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p53 Represses Class Switch Recombination to IgG2a through Its Antioxidant Function

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Ig class switch recombination (CSR) occurs in activated mature B cells, and causes an exchange of the IgM isotype for IgG, IgE, or IgA isotypes, which increases the effectiveness of the humoral immune response. DNA ds breaks in recombining switch (S) regions, where CSR occurs, are required for recombination. Activation-induced cytidine deaminase initiates DNA ds break formation by deamination of cytosines in S regions. This reaction requires reactive oxygen species (ROS) intermediates, such as hydroxyl radicals. In this study we show that the ROS scavenger N-acetylcysteine inhibits CSR. We also demonstrate that IFN-γ treatment, which is used to induce IgG2a switching, increases intracellular ROS levels, and activates p53 in switching B cells, and show that p53 inhibits IgG2a class switching through its antioxidant-regulating function. Finally, we show that p53 inhibits DNA breaks and mutations in S regions in B cells undergoing CSR, suggesting that p53 inhibits the activity of activation-induced cytidine deaminase. The Journal of Immunology, 2010, 184: 6177–6187.

In mature B cells the IgH chain locus undergoes two major genetic alterations. Somatic hypermutation (SHM) and class switch recombination (CSR) shape the Ab repertoire by increasing the affinity for Ag, and changing the isotype of the expressed Ig, respectively. CSR diversifies the effector function of the humoral immune response by changing the expressed Ig isotype from IgM to IgG, IgE, or IgA. Both SHM and CSR are dependent on the activation-induced cytidine deaminase (AID) enzyme (1, 2). During CSR, AID initiates the formation of DNA ds breaks (DSBs) in the switch (S) regions that are located upstream of each of the constant regions (Cκ), which encode the different Ig isotypes (with the exception of Cδ, encoding IgD) (3–6). AID deaminates cytidines in the S regions, thereby generating DNA ds breaks, which are removed by the base excision and mismatch repair systems, resulting in either mutations or DSBs (5, 7–11). AID-instigated DSBs in Sκ and in the downstream S regions are recombined by nonhomologous or microhomology-mediated end joining, generating the S-S junction and resulting in the excision of the intervening DNA sequences (12).

We have previously shown that AID-dependent S region blunt DSBs occur predominantly in the G1-phase of the cell-cycle (13). How this cell-cycle restriction is enforced is, however, unknown. Clearly, DNA damage response pathways are activated by CSR-associated DSBs, because efficient class switching requires DNA damage sensing proteins, such as the ataxia telangiectasia-mutated kinase (ATM), the variant histone H2AX, p53-binding protein-1, and the Mre11-Rad50-Nbs1 complex (12, 14–22). The p53 protein acts as a central role downstream in the DNA damage response pathways, essentially acting as a gatekeeper. Unresolved DNA breaks activate p53, which, depending on the context, regulates the cell-cycle, DNA repair and cell death (23).

In addition, recent reports have shown that p53 has a role in the regulation of the levels of intracellular reactive oxygen species (ROS) (24, 25). Mammalian cells are continuously subjected to ROS, which can result in proliferation, cell death or growth arrest, depending on the cellular context and the level of ROS. In cells that have sustained extensive damage, p53 can stimulate the expression of genes that increase intracellular ROS as part of a proapoptotic response (26). At low stress levels, p53 can also activate antioxidant genes that lower ROS levels as part of a protective response (24).

The role of intracellular ROS levels in B cells undergoing class switching is unknown. However, both enzymatic and spontaneous cytidine deamination are achieved through oxidation, which involves hydroxyl radicals, showing that ROS are required intermediates in this process (27, 28). In this study, we present a novel role for p53 in limiting class switching. We find that although p53 does not affect AID levels, cell-cycle progression, proliferation, or apoptosis in B cells induced to switch, it has a redox-dependent inhibitory function in CSR, resulting in inhibition of S region breaks, mutations, and isotype switching.

