A c-Myc and Surface CD19 Signaling Amplification Loop Promotes B Cell Lymphoma Development and Progression in Mice

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Malignant B cells responding to external stimuli are likely to gain a growth advantage in vivo. These cells may therefore maintain surface CD19 expression to amplify transmembrane signals and promote their expansion and survival. To determine whether CD19 expression influences this process, Eμ-Myc transgenic (c-MycTg) mice that develop aggressive and lethal B cell lymphomas were made CD19 deficient (c-MycTgCD19−/−). Compared with c-MycTg and c-MycTgCD19+/− littermates, the median life span of c-MycTgCD19−/− mice was prolonged by 81–83% (p < 0.0001). c-MycTgCD19+/− mice also lived 42% longer than c-MycTg littermates following lymphoma detection (p < 0.01). Tumor cells in c-MycTg and c-MycTgCD19−/− mice were B lineage derived, had a similar phenotype with a large blastlike appearance, invaded multiple lymphoid tissues, and were lethal when adoptively transferred into normal recipient mice. Importantly, reduced lymphomagenesis in c-MycTgCD19−/− mice was not due to reductions in early B cell numbers prior to disease onset. In mechanistic studies, constitutive c-Myc expression enhanced CD19 expression and phosphorylation on active sites. Reciprocally, CD19 expression in c-MycTg B cells enhanced c-Myc phosphorylations in early B cell numbers prior to disease onset. In mechanistic studies, constitutive c-Myc expression enhanced CD19 expression and phosphorylation at regulatory sites, sustained higher c-Myc protein levels, and maintained a balance of cyclin D2 expression over that of cyclin D3. These findings define a new and novel c-Myc:CD19 regulatory loop that positively influences B cell transformation and lymphoma progression. The Journal of Immunology, 2012, 189: 2318–2325.

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D19 is a B cell-specific transmembrane glycoprotein that by virtue of its extensive cytoplasmic domain establishes intrinsic B cell signaling thresholds and regulates BCR-dependent and BCR-independent signal transduction pathways (1). CD19 is a central signaling component of cell surface complexes that can include CD21, CD81, and CD225 (2–5). Because CD21 is a receptor for C3 complement fragment, CD19 also links the innate and adaptive arms of the immune system (6, 7). CD19 is expressed on the cell surface from the late pro-B to early pre-B cell stages, with increasing expression as B cells mature in humans (8–11) and in mice (12–14). CD19 surface density is similar on mature conventional B cells from different peripheral lymphoid tissues, and B cell activation by anti-IgM Abs, LPS, or IL-4 does not significantly alter CD19 expression (12, 15). Humans genetically deficient for CD19 expression and gene-targeted (CD19−/−) mice have B cells that are hyporesponsive to transmembrane signals and generate weak T cell-dependent humoral immune responses (16–18). By contrast, transgenic mice that overexpress CD19 have hyperresponsive B cells and develop autoantibodies with age (12, 13, 17, 19, 20). Even modest increases in CD19 expression by 15–29% induce autoantibody production in mice (21). Thus, strict regulation of CD19 expression is important for normal B cell signal transduction, development, and function.

As with normal B cells, malignant B cells responding vigorously to external and internal stimuli may gain survival and growth advantages that promote lymphoma development, survival, and proliferation (22). CD19 is expressed on nearly all malignancies of B cell origin, which includes 80% of acute lymphoblastic leukemias, 88% of B cell lymphomas, and 100% of B cell leukemias (8, 11). CD19 expression is also retained at relatively high levels on the majority of B cell tumors, including most pre-B, common, and null-acute lymphoblastic leukemias, non-Hodgkin’s lymphomas, B cell chronic lymphocytic leukemias, prolymphocytic leukemias, and hairy cell leukemias (8, 9, 11, 23). However, whether CD19 expression itself contributes to malignant B cell transformation or growth in vivo is unknown.

Because of its ubiquitous expression in B cell malignancies and importance in establishing B cell signaling thresholds, the contribution of CD19 expression to the malignant transformation of B cells and severity of lymphomas was investigated in Eμ-Myc transgenic (c-MycTg) mice (24–26). c-Myc is one of the most frequently dysregulated proteins in human malignancies. Burkitt’s lymphoma and diffuse large B cell lymphomas in humans are characterized by translocations between Ig gene promoter regions and the MYC proto-oncogene, leading to aberrant c-Myc overexpression. Similarly, c-MycTg mice, in which the MYC proto-oncogene is under the control of the IgH enhancer, develop aggressive B cell-derived lymphomas at an early age, and have a ∼90% mortality rate by 20 wk of age, with a median age of death at ∼12 wk (24, 25). c-MycTg lymphomas develop from the B220+ pre-B and immature B cell pools, and Ig gene rearrangement analyses indicate that most are monoclonal (24). Rare
genetic events coupled with c-Myc overexpression are thus required for malignant B cell transformation (25). Using c-MycG12V mice deficient in CD19 expression, a c-Myc:CD19 regulatory loop was identified in the current study that positively influences B cell transformation and lymphoma progression.

