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Differential c-Myc Responsiveness to B Cell Receptor Ligation in B Cell-Negative Selection

Nira Leider* and Doron Melamed2*†

Responsiveness of c-Myc oncogene to B cell receptor ligation has been implicated in the induction of apoptosis in transformed and normal immature B cells. These studies provided compelling evidence to link the c-Myc oncogene with the process of negative selection in B-lymphocytes. However, in addition to apoptosis, B cell-negative selection has been shown to occur by secondary Ig gene rearrangements, a mechanism called receptor editing. In this study, we assessed whether differential c-Myc responsiveness to B cell receptor (BCR) ligation is associated with the mechanism of negative selection in immature B cells. Using an in vitro bone marrow culture system and an Ig-transgenic mouse model (3-83) we show here that c-Myc is expressed at low levels throughout B cell development and that c-Myc responsiveness to BCR ligation is developmentally regulated and increased with maturation. Furthermore, we found that the competence to mount c-Myc responsiveness upon BCR ligation is important for the induction of apoptosis and had no effect on the process of receptor editing. Therefore, this study suggests an important role of c-Myc in promoting and/or maintaining B cell development and that compartmentalization of B cell tolerance may also be developmentally regulated by differential c-Myc responsiveness. The Journal of Immunology, 2003, 171: 2446–2452.

lymphopoiesis in the bone marrow (BM) is characterized by processes of proliferation differentiation and apoptosis, all regulated by lineage-specific and/or non-specific genes (1). In mice lacking genes controlling receptor assembly and signaling of specific transcriptional regulatory genes, B cell development is blocked or severely impaired, implicating the essentiality of these genes during B lymphopoiesis (reviewed in Refs.1 and 2). The c-Myc is a proto-oncogene implicated in the process of proliferation differentiation and survival of normal cells, including lymphocytes (3–5). Early studies in bursal B lymphocytes used retroviral vectors to deregulate c-Myc expression and suggested that expression of c-Myc is necessary for B cell development (6). This, however, is difficult to prove since genetic deficiency in c-Myc results in early embryonic lethality (7). In contrast, overexpression of c-Myc in the B lineage is associated with increased frequency of immature and mature B cell tumors (8), thereby supporting the notion that c-Myc is implicated with neoplastic transformation. These mice have also altered B lymphopoiesis and impaired mature B cell responsiveness to antigenic stimulation (9).

Since negative selection of immature B cell involves apoptosis, a role of c-Myc in this process has been studied, mainly using transformed immature B cell lines stimulated with anti-B cell receptor (BCR) Abs (reviewed in Ref. 10). These studies reveal that apoptosis is preceded by rapid rise and fall in c-Myc expression and cell cycle arrest and implicated a possible regulatory function of the c-Myc in the process of negative selection (11–13). The activity of c-Myc in promoting apoptosis requires the interaction of c-Myc with the Myc-associated-factor X (14), and subsequent activation of the CTCF transcription factor (15). Further details in the c-Myc-mediated apoptosis have been reviewed (5). Much less is known for the role of c-Myc in negative selection of normal B cells. Earlier studies show that BCR ligation elevates c-Myc and induces apoptosis in immature, transitional, and mature B cells (16–19). This apoptosis can be prevented by providing T cell help (16, 20). In addition, c-Myc has been shown to sensitize cells to Fas death signals (21) and in the regulation of Fas ligand expression in T lymphocytes (22). These findings are all consistent with the relationship of c-Myc expression and negative selection of B cells by apoptosis.

Another major mechanism in B cell tolerance is receptor editing. According to this model, immature B cells encountering self-Ag up-regulate V(D)J recombinase and undergo secondary L chain recombination to alter the BCR specificity (23, 24). We have previously shown that receptor editing competence is developmentally regulated and is lost with maturation (25). Other studies have shown that receptor editing competence is determined by the site of Ag encounter (26) or by the number of Jk segments available for recombination (27). Clearly, different cellular pathways and genes are activated by different tolerogenic stimuli (28). In this study, we assessed whether differential c-Myc responsiveness to BCR ligation is associated with the mechanism of negative selection in immature B cells. Our results show that c-Myc is expressed at low levels throughout B cell development and that c-Myc responsiveness to BCR ligation is developmentally regulated and is important for the induction of apoptosis but not receptor editing. Therefore, this study suggests an important role of c-Myc in promoting and/or maintaining B cell development and that compartmentalization of B cell tolerance may also be developmentally regulated by differential c-Myc responsiveness.