Materials and Methods

Mice

All mouse strains were backcrossed to C75BL/6. p53−/− mice were obtained from Dr. Stephen Jones, and were previously described (29). AID-deficient mice were obtained from T. Honjo (Kyoto University, Kyoto, Japan).
Nutlin-3 (Sigma-Aldrich) was added at the start of the culture, and the cell suspension was incubated with a flow cytometer (BD Biosciences) and analyzed using the FlowJo software (TreeStar, St. Louis, MO) and human BLyS (100 ng/ml; Human Genome Sciences, Woodbridge, VA) and IL-5 (1.5 ng/ml; BD Biosciences, San Jose, CA), and anti–mouse HRP (Jackson ImmunoResearch Laboratories, West Grove, PA) and FITC conjugated mouse–anti-mouse GL7 (BD Biosciences, San Jose, CA) were added; for IgG2b switching, HBSS (Invitrogen) and resuspended at 400,000/ml. Cells were then incubated for 2 d (8), Sestrin-1 (5-CCAGGACGAGGAACTTGG-3), Sestrin-1 FW (5-GGTTGATACGCCAAGACTTGGTGG-3), Sestrin-2a-specific probe (5-AGTGATAGAAATCTGTCAGGCT-3), a 7-amino-actinomycin D (BD Pharmingen) staining. Cells were washed with PBS, 1% FCS, 0.2% NaCl, and resuspended at 10,000/ml in 200 μl of cold PBS with 1% FCS. Cells were then washed twice with PBS, 1% FCS, 0.2% NaCl, and incubated for 30 min on ice with PE–goat F(ab')2 anti-mouse IgG1, IgG2a, IgG2b, or IgG3 (Southern Biotechnology Associates, Birmingham, AL). For Flow cytometry analysis, cells were analyzed by flow cytometry, using allophycocyanin (NAC) (Sigma–Aldrich) was dissolved in PBS and 25 μM HEPES (pH 7.0), and added at the start of the culture and again at 24 h. Infection of mice with polyclonal virus Mice were infected i.p. with 2 × 10^8 PFU/mouse polyclonal virus strain A2. Mice were sacrificed 12–20 d postinfection. Isoxyme-switched splenic germinal center B cells were analyzed by flow cytometry, using allotypicconjugated mouse–anti-mouse B220 (RA3-6B2; Caltag Laboratories, Invitrogen), and FITC conjugated mouse–anti–mouse antibodies (BD Pharmingen, San Jose, CA), in combination with either PE–conjugated goat F(ab')2 anti-mouse IgG1, IgG2a, IgG2b, or IgG3 (Southern Biotechnology Associates, Birmingham, AL). Flow cytometry For FACs analysis, cells were washed twice with PBS, 1% FCS, 0.2% NaCl, and incubated for 30 min on ice with PE–goat F(ab')2 anti-mouse IgG1, IgG2b, IgG2a, or IgG3, or PE–goat anti-mouse IgA (Southern Biotechnology Associates). For CFSE labeling, cells were washed in HBSS (Invitrogen) and resuspended at 40 × 10^6/ml. An equal volume of 2.0 μM CFSE was added and cells were incubated at 37˚C for 15 min, quenched in 100% FCS, and then washed twice with medium containing 10% FCS. For splenic B cell–subset analysis, cells were stained with anti-B220 allotypicconjugated (RA3-6B2; Caltag Laboratories), anti–CD23 PE (2G8; Southern Biotechnology Associates), anti–CD21 FITC (7666; BD Pharmingen). CFSE fluorescence and Ab staining were acquired on a LSR flow cytometer (BD Biosciences) and analyzed using the FlowJo software package (Tree Star, Ashland, OR). ROS detection by flow cytometry Splenic B cells activated for 48 h were stained with 1 μM 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H_2DCFDA) (Molecular Probes, Invitrogen) for 15 min at 37˚C, followed by a 10 min recovery period at 37˚C. Extracellular CM-H_2DCFDA was quenched with 0.2% trypsin blue. Fluorescence resulting from intracellular oxidation of CM-H_2DCFDA was assessed by flow cytometry. Dead cells were excluded by 7-amino-actinomycin D (BD Pharmingen) staining. Western blotting Cytoplasmic and nuclear extracts were prepared by resuspending cells in hypotonic buffer (10 mM HEPES pH 8.0, 1 mM EDTA, 10 mM KCl, 0.1 mM EGTA, 1 mM DTT, 2 μM pepstatin, and complete protease inhibitor mixture (Roche Applied Science, Indianapolis, IN). After 15 min incubation on ice, cells were lysed by addition of Nonidet P-40 to a final concentration of 0.625%. Supernatants were taken as cytoplasmic extracts. Nuclei were washed once with hypotonic buffer and resuspedn in hypertonic buffer (20 mM HEPES pH 8.0, 1 mM EDTA, 400 mM NaCl, 1 mM EDTA, 1 mM DTT, 2 μM pepstatin, and complete protease inhibitor mixture). For whole-cell extracts, cells were lysed in RIPA buffer (150 mM NaCl, 0.5% deoxycholate, 0.1% SDS, 1.0% NP-40, 50 mM Tris-HCl pH7.4, complete protease, and phosphatase inhibitor mixture) and subjected to three freeze-thaw cycles. Protein content of cytoplasmic, nuclear, and whole-cell extracts were determined using Bradford assay (Bio-Rad, Hercules, CA). Equal amounts of proteins were electrophoresed on 8% SDS-polyacrylamide gels or 4–20% gradient SDS-polyacrylamide gels (Pierce, Rockford, IL) and blotted onto Immobilon-P polyvinylidene fluoride membranes (Millipore, Bedford, MA). Abs were used as follows: rabbit–anti–APEX1 (33), rabbit–anti–APEX2 (33), rabbit–anti–uracil-N-glycosylase (33), rabbit–anti–phospho-185 p53 (Anaspec, Fremont CA), rabbit–anti–LC3B (Abcam, Cambridge, MA), rabbit–anti–GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA), and mouse–anti–TFBI (TATA-box binding protein; Abcam), followed by goat–anti–rabbit HRP or donkey–anti–mouse HRP (Santa Cruz Biotechnology) and enhanced chemiluminescent substrate (Pierce). RT-PCR RNA was isolated using TRI reagent (Applied Biosystems/Ambion, Austin, TX) as recommended by the manufacturer. RNA, 1–5 μg, was reverse transcribed in a 30 μl reaction with 0.6 μg of (dT)_{18} primer (Roche Applied Science) and 200 U Moloney Murine Leukemia Virus Reverse Transcriptase (Invitrogen) for 1 h at 37˚C. Germine S sequences were quantified by LightCycler PCR using SYBR Green (Roche Applied Science). PCR primers used in this study were: AID-specific (5'-ATATG-GACAGCCTTCTGAGAAGC-3'), AID-antisense (5'-TCTTCACTAA-TCAGCTATCAG-3'), Catatag-3'-hydantoin phosphotriester (5'-GTTTGGATACCCGCAGACTTGGTGG-3'), 3'-HPRT (5'-TACATG-GCAGATGCCACAGAGACTA-3'), GLY1-FW (5'-CGACGCTGTCAGTAGACT-3'), GLY1-REV (5'-CTGTCATCAGCAAGGCTC-3'), GLY2a-FW (5'-GTCCACCTTTGGTCTGCTT-3'), GLY2a-REV (5'-GCTGTATGAC-TCTACGTAGAGAG-3'), P21-FW (5'-GAAGCTTGGTCTGAGCAG-3'), P21-REV (5'-AGTGGTCTGAGCAGCAG-3'), ITG2a-REV (5'-CCAGGACGAGCAGATCGCC-3'), Sestrin-1 REV (5'-CCAGTGG- AACACTGATGC-3'). These primers do not amplify genomic DNA (not shown). Genomic DNA preparation and linker LM-PCR After culture for 2 d, viable cells were isolated by flotation on Ficoll/Hypaque gradients (ρ = 1.090), or Lympholyte (Cedar Lane, Ontario, Canada), cells were imbedded in low-melt agarose plugs, and DNA isolated as described (5). For linker ligation, 50 μl ligase buffer and 1 μl DNA were added to the plugs that were then heated to 62˚C to melt the agarose. Then, 20 μl DNA (∼200,000 cell equivalents) was added to 2 μl T4 DNA Ligase (2 Weiss units, MB Fermentas, Hanover, MD), 10 μl ds annealed linker in one times ligase buffer, 3 μl 10 times Ligate buffer, and 30 μl dH2O and incubated overnight at 18˚C. Linker was prepared by annealing 5 mmol each of LM-PCR1 (5'-GGCGGGACCCGGGAGATGCAGAC-3') and LM-PCR2 (5'-GAATTTCGCGAGAGCT-3') in 300 μl one time ligase buffer, which results in a ds oligonucleotide with a 14 nt single-stranded overhang that can only ligate unidirectionally. Ligated DNA samples were heated at 70˚C for 10 min, diluted five times in dH2O and then assayed for gapped DNA by PCR to adjust DNA input prior to LM-PCR. The primer 5'Sa (5'-GAGAGAAAATTTAGATAAATATGATACCTAGTG-3') or 5'Sa2 (5'-ATGGTTCTCTGGTCGACAAATACA-3') (Integrated DNA Technologies, Coralville, IA) were used in conjunction with linker primer (LM-PCR1) to amplify DNA breaks in Sa and Sa2a, respectively. The 3-fold dilutions of input DNA (0.5, 1.5, and 4.5 μl for Sa-LM-PCR, 1.5, 4.5, and 13.5 μl for Sa2a-LM-PCR) were amplified by HotStar Taq (Qiagen, Valencia, CA) using a touchdown PCR program (28 cycles after touchdown for Sa LM-PCR and 35 cycles after touchdown for Sa2a LM-PCR). PCR products were electrophoresed on 1.25% agarose gels and blotted onto nylon membranes (GeneScreen Plus, Perkin Elmer, Waltham, MA). Blots were hybridized with an Sa-specific oligonucleotide probe (μ probe5'-5'-AGGACCACTAAGAAGCGAAT-3') for 5'Sa LM-PCR, or an Sa2a-specific probe (Sa2a probe: 5'-CAGCTCTCAAGTGAGGCACGC-3') for Sa2a LM-PCR, end-labeled with [γ^32P]-ATP at 42˚C overnight and washed at 55˚C with 2x SSC/0.1% SDS. Semiquantitative assessment of DSB efficiency was achieved by scanning of autoradiograms, combining all three dose titration lanes for each mouse. PCR amplification and cloning of Sa-Sa3 junctions Genomic DNA was isolated from purified splenic B cells after culturing for 4 d (8). Sa-Sa3 junctions were amplified from genomic DNA by PCR.
using the Expand Long Template Taq and Pfu polymerase mix (Roche, Piscataway NJ) and the primers μ2-3(H3) and g3-2 (8). PCR products were cloned into the vector pCR4-TOPO (Invitrogen, Carlsbad, CA). For measurement of the mutation frequency and spectrum within recombined Sμ-Sγ3 segments, only clones whose Sμ or Sγ3 segments were completely sequenced were included.

