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A p53 Axis Regulates B Cell Receptor-Triggered, Innate Immune System-Driven B Cell Clonal Expansion

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Resting mature human B cells undergo a dynamic process of clonal expansion, followed by clonal contraction, during an in vitro response to surrogate C3d-coated Ag and innate immune system cytokines, IL-4 and BAFF. In this study, we explore the mechanism for clonal contraction through following the time- and division-influenced expression of several pro- and anti-apoptotic proteins within CFSE-labeled cultures. Several findings, involving both human and mouse B cells, show that a mitochondria-dependent apoptotic pathway involving p53 contributes to the high activation-induced cell death (AICD) susceptibility of replicating blasts. Activated B cell clones exhibit elevated p53 protein and elevated mRNA/protein of proapoptotic molecules known to be under direct p53 transcriptional control, Bax, Bad, Puma, Bid, and procaspase 6, accompanied by reduced anti-apoptotic Bcl-2. Under these conditions, Bim levels were not increased. The finding that full-length Bid protein significantly declines in AICD-susceptible replicating blasts, whereas Bid mRNA does not, suggests that Bid is actively cleaved to short-lived, proapoptotic truncated Bid. AICD was diminished, albeit not eliminated, by p53 small interfering RNA transfection, genetic deletion of p53, or Bcl-2 overexpression. DNA damage is a likely trigger for p53-dependent AICD because susceptible lymphoblasts expressed significantly elevated levels of both phosphorylated ataxia telangiectasia mutated-Ser156 and phospho-H2AX-Ser139. Deficiency in activation-induced cytosine deaminase diminishes but does not ablate murine B cell AICD, indicating that activation-induced cytosine deaminase-induced DNA damage is only in part responsible. Evidence for p53-influenced AICD during this route of T cell-independent clonal expansion raises the possibility that progeny bearing p53 mutations might undergo positive selection in peripherally inflamed tissues with elevated levels of IL-4 and BAFF. The Journal of Immunology, 2012, 188:000–000.

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damage can contribute to the clonal contraction of TI B cell clones, in a manner similar to that recently reported in responses to T cell-dependent (TD) stimuli (10). Third, proapoptotic molecules promoting clonal contraction might be targets for mutation by AID or reactive oxygen species (ROS) generated during clonal expansion. Thus, through understanding the mechanism for clonal contraction, we may be in a better position to understand the etiology of certain B cell disorders characterized by abnormal clonal growth.

Past studies from this laboratory have provided glimpses into possible mechanisms for the demise of human B cell clones during BCR-triggered, innate immune system-driven responses (5, 6). Two findings suggest that mitochondria-dependent intrinsic apoptosis is involved. First, Bcl-2 levels within replicating blasts decline progressively with each division (6), in a manner reminiscent of the low levels of Bcl-2 seen in germinal centers (11, 12). The level of Bcl-2 expressed is inversely related to AICD vulnerability (6). Secondly, when BAAF+, APRIL−, or exogenous PGE2-induced signals are available, dividing cells upregulate Mcl-1, a short-lived Bcl-2 family member, resulting in diminished AICD within replicating blasts (5, 6). Importantly, anti-apoptotic Mcl-1 binds with high affinity to several mitochondrial membrane-disrupting proapoptotic molecules, Bim, Puma, and truncated Bid (tBid) (13–16), suggesting that it is an important controller of mitochondria-dependent cell death.

The identities of the proapoptotic mediators present in the above BCR-triggered, innate immune system-driven clones remain undefined. Although there has been precedent for function of a FOXO3a-driven Bim pathway in promoting B cell AICD (17–20), there are reasons to suspect that a pathway involving DNA damage-promoted p53 contributes to AICD in certain settings. It may be important that replicating blasts in these BCR-triggered, innate immune system-driven clones display high levels of both AID and a marker of DNA damage: serine 1981-phosphorylated H2AX staining. Cells were stained intracellularly by exposure to specific antibodies to phospho-H2AX (Ser139) (31) and phospho-H2AX (Ser139) (32) (and also available through Fina BioSolutions, Rockville, MD), was covalently linked to streptavidin via its amino groups using the Lightning-Link streptavidin conjugation kit (Novus Biologicals, Littleton, CO). Following conjugation, culture grade BSA (0.2%) was added and the mixture was dialyzed in PBS, sterile filtered, and stored until use. At least 30 min prior to use in culture, streptavidin-AECM-dextran was premixed with biotinylated rat anti-mouse IgM mAb II/41 (Bioscience; catalog no. 13-5790-85) to yield BCR-L (each was present in culture at a final concentration of 2 and 0.5–1 μg/ml, respectively).

Cytokines and culture reagents

The recombinant human BAFF was provided by Dr. S. Kalled (Biogen Idec) or obtained from Alexis Biochemicals. Recombinant human BAFF and recombinant human IL-4 (R&D Systems) were used at concentrations of 50 and 5 ng/ml, respectively. Recombinant murine BAFF and recombinant murine IL-4 (R&D Systems) were used at concentrations of 50 and 10 ng/ml, respectively. PGE2 (Cayman Chemical) was stored as a 2 mM stock in ethyl alcohol at −70°C and diluted in culture medium just before use. Z-VAD-FMK (Sigma-Aldrich) stock (20 mM) was prepared in DMSO and diluted to a final concentration of 40 μM.