Materials and Methods

**Mouse breeding and survival analysis**

c-MycG12V (C57BL/6J-Tg[IfyMyc]22Bri/J) hemizygous mice were from The Jackson Laboratory. c-MycG8 mice were crossed with CD19-deficient mice (17), and F1 generation mice were interbred to obtain CD19 wild-type (WT), heterozygous, and deficient littermates hemizygous for the c-Myc transgene (c-MycG8, c-MycG8CD19+/−, and c-MycG8CD19−/−, respectively). c-Myc transgene presence was determined by PCR, as follows: forward primer, 5′-CACTCTCGGCTAATAGCGAAGAG-3′; reverse primer, 5′-CTGGTACGTGGTACGAGTCTCA-3′; and c-Myc transgene presented was determined by PCR, as follows: forward primer, 5′-CACTCTCGGCTAATAGCGAAGAG-3′; reverse primer, 5′-CTGGTACGTGGTACGAGTCTCA-3′, according to The Jackson Laboratory genotyping protocols. c-MycG8, c-MycG8CD19+/−, and c-MycG8CD19−/− CD19 littersmates were monitored daily for the first appearance of lymphadenopathy, usually characterized by swelling of the cervical and brachial lymph nodes resulting in the characteristic “water wings” appearance described for c-MycTg mice (25). Mice were also monitored for the rate of tumor progression and for mortality. Notably, cachexia requiring euthanasia developed in some mice without detectable lymphadenopathy. These mice were thus grouped into a survival, but not observable lymphoma, category. As required by the guidelines of the Institutional Animal Care and Use Committee of Duke University, mice were euthanized if a pre-determined level of distress was reached prior to natural death. For these mice, the date of euthanasia was used as the date of death in survival studies. Comparative statistical analysis of survival was performed by the log-rank test. Kaplan–Meier survival plots were generated using the GraphPad Prism software package. For adoptive transfer experiments, B220+ cells from c-MycG8 or c-MycG8CD19−/− mice were injected i.p. in sterile DPBS into BALB/c mice (1 × 107) or from disease-free c-MycTg and c-MycTgCD19+/- mice, respectively. 

**Reagents and Abs**

FITC-, PE-, or PE-Cy5-conjugated rat anti-mouse Abs reactive with CD19, B220, heat-stable Ag (HSA), CD43, and CD5 were from BD Pharmingen (San Diego, CA). FITC-conjugated goat anti-mouse Ab reactive with IgM was from Southern Biotechnology (Birmingham, AL). Anti-mouse Thy1.2 Ab-coated magnetic beads were from Dynal (Lake Success, NY). Rabbit polyclonal Abs reactive with c-Myc, Pax5, ERK2, cyclin D2, cyclin D3, and cyclin-dependent kinase 4 (Cdk4) were from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal Abs reactive with c-Myc-phosphorylated at threonine 58 (T58)/serine 62 (S62), CD19 phosphorylated at tyrosine 513 (Y513), and CD19 protein were from Cell Signaling Technology (Beverly, MA). Rabbit polyclonal Abs reactive with the dually phosphorylated (kinase-active) forms of ERK and JNK were from Promega (Madison, WI). Some c-MycTg mice die of B cell tumors that do not induce lymphadenopathy. During these survival studies, mice were monitored daily for the first appearance of lymphadenopathy, usually characterized by swelling of the cervical and brachial lymph nodes resulting in the characteristic “water wings” appearance described for c-MycTg mice (25). Mice were also monitored for the rate of tumor progression and for mortality. Notably, cachexia requiring euthanasia developed in some mice without detectable lymphadenopathy. These mice were thus grouped into a survival, but not observable lymphoma, category. As required by the guidelines of the Institutional Animal Care and Use Committee of Duke University, mice were euthanized if a pre-determined level of distress was reached prior to natural death. For these mice, the date of euthanasia was used as the date of death in survival studies. Comparative statistical analysis of survival was performed by the log-rank test. Kaplan–Meier survival plots were generated using the GraphPad Prism software package. For adoptive transfer experiments, B220+ cells from c-MycG8 or c-MycG8CD19−/− mice were injected i.p. in sterile DPBS into BALB/c mice (1 × 107) or from disease-free c-MycTg and c-MycTgCD19+/- mice, respectively.

