B Cell–Specific Deficiencies in mTOR Limit Humoral Immune Responses

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J Immunol published online 15 July 2013
http://www.jimmunol.org/content/early/2013/07/13/jimmunol.1201767

Supplementary Material
http://www.jimmunol.org/content/suppl/2013/07/15/jimmunol.1201767
7.DC1

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B Cell–Specific Deficiencies in mTOR Limit Humoral Immune Responses

Shuling Zhang,* Margaret Pruitt,* Dena Tran,* Wendy Du Bois,* Ke Zhang,* Rushi Patel,* Shelley Hoover,* R. Mark Simpson,* John Simmons,* Joy Gary,* Clifford M. Snapper,† Rafael Casellas,*‡ and Beverly A. Mock*

Generation of high-affinity Abs in response to Ags/infectious agents is essential for developing long-lasting immune responses. B cell maturation and Ab responses to Ag stimulation require Ig somatic hypermutation (SHM) and class-switch recombination (CSR) for high-affinity responses. Upon immunization with either the model Ag 4-hydroxy-3-nitrophenylacetyl hapten (NP) conjugated to chicken γ globulin lysine (NP-CGG) or heat-killed Streptococcus pneumoniae capsular type 14 protein (Pn14), knock-in (KI) mice hypomorphic for mTOR function had a decreased ability to form germinal centers, develop high-affinity anti-NP–specific or anti-Pn14–specific Abs, and perform SHM/CSR. Hypomorphic mTOR mice also had a high mortality (40%) compared with wild-type (WT) (0%) littermates and had lower pneumococcal surface protein A–specific Ab titers when immunized and challenged with live S. pneumoniae infection. Mice with mTOR deleted in their B cell lineage (knockout [KO]) also produced fewer splenic germinal centers and decreased high-affinity Ab responses to NP-CGG than did their WT littermates. CSR rates were lower in mTOR KI and KO mice, and pharmacologic inhibition of mTOR in WT B cells resulted in decreased rates of ex vivo CSR. RNA and protein levels of activation-induced cytidine deaminase (AID), a protein essential for SHM and CSR, were lower in B cells from both KI and B cell–specific KO mice, concomitant with increases in phosphorylated AKT and FOXO1. Rescue experiments increasing AID expression in KI B cells restored CSR levels to those in WT B cells. Thus, mTOR plays an important immunoregulatory role in the germinal center, at least partially through AID signaling, in generating high-affinity Abs. The Journal of Immunology, 2013, 191: 000–000.

The mechanistic target of rapamycin (mTOR, MTOR) regulates cell growth and metabolism through its activity as a serine-threonine kinase. MTOR forms two protein complexes, mTORC1 and mTORC2, which are involved in phosphorylating many downstream targets, including S6K, 4EBP1, and AKT (1, 2). Rapamycin and its analogs inhibit mTOR activity, which are widely used as immunosuppressants during organ transplantation, and have been increasingly used to prevent graft-versus-host disease after bone marrow transplantation (3). mTOR inhibition is pleiotropic, having differential effects on various immunocompetent cells (4–6). Many studies focused on proliferation/activity of dendritic and T cell populations as the primary targets of immunosuppression (3, 7, 8). In this study, we chose to focus on B cells. We recently developed a potential mouse model of chronic immunosuppression by transcriptionally inactivating a knock-in (KI) allele of mTOR; spleens of these hypomorphs were disproportionately small relative to their total body weight, and mTOR protein levels were reduced by 70%. Unexpectedly, we found several effects of this KI on B cell differentiation, migration, and homeostasis, in addition to increases in induced Foxp3+ T regulatory cells (9). Similarly, rapamycin was also shown to promote the expansion of Foxp3+ regulatory T cells after organ transplantation (10). In KI mice, B cell proliferation was less impaired in response to LPS than to either anti-IgM or anti-CD40, suggesting that innate immune responses of the mTOR-deficient mice were more intact than were their adaptive responses (9).