B cell sources, purification, and culture

Human follicular cells. De-identified tonsils from elective tonsillectomy were used according to Institutional Review Board guidelines (with the cooperation of the Department of Pathology, New York Eye and Ear Infirmary, New York, NY and the Department of Pathology, Northern Westchester Hospital, New York, NY). De-identified spleens were obtained from the National Disease Research Interchange and Cooperative Human Tissue Network, processed, and stored at −150°C (6). The follicular (FO) subset designation is used in this study to represent cells of the conventional mature B cell subset, as distinguished from B1 cells and MZ cells. As we described earlier (5, 6), human FO (BC2) cells were selected from human tonsil, or occasionally from normal human spleen, on the basis of their high density, and hence relatively resting state, upon Percoll density gradient centrifugation, as well as their CD27 and CD43 negativity upon staining with mAb/dextran at limiting concentration of 0.01 μg/ml (30, 31) (and also available through Fina BioSolutions, Rockville, MD). For lysate preparation, cells were cultured at 1 × 10^7 cells/200 μl in enriched medium (5). For lysate experiments, purified resting B cells from the peripheral blood of one of the authors was obtained by negative depletion using a magnetic bead purification kit (Miltenyi Biotech). To monitor replication, FO B cells were labeled with 1 μM CFSE and cultured in 96-well plates at a concentration of 0.5 × 10^3 to 10^4 cells per 200 μl in an enriched medium (5). For lysis preparation, cells were cultured at 1 × 10^7 cells/200 μl in enriched medium. Mouse B cells. Studies with mice were performed following review and in concordance with Institutional Animal Use and Care Committee guidelines. Age-matched male wild-type C57BL/6/JTac mice and congenic B6.129.SgSg(Bcl-2 & H2AΔ20) N12 mice with a homozygous p53 mutation (33) were obtained from Taconic Farms. Mice of the congenic C57BL/6 mouse strain with deletion of gene for AID (AICDA) generated in the laboratory of Dr. Tasuko Honjo (34) (also on the C57BL/6 background) were the gift of Dr. Betty Diamond. All mouse spleen cells were subjected to density gradient centrifugation as above to obtain high-density lymphocytes. B cells were subsequently purified by negative selection (Miltenyi Biotech B cell isolation kit; catalog no. 130-090-862), labeled with CFSE, and cultured as above.

Assessment of culture viability and replication

Methods described previously were employed (5, 6). In brief, CFSE-labeled cells were harvested into cold PBS, fixed in 1–2% formaldehyde; and analyzed by flow cytometry (FACScan or FACSCalibur with CellQuest data analysis software or FlowJo 7.6.1 software) following exclusion of very low forward light scatter (FSC) events (debris), with an exception shown in Fig. 1A. The relative yield and relative viability of cells within each division subset were calculated by gating total cells (viable and apoptotic) into various division subsets and subsequently determining percentage viability within each subset on the basis of FSC/SSC (3).
Ab (or with control IgG) for 20–40 min, washed, and, if needed, exposed to secondary labeled anti-IgG for 20–30 min. Washed cells were refixed in 2% formaldehyde prior to flow cytometry. Abs used for the above assays included: anti-Bid (mouse mAb 7A3; Cell Signaling Technology), anti–p-H2AX-Ser139/140 mouse mAb 3F2 (Thermo Scientific), followed by PE- goat F(ab’); anti-mouse IgG preabsorbed against human IgG; anti-p53 (PE-anti-p53 [clone DO-7]) and PE-IgG2b mAb control [clone 27-35]; BD Pharmingen staining kit; PE-conjugated anti-p-ATM-Ser198/128 mouse mAb 1B11 (MultiCure) and anti–Mcl-1 (mouse mAb clone 22; BD Pharmingen) or MOPC-21 mouse IgG1 mAb control (directly labeled with Zenon Alexa Fluor 647-R-PE mouse IgG1 labeling reagent).

Immunoblotting studies
Lyseus were prepared in M-PER lysis solution (Pierce) or in RIPA lysis buffer plus 1 mM EDTA plus protease/phosphatase inhibitor mix (Roche). Lysates were quantified for protein, electrophoresed, and blotted onto nitrocellulose or polyvinylidene difluoride membranes as described (6). Western blotting was performed with the following: rabbit anti-Bax and rabbit anti-Bim polyclonal Abs (pAb; BD Pharmingen); rabbit anti-Bad pAb and mouse anti-Bid (mAb 7A3) (Cell Signaling Technology); anti–Puma (rabbit mAb EP512Y; Epitomics); anti–p53 (mouse mAb DO-1) (a gift of Dr. Roger Grand, University of Birmingham, Birmingham, U.K.); anti-total H2AX (rabbit mAb D17A5; Cell Signaling Technology); anti–phospho-H2AX-Ser139/140 (mouse mAb 3F2; Thermo Scientific) and loading control anti-actin mAb (Novus Biologicals) or anti-catalase pAb (6). In each experiment, blots were sequentially stripped and reanalyzed for additional proapoptotic molecules and loading control protein. The latter was detected with standard ECL detection reagent; other blots used SuperSignal Pico or Femto high-sensitivity substrate (Pierce). In analysis of Bax, Bad, Bid, Bin, and Puma, standardized values of a given protein were obtained as described (36) and then expressed as a percentage of the maximum level attained within that experiment.

Quantitative RT-PCR of Bim, Bid, Bad, and p53 mRNA levels
For quantitative analysis of mRNA levels within B cells, mRNA was isolated from harvested cells (≥106) using a Mini-Prep Qiagen kit. From each mRNA, cDNA synthesis was performed using oligo(dT) primers from a cDNA kit (Invitrogen). Primers and probes were designed from universal probe library (Roche). Bim, probe no. 63 and primer sequence (forward, 5’TGGTGAAGGCGTGAATTCC-3’; reverse, 5’TGGCCTGCTGTT-GTAATTATC-3’); Bid, probe no. 44 and primer sequence (forward, 5’TGGTAACGGAGTTGATGCC-3’ and reverse, 5’GGCTGGACCCCTTGAAGT-3’). Quantitative RT-PCR was executed at least in triplicates using cDNA as a template with Eurogentec MasterMix. The conditions for quantitative PCR were as follows: initial uracil-N-glycosylase activation at 50˚C for 2 min; HotGoldStar activation (and uracil-N-glycosylase inactivation) at 95˚C for 10 min; followed by 45 cycles, each at 95˚C for 15 s (denaturation) and 60˚C for 1 min (annealing and extension). Fold change was calculated by comparing treated versus untreated groups. Endogenous control for assay was β-actin, used as described previously (10). Data were analyzed using RQ Manager version 1.2. Fold change was calculated by comparing treated versus untreated groups.