**In vitro proliferation**

Primary tumor cells from c-MycG8 or c-MycG8CD19−/− mice were mixed at a 1:1 ratio and then labeled with eFluor 670 cell proliferation dye (eBioscience), according to the manufacturer’s instructions. The level of fluorescence intensity was then assessed by flow cytometry (excitation 633 nm/emission 670 nm) immediately after labeling of cells, or following a period of cell culture.

**Comparison of tissue B cell numbers and cell surface protein expression levels**

Leukocytes were isolated from the blood, bone marrow (bilateral femurs), spleens, cervical lymph nodes, and peritoneal cavities of 6- to 8-week-old disease-free c-MycG8, c-MycG8CD19−/−, or transgenic littermates. Cells were then labeled with predetermined optimal concentrations of Abs reactive with cell surface molecules of interest to distinguish lymphocyte populations and to evaluate mean fluorescence intensity values by flow cytometry analysis.

**BrdU incorporation**

Lymphoma-free c-MycG8 and c-MycG8CD19−/− mice, or WT littermates, were injected i.p. with sterile-filtered BrdU (100 μg/g body weight) in PBS. After 3 h, tissues were harvested and single leukocyte populations were assessed for the percentage of BrdU-positive B cells. Briefly, cells were fixed in 0.5% paraformaldehyde, permeabilized with 3 N HCl containing 0.5% Tween 20, neutralized with 0.1 M disodium tetraborate, and stained with FITC-conjugated anti-BrdU Ab (BD Biosciences) and PE-conjugated anti-mouse B220 Ab, followed by flow cytometry analysis.

**Analysis of protein phosphorylation and expression levels**

B cells were isolated from single-cell suspensions of splenocytes from disease-free c-MycG8 and c-MycG8CD19−/− mice, or from CD19+/+ and/or CD19−/− littersmates to a purity of ≥95% by removal of T cells and NK cells with magnetic beads, and any residual monocytes by reverse fluorescence. A total of 5 × 10⁶ B cells was lysed in a buffer containing 1% Nonidet P-40, 150 mM NaCl, 50 mM Tris-HCl (pH 8.0), 1 mM Na orthovanadate, 2 mM EDTA, 50 mM NaF, and 1 mM PMSF, with solubilized proteins separated on 10% SDS-PAGE gels, followed by transfer to nitrocellulose membranes. Immunoblottting was performed with Abs specific for CD19 phosphorylated at regulatory tyrosines on the cytoplasmic tail, or for c-Myc and Pax5 proteins. Phospho-CD19 blots were then stripped and reprobed with Abs reactive with CD19 protein. For analysis of ERK and JNK activation, immunoblotting was performed with Abs specific for the kinase-active (dually phosphorylated) forms of ERK or JNK, followed by reprobing with Abs reactive with ERK2 protein. c-Myc phosphorylation was assessed by immunoblotting using Abs reactive with c-Myc when phosphorylated at T58/S62. The membranes were then reprobed with Abs reactive with c-Myc and ERK2 proteins. D-type cyclin and Cdk4 expression was assessed by immunoblotting with Abs specific for these proteins, followed by reprobing with Abs for ERK2. Densitometry analysis of protein bands in immunoblots was performed using NIH Image software version 1.60.

**cDNA gene arrays**

GEArray Q Series mouse gene expression arrays specific for genes in the cell cycle pathway were from SuperArray (Bethesda, MD). Purified splenic B cells (1 × 10⁶) were isolated from disease-free MycG8CD19−/− and c-MycG8CD19−/− littermates, and total RNA was isolated using a RNeasy kit (Qiagen, Valencia, CA). RNA (2.5 μg) was then subjected to cDNA synthesis and hybridization to the arrays, according to the manufacturer’s instructions. Reagents used for cDNA synthesis not provided in the kits included [32P]dCTP (ICN) and SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA). cDNA probe hybridization was analyzed using a phosphorimager. Gene density analysis was performed using the ScanAlyze program, version 2.5 (M. Eisen, Stanford University).

**Results**

**CD19 deficiency prolongs the life span of c-MycG8 mice**

The influence of CD19 expression on survival was evaluated for c-MycG8 mice, and c-MycG8 littermates heterozygous for CD19 expression (c-MycG8CD19+/−) or lacking CD19 expression (c-MycG8CD19−/−). The life span of c-MycG8CD19−/− mice was significantly prolonged in comparison with both c-MycG8 and c-MycG8CD19+/− littermates (p < 0.0001, Fig. 1A). The median age of mortality for c-MycG8CD19−/− mice was 24.3 wk, compared with 13.4 wk for c-MycG8−/− littermates (81% increase) and 13.3 wk for c-MycG8CD19+/− littermates (83% increase). Nevertheless, a 100% penetrance of lymphomas occurred in c-MycG8 CD19−/− mice, as all succumbed to disease by 76 wk of age. This indicates that although CD19 expression is not required for c-Myc–derived lymphomas to occur, it accelerates malignant transformation and/or disease severity.