Results

p53 inhibits IgG2a class switching

To examine if p53 has a role in CSR, splenic B cells from p53−/− and WT littermates were stimulated to switch to several different isotypes in vitro by culturing with LPS, anti-IgD dextran, and various cytokines (see Materials and Methods). Surface expression of switched Ig isotypes and proliferation were assayed by flow cytometric analysis of CFSE-stained splenic B cells cultured for 2.5 d. Switching to most isotypes was not affected by the absence of p53, but IgG2a switching was increased by 2.2-fold in p53−/− B cells relative to WT cells (Fig. 1). A slight but consistent increase in IgG2b and IgA switching was also observed. No appreciable differences in the number of cell divisions, as assessed by dilution of the CFSE signal, were observed in p53−/− versus WT B cells for any of the culture conditions (Supplemental Fig. 1A). Also, the cell-cycle profiles of WT and p53−/− splenic B cells induced to undergo CSR were indistinguishable (Supplemental Fig. 1B), indicating that the inhibitory effect of p53 on IgG2a switching is not mediated through perturbation of proliferation or the cell-cycle. The absolute numbers and proportions of total B cells and newly formed, follicular, and marginal zone B cell subsets in the spleens of p53−/− mice were not different from WT littermates (Supplemental Fig. 2).

Increased in vivo IgG2a switching in polyoma virus-infected p53−/− mice

To determine whether p53 has an effect on isotype expression during an immune response in vivo, we infected p53−/− and WT littermate mice with polyoma virus, which elicits a robust protective Ab response that is characterized by the induction of predominantly IgG2a and IgG2b Abs (34). Splenic germinal center B cells were analyzed by flow cytometry 12–20 d post-infection. A 2-fold increase in IgG2a-expressing splenic germinal center B cells was observed in polyoma virus infected p53−/− mice compared with infected WT littermates (p = 0.018), whereas the proportions of germinal center B cells expressing IgG1, IgG2b, or IgG3 were not significantly different (Fig. 2). Thus, p53-deficiency appears to specifically increase IgG2a CSR in vitro and in vivo, but to have little effect on other isotypes.

Increased p53 levels inhibits CSR to multiple isotypes

To determine the consequences of increased p53 levels on CSR, we assessed in vitro class switching in Nutlin-3–treated splenic B cells. Nutlin-3 is a potent MDM2 antagonist, stabilizing p53 by inhibiting MDM2-mediated degradation of p53, thereby strongly activating the p53 pathway (35). In B cells activated for IgG2a switching, Nutlin-3 treatment resulted in a 5-fold increase in p21 mRNA expression, which is a direct transcriptional target of p53 (Fig. 3B). Nutlin-3 treatment significantly inhibited CSR to IgG2a, IgG2b, IgG3, and IgA, indicating that supraphysiological p53 levels decrease switching to multiple Ig isotypes (Fig. 4A). Cell proliferation was largely unaffected by this inhibitor at the concentration used in this study and did not account for the decreased switching, as determined by assessing switching in each cell division using CSFE staining (Fig. 4B; data not shown).

Cell-death and survival of switching splenic B cells are not affected by p53-deficiency

In addition to regulation of the cell-cycle, p53 also coordinates cell-death pathways, such as apoptosis and autophagy (36, 37). However, in splenic B cells activated for CSR, we detected no effect of p53-deficiency on cell-death, as measured by 7-amino-actinomycin D staining (Supplemental Fig. 3A), or on autophagy, as determined by LC3-I/II immunoblotting (Supplemental Fig. 3B). These results show that p53 does not act in CSR through its well-established functions in the regulation of the cell-cycle and cell-death.

AID expression and germline S region transcription in activated p53−/− splenic B cells is unaltered

The amount of CSR observed is related to the expression level of AID, which in turn correlates with aicda mRNA levels (38, 39). However, by immunoblotting we found no effect of p53 deficiency on levels of AID protein in splenic B cells activated to undergo

![Figure 1](http://www.jimmunol.org/)

**Figure 1.** p53-deficient splenic B cells show increased IgG2a switching in culture. CFSE-loaded splenic B cells from p53−/− and WT littermate mice were cultured for 2.5 d with LPS, cytokines, and/or anti-δ-dextran to induce Ig class switching to the indicated isotypes. A, Cells were analyzed for CSFE-dilution and surface Ig by flow cytometry; representative FACS plots are shown; percentages of switched cells are indicated within the gates. B, Data from eight experiments (eight sets of mice) were normalized to the percent of WT littermate (WT) switching, indicated as 100%. Mean percentages of switching in p53−/− relative to WT (+ SEM) for the different isotypes are shown. Significance was determined by the one-sample t test.
EFFECT OF p53 ON Ig CLASS SWITCHING

FIGURE 2. Polyoma virus infected p53−/− mice show increased in vivo IgG2a switching. p53−/−, aid−/−, and WT littermate mice were inoculated i.p. with 2 × 10⁶ PFU of polyoma virus strain A2. Splenic germinatal center B cells were analyzed for surface isotype expression by flow cytometry 12–20 d after inoculation. A, FACS plots show surface Ig expression on B220⁺ GL7⁺ splenic B cells from an experiment in which cells were harvested 14 d postinfection. Percentages of switched cells are depicted next to the gates. B, Data points and bars represent individual mice and means (n = 7 for WT, n = 5 for p53−/−) of the percentages of isotype switched cells within the B220⁺ GL7⁺ splenic germinatal center B cell subset. Three independent experiments were performed using: 3, 2, and 2 WT mice, 1, 2, and 2 p53−/− mice, and in the second experiment (shown) 2 aid−/− mice were included. Significance was determined by the Mann-Whitney U test.