p53 small interfering RNA treatment
Prior to small interfering RNA (siRNA) transfection, B cells were preactivated with low-dose BCR:CD21-L plus IL-4 (with or without BAFF) for 2–3 d. Recovered cells (in some cases enriched for viable cells by Ficoll-Hypaque centrifugation) were washed, and 1–5 × 106 cells were subjected to nucleoefection with 2 µg p53 siRNA (Qiagen–validated siRNA [Hs_TP53_9]) or 2 µg AllStars control siRNA (Qiagen) (or, alternatively, 1.5 µg siRNA representing p53 Stealth RNA interference [TP53HS10905-7] and GCC-rich ‘control RNA interference [Invitrogen] using program L-15 of an Amaxa nucleoefection device. Following re-equilibration (6–12 h in RPMI 1640 plus 10% FCS plus Glutamax plus PenStrep), nucleofected cells were restimulated with BCR:CD21-L plus IL-4 plus BAFF for an additional 3 d in medium containing Primocin (Amaza). At that time, cell cultures were assessed by flow cytometry for viability and cell size (light scatter). Cell recovery was determined in most experiments by introducing a known number of standard calibrate beads on an Abbott tube plate prior to analysis. In early optimization experiments, nucleoefection of Amaza pmaxGFP plasmid under the above conditions resulted in 40–70% GFP+ cells within the gated viable subset at 24 h following nucleoefection.

Results
Kinetics of AICD in human FO lymphocytes stimulated by surrogate C3d-coated Ag and innate immune system cytokines
The representative experiment in Fig. 1A reveals the time course for the dynamic clonal expansion/contraction response of CFSE-labeled quiescent human tonsil FO (B2) cells upon culture with a limiting dose of surrogate C3d-Ag (BCR:CD21-L) and IL-4 plus BAFF. The histograms confirm our prior findings that the greatest proportional yield of viable progeny in this TI response is seen between days 4 and 5 of the response (6). At day 5, apoptotic progeny (shrunken cells with low CFSE) are clearly evident. Following day 5 of activation, the proportion of gated intact apoptotic cells increases with time, as does the detection of fragmented cells of very low FSC (debris, or D) (Fig. 1A). The rise in debris indicates that percentage viability values, calculated on the basis of gated intact viable and apoptotic cells (Fig. 1B), are undoubtedly an overestimate at late culture intervals. Nevertheless, as we have described earlier (5, 6), through evaluating percentage viability as a function of division it is readily apparent that AICD affects primarily cells with two or more divisions (Fig. 1B). The AICD of progeny is not unique to tonsil FO cells following in vitro activation, but it is also evidenced during BCR:CD21-L plus IL-4 plus BAFF-induced clonal expansion of purified peripheral blood B cells and isolated splenic FO B cells (Fig. 1C) (6). In an earlier study we demonstrated that progeny gated as apoptotic, on the basis of FSC/SSC, express high levels of active caspase 3 (23). The importance of intracellular caspases in mediating the above AICD is presently shown by the significantly greater recovery of viable progeny in cultures pulsed on day 4 with Z-VAD, a pan-caspase inhibitor (Fig. 1D).

Importantly, several possibilities for this in vitro AICD have been excluded. The AICD is not due to limiting nutrients following the burst of proliferation: cultures pulsed with CD40-L or with TLR9-engaging CpG (oligodeoxynucleotide 2006) manifest considerably greater cycling and substantially lesser AICD through day 7 (data not shown). Furthermore, supplementing cultures with fresh medium or reducing the cell density does not affect AICD (data not shown). Additionally, membrane death receptors, Fas, TNFfr, and DR, do not appear relevant: in several experiments, Fas was undetectable by immunostaining with Ab that detected Fas on CD40-L–stimulated cells (36), and AICD was unaffected by neutralizing anti–TNF-α mAb or TRAIL–Fc (data not shown). In the following experiments, we explore other explanations for the declining viability of replicating blasts in these TI clones.

Expression of several proapoptotic Bcl-2 family members rises during BCR-triggered, innate immune system-driven clonal expansion
To obtain greater insights into the mechanism for the above AICD, we monitored cultures prior to (days 3–4) and after (days 5–6) AICD was evident for expression of several molecules implicated in intrinsic, mitochondria-dependent apoptosis. This involved immunoblotting and/or immunostaining for FOXO-regulated Bim (38, 39) and p53-regulated Bax, Bid, Bad, Puma, and caspase 6 (13, 25–27). Expression of proapoptotic molecules within lysates of cells exposed to low-dose BCR:CD21-L plus growth-promoting IL-4 with or without BAFF was compared with that within cultures exposed to BCR:CD21-L alone (Fig. 2A–D). B cells exposed solely to this low dose of BCR:CD21-L typically manifest sustained viability through day 6, but minimal growth (6, 31). We additionally examined proapoptotic molecules, alongside anti–apoptotic Bcl-2 and Mcl-2, by two-color intracellular immuno-fluorescent staining of CFSE-labeled cultures (Fig. 2E–G). In this
case, we compared relative expression of apoptosis-regulating proteins within dividing, apoptosis-vulnerable blasts and non-dividing, apoptosis-resistant blasts (6).

**Profile prior to AICD.** Despite the relatively high cell viability at days 3–4 (Fig. 1A, left column), these cells showed a pronounced upregulation of several proapoptotic molecules reported to be downstream of p53, that is, Bax, Bid, Bad, and Puma (Fig. 2A–C). As discussed in an earlier report (6), it is highly likely that apoptosis is thwarted in these day 3 lymphoblasts by the concomitant high expression of several anti-apoptotic members of the Bcl-2 family: Bcl-2, Bcl-xL, and Mcl-1. The levels of FOXO-regulated Bim were not significantly increased. Indeed, the most proapoptotic isoforms, BimL and BimS, were less evident in stimulated cultures containing growth-promoting cytokines than those without (Fig. 2A, 2C, bottom row).