During these survival studies, mice were monitored daily for the onset of lymphoma as assessed by the visual enlargement of one or more peripheral lymph nodes until the time of death. The mean age at which visible lymphoma onset occurred in c-MycG8CD19−/− mice was significantly delayed in comparison with c-MycG8 littermates (40% increase, p < 0.01, Fig. 1B), and there was a prolonged survival period between initial lymphoma detection and death (49% increase, p < 0.01). This represented a 42% greater mean life span for c-MycG8CD19−/− mice that had lymph node involvement (p < 0.01).

Some c-MycG8 mice die of B cell tumors that do not induce lymphadenopathy, but rather invade solid organs or the CNS,
c-MycTg mice. The log-rank test was used to determine the level of significance between curves in the Kaplan–Meier survival plots. Numbers (arrows) indicate median ages of death for c-MycTg and c-MycTgCD19−/− littermates. (B) CD19 expression accelerates lymphoma development and progression in c-MycTg mice. Values represent mean (±SEM) age at disease onset, duration of disease, and age at death (or euthanasia) for mice of each genotype in the pool of littermates described in (A). "Observable lymphoma" includes all mice with detectable lymphadenopathy prior to death, whereas "Wasting disease" includes all mice that succumbed to disease without detectable lymph node involvement. Statistical analysis used the Student t test. Significant differences in means between genotypes are indicated. **p ≤ 0.01.

B cell numbers are similar between c-MycTg and c-MycTgCD19−/− mice

Reduced tumorigenesis in c-MycTgCD19−/− mice did not result from reduced B cell numbers. Total B220low B cell numbers, including pro/pre- plus immature B cells in the bone marrow and immature B cells in all other tissues, were similar between c-MycTg and c-MycTgCD19−/− mice (Fig. 4A). Notably, mature

FIGURE 1. CD19 expression promotes lymphomagenesis in c-MycTg mice. c-MycTg, c-MycTgCD19+/−, and c-MycTgCD19−/− littermates were monitored daily for the onset of lymphadenopathy and mortality, or a moribund state requiring euthanasia. (A) CD19 expression accelerates death of c-MycTg mice. The log-rank test was used to determine the level of significance between curves in the Kaplan–Meier survival plots. Numbers (arrows) indicate median ages of death for c-MycTg and c-MycTgCD19−/− littermates. (B) CD19 expression accelerates lymphoma development and progression in c-MycTg mice. Values represent mean (±SEM) age at disease onset, duration of disease, and age at death (or euthanasia) for mice of each genotype in the pool of littermates described in (A). "Observable lymphoma" includes all mice with detectable lymphadenopathy prior to death, whereas "Wasting disease" includes all mice that succumbed to disease without detectable lymph node involvement. Statistical analysis used the Student t test. Significant differences in means between genotypes are indicated. **p ≤ 0.01.

FIGURE 2. B cell blasts dominate the blood and lymphoid tissues of c-MycTg and c-MycTgCD19−/− mice with high tumor burden. Immunofluorescence staining followed by flow cytometry analysis was performed on blood(A) and lymph node (B) cells isolated from representative c-MycTg and c-MycTgCD19−/− mice with lymphadenopathy that had reached humane endpoint criteria. Blood and lymph nodes from WT littermates were assessed for comparison. (A) The left panels show blood cells with the FSC and side scatter (SSC) profiles of lymphocytes. Gated regions in the middle panels indicate the percentage of B220+ cells of the total lymphocytes shown at left. The vertical dashed line in the panels at right is shown to delineate cells that were positive or negative for CD19 expression based on control Ab staining. (B) Gated regions indicate the percentage of B220+ cells out of total lymph node cells. These results are representative of ≥5 mice of each genotype.
B cell numbers were reduced in the spleen and lymph nodes of c-MycTgCD19^{−/−} mice compared with c-MycTg littermates, as occurs in CD19^{−/−} mice (17). However, reduced B220^{high} B cell numbers do not explain the enhanced survival of c-MycTgCD19^{−/−} mice, as lymphomas in c-MycTg mice generally derive from B220^{low} B cells (24, 25). Also, the level of short-term proliferation of B cells in vivo was similar between lymphoma-free c-MycTg and c-MycTgCD19^{−/−} mice as determined by BrdU uptake (Fig. 4B). Therefore, the reduced lymphomagenesis and enhanced survival of c-MycTgCD19^{−/−} mice were not due to reductions in early B cell numbers or to detectable changes in B cell proliferation prior to disease onset.