CSR under several different conditions (Supplemental Fig. 4A). Concomitantly, no differences were found in the aicda transcript levels, as measured by semiquantitative RT-PCR (Supplemental Fig. 4B). S region transcription is also required for CSR (12). To address whether this is inhibited by p53, we measured γ1 and γ2a germline transcripts after stimulation with LPS and IL-4 or LPS and IFN-γ, respectively (Supplemental Fig. 4C). Neither γ1 nor γ2a germline transcripts were increased in p53−/− splenic B cells.

S region DSBs are increased in switching p53−/− splenic B cells

Because S region DSBs are required for CSR, we asked whether they are increased in p53−/− B cells induced to switch. As shown in Fig. 5, the frequency of AID-dependent DSBs in Sµ and Sγ2a is increased by 2- and 2.3-fold, respectively, in p53−/− cells relative to WT cells stimulated to switch to IgG2a (LPS and IFN-γ) for 2 d. There was little or no increase in Sµ DSBs in cells induced with LPS and IL-4 to switch to IgG1. These results are consistent with the increased CSR to IgG2a, but not to IgG1, in p53−/− cells. We have previously shown that AID-dependent Sµ DSBs are restricted to the G1 phase of the cell cycle in splenic B cells induced to switch in culture (13). As p53 is essential for the G1-S checkpoint, we asked whether the increased S region DSBs were due to delayed repair, but found that the Sµ DSBs in p53−/− cells are still almost entirely restricted to G1 phase (data not shown).

Increased mutation frequencies at S-S junctions

Mutations are frequently found in the nucleotides surrounding the junctions of recombinant switch regions (S-S junctions). We examined whether the increased S region breaks in p53−/− B cells were accompanied by an increase in S region mutations by sequencing Sµ-Sγ3 junctions from p53−/− and WT B cells induced to switch to IgG3. Indeed, we observed a 3.1-fold increase in mutation frequencies in cloned Sµ-Sγ3 junction fragments from p53−/− cells induced to switch to IgG3 (mutation frequency: WT = 30.4 × 10⁻⁴ versus p53−/− = 95.2 × 10⁻⁴, p = 0.00001). There is no apparent difference in the distribution of the mutations between WT and p53−/− cells relative to the Sµ-Sγ3 junctions (Fig. 5E). Taken together with the finding of increased S region DSBs in p53−/− cells, these data suggest that p53 affects switching by inhibiting AID activity and/or stimulating DNA repair.

Base excision repair is unperturbed in p53−/− splenic B cells

Our data indicate that S region DSBs and mutations are increased in p53−/− cells. This could be due to an increase in activities that increase mutations and breaks, or alternatively, to a decrease in DNA repair activity. An essential component of base excision repair (BER), DNA polymerase β (Polβ), was shown to be stimulated by p53 in cell-free repair experiments (40). Moreover, Polβ levels and activity correlate with p53 levels and function in several human cell lines and mouse embryonic fibroblasts (41). Polβ-deficient B cells show a modest increase (1.6-fold) in IgG2a switching, but no changes in other isotypes. This finding, along with the finding that Polβ associates with S regions in B cells undergoing CSR, suggests that Polβ attempts to repair AID-instigated lesions but is simply overwhelmed by the large number of lesions (42). We considered the possibility that the role of p53 in CSR could be mediated through its effects on Polβ levels and activity. To address this issue, nuclear protein extracts from WT and p53−/− B cells activated for IgG2a switching were tested for BER competence. A 43 nt ds oligonucleotide containing a single tetraphydrofuran moiety (mimics an abasic site) within one of the strands was incubated with nuclear extract in the presence of [³²P]α-dCTP. Abasic site cleavage and Polβ activity, followed by
ligation, results in the incorporation of radioactive dCTP into the 43 nt oligonucleotide. As shown in Supplemental Fig. 5A, p53 deficiency does not affect the efficiency of BER in these extracts. Consistent with this result, immunoblotting experiments showed that p53 does not affect Polβ levels in splenic B cells, nor the levels of AP endonucleases (Apex1 and Apex2) (Supplemental Fig. 5B, 5C), which we have shown to be important for CSR and for Sμ DSBs (33). Moreover, using a [32P]γ-ATP end-labeled single-stranded oligonucleotide containing a tetrahydrofuran residue, we were unable to detect any differences in the abasic site cleavage activity in nuclear protein extracts from WT or p53−/− B cells (data not shown). Thus, we find no evidence for reduction in BER activity in p53−/− B cells.

IFN-γ activates p53 in switching B cells

It has been shown in several different cell types that IFN-γ activates p53, which can result in cell-cycle arrest and even senescence (43–46). Because IFN-γ is used to stimulate IgG2a switching, we hypothesized that the isotype-specific effect of p53 is due to enhanced activation of p53 in the presence of IFN-γ. Note that in Nutlin-3–treated cells, all isotypes were reduced, suggesting that if p53 were induced under other CSR conditions, these other isotypes would also be enhanced in its absence. DNA damage induces phosphorylation of p53 on Ser18, the mouse equivalent of Ser15 in human p53, which is a substrate for ATM and ATM- and Rad3-related kinases (47–49). We assessed p53 Ser-18 phosphorylation in naive ex vivo splenic B cells and in splenic B cells activated in vitro with either LPS and anti-δ-dextran or LPS and anti-β-dextran and IFN-γ. Activated splenic B cells irradiated at 5 Gy were used a positive control. Gapdh was used for protein loading control. This experiment was performed twice, using cells from one mouse each time. B, Quantitative PCR analysis for p21 mRNA expression in WT splenic B cells stimulated for 48 h with LPS and anti-δ-dextran or LPS and anti-β-dextran and IFN-γ. Cells treated with 2.5 μM Nutlin-3 were used as a positive control, p53−/− cells as a negative control. Bar graph depicts relative p21 mRNA abundance normalized to WT splenic B cells stimulated with LPS and anti-β-dextran. Significance was determined by the one-sample t test. Four independent experiments were performed, using four sets of mice. C, Quantitative PCR analysis for Sestrin 1 mRNA expression relative to hprt mRNA in WT splenic B cells stimulated to switch to the indicated isotypes as described in Materials and Methods. Data are from one experiment, PCR was performed in triplicate.
more p53 Ser18 phosphorylation. Stimulation of splenic B cells to switch to other Ig isotypes, using different cytokines, results in smaller increases in p53 Ser-18 phosphorylation than does IFN-γ treatment (data not shown). Although IFN-γ stimulates p53 phosphorylation, we did not observe an increase in p53 protein or mRNA levels in these cultures (data not shown).