**Profile when AICD is manifest (days 5–6).** Further insights into the mechanism for AICD came from analyzing the repertoire of expressed proapoptotic proteins, in the period after the initial burst of replication. First, Bim levels generally declined in late stage cytokine-supplemented cultures, as compared with cultures with BCR:CD21-L alone (Fig. 2A, 2C, bottom row), further suggesting...
FIGURE 2. Profile of pro- and anti-apoptotic molecules displayed in BCR:CD21-L–triggered B cells receiving costimuli from IL-4 with or without BAFF. (A–D) Immunoblotting analyses of SDS-PAGE separated proteins in lysates of human FO cells cultured for 3–4 or 5–6 d with BCR:CD21-L and medium, IL-4, or IL-4 plus BAFF. (A) Lysates from a representative single experiment were analyzed for Bax, Bid, Bim, and Bad by sequential stripping and reblotting. In this experiment, day 3 and day 5 lysates were contemporaneously transferred to different blots, with subsequent simultaneous analysis. (B) Immunoblots from two separate experiments evaluating Puma within day 4 lysates. (C) Pooled analysis of the relative expression of Bax, Bid, Bad, Puma, and BimL and BimM isoforms, within multiple experimental lysates collected at days 3–4 (prior to AICD) and at days 5–6 (AICD evident). Within each experiment, densitometric data for expression of the test molecule and loading control protein were obtained; values for the given test molecule were standardized on the basis of loading control in each experiment and calculated as percentage of maximum observed; and finally, all the latter values were from multiple experiments and are expressed as means ± SEM. The p values for significance from the Student t test are shown. The shaded bar below each set of pooled data indicates the relative propensity of cultures to display AICD, as shown in Fig. 1 and elsewhere (6). (D) Bcl-2/Bax expression ratios were obtained for the differing culture conditions by dividing the mean values for Bcl-2 expression (as percentage of maximum from a past analysis (6) of the same lysates here analyzed for Bax) by the mean values (percentage of max) for Bax expression. (E and F) Immunocytotfluorimetric analysis of selected proapoptotic and anti-apoptotic molecules within CFSE-labeled blasts in 5 d cultures activated by BCR:CD21-L plus IL-4 plus BAFF. (E) Following intracellular staining with mAb to intact Bid, caspase 6, Puma (red), or Bcl-2 or Mcl-1 (green), or, alternatively, IgG control (black), viable-gated cells were analyzed for expression of each protein within cells of differing division status by two-color flow cytometry. (F) Shown are the calculated values for PE fluorescence intensity above IgG control background, for each CFSE-determined division. Results are representative of a minimum of three experiments. (G) Means ± SEM of percentage of maximum values for Bid expression in diverse division subsets (n = 6 experiments).
that Bim is not a primary inducer of AICD late in these cultures. This was preceded by significantly diminished Bim mRNA on day 4 (Fig. 3). Second, both Bax and Bad appear elevated compared with levels detected in cells responding to BCR:CD21-L alone (Fig. 2A). Although these differences did not reach statistical significance in the pooled experiments (Fig. 2C), we attribute this to 1) the notably greater cell death and ensuing protein degradation within the cytokine-supplemented cultures at this late interval (cells with most elevated levels have succumbed to apoptosis), and 2) the fewer experimental lysates analyzed at this late interval. In support of this conclusion, Bax mRNA was significantly elevated within day 4 cultures (Fig. 3), just prior to obvious AICD (Fig. 1).

Third, proapoptotic Puma was not only upregulated at day 4, as assessed by immunoblotting (Fig. 2B, 2C, top row), but also strongly expressed within viable day 5 replicating lymphoblasts, as discerned by intracellular staining (Fig. 2E, 2F). Fourth, caspase 6, a protease reported to lower the threshold for apoptosis, was strongly evident in the day 5 replicating blasts (Fig. 2E, 2F). Finally, tBid, a truncated highly proapoptotic form of Bid (13), appeared to be actively formed within the replicating clones, as detailed below.

Evidence for tBid generation in replicating blasts. Although indirect, there is substantial evidence suggesting that Bid is fragmented to tBid during this TI B cell clonal expansion. First, prior to the proliferative burst, the nonapoptotic Bid form (21 kDa) was highly expressed in cytokine-supplemented cultures (particularly those with both IL-4 and BAFF). This major 21-kDa band vanished below.

FIGURE 3. Replicating B cells stimulated with BCR:CD21-L plus IL-4 plus BAFF exhibit elevated mRNA levels of Bid and Bax, but repressed Bim mRNA. (A) Cultures stimulated with BCR:CD21-L alone or with growth-promoting IL-4 and BAFF were harvested on day 4, mRNA isolated, and cDNA was prepared with oligo(dT) primer. Quantitative PCR (q-PCR) was performed with primers specific for Bim, Bid, Bax, and β-actin. Values for fold difference (Δ) of the above were obtained through comparison with β-actin. Values for fold difference (Δ) were obtained by comparing ΔCt values for each proapoptotic mRNA in cytokine-supplemented cultures with the respective ΔCt values in control cultures with BCR:CD21-L alone. The p values represent the significance of comparisons between Δ values in cytokine-supplemented cultures versus those in control cultures in a total of four replicate experiments, using a two-tailed Student t test. (B) Bid mRNA levels in divided blasts are comparable to Bid mRNA in undivided blasts. For semiquantitative RT-PCR of Bid, RNA from sorted nondivided and divided blasts on day 5 of activation showed Bid mRNA levels to be sustained during division (Fig. 3B). A third line of evidence supporting the formation of tBid is that levels of intact Bid significantly decline in viable divided blasts, as compared with the undivided blasts within the same cultures (Fig. 2E–G). Finally, there clearly exists an inverse relationship between level of intact Bid and lymphoblast susceptibility to AICD: cells with the most extensive division manifest the lowest levels of intact Bid (Fig. 2G) and the greatest susceptibility to AICD (5, 6). Taken together, the above findings strongly suggest that cleavage of intact Bid into labile, but highly proapoptotic, tBid contributes to the high apoptosis susceptibility of progeny within these TI human B cell clones.