Constitutive c-Myc expression augments CD19 surface density
Because genetic alterations in CD19 expression have dramatic effects on B cell function in humans and mice (12, 13, 16–20), whether CD19 expression levels are altered in c-MycTg mice was therefore assessed. B cell subset analysis in c-Myc Tg mice revealed that surface CD19 expression was detectable at higher levels on bone marrow CD43^{−/−}B220^{low} pre-B cells, on HSA^{high}
B220<sub>low</sub> immature B cells in the bone marrow and spleen (42 and 37%, respectively, \( p < 0.01 \)), and on HSA<sub>low</sub>B220<sub>high</sub> mature bone marrow and spleen B cells (14 and 17%, respectively, \( p < 0.01 \)) as determined by immunofluorescence staining (Fig. 5A). A similar increase in CD19 expression on mature blood B cells was observed in c-Myc<sup>Tg</sup> and c-Myc<sup>Tg</sup>CD19<sup>−/−</sup> mice. Bone marrow (bilateral femurs), spleens, cervical lymph nodes, and blood were isolated from 6- to 8-wk-old lymphoma-free c-Myc<sup>Tg</sup> (\( n = 6 \)) and c-Myc<sup>Tg</sup>CD19<sup>−/−</sup> (\( n = 7 \)) mice. Isolated leukocytes or whole blood was then labeled with B220 Abs to determine mean (±SEM) numbers of leukocytes, B cells, B220<sub>high</sub> mature B cells, and B220<sub>low</sub> pre-B/immature B cells in each tissue. Blood analysis represents cells per milliliter. Statistical analysis used the Student t test, with significant differences between means indicated, ** \( p < 0.01 \). (B) B cell proliferation rates were similar in c-Myc<sup>Tg</sup> and c-Myc<sup>Tg</sup>CD19<sup>−/−</sup> mice. BrdU incorporation was measured for tissue B cells isolated from lymphoma-free c-Myc<sup>Tg</sup> and c-Myc<sup>Tg</sup>CD19<sup>−/−</sup> mice and their WT littermates (\( n = 4 \) per group). Mean values (±SEM) indicate percentages of BrdU-positive B cells of total B cells in each tissue.

**FIGURE 4.** B cell development and proliferation in c-Myc<sup>Tg</sup> and c-Myc<sup>Tg</sup>CD19<sup>−/−</sup> mice. (A) Normal B cell numbers in c-Myc<sup>Tg</sup> and c-Myc<sup>Tg</sup>CD19<sup>−/−</sup> mice. Bone marrow (bilateral femurs), spleens, cervical lymph nodes, and blood were isolated from 6- to 8-wk-old lymphoma-free c-Myc<sup>Tg</sup> (\( n = 6 \)) and c-Myc<sup>Tg</sup>CD19<sup>−/−</sup> (\( n = 7 \)) mice. Isolated leukocytes or whole blood was then labeled with B220 Abs to determine mean (±SEM) numbers of leukocytes, B cells, B220<sub>high</sub> mature B cells, and B220<sub>low</sub> pre-B/immature B cells in each tissue. Blood analysis represents cells per milliliter. Statistical analysis used the Student t test, with significant differences between means indicated, ** \( p < 0.01 \). (B) B cell proliferation rates were similar in c-Myc<sup>Tg</sup> and c-Myc<sup>Tg</sup>CD19<sup>−/−</sup> mice. BrdU incorporation was measured for tissue B cells isolated from lymphoma-free c-Myc<sup>Tg</sup> and c-Myc<sup>Tg</sup>CD19<sup>−/−</sup> mice and their WT littermates (\( n = 4 \) per group). Mean values (±SEM) indicate percentages of BrdU-positive B cells of total B cells in each tissue.

**FIGURE 5.** Constitutive c-Myc expression enhances CD19 surface density. (A) CD19 expression by normal B cell subsets in c-Myc<sup>Tg</sup> mice. Bone marrow, spleen, and peritoneal cavity (Per. cavity) in each tissue. Blood analysis represents cells per milliliter. Statistical analysis used the Student t test, with significant differences between means indicated, ** \( p < 0.01 \). (B) CD19 expression by blood B cells from c-Myc<sup>Tg</sup>, c-Myc<sup>Tg</sup>CD19<sup>−/−</sup>, or nontransgenic CD19<sup>−/−</sup> littermates. Blood leukocytes were stained to identify CD19<sup>+</sup> B cells as in (A) by flow cytometry analysis. Results represent those from \( 3 \) mice of each genotype. (C) CD19 expression by malignant B cells in c-Myc<sup>Tg</sup> mice. Immunofluorescence staining of CD19 and B220 expression on spleen B cells from a representative c-Myc<sup>Tg</sup> mouse with aggressive lymphadenopathy and splenomegaly, in comparison with B cells from a WT littermate. The dashed line indicates the mean MFI for CD19 expression in WT. Similar results were observed in three mice for each group.