**FIGURE 5. S region DSBs are increased in p53−/− splenic B cells induced for IgG2a switching.** Sμ and Sy2a LM-PCR were performed on 3-fold dilutions of GAPDH-normalized DNA isolated from WT, p53−/−, and aid−/− splenic B cells that had been stimulated for 2 d with LPS and IL-4 or LPS and IFN-γ. Blunt DSBs in Sμ and Sy2a were assessed by use of 5′ Sμ or 5′ Sy2a primer, respectively, in combination with a linker-specific primer. PCR products were blotted and hybridized with an internal Sμ or Sy2a probe, respectively. A, Sμ DSBs in cells activated for IgG1 switching. B, Sμ DSBs in cells activated for IgG2a switching. C, Sy2a DSBs in cells activated for IgG2a switching. D, Bar graph depicts means (+ SEM) of densitometry measurements of autoradiographic films, normalized to WT, which was set at 1.0 (n = 4 for all experiments, except for Sy2a DSBs under IgG2a conditions, n = 3). All three titration lanes were scanned together. Each replicate experiment was performed on material from a separate set of mice. Two independent experiments using two sets of mice were performed. E, Mutations in Sμ-Sy3 junctions from WT and p53−/− splenic B cells activated to undergo IgG3 switching. Mutation frequency per 50-nucleotide segment is plotted for WT (black bars) and p53−/− (white bars). Overall mutation frequency is 3.1-fold increased in p53−/− versus WT (p = 0.00001). Total mutations/nucleotides analyzed for WT: 31/10,231; for p53: 30/3182.

**p53 Ser18 phosphorylation regulates IgG2a switching**

To test whether phosphorylation of p53 on Ser18 impacts CSR, we examined CSR in cultured splenic B cells obtained from mice harboring knock-in alleles in which p53 Ser18 was mutated to Ala (p53 S18A) (30, 50). IgG2a was increased by 1.8-fold in p53 S18A knock-in splenic B cells, slightly less than the effect of p53 knockout, indicating that p53 Ser18 phosphorylation is important for the inhibition of IgG2a class switching in IFN-γ-treated B cells, although other amino acids might also be involved (Fig. 6). Also, a slight but significant increase in IgA switching was observed in p53 S18A B cells, similar to what was found in p53−/− B cells.

**Increased levels of ROS in p53−/− cells and reduction of IgG2a switching in p53−/− splenic B cells treated with the antioxidant NAC**

In a number of different cell types, IFN-γ treatment results in increased ROS production, which in turn can activate p53 (43, 44, 51, 52). In phagocytic cells, IFN-γ induces the expression of several NADPH oxidases, which are responsible for the production of ROS as part of a microbicidal host response (53). IFN-γ might also increase mitochondrial ROS production in B cells. ROS-induced DNA damage can activate the p53 pathway in an ATM-dependent manner (54). p53 induces the expression of antioxidant genes, thereby decreasing the levels of intracellular ROS (24, 55). Following from this, one would expect increased levels of intracellular ROS in IFN-γ-treated p53−/− splenic B cells relative to WT cells. Using the fluorescent probe CM-H2-DCFDA to assess intracellular ROS levels, we established that this is indeed the case (Fig. 7A). Other CSR induction conditions also cause increased ROS levels (not shown), most likely because B cell activation stimulates mitochondria, which generate ROS (53, 56, 57). However, we find that ROS levels are higher in cells in which the induction conditions include IFN-γ.

We also examined levels of the mRNA for Sestrin 1, an antioxidant protein that is induced by ROS and is also transcriptionally upregulated by p53 (58). We found that Sestrin 1 mRNA is expressed most highly in cells treated for 2 d to induce IgG2a switching, but to a lesser extent in cells treated to switch to other isotypes (Fig. 3C). Although we find a small but significant increase in IgA CSR, Sestrin 1 mRNA is not induced under IgA switching conditions. We speculate that ROS might also be elevated in these cultures, but because p53 is known to induce other antioxidant genes besides Sestrin 1 (22, 55), this might account for the effect of p53 on IgA CSR. Taken together, our results suggest that ROS levels are increased during CSR, and that this effect is strongest in cells in which the activation conditions include IFN-γ.

Addition of the thiol-containing antioxidant NAC to the B cell cultures decreased the levels of intracellular ROS, as expected (Fig. 7A). NAC is a low m.w. thiol-containing compound that detoxifies ROS and enhances glutathione synthesis, which also reduces ROS (59). In preliminary experiments, we optimized the amount of NAC to add (not shown). Importantly, the increase in IgG2a switching in p53−/− relative to WT splenic B cells was nearly abolished when 10 mM NAC was added to the cultures (Fig. 7B; data not shown). Specifically, NAC treatment inhibited IgG2a CSR in WT cells by 36%, but inhibited CSR in p53−/− cells to a greater extent, by 53% (two independent experiments), suggesting that the antioxidant function of p53 might be responsible for the inhibitory effect on IgG2a switching.
WT cells induced to switch to several isotypes. CFSE-stained mouse splenic B cells were activated to induce class switching to IgG1, IgG2a, IgG2b, IgG3, and IgA. We found that 10 mM NAC inhibits class switching by 30–80%, depending on the isotype, with the exception of IgG2b (Fig. 7C, 7D). As shown by CFSE-dilution, NAC has no effect on cell-division rate, except under IgG2b induction conditions (Fig. 7C).

Because ROS also function as second messengers in several signaling cascade pathways, we analyzed whether NAC affected Ig germline transcription. Germline transcripts for γ1 and γ2a were not affected by NAC treatment (Supplemental Fig. 6A). Also, aicda mRNA levels were similar in NAC-treated versus untreated control B cell cultures stimulated for IgG1 and IgG2a switching (Supplemental Fig. 6A).

We have previously shown that formation of S region DSBs requires the BER pathway enzymes uracil-N-glycosylase and Apex1/Apex2 (5, 33). Both the levels and the subcellular localization of these enzymes are unchanged upon NAC-treatment of B cells stimulated for CSR (Supplemental Fig. 6B). However, by use of LM-PCR we found that AID-dependent blunt DSBs in Spα are ~2-fold decreased in NAC-treated B cells compared with untreated cultures (Fig. 7E), consistent with the decrease in CSR on NAC treatment. We conclude that NAC inhibits the formation of S region DSBs, but does not affect S region germline transcription or the expression and subcellular localization of the proteins involved in DSB-formation during CSR.