In this study, we examined the humoral immune responses of mTOR KI mice to infection with Streptococcus pneumoniae, one of the most common bacterial infections arising, as a result of immunosuppression, in both marrow and solid organ transplant patients (11, 12), as well as in patients undergoing chemotherapy (13). Mediation of infection requires the formation of germinal centers (GCs) within splenic follicles as an essential event in the generation of high-affinity, Ab-secreting plasma cells and memory B cells (14, 15). In this study, GC formation and GC B cell functions were compared between mTOR hypomorphs (KI) and wild-type (WT) littermates, as were their ability to produce high-affinity Ab isotypes in response to the model T cell–dependent Ag 4-hydroxy-3-nitrophenylacetyl hapten (NP) conjugated to chicken γ globulin lysine (NP-CGG) or S. pneumoniae infection. In addition, to address the role of mTOR in B cells in these responses,
we examined the humoral responses of conditional B cell knock-out (KO) of mTOR [mTOR-floxed hypomorphs were crossed to CD19<sup>cre</sup> mice (16)] immunized with NP-CGG.

Ig somatic hypermutation (SHM) and class-switch recombination (CSR) are the primary effectors of Ab diversity and occur following stimulation of mature B cells by a cognate Ag within the GCs of peripheral lymphoid organs. SHM and CSR initiation requires activation-induced cytidine deaminase (Aicda; AID), which deaminates cytidine residues in DNA to produce uracil, thus generating U:G mismatches (17–19). SHM and CSR are obligatory for the production of high-affinity Abs, we performed both in vivo and ex vivo experiments to determine whether these mechanisms are intact in our mTOR KO and KO mice.

Materials and Methods

Mice

Mice were bred in conventional facilities with food and water ad libitum. All animals were treated in accordance with the guidelines provided by the Animal Care and Use Committee of the National Cancer Institute for protocol numbers LG-009, LG-017, and LG-023. mTOR KO offspring carrying neo-mTOR (mTOR KO) were identified by PCR of tail DNA using forward (5'-CA-GACGAGGAGGAAGAACAG-3') and reverse (5'-CCAGCCTCTTCTCAGCATCACTCTTG-3') primers; 239- and 273-bp fragments were amplified from WT and KO mice, respectively, as described (9). mTOR KO mice were bred with β-actin cre mice; the resultant progeny deleted neo ubiquitously while retaining the BALB/c allele of mTOR. These KO neo<sup>−/−</sup> mice have the same amount of mTOR protein and make similar amounts of Ig Ab in response to Ag as do WT mice (9). Conditional B cell KO mice (mTOR KO) were generated by crossing CD19<sup>cre</sup> mice (16) with the KO neo<sup>−/−</sup> harboring mTOR<sup>Bosch</sup> (Supplemental Fig. IA, IB); the resultant progeny deleted the mTOR allele specifically in CD19<sup>+</sup> B cells (KO mice: mTOR<sup>fl/fl</sup> CD19<sup>cre/+</sup>; WT: mTOR<sup>+/+</sup> CD19<sup>cre/+</sup>). mTOR KO offspring carrying the mTOR deletion in CD19<sup>+</sup> B cells were identified by PCR of tail DNA using forward (5'-CA-GACGAGGAGGAAGAACAG-3') and reverse (5'-CA-GACGAGGAGGAAGAACAG-3') primers; 239- and 273-bp fragments were amplified from WT and KO mice, respectively (as described in Supplemental Fig. IB) (9). Spleen weights from WT and KO mice analyzed (as described in Supplemental Fig. IB) (9). Spleen weights from WT and KO mice were adjusted for total body weight similar to those of WT mice (Supplemental Fig. IC). AID KO (Aicda<sup>−/−</sup>) mice backcrossed to BALB/c mice were originally obtained from Muramatsu et al. (19, 28). AID KO mice and their WT littermates were aged in our conventional (non-SPF) facility to assess their survival. One group of 17 AID KO mice was weaned onto bacon-flavored chow containing the antibiotic metronidazole (138 mg/kg).

Reagents

Pneumococcal surface protein A (PspA) was purified as described (29). LPS and L-glutamine were purchased from Sigma-Aldrich. α-β-dextran was purchased from Fina BioSolutions. Recombinant mouse IL-4 was purchased from PeproTech.