c-Myc and CD19 influence the phosphorylation of one another at regulatory sites

CD19 regulates signal transduction by amplifying Src family kinase activity (27, 28) and through the Ras signaling pathway (29–32). CD19 phosphorylation at regulatory tyrosines in its cytoplasmic tail, especially the signal-initiating Y513 motif, is required for its regulatory activity (27, 28, 33–35). Therefore, the effect of c-Myc overexpression on CD19 phosphorylation was examined by immunoblot analyses. Compared with WT littermates, CD19 was hyperphosphorylated at Y513 in spleen B cells from c-Myc<sup>Tg</sup> mice (Fig. 6A). Total CD19 protein was also in-
assessed by immunoblotting using Abs specific for CD19 phosphorylated at Y513 (pCD19) and Pax5 protein. Phospho-CD19 blots were stripped and reprobed with Abs reactive with total CD19 protein. Brackets at right indicate the multiple bands characteristic of CD19 protein migration on SDS-PAGE gels. Two experiments producing similar results are shown. (B) CD19 expression drives c-Myc hyperphosphorylation in c-MycTg mice. Lysates of purified spleen B cells from lymphoma-free c-MycTg, c-MycTgCD19−/−, WT, and CD19−/− littermates were assessed by immunoblotting using Abs reactive with c-Myc phosphorylated at T58/S62 (pc-Myc). Membranes were subsequently stripped and reprobed with Abs reactive with total c-Myc protein, as well as with total ERK2 protein to indicate equivalent protein loading. Brackets at right indicate the multiple bands characteristic of c-Myc protein migration. Two of three experiments producing similar results are shown. (C) Constitutive c-Myc and CD19 expression drives ERK activation. Lysates of purified spleen B cells from c-MycTg and c-MycTgCD19−/− littermates were assessed by immunoblotting using Abs specific for the kinase-active forms of ERK1/2 or JNK1/2 (pERK1/2 and pJNK1/2, respectively). The membranes were stripped and reprobed using Abs reactive with total ERK2 protein. Two experiments producing similar results are shown.

FIGURE 6. Constitutive c-Myc expression drives CD19 phosphorylation, whereas CD19 expression reciprocally drives c-Myc phosphorylation. (A) Constitutive c-Myc expression in B cells drives CD19 hyperphosphorylation. Lysates of purified spleen B cells from lymphoma-free c-MycTg and WT mice were assessed using immunoblotting using Abs specific for CD19 phosphorylated at Y513 (pCD19) and Pax5 protein. Phospho-CD19 blots were stripped and reprobed with Abs reactive with total CD19 protein. Brackets at right indicate the multiple bands characteristic of CD19 protein migration on SDS-PAGE gels. Two experiments producing similar results are shown. (B) CD19 expression drives c-Myc hyperphosphorylation in c-MycTg mice. Lysates of purified spleen B cells from lymphoma-free c-MycTg, c-MycTgCD19−/−, WT, and CD19−/− littermates were assessed by immunoblotting using Abs reactive with c-Myc phosphorylated at T58/S62 (pc-Myc). Membranes were subsequently stripped and reprobed with Abs reactive with total c-Myc protein, as well as with total ERK2 protein to indicate equivalent protein loading. Brackets at right indicate the multiple bands characteristic of c-Myc protein migration. Two of three experiments producing similar results are shown. (C) Constitutive c-Myc and CD19 expression drives ERK activation. Lysates of purified spleen B cells from c-MycTg and c-MycTgCD19−/− littermates were assessed by immunoblotting using Abs specific for the kinase-active forms of ERK1/2 or JNK1/2 (pERK1/2 and pJNK1/2, respectively). The membranes were stripped and reprobed using Abs reactive with total ERK2 protein. Two experiments producing similar results are shown.

Discussion

To our knowledge, these studies demonstrate for the first time that CD19 and c-Myc expression synergize functionally to influence significantly altered the pattern of cyclin gene and protein expression in c-MycTg B cells.

Because c-Myc is a potent regulator of cell cycle progression, the influence of CD19 deficiency on c-Myc function may culminate in the altered expression of cell cycle regulatory genes. To assess this, cDNA arrays were used to quantify cell cycle gene expression by spleen B cells from lymphoma-free c-MycTg and c-MycTgCD19−/− mice. Although most cell cycle genes were expressed at nearly identical levels between genotypes, c-MycTgCD19−/− B cells exhibited marked reductions in cyclin D2 expression and reciprocally augmented expression of cyclin D3 relative to c-MycTg B cells (Fig. 7A). Expression of a key catalytic binding partner of D-type cyclins, cyclin-dependent kinase 4 (Cdk4), was not altered in c-MycTgCD19−/− B cells. Parallel alterations in cyclin D2 and cyclin D3 were observed at the protein level between c-MycTg and c-MycTgCD19−/− B cells (Fig. 7B). Thus, CD19 deficiency significantly altered the pattern of cyclin gene and protein expression in c-MycTg B cells.