Materials and Methods

Mice

Mice used in these experiments were 3-83Tg B10.D2Sn/J, encoding a BCR reactive to the mouse class I MHC Ags Kk and Kb (a gift from Dr. D.

1 Abbreviations used in this paper: BM, bone marrow; BCR, B cell receptor; Tg, transgenic; RAG-2, recombination-activating gene 2.

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Nemazee, The Scripps Institute, La Jolla, CA (29) or nontransgenic (Tg) littermates. In some experiments, 3-83Tg mice deficient in CD19 (a gift from Dr. R. Rickert, University of California, San Diego, CA (30)) were used. All Tg mice were on a nondepleting H-2* genetic background. Mice were housed and bred at the animal facility of the Technion, Faculty of Medicine, and used at 4–10 wk of age.

Cells and BM cultures

B cell precursors from 3-83Tg and non-Tg mice were grown in vitro as described previously (31, 32). Briefly, BM cells were depleted of erythrocytes and were cultured in IMDM (Life Technologies, Gaithersburg, MD) supplemented with 10% FCS, 5 × 10⁻⁵ M 2-ME, and 50–100 U/ml rIFN-γ (33) at a concentration of 2 × 10⁵ cells/ml for 5–6 days. These culture conditions allow preferential growth of IL-7 responsive B cell precursors. After 5–6 days the culture contains >98% of B220⁺ cells (32). These primary cultures were used to sort immature and transitional B cells. Secondary cultures were performed by plating the B cell precursors grown in primary cultures in the presence of adherent 2000-rad treated op42 stromal cells and IL-7 for 7–10 days as we have described elsewhere (25, 31). These secondary cultures allow expansion of early B cell precursors (34) and were used to sort proB and preB cells. In some experiments, Tg B cells were stimulated with mAb anti-3-83 clonotype 54.1 at 20 μg/ml (35) or with 10 μg/ml F(ab)², rabbit anti-mouse IgG (H + L; Zymed Laboratories, San Francisco, CA). WEHI 231 cells were grown in supplemented IMDM. In some experiments, phosphothioate c-Myc antisense or nonsense oligonucleotides were added to the cultured cells at a final concentration of 10 μM. Sequences of c-Myc antisense and nonsense were as described previously (19). Before their use, antisense and nonsense oligonucleotides were treated with CpG methylase SsI (New England Biolabs, Beverly, MA) to prevent potential interaction and activation of the B cells mediated by CpG motifs.

Immunoblotting for c-Myc protein

Determination of c-Myc protein expression in B cells was performed as described elsewhere (19, 22). Briefly, 4–6 × 10⁵ cells were washed with PBS and lysed with lysis buffer containing protease inhibitors. Equal volumes of samples were loaded and separated on 10% SDS-polyacrylamide gel and blotted to a Millipore polyvinylidene fluoride membrane (Millipore, Bedford, MA). Membranes were blocked with 5% milk and reacted with polyclonal rabbit anti-mouse c-Myc or with polyclonal goat anti-mouse actin (both from Santa Cruz Biotechnology, Santa Cruz, CA) to prevent potential interaction and activation of the B cells mediated by CpG motifs. Primary antibodies were detected using horseradish peroxidase-conjugated goat anti-rabbit IgG or anti-guinea pig IgG (Amersham, Arlington Heights, IL) and ECL reagent (Amersham). The results are expressed as a semi-quantitative estimate, where signal intensity of c-Myc or RAG-2 product was normalized to that of Gap control signal, as we have described elsewhere (31).