**S-S junctions are normal in p53−/− splenic B cells**

We considered the possibility that end joining might be altered due to the increased ROS levels, as it has been shown that increased ROS can reduce the levels and activity of DNA-dependent DNA protein kinase (DNA-PKcs) (60). To assess this, we examined the sequences of Spα-Sy3 junctions from p53−/− and WT B cells induced to switch to IgG3. S-S junctions from B cells deficient in DNA-PKcs show increased lengths of microhomologies, suggesting defective nonhomologous end joining (61). However, we found no differences in the frequency of blunt S-S junctions or lengths of junction microhomology between p53−/− and WT B cells (Supplemental Table I). These data suggest that end-processing and recombination of S regions are normal in p53−/− B cells.

**Increased ROS levels modestly enhance IgG2a class switching**

We asked whether increased ROS levels in switching B cells could enhance class switching. To that end, we tried several different reagents, such as adding H2O2 and l-buthionine sulfoximine to the cultures. These experiments can be problematic as these chemicals are highly toxic to activated B cells (data not shown). Nonetheless, lowering the concentration of β-mercaptoethanol in the culture medium from 50 μM to 20 μM resulted in a modest (1.3-fold, \( p = 0.017 \)) increase in IgG2a switching, whereas switching to other isotypes was not affected. Further lowering of β-mercaptoethanol concentrations inhibited class switching, as it decreased cell proliferation (data not shown). This result is consistent with our hypothesis that increased ROS levels cause the increased CSR to IgG2a that we observe in p53−/− cells.

**Discussion**

Although it has been shown that p53 prevents the formation of chromosomal translocations that derive from CSR activity (62), whether p53 affects the CSR mechanism itself has not been previously addressed. This is important because it is not known if the response to genotoxic stress in the form of programmed DSBs limits gene diversifications that are an integral part of the adaptive immune system. In human germinal center B cells, which

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**FIGURE 6.** p53 Ser18 phosphorylation regulates IgG2a switching. CFSE-loaded splenic B cells from p53S18A and WT control mice were cultured for 2.5 d with LPS, cytokines, and/or anti-δ-dextran to induce Ig class switching to the indicated isotypes. Class switching was determined by flow cytometry for surface Ig staining. A, Representative FACS plots are shown. Upper panel shows equal CFSE loading in WT B cells from the same colony and p53S18A splenic B cells. Percentages of switched cells are indicated within the gates. B, Bar graph depicts data from four sets of mice, which were all analyzed in one experiment. Percent CSR in p53S18A relative to WT (+ SEM) for the different isotypes are shown. Significance was determined by the one-sample t test.

**NAC inhibits S region breaks and class switching in WT splenic B cells**

To further establish the importance of the redox state of B cells for CSR, we tested the effect of adding 10 mM NAC to cultures of
presumably represent the population that undergoes CSR and SHM in vivo, it has been shown that Bcl6 represses transcription of p53 and p21, suggesting that the induction of apoptosis and cell-cycle arrest might be blunted in cells sustaining DNA DSBs occurring during CSR and SHM (63, 64).

In this study, we show that p53 inhibits IgG2a class switching in vitro and in vivo, and that this is not mediated through its canonical effects on the cell-cycle or cell death, but most likely through regulating the redox state of switching B cells. These results raise the question of how intracellular ROS might affect S region DSBs and class switching. Interestingly, the chemical pathway resulting in deamination of cytidine to uracil involves an oxidation reaction. ROS, such as hydroxyl radicals (•OH), are required intermediates in this reaction (27, 28). We reasoned that

![Figure 7](http://www.jimmunol.org/)

**FIGURE 7.** Increased ROS in switching p53−/− splenic B cells; NAC inhibits CSR and abolishes increased IgG2a switching in p53−/− splenic B cells. A. Shown are overlay histograms of WT (red profile) and p53−/− (blue profile) splenic B cells loaded with the CM-H2-DCFDA fluorescent ROS probe after activation with LPS and anti-δ-dextran and IFN-γ for 48 h. Treatment with 10 mM (blue profile) and 20 mM NAC (green profile) decreases intracellular ROS in activated WT and p53−/− splenic B cells. B, CFSE-loaded cells were activated for IgG2a switching and treated with 10 mM NAC. IgG2a switching was assessed by flow cytometry for surface Ig staining after 2.5 d. Representative FACS plots of three independent experiments are shown. Percentages of IgG2a switched cells are given within the gates. C, CFSE-loaded WT splenic B cells were activated with LPS and cytokines and/or anti-δ-dextran to induce CSR to the indicated Ig isotypes. CSR was assessed by flow cytometry for surface Ig staining. Percentages of switched cells are indicated within the gates. Top row shows untreated control cultures, middle row shows cultures treated with 10 mM NAC. Bottom row shows overlay histograms of CSFE fluorescence in control cultures (red profiles) and 10 mM NAC-treated cultures (blue profiles). Representative FACS plots of three experiments are shown. D, Bar graph shows data from the three experiments normalized to untreated cells, shown as 100%. Means (+ SEM) of percentages of switched cells in NAC-treated cultures relative to untreated cultures are shown. Significance was determined by the one-sample t test. E, Sμ LM-PCR was performed on 3-fold dilutions of Gapdh-normalized DNA from WT and aid−/− splenic B cells activated with LPS and anti-δ-dextran with or without 10 mM NAC for 2 d. LM-PCR products were blotted and hybridized with an internal Sμ probe. Densitometry scanning was performed on all three lanes representing the 3-fold dilutions. Untreated sample was set at 1.0 to determine relative density. Representative result of two experiments is shown.
the intracellular redox state, and perturbations thereof, could potentially influence the cytidine deaminase activity of AID in B cells, which could be the basis of the observed effect of p53 deficiency on IgG2a class switching. Our finding that both S region mutations and DSBs are increased in p53−/− cells supports the hypothesis that the increased levels of ROS in these cells stimulate AID activity. Most importantly, these data suggest that the role of p53 in suppressing chromosomal translocations and B cell oncogenesis might be due to its ability to inhibit AID-induced DNA mutations and breaks, in addition to its ability to prevent selection and outgrowth of cells that show dysregulated growth.

Alternatively, it might be that increased ROS levels in IFN-γ-stimulated p53-deficient B cells cause DNA lesions that lead to strand breaks in S regions, which in concert with AID-induced breaks, stimulate CSR. Of note, we did not observe any class switching activity in aid−/− p53−/− double knock-out B cells (data not shown), showing that AID is absolutely required, and that potential ROS-initiated DNA strand breaks are not sufficient to support class switching.