Immunizations

Mice (8–10 wk old) were immunized i.p. with 2 × 10⁸ CFU heat-killed*S. pneumoniae* capsular type 14 protein (Pn14) (29) in saline or 100 μg NP<sub>3</sub>-CGG (Biosearch Technologies; catalog no. N-5055) adsorbed on 25 μg alum (Rehydragel HPA; Reheis). Control mice were injected with the same volume of saline. Mice injected with Pn14 were boosted 14 d after the first injection with 2 × 10⁷ CFU/mouse. Measurements of anti-NP and anti-PspA Ig isotype titers were determined from sera of mice.

Preparation and infection of Pn14

A frozen stock of Pn14 was thawed and plated on trypticase soy agar plus 5% sheep blood (TSA II; BD Diagnostic Systems) for a 24-h incubation at 37°C under anaerobic conditions (BD BBL GasPak Plus Anaerobic System Envelopes with Palladium Catalyst); the isolated colonies were transferred to 15 ml Bacto-Todd Hewitt Broth (BD Diagnostic Systems) for 18 h without oxygen or shaking. Pn14 challenge titers were determined by 10-fold serial dilution and plating onto the blood agar. Three-month-old mice were challenged with 1 × 10⁷ CFU/mouse of live Pn14 in a final volume of 200 μL PBS by i.p. injection. Fourteen days postbacterial immunization, mice were bled to determine Ab titers. On day 15, the mice were rechallenged i.p. with a high dose (1 × 10⁷ CFU/mouse) of live Pn14; they were bled at 15 h postchallenge to determine bacteremia levels. Survival was monitored for 15 additional days.

Measurement of serum Ag-specific Ig isotype titers and avidities

Serum samples were prepared at a 1:50,000 dilution for total IgG or at a 1:10,000 dilution for total IgM. Quantification of IgG and IgM was performed by ELISA, according to the manufacturer’s protocols (Immunology Consultants Laboratory Mouse IgG ELISA: E-900G and Mouse IgM ELISA; E-900M).

ELISA plates (Dynex Immulon 4HBX) were coated with 5 μg/ml (50 μL/well) PspA or 10 μg/ml (50 μL/well) NP<sub>3</sub>-BSA (Bioase Technologies; cat. no. 5050-10) in PBS overnight at 4°C. Plates were blocked with PBS plus 1% BSA for 1 h at 37°C. Two-fold dilutions of serum samples, starting at a 1/200 serum dilution, in PBS plus 0.5% BSA were added for 1 h at 37°C, and plates were washed three times with PBS plus 0.1% Tween 20. Alkaline phosphatase-conjugated polyclonal rat anti-mouse IgM and IgG isotype-specific Abs (Southern Biotech) were added and incubated at 37°C for 1 h. Plates were washed three times with PBS plus 0.1% Tween 20. Substrate (p-nitrophenyl phosphate, disodium; Sigma-Aldrich), at 1 mg/ml in 0.1 M glycine buffer (2 mM MgCl<sub>2</sub>, 1 mM ZnCl<sub>2</sub> [pH 10.4]), was added for 30 min at room temperature for color development. Color was read at an absorbance of 400 nm.

Avidity of anti-PspA Abs was determined by modifying this immunoassay to include an 8-min wash with 6.5 M urea (30). The percentage of avidity was calculated by dividing the OD of the urea-washed samples by the OD of the samples not washed with urea to obtain the percentage of IgG bound after washing. The relative avidities of anti-NP Abs were determined using an ELISA with BSA coupled to NP<sub>3</sub>-BSA and NP<sub>4</sub>-BSA. The relative avidity of the Abs was determined by the level of Abs using NP<sub>3</sub>-BSA, which measures only high-affinity anti-NP<sub>3</sub>-BSA, and NP<sub>4</sub>-BSA, which measures both high- and low-affinity anti-NP<sub>4</sub> (31).