RT-PCR analysis

cDNA templates were generated from total RNA, purified using the RNAzol method (Tel-Test, Friendswood, TX) and reverse transcribed using random primers. Expression levels of c-Myc, recombination-activating gene 2 (RAG-2), and control Gus mRNA were determined by a RT-PCR assay. The PCR conditions and primer sequences for RAG-2 and Gus are as described previously (31). Primer sequences of c-Myc were: forward, 5'-TTCAGCGCGTGCAGGCTGTGCT-3' and reverse, 5'-GTGGGCTCCGGATGATG-3'. PCR conditions for c-Myc were 40 s at 94°C, 40 s at 65°C, and 60 s at 72°C for 30 cycles. The PCR was performed using serial dilutions of cDNA and the PCR products were fractionated on 1% agarose gels. Gels were scanned using an UVIDoc Gel Documentation System and images were analyzed using UVIDoc software (UVItec, Cambridge, U.K.) to quantify signal intensity. The results are expressed as a semi-quantitative estimate, where signal intensity of c-Myc or RAG-2 product was normalized to that of Gap control signal, as we have described elsewhere (31).

Flow cytometry and cell separation

Single-cell suspensions from BM cultures were stained for surface marker expression using FITC, PE, and biotin-conjugated mAbs and visualized with streptavidin TriColor (Caltag Laboratories, San Francisco, CA). Abs used for cell staining were: goat anti-mouse IgM (Caltag Laboratories); mAbs to IgD, AMS 9:1; CD43, S7 (BD Biosciences Pharmingen, San Diego, CA); and B220, RA3-6B2 (Caltag Laboratories). In some experiments, IgM⁺IgD⁻ and IgM⁻IgD⁺ cells were sorted as we have described previously (25). Cell sorting and data collection were performed using FACSCalibur and CellQuest software (BD Biosciences Immunocytometry Systems, Mountain View, CA). In some experiments, B cell precursors were stained for IgM, IgD, B220, or CD43 and separated using streptavidin, anti-FITC, or anti-PE MACS microbeads (Miltenyi Biotec, Auburn, CA). Sorted cells were stimulated with anti-BCR or used as unstimulated for preparation of RNA and protein.

Apoptosis assay

Apoptosis in cultured B cells, mediated by anti-BCR Abs, was determined by the TUNEL assay (Boehringer Mannheim, Mannheim, Germany) as described previously (31). As a positive control for the activity of the assay, in each experiment some samples were treated with the apoptosis-inducing drug Beauvericin (cyclo-[β-α-hydroxyisovaleryl-L-methylphenylalanyl]-); Sigma-Aldrich, St. Louis, MO) (31) at a final concentration of 5 μM.

Statistical analysis

The statistical significance of differences between experimental groups was determined using the unpaired two-tailed Student’s t test with differences considered significant at p < 0.05.

Results

The determination of c-Myc expression by RT-PCR is in correlation with the protein levels

In this study, we attempted to study the expression of c-Myc during B cell development and the establishment of self-tolerance. Since some of the described experiments were limited by the number of available cells, it was difficult to detect c-Myc protein or mRNA by Western or Northern blots, respectively, as described previously (11, 13). To overcome this limitation, we adopted the RT-PCR approach to amplify c-Myc mRNA. At first, we used the transformed immature B cell line WEHI 231 to determine the correlation between c-Myc expression detected by RT-PCR and that detected by Western blot. WEHI 231 cells were stimulated with anti-BCR Abs for 2–8 h and c-Myc expression in each time point was determined by Western blot using c-Myc-specific Abs or by RT-PCR. As has been described before (12), we found in both methodologies that c-Myc expression was rapidly induced upon stimulation, peaked after 2–3 h, and declined thereafter (Fig. 1). Upon quantitation, both methodologies revealed a 3–to 4-fold increase in c-Myc expression after BCR ligation (data not shown). We concluded that the RT-PCR of c-Myc mRNA can be used as an efficient method to probe for c-Myc expression.