Significance of diminished Bcl-2 expression for AICD

In the context of a notable upregulation of several proapoptotic molecules within replicating blasts, it is warranted to reconsider expression of the opposing anti-apoptotic molecules, namely Bcl-2 and Mcl-1. We have previously shown that Bcl-2 levels precipitously decline with each successive division, whereas Mcl-1 levels rise, in a BAFF-dependent manner (6). The opposing trends are also evident within the new experiment in Fig. 2E and 2F. Given previous evidence that the Bcl-2/Bax ratio is strongly linked to B cell survival (43), we compared levels of Bcl-2 and Bax in cultures before and after AICD had commenced (day 3 versus day 5). Fig. 2D shows that whereas the Bcl-2/Bax ratio in cells exposed only to BCR:CD21-L remains high throughout 5–6 d culture, this ratio notably drops in cytokine-supplemented cultures beginning to manifest AICD. Thus, despite the BAFF-driven elevation in Mcl-1, the decline in Bcl-2 appears to jeopardize the survival of replicating blasts under these stimulation conditions.

To further test this contention, we established similar culture conditions for stimulating a TI response from B cells from wild-type (WT) and Bcl-2 transgenic mice (35). In these latter experiments, cultures were stimulated with a limiting dose of BCR-L (anti-mouse IgM/dextran at 0.5–1 μg/ml) plus IL-4 and BAFF. The data in Fig. 4 clearly demonstrate that presence of the Bcl-2 TG augments the yield of viable day 5 progeny. The bonus effect of Bcl-2 overexpression in these mouse B cell cultures was most prominent in responses to BCR-L plus IL-4 (Fig. 4, left panels), but nevertheless was also evident in responses to BCR-L plus IL-4 plus BAFF (Fig. 4, right panels). Taken together, we conclude that a rise in proapoptotic molecules, combined with declining Bcl-2 expression, makes these TI clones highly susceptible to AICD.
**p53 is upregulated during TI stimulation of human B cells**

Although levels of p53 protein in normal cells are typically quite low due to ubiquitination of p53 by Mdm2 (44, 45), the evidence for heightened expression of molecules known to be direct transcriptional targets of p53 led us to monitor p53 protein expression. Elevated p53 expression was confirmed with the use of quantitative PCR of cDNA (Fig. 6A) and by a 40% decline in p53 protein (as reflected by mean fluorescence intensity [MFI] of PE-anti-p53 intracellular staining) (Fig. 6B). Nevertheless, this partial decline in p53 expression was associated with a significant increase in cytokine viability, cell size, and cell yield (Fig. 6B). Treatment with specific vimentin siRNA failed to affect p53 levels, while effectively downregulating vimentin (Fig. 6A), and it had no effects on cell viability or size, as compared with parallel cultures treated with control siRNA (data not shown). These findings are consistent with a p53 effect on cell viability and/or division. We were unable to reliably monitor the effects of p53 siRNA transfection on CFSE-labeled cultures given that even control nucleofected cells failed to regain the strong proliferative burst seen in unmanipulated cultures.

As an alternative test for assessing the functional relevance of p53 in mediating AICD, we compared viability and cell yield within cultures of CFSE-labeled B cells from p53-deficient (p53 knockout [KO]) or WT mice. Purified high-density splenic B cells were stimulated either with mouse-specific BCR-L and IL-4 (Fig. 7A, 7C, 7D, 7G) or with BCR-L plus IL-4 and BAFF (Fig. 7B, 7E, 7F, 7H). (In Supplemental Fig. 2, an experiment testing various BCL-L doses is shown; a dose of 0.5 or 1 μg/ml was used for all subsequent experiments.) These studies with p53 KO B cells consistently showed (five of five experiments) that overall culture viability was increased when p53 was absent (Fig. 7A, 7B). When CFSE-labeled division subsets were analyzed, heightened viability was typically seen at all divisions, but this was statistically significant or of borderline significance (Fig. 7D, 7F). Despite the above, it was also evident that at least half of the p53-deficient, highly divided lymphoblasts succumbed to death in cultures optimally stimulated with BCR-L plus IL-4 plus BAFF. This finding might suggest that p53 has only a minor proapoptotic function. Nevertheless, an alternative possibility is that the highly divided lymphoblasts within the p53-deficient cultures are dying by “mitotic catastrophe.” The latter is a death that occurs in cells with defective checkpoints and has been observed in replicating p53-deficient cells during mitosis, due to the lack of p53-imposed cell cycle brakes that permit repair of DNA damage (49–51).
Although not definitive, there is some support for mitotic catastrophe within p53-deficient B cell cultures activated with BCR-L plus IL-4 plus BAFF. First, the viability bonus attributed to p53 deficiency was less apparent in day 6 cultures as compared with day 4 and day 5 cultures (Fig. 7G, 7H). Second, greater levels of cyclin B1 were evident in a lysate of activated day 5 p53 KO cultures as compared with WT cultures upon immunoblotting (Supplemental Fig. 3). Cyclin B1 is characteristically elevated in cells undergoing mitotic catastrophe due to loss of a cell cycle brake in G2/M (52). Although further studies are necessary before we can unequivocally conclude that p53-deficient clones die by mitotic catastrophe, this process termed the “Achilles heel” of p53-deficient cells (51) may contribute to the eventual demise of p53-deficient blasts that avoid earlier apoptosis. Taken together, our results indicate that a p53-mediated proapoptotic pathway is at least in part responsible for the reduced viability of lymphoblasts in WT cultures.

Viable replicating B lymphoblasts in TI clones express high levels of phosphorylated ATM and H2AX

One primary route whereby p53 protein levels rise is through p53 phosphorylation (stabilization) by ATM. Once phosphorylated, p53 is resistant to ubiquitination by Mdm2 (53, 54). Thus, recent findings that activated (Ser1981-phosphorylated) ATM is highly upregulated within blasts of these TI cultures (55) is consistent with an active p53 axis. Insomuch as ATM can be activated both through its detection of DNA double strand breaks and through oxidative stress (54), in this study we probed TI blasts for a more definitive indicator of DNA damage: phosphorylation of the histone H2AX (56). Results from two representative experiments in which CFSE-labeled blasts in day 5 cultures were stained for p-ATM-Ser1981 and p-H2AX-Ser139 are shown in Fig. 8A (left panels, human B cells activated with BCR:CD21-L plus IL-4 plus BAFF).
BAFF; right panels, mouse B cells activated with BCR-L plus IL-4 plus BAFF). Although all cells were positive for p-ATM and p-H2AX, dividing lymphoblasts clearly exhibited the highest levels of these phosphoproteins, both in human and mouse cultures.