Immunohistochemical labeling

Slides of spleen sections (prepared by Histoserv) were first deparaffinized in xylene and rehydrated through a graded ethanol series. The slides were blocked for endogenous peroxidase with freshly prepared 30% H<sub>2</sub>O<sub>2</sub> in ethanol and blocked with 10% normal rat serum in 0.1% BSA in PBS. Slides were incubated with either biotin-labeled anti-CD45R/B220 (BD Bioscience) or biotinylated peanut agglutinin (PNA) (Vector Laboratories), washed, and subsequently incubated with peroxidase-conjugated avidin-biotin complex mix and DAB Chromogen buffer (Vector Laboratories). The slides were counterstained with Hematoxylin Nuclear Counterstain (Vector Laboratories) and dehydrated. The procedure of double immunolabeling with anti-CD3 (labeled with avidin-biotin complex conjugate with alkaline phosphatase, developed with VECTOR Blue Chromogen; AbD Serotec) and anti-B220 (BD Bioscience) was similar and was performed by the Laboratory Animal Sciences Program, Science Applications International Corporation-Frederick, National Cancer Institute-Frederick. Stained slides were scanned, and GC area and number were measured using color deconvolution analysis software (Aperio Technologies, Vista, CA).

Cell purifications

CD43<sup>+</sup> B cells were purified from spleens using MACS kits (Miltenyi Biotec) following the removal of RBCs with ACK lysis buffer. Cells were cultured in RPMI 1640 supplemented with 10% FBS, penicillin (100 U/ml), streptomycin (100 μg/ml), 1% γ-glutamine, and 2-ME (5 × 10⁻³ M) (B cell culture media). WEST-1 reagent (Roche 1644807) was used to measure cell viability/proliferation.

Flow cytometry analysis

All unlabeled Abs, PE, FITC, allophycocyanin, or PerCP conjugates were purchased from BD Pharmingen or eBioscience. Cells were separated from spleens or lymph nodes (LNs), and RBCs were removed using ACK lysis buffer. Stained cells were resuspended in FACS buffer (PBS with 0.5% BSA, 2 mM EDTA) to a final concentration of 10⁷ cell/mL. A total of 100

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μl cells was blocked with Fc blocker (anti-CD16/32) for 15 min at 4°C, incubated with single Abs or an Ab mixture at 4°C for 30 min, and washed twice with FACS buffer. Cells were fixed with 400 μl FACS buffer containing 1% paraformaldehyde and analyzed on a FACS-Calibur with FlowJo 8.7 and ModFit LT software.

**Measurement of cell division by CFSE dilution**

Cells were stained with 1 μM CFSE (Vybrant CFDA-SE; Invitrogen). After labeling, cells were washed twice with FACS buffer medium. CFSE-loaded B cells were cultured in B cell culture medium for 72 h at 0.5 × 10^6 cells/well in six-well plates. Cells were analyzed on a FACS-Calibur using FlowJo 8.7 and ModFit LT software.

**Apoptosis and cell cycle assays**

Purified B cells stimulated for 48 h with LPS alone, LPS + IL-4, or LPS + α-ε-dextran were counted by annexin V and 7-aminoactinomycin D (BD Pharmingen Apoptosis Detection Kit) or PI/RNase Staining Buffer (BD Pharmingen Cell cycle detection kit) using the manufacturer’s suggested protocols. Cells were analyzed on a FACS-Calibur using FlowJo 8.7 and ModFit LT software.