FIGURE 7. CD19 regulates D-type cyclin expression in c-MycTg B cells. (A) CD19 expression drives cyclin D2 but inhibits cyclin D3 transcription in c-MycTg B cells. Cell cycle pathway gene expression arrays were used to screen RNA transcripts from purified splenic B cells from lymphoma-free c-MycTg or c-MycTgCD19−/− littermates. The positions of cyclin D2 and cyclin D3 on the arrays are directly below the underlined numbers 1 and 2, respectively. The position of Cdk4 on the arrays is directly below the underlined number 3. The bar graph shows relative cDNA hybridization to the genes on the arrays. (B) CD19 expression drives cyclin D2 protein production, but inhibits cyclin D3 production in c-MycTg B cells. Lysates of purified spleen B cells from lymphoma-free c-MycTg, c-MycTgCD19−/−, WT, and CD19−/− littermates were assessed by immunoblotting using Abs specific for cyclin D2, cyclin D3, or Cdk4, followed by reprobing of the membranes with Abs reactive with ERK2 as a loading control. The bar graph shows the mean (±SEM) relative density of protein bands from three independent immunoblotting experiments. Statistical analysis was performed using the Student t test, with significant differences between genotypes indicated, **p < 0.01.
spontaneous B cell lymphoma onset and severity. CD19 deficiency significantly prolonged the life span of c-Myc\textsuperscript{Tg} mice, with a >80% increase in the median age of survival when compared with CD19-expressing littermates (Fig. 1A). CD19 expression at a threshold level regulated lymphomagenesis, because the median survival age was similarly short in c-Myc\textsuperscript{Tg} mice with either WT or heterozygous (50% reduced) levels of CD19 expression. Importantly, CD19 deficiency influenced survival through a significantly delayed age of lymphoma onset and a prolonged period of survival after lymphoma detection, both of which were aspects of c-Myc\textsuperscript{Tg}CD19\textsuperscript{+/−} mice (Fig. 1B). Supporting these data, it was recently demonstrated that adoptively transferred B lymphoma cell lines were more tumorigenic when CD19 was expressed, and that CD19 expression in B lymphoma cell lines positively correlated with c-Myc protein expression and stability (40). Thereby, the malignant outcome of c-Myc translocations in B cells is significantly influenced by their expression of CD19.

Mechanistically, CD19 deficiency reduced c-Myc phosphorylation at S62 in c-Myc\textsuperscript{Tg} mice (Fig. 6B), a regulatory site that cooperates with T58 to balance c-Myc protein stability (39). S62 phosphorylation precedes that of T58, and functions to directly stabilize c-Myc protein. In turn, T58 phosphorylation is accompanied by dephosphorylation of S62, which enhances c-Myc degradation via the ubiquitin/proteasome pathway. Supporting the latter, tumor penetrance is enhanced in mice expressing mutant T58A c-Myc proteins relative to WT c-Myc (41). Reduced c-Myc phosphorylation in B cells from c-Myc\textsuperscript{Tg}CD19\textsuperscript{+/−} mice most likely reflects a lack of initial S62 phosphorylation because this is mediated by ERK activity, and ERK activation was impaired in c-Myc\textsuperscript{Tg}CD19\textsuperscript{+/−} B cells (Fig. 6C). Thus, the direct effects of CD19 expression on c-Myc phosphorylation and stability explain at least in part why CD19 facilitates the development and progression of malignant B cells, as also reported by Chung et al. (40), whereby constitutive and stabilized c-Myc protein dysregulates multiple signaling pathways.