In the absence of DNA-PKcs, AID-dependent chromosomal IgH breaks accumulate in switching B cells. Interestingly, it was shown that p53 only limits chromosomal IgH breaks in LPS-stimulated DNA-PKcs-deficient B cells and not in CD40-stimulated B cells (65). The authors suggested that this might be because CD40 ligation might cause stronger Bcl6 upregulation and concomitant p53 suppression than LPS stimulation. However, we find that IgG2a switching is similarly increased in LPS and IFN-γ and anti-CD40 and IFN-γ-treated p53−/− B cells, compared with WT (data not shown).

We speculate that the isotype-specific effect for IgG2a is probably due to a combination of increased p53 activation on IFN-γ treatment, which is used to induce IgG2a CSR, and the increased production of ROS in IFN-γ-treated cells. We found that induction of p53 Ser18-phosphorylation is stronger on IFN-γ treatment than under conditions used to stimulate switching to other isoatypes, and that p53 Ser18-phosphorylation is important for the inhibitory effect on IgG2a switching, as demonstrated by the increased in vitro IgG2a switching of p53 S18A knock-in B cells. A concomitant increase in the expression of the p53 target gene sestrin 1 was found in IFN-γ-treated B cells, relative to other induction conditions (Fig. 3C). However, the finding that mutations are increased in segments surrounding S-S junctions in cells induced to switch to IgG3 suggests that p53 also represses AID activity in the absence of IFN-γ, although apparently to a lesser degree.

The induction of p21 observed in WT B cells treated with LPS and IFN-γ did not result in a measurable effect on proliferation or the cell-cycle, although if only a small proportion of the cells were affected we would not have detected it. Also, p21 induction does not appear to activate the cell-cycle checkpoint kinase Chk2, as we find that class switching is unaltered in chk2−/− splenic B cells (data not shown). The finding that Chk2 does not regulate CSR was also recently reported by Jankovic et al. (66).

A low level of p53 activation has been shown to be important for some of its functions. For instance, p53 regulates stem cell renewal and pluripotency potential in the absence of any obvious stressors (67). Also, p53 affects reproductive success by regulating the expression of LIF, which is essential for fetal implantation (68). These functions are not typically associated with DNA damage, indicating that p53 also acts in normal homeostatic processes. Based on the results presented in this study, CSR can be added to the ever-growing list of processes modulated by p53.

In a broader perspective, how would the organism benefit from the p53-restricted IgG2a switching in B cells? The IgG2a isotype is involved in the pathology of most of the Ab-mediated autoimmune disease models in the mouse, so one can envision that limiting IgG2a switching might be beneficial. Aging p21-deficient animals develop autoimmune disease (69), and Ag-induced arthritis is more severe in p53−/− mice, without altering the Ag-specific IgG responses (70). On polyoma virus challenge, p53-deficient animals have significantly more IgG2a-expressing germinal center B cells in the spleen than polyoma-infected WT littermates, but this did not result in a measurable increase in virus-specific IgG2a titers in the serum (data not shown). This could be due to compensatory mechanisms. We did not detect any differences in the serum titers of spontaneous antinuclear Abs (ANAs) in eight WT versus p53−/− mice (data not shown). Spontaneous ANA titers usually rise with age, but due to the high incidence of malignant tumors in p53−/− mice, we could not assess ANA titers in older mice. Taken together with our data, these results indicate that p53 regulates the switching event per se, but it is unclear whether it affects subsequent production of Abs.

IgG2a CSR appears to be particularly sensitive to increased ROS levels. Increasing intracellular ROS by culturing B cells in a lower concentration of the reducing agent β-mercaptoethanol resulted in a slight, but consistent and significant increase in IgG2a switching, showing that this isotype is particularly sensitive to changes in the intracellular redox state. In addition to our finding that ROS levels appear highest in IFN-γ-treated cells, it is also possible that this sensitivity is due to the fact that the Sy2a switch region has the lowest number of AID hotspots of all S regions, which might make AID-instigated lesions particularly limiting for IgG2a switching (42). Increased efficiency of AID-mediated cytidine deamination might therefore especially impact IgG2a switching. Consistent with this, we previously found that DNA Polβ deficiency, the major DNA polymerase involved in correcting AID lesions as part of the BER pathway, results in a small but consistent increase in IgG2a switching, specifically (42).

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Disclosures

The authors have no financial conflicts of interest.

References


Corrections


The sixth author’s institutional affiliation was published incorrectly. Dr. Nahla El Falaky’s affiliation should read Department of Medical Microbiology and Immunology, Faculty of Medicine, Cairo University, Cairo, Egypt.

The corrected author line and affiliations footnote are below. These have also been corrected in the online version of the article, which now differs from the print version as originally published.

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www.jimmunol.org/cgi/10.4049/jimmunol.1190058
Supplementary Data and Figure legends

Table S1: Lengths of S\textsubscript{\textmu}-S\textgreek{3} junctional microhomology in WT and p\textgreek{53}--/ B cells*

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*Junctions were amplified from splenic B cells were induced to switch to IgG3 for 4 days.
**Lengths of microhomology between S\textsubscript{\textmu} and S\textgreek{3} at junction.
Supplementary Figure S1. Proliferation and cell-cycle distribution are not altered in switching p53-/− B cells. CFSE-loaded splenic B cells from p53-/− and wild-type littermates were activated with LPS, cytokines and/or anti-δ-dextran to switch to the indicated Ig isotypes. (A) Shown are overlay histograms of CSFE fluorescence at the start of culture (upper panel) and after 3 days of culture. This analysis was performed for each of the 8 independent experiments, using 8 sets of mice, reported in Fig 2. (B) Splenic B cells from p53-/− and wild-type littermates were activated to undergo CSR to the indicated Ig isotypes. After 2 days, cells were fixed in 70% ethanol and stained for DNA content with propidium iodide (PI). Shown are overlay histograms of PI fluorescence, wild-type is represented by the red profiles, p53-/− is represented by the blue profiles. Two independent experiments, using 2 sets of mice, were performed.

Supplementary Figure S2. Splenic B-cell populations are normal in p53-/− mice. Ex vivo splenocytes from p53-/− and wild-type littermates were counted and analyzed by flow cytometry for B-cell subset distribution. Cells were stained for B220, CD21, and CD23. (A) Upper panel shows FACS histograms for fluorescence intensity of B220 staining. Percentages of positive cells are indicated above the gates. Lower panel show CD21 versus CD23 FACS plots within a B220+ gate. The CD21hi CD23lo population represents marginal zone B cells (MZ), the CD21lo CD23hi population represents follicular B cells (Foll), and the CD21lo CD23lo population represents newly formed B cells (NF). (B) Bar graph depicts absolute numbers of splenic B-cell subsets in wild-type (black bars) and p53-/− mice (white bars). (C) Bar graph shows means (+SEM) of percentages of B220+ cells of the total splenocytes and the percentages of
B-cell subsets within the B220+ gate. Wild-type is shown as black bars, p53-/ is shown as white bars. Three independent experiments, using 3 sets of mice, were performed.