The c-Myc is expressed at low levels during normal B cell development

To determine c-Myc expression in B lymphopoiesis, we used our BM culture system. Cells grown in primary cultures are enriched with immature (IgM⁺IgD⁻) and transitional (IgM⁺IgD⁺) stages, whereas secondary cultures (in the presence of stroma cells) are enriched with the earlier stages proB (B220⁻CD43⁻IgM⁻) and preB (B220⁺CD43⁻IgM⁻) as described elsewhere (32, 34). By

FIGURE 1. Determination of c-Myc protein and mRNA expression in WEHI 231 cells by Western blot and by RT-PCR. WEHI 231 cells were stimulated for 2–8 h with 10 μg/ml F(ab)² rabbit anti-mouse IgG (H + L). Following stimulation, cells were collected and used to determine c-Myc protein by Western blot (top) and c-Myc mRNA by RT-PCR (bottom) as described in Materials and Methods. The results shown are representative of five different experiments.
using magnetic beads, we sorted proB, preB, immature, and transitional B cells that were grown in vitro as well as mature B cells from adult mouse spleen. The expression level of c-Myc protein in each sorted population was determined by Western blot (Fig. 2, top). We found that c-Myc is expressed at low levels throughout B cell development but was significantly increased in mature B cells. Quantitation of c-Myc expression by RT-PCR essentially confirms these observations (Fig. 2, bottom). Both methodologies revealed that c-Myc expression in transformed WEHI 231 cells was 10- to 12-fold higher than that found in normal B cells. These findings may implicate an important function of c-Myc in maintaining and/or promoting the normal process of B cell development.

Developing 3-83Tg B cells express low levels of c-Myc, but exhibit differential c-Myc responsiveness upon BCR ligation

To study the expression of c-Myc in B cell tolerance we used the 3-83Tg mouse system. A significant advantage of the Tg system is the expression of a single clonotypic receptor, allowing us to efficiently control B cell stimulation without the involvement of compensating mechanisms that may be found in a polyclonal repertoire. However, the presence of a rearranged receptor accelerates B cell development, essentially skipping the proB and preB stages, as found both in vivo and in vitro (32). To study c-Myc expression in the 3-83Tg system, B cell precursors grown in BM cultures were sorted to immature (IgM⁺ IgD⁻) and transitional (IgM⁺ IgD⁺) stages, whereas mature 3-83Tg cells were sorted from adult spleen. Sorted cells were then stimulated with mononclonal anti-3-83 clonotypic Ab (54.1) to mimic Ag binding as we have shown before (31). The expression of c-Myc protein and mRNA were determined in unstimulated and stimulated cells by Western blot and by RT-PCR (Fig. 3). The results clearly show that c-Myc expression in 3-83Tg system is similar to that found in non-Tg cells (Fig. 2). In the absence of stimulation, we found that c-Myc was expressed at low levels in immature and transitional 3-83Tg cells, but increased in mature splenic cells. However, stimulation with anti-BCR Abs results in elevated c-Myc expression in all cells as revealed by both c-Myc protein detection (Fig. 3, top) and RT-PCR of c-Myc mRNA (Fig. 3, bottom). Quantitation of c-Myc expression by RT-PCR revealed significant differences in c-Myc responsiveness in the stimulated cells. Although transitional and mature cells elevated c-Myc by 3- to 5-fold, immature cells had only a 2- to 3-fold increase (Fig. 3, bottom, p < 0.05). We conclude that c-Myc responsiveness to BCR ligation is differentially regulated during B cell development and is increased with maturation.

Differential c-Myc responsiveness of early and late 3-83Tg immature B cells to BCR ligation

We have previously shown that tolerance is developmentally regulated in the immature B cell population. Although immature cells at the early stage (IgM⁺ IgD⁻) undergo receptor editing in response to BCR ligation, immature cells at a later stage (IgM⁺ IgD⁺) encountering Ag are negatively selected by apoptosis (25). To test for a possible role of c-Myc in the differential tolerance responsiveness, we first monitored c-Myc expression in early and late 3-83Tg immature cells upon stimulation. Immature cells grown in BM cultures were sorted to early IgM⁺ IgD⁻ and late IgM⁺ IgD⁺ populations (Fig. 4A). Sorted cells were stimulated with anti-BCR Ab and c-Myc expression was determined temporarily 1-8 h after stimulation (Fig. 4B). The results clearly show differential c-Myc responsiveness between the two cell populations. Although the more developed IgM⁺ IgD⁺ cells have a robust c-Myc expression upon BCR ligation, reaching a 4- to 6-fold increase 2 h after stimulation and dropping thereafter (Fig. 4B, right), only mild c-Myc responsiveness was observed in the early...
IgM<sup>lo/IgD<sup>-</sup> cells. These findings support our hypothesis that c-Myc responsiveness is developmentally regulated and therefore may be involved in the determination of B cell tolerance.