To better understand the timing of DNA damage within these cultures, B cells were stained for p-ATM-Ser1981 and p-H2AX-Ser139 both prior to culture and at days 2–3 following culture with BCR:CD21-L, with or without supplementary cytokines. The representative experiments in Fig. 8B show that whereas levels of p-H2AX remain low in cultures exposed to BCR:CD21-L alone, expression of this indicator of DNA damage significantly rises in cultures with added IL-4 and BAFF. When a small proportion of blasts has begun to divide (day 3), a subset of undivided cells and all the divided blasts express high levels of p-H2AX. Interestingly, p-ATM was more prominent in freshly isolated cells than was p-H2AX, perhaps reflecting the capacity of oxidants such as H2O2 to activate ATM phosphorylation (54). Levels of p-ATM did not further increase in cells exposed to BCR:CD21-L alone, but rose substantially in cells receiving growth-promoting stimuli from IL-4 and BAFF, particularly in dividing blasts. Taken together, the representative experiment in Fig. 8B shows that B cells exhibit some ATM activation even prior to culture, but DNA damage, as shown by p-H2AX, is minimal until cells begin showing signs of DNA replication/division.

The immunoblotting evidence in Fig. 8C and 8D provides further support for the conclusion that H2AX phosphorylation is most marked in BCR:CD21-L–triggered B cells exposed to growth-promoting cytokines. Although the ratio of p-H2AX to total H2AX was elevated in IL-4–supplemented cultures at day 3, evidence for p-H2AX phosphorylation was substantially more pronounced at day 5 when many cells had divided. Interestingly, blotting with the anti–p-H2AX mAb not only revealed an expected 15-kDa band for H2AX, but also a relatively intense band between the 52- and 225-kDa molecular mass markers. In both experiments, the band was most notable within cultures exposed to IL-4 and BAFF. Although its identity remains unclear, there is a possibility that the high-molecular mass band reflects a complex of p-H2AX and other proteins known to aggregate at sites of DNA damage (58, 59). This possibility is consistent with the decline in 15-kDa total H2AX, as well as 15-kDa pH2AX (as compared with actin loading control) within cultures supplemented with both IL-4 and BAFF (Fig. 8C). Although a similar high-molecular mass band was not noted with the anti-H2AX immunoblotting mAb that recognizes total H2AX (data not shown), it is possible that the epitope engaged by the latter mAb is modified or blocked in the putative complex. Taken together, although the identity of the molecule(s) in the upper molecular mass band is unclear, both the available evidence from intracellular staining and the ratiometric analysis of monomeric (15-kDa) pH2AX/H2AX levels show that B cells within cytokine-supplemented cultures exhibit greater p-H2AX-Ser1981 phosphorylation and hence greater DNA damage. Several PI3K-like kinases, ATM, ATR, and DNA-PK, could be involved in initiating the phosphorylation of the H2AX histone at sites of DNA damage. Evidence of the concomitant up-regulation of p-ATM-Ser1981 by both immunostaining (Fig. 8A, 8B) (7) and immunoblotting (Supplemental Fig. 4) suggests that activated ATM at a minimum is involved.

**B cells genetically deficient in AID exhibit lesser, but not ablated, AICD**

Although H2AX phosphorylation in part undoubtedly reflects DNA strand breaks known to occur during the process of DNA replication (60), the evidence that AID is highly expressed and active within replicating human blasts stimulated within BCR:CD21-L plus IL-4 with or without BAFF (7) strongly suggests that a component of the evidenced DNA damage reflects AID-induced enzymatic activity. To test the hypothesis that AID function is contributing to AICD within T1 clones, we compared the in vitro...
responses of B cells from WT mice and mice with deleted Aicda (AID-KO) to a similar mode of TI activation. The data in Fig. 9 show that although AICD remains prominent in AID-KO cultures, AID deficiency does have a minor, yet reproducible, impact on the percentage viability of highly divided lymphoblasts and their total yield. This is weakly apparent upon the overlay of histograms from viable and apoptotic CFSE-labeled blasts (Fig. 9A, 9B). It is further evidenced by the greater percentage viability (Fig. 9C, 9E)
and greater total viable cell yield (Fig. 9D, 9F) of AID-KO cultures versus WT cultures. Whereas in BCR-L plus IL-4-activated cultures, the prosurvival effects of AID deficiency are consistently noted only in the most highly divided B lymphoblasts (Fig. 9C, 9D), within cultures exposed to both IL-4 and BAFF, the prosurvival effects of AID deficiency appear to extend to both undivided and divided lymphoblasts (Fig. 9E, 9F). This might relate to the fact that BAFF synergizes with IL-4 in promoting AID expression/activity (7, 61, 62). Taken together, these mouse B cell studies indicate that although AID function contributes to AICD within BCR-triggered, IL-4 and BAFF-driven clones, AID function is not obligatory for AICD of the replicating B lymphoblasts.

**Discussion**

The present study provides novel evidence that p53—a transcription factor, coined “guardian of the genome and policeman of the oncogenes” (63, 64)—has a functional role in regulating lymphoblast survival during an in vitro TI immune response of mature B lymphocytes. Importantly, the synergistic stimulator that generate susceptible clones, Ag, C3d, IL-4, and BAFF are abundant in certain in vivo settings where B cell foci are prominent (65–67), suggesting that these findings may have physiologic relevance. A functional role for p53 in promoting AICD was supported by several lines of data: 1) elevated levels of p53 protein, as well as upregulated mRNA and/or protein of several proapoptotic mole-
cules known to be under the positive transcriptional control of p53, that is, Bax, Bad, Puma, Bid, and caspase 6 (25–27, 68); 2) augmented lymphoblast survival upon p53 siRNA treatment (human) or p53 gene deletion (mouse); and 3) correlation of p53 pathway upregulation with heightened levels of DNA damage. p53 can transactivate genes for proapoptotic proteins involved in mitochondria-dependent intrinsic apoptosis as well as genes for membrane death receptors, Fas and DR. Nevertheless, these studies with B lymphoblasts from TI clones showed evidence for the former but not the latter. Such findings are consistent with evidence that p53 transcriptional targeting is highly regulated (69). Importantly, in recent years it has become clear that p53 can also promote mitochondria-dependent apoptosis through its direct functions in the cytoplasm (28, 70, 71). This latter function of p53 may also influence TI clonal contraction. Future studies are needed to clarify its relative importance.

