounts of RNA were treated with DNase at 37°C for 15 minutes and reverse transcribed by Superscript III (Invitrogen) with random hexamers (Promega). Eighteen microliters of master mix containing cDNA and SYBR Green was added to 2 μL of a 100 μmol/L forward and reverse primer. PCR and detection were conducted in an ABI prism 7000 thermocycler (Applied Biosystems). Results were quantified using the ΔΔCt method (58). Primer sequences are provided (Supplementary Table S2) or have been described previously (21).

Cell-Cycle Analysis

A total of 5 × 10⁶ cells were fixed by the dropwise addition of 4.5 mL of ice-cold 95% ethanol during slow vortexing and placed at 4°C for 24 hours. Washed cells were resuspended in 300 μL of PBS-2% FBS containing 10 μg/mL of propidium iodide and 250 μg/mL RNAase A for 30 minutes before analysis. Five thousand single-cell events

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were captured with a flow cytometer and analyzed using Modfit software (Verity Software House).

**Immunofluorescence**

Four-micrometer fresh-frozen sections were fixed in ice-cold acetone, washed and incubated with rat anti-mouse Gr1 (BD Pharmingen) and F4/80 (AbD Serotec) antibodies, incubated with goat anti-rat Alexa 647 secondary antibody (Invitrogen), and mounted in Prolong Gold with 4′,6-diamidino-2-phenylindole (DAPI; Invitrogen). Images were acquired using an Olympus BX-51 fluorescent microscope and analyzed using SPOT advanced software (Diagnostic Instruments).

**Senescence-Associated β-Galactosidase Staining**

Eight-micrometer sections were cut from samples embedded in Tissue-Tek OCT (Sakura Finetek) and stained with the use of the Senescence-Associated β-Galactosidase staining kit (Cell Signaling) according to the manufacturer’s instructions.

**Statistical Analysis**

Survival curves were plotted by the Kaplan-Meier method. The log-rank test was used to assess differences and nominal P values were calculated.

**Disclosure of Potential Conflicts of Interest**

R.W. Johnstone has a commercial research grant and has received honoraria for service on the speakers’ bureau for Novartis. G.A. McArthur has commercial research grants from Novartis and Pfizer. No potential conflicts of interest were disclosed by the other authors.

**Authors’ Contributions**


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Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): G. Poortinga, K.L. Stanley, M. Bots, G.A. McArthur


**Acknowledgments**

The authors thank Daniela Cardozo, Susan Jackson, Rachel Walker, Anthony Natoli, and Dr. Petranell Ferraio for technical assistance, Carleen Cullianane and A/Prof. Ygal Haupt for helpful discussion, and Louise Purton and Carl Walkley for critical reading of the manuscript. Everolimus and placebo preparations were kindly provided by Dr. Heidi Lane (Novartis Institute for BioMedical Research, Basel, Switzerland).

**Grant Support**

This work was supported by grants from the National Health and Medical Research Council of Australia to R.D. Hannan (166908 and 251688), R.B. Pearson (509087 and 400116), M.J. Smyth (454569), R.W. Johnstone (251608 and 566702), and G.A. McArthur (400120 and 568876) and from Cancer Council Victoria (to M. Wall, R.B. Pearson, and R.W. Johnstone), the Leukaemia Foundation of Australia (to C.J. Chan, R.W. Johnstone, and R.D. Hannan), the Victorian Cancer Agency (to M. Wall and R.W. Johnstone), the Susan G. Komen for the Cure Foundation (to R.W. Johnstone), the Australian Rotary Health Foundation (to R.W. Johnstone), and the National Cancer Institute (to S.W. Lowe). G.A. McArthur received the Sir Edward Dunlop Fellowship of the Cancer Council of Victoria.

Received July 30, 2012; revised October 22, 2012; accepted October 23, 2012; published OnlineFirst December 14, 2012.

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