**c-Myc antisense oligonucleotides inhibit apoptosis of immature 3-83Tg B cells upon BCR ligation**

As we have previously shown (25), 3-83Tg late immature B cells undergo apoptosis upon BCR ligation. This negative selection mechanism has been shown in transformed WEHI and normal B cells to be regulated by the rapid elevation in c-Myc (11, 13, 19) and could be inhibited by blocking c-Myc protein synthesis using the c-Myc antisense approach (11, 19). To test whether c-Myc antisense can block apoptosis of immature 3-83Tg cells that are grown in vitro, immature B cells were stimulated with anti-BCR Abs in the absence or presence of c-Myc antisense, and apoptosis was determined by TUNEL assay. Fig. 5 clearly shows that this experimental maneuver could rescue immature 3-83Tg cells from apoptosis mediated by anti-BCR Ab. In control cultures, we obtained a spontaneous apoptosis rate of 9–15%, whereas in the presence of anti-BCR Ab the apoptosis rate was increased to 30–40%. However, the addition of c-Myc antisense completely inhibited apoptosis in the cultured B cells, reaching levels that are not different from those obtained in untreated cultures (9–12%; Fig. 5, A and B). No inhibition of apoptosis was found in the presence of nonsense control oligonucleotides (Fig. 5, A and B). Notably, in agreement with a previous report (19), c-Myc antisense efficiently inhibited spontaneous apoptosis. To confirm that c-Myc antisense oligonucleotides effectively inhibit c-Myc protein synthesis, treated cells were lysed and c-Myc protein expression was determined by Western blot. The results in Fig. 5C show that c-Myc antisense, but not nonsense oligonucleotides, blocked the c-Myc responsiveness mediated by BCR cross-linking.

**c-Myc antisense oligonucleotides do not alter RAG-2 expression in immature 3-83Tg cells upon BCR ligation**

To test whether c-Myc is involved in the process of receptor editing, we studied the induction of the RAG-2 gene in 3-83Tg cells stimulated with anti-BCR Abs and treated with c-Myc antisense (Fig. 6). As we have previously shown, immature 3-83Tg cells up-regulate RAG expression in response to BCR ligation (Refs. 25 and 31 and Fig. 6). However, in contrast to the efficient ability to block apoptosis, the addition of c-Myc antisense had no effect on the induction of receptor editing. We found that RAG-2 expression in stimulated cells was not significantly altered in the presence of c-Myc antisense (Fig. 6). Furthermore, these results also suggest that cells protected from apoptosis by c-Myc antisense fail to up-regulate the recombine genes and to undergo receptor editing. We conclude that developmental progression, previously shown to compartmentalize immature B cell tolerance (25), also compartmentalizes c-Myc responsiveness. Thus, c-Myc responsiveness is required for apoptosis, but has no significant effect in the induction of receptor editing.
Immature 3-83Tg/CD19−/− cells that undergo Ag-independent receptor editing express low levels of c-Myc

The fact that c-Myc antisense had no effect on RAG-2 expression may suggest that V(D)J recombination, during the process of receptor editing, is not regulated by the c-Myc. To support this hypothesis, we determined c-Myc expression in immature 3-83Tg cells deficient in CD19, previously shown by us to fail positive selection and to undergo spontaneous, Ag-independent receptor editing, is developmentally regulated and that immature B cells proceed from the early immature stage that is apoptosis competent to a late immature stage that is apoptosis resistant and receptor editing incompetent (25). As shown here, we found differential c-Myc responsiveness to BCR ligation between these two subpopulations (Fig. 4). In agreement with this, we used the 3-83Tg system to show that B cell tolerance is developmentally regulated and that immature B cells proceed from the early immature stage that is apoptosis resistant and receptor editing incompetent (25). As shown here, we found differential c-Myc responsiveness to BCR ligation between these two subpopulations (Fig. 4). In agreement with earlier studies in transformed and normal immature cells (11, 12, 15) that lack of c-Myc blocked thymocyte development at the early double-negative stage in c-Myc−/− chimeric mice (39). Thus, it is possible that c-Myc integrates signals for proliferation, differentiation, and survival during lymphocyte development, thereby controlling cell cycle regulation. This may be mediated by regulation or interaction with other transcription factors that regulate cell cycle entry or transit (reviewed in Ref. 5) or in providing survival signals (5, 40). Our results showing constant expression of c-Myc in all stages of B cell development are in agreement with this.