Until now, direct evidence that p53 influences B lymphoblast survival during a normal immune response has been limited. This is perhaps surprising given the established roles of p53 in regulating cell cycle progression and survival following cell stress in many lineages (64). Certainly, p53 function is anticipated within T cell-dependent germinal centers given the high levels of AID-induced DNA damage and oxidative stress within rapidly replicating centroblasts. Quite consistent with this, a study by Martinez-Valdez et al. (12) found that germinal centers express elevated p53 protein as well as proapoptotic proteins such as Bax. Nevertheless, the importance of p53 in such T cell-dependent foci became obscured by other findings suggesting that p53 function within germinal centers was under strong negative control. First, p53 mutations were rare in most B cell malignancies of germinal center origin compared with an average rate of ∼50% in non-B cell malignancies (72). Second, the major germinal center master regulator, Bcl-6, was found to actively repress transcription of p53 mRNA (73), as well as transcription of ATR, a kinase that phosphorylates and stabilizes p53 protein following single-stranded DNA damage (74). Third, there was evidence that FOXO-regulated Bim, but not p53-regulated Bid, was important in germinal center apoptosis (18). Finally, mice with genetic deficiency in p53 readily succumbed to tumors of non-B cell lineages, rather than B cell tumors (75), suggesting that p53 function in B cells is distinctly regulated. A possible reconciling explanation is that transcription from the p53 gene is significantly downregulated within germinal centers (making the p53 gene less vulnerable to mutation), but that low levels of translated p53 protein are significantly stabilized by p53-modifying kinases, ATM and DNA-PKA. The latter are not subject to Bcl-6 repression and are activated by DNA damage/cell stress (21, 76, 77).

Of relevance to TI responses and p53 activity, two earlier studies with p53-deficient murine B cells showed that p53 can slow cell cycle progression in response to LPS and IL-4 (78, 79). Because no difference was observed in the viability of cultured p53-deficient and WT B cells at day 3, the authors concluded that p53 did not
influence mature B lymphocyte survival. Consistent with the above, we also observed no difference in viability between WT and p53-deficient B cells at this early culture interval. Nevertheless, significant differences emerged as clonal expansion progressed through day 5 of the response.

Thus, the present report provides the first direct evidence, to our knowledge, that p53 has a functional role in regulating B lymphoblast viability during clonal expansion. Findings reported by Hao et al. (80) as this manuscript was in revision are consistent with our conclusions that p53 plays a role in regulating the survival of B cells during TI B cell responses and, furthermore, suggest that this occurs in vivo. In the latter study, mice with genetic ablation of Mule (a ubiquitin ligase which targets p53, as well as other proteins such as Mcl-1; see Ref. 81) exhibited significantly diminished levels of serum IgM and IgG3 in response to the TI stimulus, TNP-Ficoll, while exhibiting relatively normal IgM and IgG responses to the TD stimulus, NP-CGG. Interestingly, cultures of mule-deficient B cells yielded higher readily divided lymphoblasts in response to LPS plus IL-4 and showed an abnormal increase in Puma mRNA as compared with WT B cells, which was not apparent in the absence of p53 (80). Taken together, the data from the current study involving both human and mouse B cells and the recent in vivo and in vitro murine studies of Hao et al. (80) strongly argue that p53 has a functional role in regulating B cell survival during a TI clonal expansion.

A predilection for TI B cell clones to succumb to p53-mediated apoptosis, a result of sustained DNA damage, sheds some light on several past observations. Mice deficient in p53 exhibit an expanded splenic marginal zone and increased incidence of marginal zone lymphoma (75). p53-deficient lymphoblasts switch more effectively to IgG2a than do WT B cells following in vitro stimulation with LPS plus cytokines or in vivo stimulation of mice with polyoma virus (82). Ig-associated gene translocations occur more frequently in LPS plus IL-4–stimulated B cell cultures from p53-deficient mice than in similarly stimulated normal B cells or B cells from mice genetically deficient in both p53 and AID (83). Finally, in humans, p53 mutations are more common in malignancies that likely originate outside of germinal centers: B–chronic lymphocytic leukemia and related prolymphocytic leukemia (72, 84–86), MALT lymphoma (72, 87, 88), and marginal zone lymphoma (89, 90).

In the present model for TI B cell clonal expansion, it appears likely that Puma and tBid are the important triggers for the oligomerization of apoptosis inducers, Bax or Bak, within the mitochondrial membrane and ensuing AICD (91). Although the evidence for Puma involvement is only indirect, other recent evidence shows that Puma has a critical role in determining the fate of mitogen-stimulated B cells in vitro and the development of B cell memory in vivo (16). Interestingly, although Puma is transcriptionally upregulated by p53 upon DNA damage, it can also be upregulated during cytokine growth withdrawal by FOXO3a (29).

The latter pathway may be less relevant in our IL-4– and BAFF-supplemented cultures given that mRNA and protein for another FOXO3a-regulated molecule, the apoptosis-activator Bim (92), is downregulated in these cultures. Diminished Bim mRNA may represent optimal activation of the PI3K/Akt pathway by IL-4 (93) since Akt phosphorylates FOXO3a and promotes its exclusion from the nucleus (94, 95). Consistent with the above interpretation, lysates of cultures stimulated with BCR:CD21-L plus BAFF, in the absence of IL-4, expressed significantly greater levels of the various Bim isoforms (P.K.A. Mongini, unpublished observations).