Supplementary Figure S3. Cell-death and survival of switching splenic B cells are not affected by p53-deficiency. Splenic B cells from p53-/ and wild-type littermates were stimulated for CSR to the indicated Ig isotypes for 2.5 days. Percentages of dead cells were determined by flow cytometry using 7-amino-actinomycin D (7-AAD) staining. (A) FACS histograms of 7-AAD fluorescence intensity are shown. Percentages of 7-AAD positive events are shown above the gates. (B) Autophagy was assessed by western blot analysis for the protein LC3B; the faster migrating LC3-II isoform is converted from the LC3-I isoform through lipidation, and is an indicator of autophagy (72). Splenic B cells from p53-/ and wild-type littermates were activated to undergo CSR to IgG1 and IgG2a. Whole cell extracts were prepared after 48 h of stimulation. Gapdh was used as protein loading control. Experiments in both A and B were performed once each.

Supplementary Figure S4. AID expression and S region germline transcription are normal in activated p53-/ splenic B cells. (A) Western blot analysis of AID expression in p53-/ and wild-type splenic B cells activated to undergo CSR to the indicated Ig isotypes. Whole cell extracts were prepared after 48 h of stimulation. Activated splenic B cells (LPS + anti-δ-dextran) from aid-/ mice were included as a negative control. (AID is indicated; the upper band appears variably and is not AID). Gapdh was used as protein loading control. Two independent experiments were
performed, using two sets of mice. (B) Semi-quantitative RT-PCR analysis of aicda mRNA expression in splenic B cells from p53-/− and wild-type littermate mice. B cells were stimulated with LPS+IFNγ (IgG2a condition) and LPS+IL-4 (IgG1 condition) for 48 h. Shown are threefold dilutions of cDNA, normalized by RT-PCR for the hprt housekeeping gene. One experiment was performed; the aicda cDNA was also analyzed at 36 h with similar results. (C) Semi-quantitative RT-PCR analysis of γ1 (upper panel) and γ2a germline transcription (lower panel). Splenic B cells from p53-/− and wild-type littermates were stimulated with LPS+IL-4 and LPS+IFNγ for 36 and 48 h. Shown are threefold dilutions of cDNA, normalized by RT-PCR for the hprt housekeeping gene. The experiment was performed once.

**Supplementary Figure S5. Base excision repair (BER) is unperturbed in p53-/− splenic B cells.** (A) Repair assay to assess BER competence in nuclear protein extracts from activated wild-type and p53-/− splenic B cells. One and 2 μg of nuclear protein extract was incubated with [32P]α-dCTPs and ds oligonucleotides containing a single tetrahydrofuran-moiety, mimicking an abasic site. Incorporation of [32P]α-dCTP in the repaired 43-nt oligonucleotide and the cleaved 31-nt oligonucleotide was assessed by autoradiography after electrophoresis of samples on a denaturing 20% acrylamide gel. For the indicated samples, T4-ligase was added to the reaction to complete repair, as ligase activity appears to be limiting in the nuclear extracts. Mock-treated ds oligonucleotide was run as a negative control. (B) Western blot analysis of DNA Polβ expression in whole cell extracts from unstimulated (ex vivo) B cells and LPS + IFNγ stimulated (48 h) B cells from p53-/− and wild-type littermate mice. Gapdh was
used as protein loading control. (C) Western blot analysis of Apex1 and Apex2 protein expression in whole cell extracts from LPS+IL-4 and LPS+IFNγ stimulated (48 h) p53-/− and wild-type splenic B cells. Each experiment in Fig S5 was performed once.

Supplementary Figure S6. NAC treatment does not affect expression of AID, base excision repair proteins or germline transcription. (A) Semiquantitative RT-PCR analysis of germline γ1 and γ2a transcripts, and aicda mRNA expression in LPS+IL-4 and LPS+anti-δ-dextran+IFNγ stimulated wild-type splenic B cells (48 h) treated with 10 mM NAC. Shown are threefold dilutions of cDNA, normalized by RT-PCR for the hprt housekeeping gene. (B) Western blot analysis of Apex1, Apex2 and Ung expression in cytoplasmic and nuclear protein extracts from wild-type splenic B cells stimulated with LPS+IL4 and LPS+anti-δ-dextran (48 h) and treated with 10 mM NAC. Gapdh was used as a cytoplasmic protein loading control. Tbp1 was used as a nuclear protein loading control. Each experiment was performed once.
Figure S1

A

Ex vivo B cells

LPS + IL-4 (IgG1)

LPS + anti-δ-dextran + IFNγ (IgG2a)

LPS + TGFβ (IgG2b)

LPS + anti-δ-dextran (IgG3)

LPS + anti-δ-dextran + IL-4 + IL-5 + TGFβ (IgA)

CFSE

B

Wild-type p53^+/−

Wild-type p53^−/−
Figure S2

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Figure S3

**A**

- LPS + IL-4 (IgG1)  
  - Wild-type: 7.4  
  - p53−/−: 8.2

- LPS + anti-δ-dextran + IFNγ (IgG2a)  
  - Wild-type: 3.9  
  - p53−/−: 4.2

- LPS + TGFβ (IgG2b)  
  - Wild-type: 4.5  
  - p53−/−: 5.4

- LPS + anti-δ-dextran (IgG3)  
  - Wild-type: 5.1  
  - p53−/−: 4.9

- LPS + anti-δ-dextran + IL-4 + IL-5 + TGFβ (IgA)  
  - Wild-type: 9.9  
  - p53−/−: 7.3

**B**

- IgG1  
  - Wildtype  
  - p53−/−
- IgG2a  
  - Wildtype  
  - p53−/−

**Western Blot**

- LC3-I  
- LC3-II  
- Gapdh
**Figure S4**

**A**

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Figure S5

A

+ T4-Ligase

neg. wildtype p53-/- wildtype p53-/- marker

repair

cleavage

1 μg 2 μg

B

Ex vivo B cells LPS + IFNγ

wildtype p53-/- wildtype p53-/-

Polymerase β

Gapdh

C

LPS + IL-4 LPS + IFNγ

wildtype p53-/- wildtype p53-/- apex2Y/-

Apex1

Apex2
Figure S6

A

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<td>10 mM NAC</td>
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- germline γ1
- germline γ2a
- aicda
- hprr

B

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