Supporting the existence of a c-Myc:CD19 amplification loop in c-Myc\textsuperscript{Tg} mice, constitutive c-Myc expression also augmented CD19 expression levels on normal pre-B, immature, and mature B cells (Figs. 5A, 5B, 6A). Although the mechanism through which constitutive c-Myc expression resulted in augmented CD19 expression is currently unknown, it has been previously shown that the surface expression level of CD19 on pre-B cell colonies derived from cyclin D2-deficient mice is decreased when compared with pre-B cell colonies derived from WT mice (42), and cyclin D2 was upregulated dramatically in c-Myc\textsuperscript{Tg} B cells relative to WT B cells (Fig. 7). Increased CD19 expression correlated with enhanced CD19 Y513 phosphorylation (Fig. 6A). Of the nine conserved tyrosines within the CD19 cytoplasmic domain, Y513 phosphorylation precedes that of the other sites (27, 28). Phosphorylated Y513 is then bound by and amplifies the activity of Src-family kinases, which in turn phosphorylate CD19 at other sites (27, 28), including Y391, which recruits and activates Vav (29–32). Y513 also shares binding activity with PI3K, bringing it to the cell membrane (43, 44). Thus, heightened CD19 expression and phosphorylation in c-Myc\textsuperscript{Tg} mice most likely amplified important B cell signaling and survival pathways that enhanced lymphomagenesis. Malignant transformation remained a c-Myc–dependent process, however, rather than enhanced CD19 expression independently driving tumor development, because B cell lymphomas have never been observed in CD19\textsuperscript{Tg} mice that overexpress CD19 by 3-fold (12, 13, 17, 19, 20).

Although the short-term rate of B cell proliferation in vivo was similar between c-Myc\textsuperscript{Tg} and c-Myc\textsuperscript{Tg}CD19\textsuperscript{+/−} mice (Fig. 4B), CD19 regulated the ratio of cyclin D2 and cyclin D3 expression (Fig. 7). These cyclins critically govern the G1-S–phase transition of cell cycle in B cells, and cyclin D2 expression relies on ERK activity (45). That CD19 deficiency reduced ERK activity in c-Myc\textsuperscript{Tg} B cells (Fig. 6C) supports the observed reduction of cyclin D2 expression (Fig. 7). Cyclin D2 expression requires c-Myc transcriptional activity in a Burkitt’s lymphoma-like cell line (46), and c-Myc targets the cyclin D2 promoter (47). Whereas cyclin D2 is the predominant D-type cyclin expressed by normal B cells (48), both cyclin D2 and cyclin D3 are expressed in Burkitt’s lymphomas (49). Importantly, cyclin D3 can compensate for cyclin D2 deficiency in promoting B cell proliferation, but this substitution may prolong S-phase transition (48). Thus, reciprocally enhanced cyclin D3 expression despite a reduction in cyclin D2 may explain why lymphomas still developed with high penetrance in c-Myc\textsuperscript{Tg}CD19\textsuperscript{+/−} mice, but at a significantly delayed rate and with less severity. Because changes in cyclin expression were observed in nonmalignant B cells, even subtle changes in cell cycle progression may affect the outcome of malignant conversion or provide a growth advantage to premalignant clones. The impact of these differences may become less obvious once rapidly dividing malignant clones evolve during progression to aggressive lymphoma.

These data define a novel, interdependent pathway between c-Myc and CD19 that influences both normal and malignant B cell function. Notably, however, the similar aggressiveness of adoptively transferred B cells from c-Myc\textsuperscript{Tg} and c-Myc\textsuperscript{Tg}CD19\textsuperscript{+/−} mice with end-stage disease (Fig. 3) suggests that CD19 expression may no longer have a significant impact on proliferation once aggressive tumors are established, highlighting the importance of CD19 expression during early lymphoma development. CD19 expression is also likely to contribute to tumorigenesis and progression by influencing tumor cell survival and dissemination, intracellular activation of negative regulatory pathways, and secretion of immunosuppressive cytokines or soluble factors, all of which may have significant effects on disease outcome. Alternatively, CD19 deficiency in the B cell lineage may have secondary consequences that affect lymphoma development and progression. For example, CD19 deficiency leads to significant developmental changes within some B cell subsets, with accompanying effects on T cell-dependent and T cell-independent immune responses (50, 51). As such, B cell CD19 deficiency may also negatively influence antitumor immune responses by altering T cell activation or innate cell functions that influence lymphoma development and progression.

The biochemical analyses carried out in this study suggest the following operational model of lymphomagenesis resulting from this c-Myc:CD19 pathway: constitutive c-Myc overexpression due to chromosomal translocations induces a signaling program that relies significantly on CD19. In this process, c-Myc induces CD19 overexpression and hyperphosphorylation (Fig. 6A), which activates the Ras-ERK signaling cascade (Fig. 6C) and promotes c-Myc phosphorylation at the stabilizing S62 site (Fig. 6B). Stabilized c-Myc in turn governs a unique genetic program dominated by cyclin D2 overexpression that enhances lymphomagenesis. Thus, retention of CD19 expression by most B cell malignancies may confer a growth advantage that further subverts tumor immunity. Thereby, drugs that inhibit CD19 amplification of Src-family kinase or Ras-ERK activity may improve current lymphoma therapies.

Disclosures
T.F.T. is a paid consultant and shareholder for Angelica Therapeutics.

References