The evidence for involvement of tBid in promoting AICD is also indirect, yet worth noting. Although tBid levels were difficult to reliably observe, intact Bid protein (but not mRNA) precipitously declined during successive divisions within IL-4– and BAFF-supplemented cultures. Bid cleavage to the highly proapoptotic, labile tBid can be achieved by number of proteases, for example, caspases 8 and 10, calpain, granzyme B, and cathepsins (96). Importantly, all have been reported in activated B cells (97–99). Thus, although in vivo studies with Bid-deficient mice suggest that tBid has minimal to no effect on germinatal center formation (18), there is a strong possibility that this molecule is relevant in precipitating the clonal contraction of TI clones.

In the context of Puma and tBid, it is undoubtedly relevant that Mcl-1 can bind both with high affinity and thereby block intrinsic apoptosis (13, 100). The significance of Mcl-1 is more apparent by the precipitous decline of Bcl-2. Whereas the latter anti-apoptotic protein is highly expressed in resting B cells and nondividing activated blasts, its levels drop with each successive division (present study and Ref. 6). This phenomenon may help explain why an in vivo-expressed Bcl-2 TG helped prevent deletion of autoreactive mouse B cells within peripheral tissues (101, 102) and, furthermore, had a greater effect at augmenting B cell memory to TI Ags, as compared with TD Ags (103). Prior findings from this laboratory suggest that deletion of autoreactive B cells by p53-driven AICD might be averted and T1 memory increased in tissues with elevated concentrations of Mcl-1–augmenting BAFF, APRIL and PGE2 (5). Such a scenario might in part explain why salivary gland-targeted Sjögren’s syndrome is highly linked to APRIL titers (104) and why SLE autoantibody titers decline in mice treated with inhibitors of Mcl-1–upregulating COX-2 (105).

Note that p53 has been implicated in the negative regulation of Bcl-2 both through direct repressive effects at the Bcl-2 promoter (106) and through induction of Bcl-2 inhibitory micro-RNA: miR-15, miR-16, and miR-34 (107–109). Nevertheless, because Bcl-2 is subject to other means of regulation (110–112), one cannot conclude that the delay in Bcl-2 reflects an augmented p53 axis. This will require further study.

In this study, we made efforts to replicate the human TI response to low-dose BCR:CD21-L plus IL-4 with or without BAFF with mouse cultures stimulated with higher dose BCR-L plus IL-4 with or without BAFF to more readily study the effects of AID on the process of AICD. The resulting experiments showed that AID function only slightly contributes to the clonal contraction of mouse B cell clones. Interestingly, although reduced AICD in p53-deficient cultures extended to all division subsets, the protective effect of AID deletion was noted predominantly in the most highly divided cells. This is consistent with the strong linkage between AID expression/function and cell division (7, 113).

There is a strong possibility that the present findings with mouse B cells do not adequately mirror the AICD-inducing effects of functional AID within human B cell clones. First, dividing mouse lymphoblasts recovered from BCR-L–, IL-4–, and BAFF-activated cultures manifest little, if any, intracellular staining with an anti-mouse AID mAb (clone ZA001) (H. Lee and P.K.A. Mongini, unpublished results), whereas replicating blasts of human B cell cultures show high AID expression upon staining with the same mAb or an additional human AID-specific rat mAb (7). One possible explanation for these findings is that stronger BCR engagement in the mouse B cell cultures may have dampened AID transcription (114). (Mouse cultures were stimulated with 1 μg/ml anti-IgM/dextran, whereas human B cell cultures were stimulated with 0.01 μg/ml anti-IgM/anti-CD21/dextran.) Second, in addition to diminished AID protein, AID function is less evident in the mouse cultures than in human cultures: substantially fewer IgG3 switched progeny are typically recovered from the above TI-activated mouse B cell cultures (P.K.A. Mongini, unpublished results) than from activated human B cell cultures (7). Thus, taken
together, it remains quite possible that AID activity has a significantly greater contribution to p53-mediated AICD within the studied TI human B cell clones than revealed in the present mouse cultures. Importantly, replicating mouse B cells are not resistant to AICD following AID-induced DNA damage. Zaheen et al. (10) recently demonstrated that AID activity significantly constrains clonal size within BCR and CD40-stimulated mouse B cell cultures.

In conclusion, the present evidence that a p53 axis governs the survival of replicating B lymphoblasts during the above TI response may illuminate the stimulatory environment where premalignant or transformed B cells with altered genes of the p53 axis arise. We propose that inflamed peripheral tissues laden with C3dg-coated foreign or self Ag and IL-4(IL-13) and BAFF-releasing cells of the innate immune system (65, 115–121) may be such a milieu. In these sites, TI B cell clonal expansion would be promoted, but there would be strong positive selection pressure for B lymphocytes to escape p53-mediated AICD. Both the high AID expression/activity elicited by these stimuli (7) as well as the oxidative stress of the proliferative burst should induce mutations. Those within p53 and other genes of the p53 axis will be selected for and may represent one step in a series toward full malignancy, or alternatively the defining event. Such a stimulatory milieu might explain why the p53 gene is deleted in ∼30% of patients with benign CDS monobclonal B cell lymphocytosis (122). It might also help explain why a high proportion of CDS B-chronic lymphocytic leukemia clones display alterations in genes encoding p53 pathway proteins (84, 123, 124) and/or have lost one of several p53-expressing leukemia clones display alterations in genes encoding p53 expression/activity elicited by these stimuli (7) as well as the oxidative stress of the proliferative burst should induce mutations. Those within p53 and other genes of the p53 axis will be selected for and may represent one step in a series toward full malignancy, or alternatively the defining event. Such a stimulatory milieu might explain why the p53 gene is deleted in ∼30% of patients with benign CDS monobclonal B cell lymphocytosis (122). It might also help explain why a high proportion of CDS B-chronic lymphocytic leukemia clones display alterations in genes encoding p53 pathway proteins (84, 123, 124) and/or have lost one of several p53-regulated miRNA genes that negatively control Bcl-2 (125–128).

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Disclosures

The authors have no financial conflicts of interest.

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