One of the open-ended questions in B cell development is the regulation of self-tolerance. This has been shown to occur mainly by apoptosis or by receptor editing (23, 24, 41, 42). Earlier studies provided evidence for a rapid induction and a fall in c-Myc following BCR ligation, eventually leading to apoptosis in transformed immature B cell lines (10). Inhibition of c-Myc synthesis or its transcriptional targets rescued these immature B cells from apoptosis, thereby implicating an important role for the c-Myc in negative selection (11, 15 and our unpublished data). In previous studies, we used the 3-83Tg system to show that B cell tolerance is developmentally regulated and that immature B cells proceed from the early immature stage that is apoptosis resistant and receptor editing incompetent (25). As shown here, we found differential c-Myc responsiveness to BCR ligation between these two subpopulations (Fig. 4). In agreement with earlier studies in transformed and normal immature cells (11, 12, 15–17, 19), receptor ligation induced rapid c-Myc synthesis, followed by apoptosis in the late immature cells (IgMhiIgD−) that could be blocked by c-Myc antisense (Fig. 5). In contrast, only mild c-Myc

Discussion

The present study attempts to investigate the expression of c-Myc in the B lineage and its role in the regulation of B cell tolerance. Using an in vitro culture system and the 3-83Tg mice, this study suggests that c-Myc is important in promoting B cell development and that differential c-Myc responsiveness to BCR ligation may contribute to the regulation of negative selection. Hence, receptor editing and apoptosis are determined not only by the developmental stage, but also by the competence to mount c-Myc responsiveness.

B cell development in the BM proceeds through processes of proliferation and differentiation, and most of these newly formed B cells are lost due to failure to fulfill appropriate developmental requirements (1). Since the c-Myc has been linked with normal cell growth and apoptosis (3–5), it is not surprising to detect c-Myc in developing B cells (Fig. 2). Remarkably, we and others (15) show that c-Myc expression levels in normal cells are significantly low relative to those expressed in the widely used transformed immature B cell line WEHI 231. This may arise from deregulation of the c-Myc gene or continuous activation resulting from the transfection of this cell line (4). Because levels of c-Myc did not significantly change during development, we speculate that c-Myc is important for maintenance and/or for promoting of B cell development. This, however, is difficult to prove since c-Myc deficiency causes embryonic lethality (7). On the other hand, overexpression of c-Myc in the B lineage results in increased protein synthesis and cell size of developing cells (37), as well as accumulation of early precursors in the BM (9). In addition, c-Myc overexpression is associated with increased immature and mature B cell tumors involving chromosomal translocations (8). Earlier studies in chicken bursal B lymphocytes showed that retroviral deregulation of c-Myc induces outgrowth of a population of cells bearing phenotypic characteristics of bursal stem cells (6). More recent studies showed that Tg expression of the c-Myc antagonist, Mad1, modulated T cell development (38) and that lack of c-Myc blocked thymocyte development at the early double-negative stage in c-Myc−/− chimeric mice (39).

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responsiveness was found in early immature cells (IgMlowIgD−) and receptor editing was unaffected by c-Myc antisense (Fig. 6). These results suggest that developmental progression of immature B cells and the alternation in apoptosis sensitivity are linked with the susceptibility of the cell to mount c-Myc responsiveness upon BCR ligation. Such responsiveness might be important for activation of subsequent genes and transcription factors that are regulated by c-Myc, although its exact role in the apoptotic pathway is not fully defined. Cell death by c-Myc has been shown to be associated with activation of certain Jun kinases and caspase-3, which is crucial for the chromatin collapse and nucleosomal DNA degradation, and in stimulating synthesis of other apoptotic-inducing proteins such as ornithine decarboxylase and α-prothymosin (reviewed in Refs. 5 and 10). In addition, several cell cycle regulating genes, such as cyclin A and Cdc25A, and transcription factors such as CTCF or P19ARF have been shown to be c-Myc targets in promoting apoptosis (5, 15). Other potential myc target genes have been shown using microarray studies (43). Hence, differential c-Myc responsiveness that is developmentally regulated may be linked with differential susceptibility to BCR-mediated apoptosis and receptor editing competence obtained in developing B cells (25). This hypothesis is supported by showing that late immature B cells, protected from apoptosis by c-Myc antisense (Fig. 6) or by overexpressing bcl-2 (25), do not reactivate receptor editing in response to receptor ligation. In addition, c-Myc responsiveness increases along with maturation in transitional and mature B cells (Fig. 3), developmental stages that fail to activate receptor editing (44, 45) and undergo apoptosis in response to BCR ligation (16, 18–20). Thus, it is possible that c-Myc responsiveness is involved in developmental compartmentalization of B cell tolerance.

The inhibition of c-Myc synthesis by antisense oligonucleotides had no effect on RAG expression (Fig. 6). This may suggest that the RAG-2 gene is not regulated by the c-Myc. Looking into the RAG-2-regulating elements clearly show that canonical c-Myc-binding regions are absent (46). This, however, does not exclude other types of regulation or binding of c-Myc to noncanonical sequences, as suggested for the expression of Fas ligand in T cells (22). Since V(D)J recombination is cell cycle regulated and suppressed in cycling cells (47), it contradicts the biological activity of c-Myc, which promotes entry into the cell cycle (3–5). Hence, it is less likely that c-Myc is involved in RAG gene regulation or in the V(D)J recombination activity. This is supported by showing that immature 3-83Tg/CD19−/− B cells, which undergo spontaneous, Ag-independent editing (36), did not express elevated c-Myc levels (Fig. 7). Furthermore, in mice overexpressing c-Myc in the B lineage, V(D)J recombination proceeds and results in the generation of mature B cells. The development of B cells in these mice is perturbed and favors cell proliferation over cell differentiation (9). In addition, Ig− cells lines isolated from these mice (48), as well as EBV-transformed B lymphoma cells (49), express RAG genes despite the high levels of c-Myc expression in these cells. It is therefore possible that receptor editing and c-Myc responsiveness are independently regulated by BCR signaling in immature B cells. This may suggest that the alternation in the mechanism of tolerance, from receptor editing to apoptosis, is accompanied both by the ability to mount c-Myc responsiveness and by the failure to reprogram V(D)J recombinase.

In mature B cells the c-Myc is constantly expressed (50) but is significantly up-regulated upon BCR ligation (19). This up-regulation is probably required to induce cell proliferation, since c-Myc has been shown to control genes involved in growth and cell cycle regulation of stimulated B cells (17). Inhibition of c-Myc expression in mature B cells via conditional LoxP/cre-based mutation, severely impairs proliferation of stimulated B cells (51). Hence, the ability of mature B cells to mount a c-Myc response upon BCR ligation is a critical step for activation. As we show here, this ability is developmentally regulated and increased with maturation (Fig. 3). However, mature B cells stimulated via the BCR undergo apoptosis as a consequence of lack of T cell help, a mechanism thought to be important for establishment of peripheral tolerance (1). This apoptosis is mediated via Fas signaling and can be prevented by anti-CD40 Abs (1) shown to inhibit induction of CTCF transcription factor (15). In these apoptotic cells, c-Myc appears to mediate mitochondrial dysfunction (52) and is linked with the Fas signaling pathway (5, 21). Thus, the pivotal c-Myc role in determination of activation or apoptosis in mature B cells following BCR ligation lies in the presence or absence of T cell help.

Finally, much of the data and the conclusions drawn here are based on the Ig-Tg experimental system. It is important to note that this artificial system significantly perturbs the normal process of B cell development, as we have described previously (32). Nevertheless, the utilization of a monoclonal repertoire facilitates our ability to monitor biological process, such as V(D)J recombination and receptor signaling, that are subjected to compensating selecting mechanisms present in a non-Tg system. By taking the advantage of the Ig-Tg system, our data suggest that c-Myc responsiveness is developmentally regulated and plays an important function in B cell negative selection. This, however, is yet to be shown in a non-Tg system.

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