**SHM analysis**

B cells were prepared from spleen or LNs, and RBCs were removed with ACK lysis buffer. GC cells from immunized mice were sorted using FITC (FITC), FAS (PE), and B220 (allophycocyanin) (FACSdiva flow cytometer cell sorter). GC sorted cells (~50,000 cells) were resuspended in 50 μl lysis buffer (10 mM Tris, 0.1 mM EDTA, and 0.5 mg/ml proteinase K) and incubated at 50°C for 2.5 h and then protease K was inactivated at 95°C for 10 min. The Igδ4 μ4 intronic region was amplified with primers V1: 5′-AGCCTGACATCTGGAG-3′ and V2: 5′-TAGTTGGAACACATTCTCAG-3′ using 20 μl digested cell solution in a 50-μl reaction for the first-round PCR. The region was amplified in the second-round PCR using 5 μl reaction 1 with primers V3 (5′-CTGACATCTGGAGGCTC-3′) and V4 (5′-GCTGTACACATCTGGAGGCTC-3′) amplification (amplification protocol: first-round PCR, 30 cycles of 30 sec at 95°C, 30 sec at 55°C, and 2 min at 72°C; second-round PCR, 30 cycles of 30 sec at 95°C, 30 sec at 65°C, and 2 min at 72°C). Taq-PFU (19/1) was used for PCR. Switch μ (Sp μ) mutations were detected by PCR amplification of a 650-bp genomic DNA fragment from ex vivo B cells activated with LPS + IL-4 (110 μl) using the primers Sp(B)-5′-GTAAGAGGAGCCACAGGCTAAG-3′ and Sp(D)-5′-CATCGAGATCTGGAGCC-3′ at 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s. PCR products were purified from gel slices, ligated into TA vectors, and sequenced with M13 forward and reverse primers. The data were analyzed with the Web-based SHMTool (32). Mutations were counted by two methods to provide a more accurate estimate of point mutation frequency (Table I). A single B cell clone produces individual mutation products were purified from gel slices, ligated into TA vectors, and sequenced with M13 forward and reverse primers.

**CSR analysis**

CD3+ resting B cells were obtained from spleens and LNs using magnetic CD43 beads; CD19+ CD43- resting B cells were further purified from CD43- cells using magnetic CD19+ beads, according to the manufacturer’s instructions (Miltenyi Biotec). The cells (0.5 × 10^6/well) were cultured in six-well plates in 5 ml B cell culture medium and incubated at 37°C for 72 h. To induce specific isotype switching, B cells were stimulated with 50 μg/ml anti-CD40 (Sigma-Aldrich, L2828-25MG) plus either 20 ng/ml IL-4 (Peprotech; 214-14) for induction of IgG1 switching or 3 ng/ml α-β-dextran for induction of IgG2 switching.

**RNA preparation and real-time RT-PCR**

Total RNA was isolated from cells with TRIzol reagent (Invitrogen). cDNAs were made with TaqMan Reverse Transcription Reagents (Applied Biosystems; cat. no. N8080234), and real-time PCR was performed using SYBR Green PCR Master Mix (cat. no. 4309155; ABI 7500) with the following primers: mTOR: forward primer: 5′-CTCTGGCGCAAGATGCTCATC-3′; reverse primer: 5′-TGTTGCTCCAGCTTGCGAAGA-3′; β-actin: forward primer: 5′-CTCTTGAGGACTTTGATGAC-3′; reverse primer: 5′-GACCCTTTGCAAAACATCT-3′; reverse primer: 5′-GGAGAAGATCTTCGAGA-3′; polh (DNA polymerase γ): forward primer: 5′-CAACATGGGCTGGACATA-3′; reverse primer: 5′-GAGAATAGATTCACGCTGC-3′; reverse primer: 5′-GATGTTCATTCAAGTGGC-3′; Exon:1: forward primer: 5′-CCAATGCCCACAAAGATTAAT-3′; reverse primer: 5′-ACAGACTCTCATAGCAGC-3′; Msh2: forward primer: 5′-AAACAATTGAAAAACTGGTCTGTG-3′; reverse primer: 5′-CCTCTTGAGACTTACCCG-3′; reverse primer: 5′-TGTTGTTTGGCTACATTGG-3′; reverse primer: 5′-TGTTAGGCCTATGTGACTGA-3′; and 18S RNA: reverse primer: 5′-GCCGTAGAGTTAATCTTTG-3′; reverse primer: 5′-CATTCTGGCAAATGCTTCG-3′.

**PCR of germine clones**

PCR was performed with the following primers to obtain the indicated product sizes: (μ) ImF and CmR, 245 bp; (γ3) IgF and CgR, 323 bp; and (γ1) IgI and CgR, 429 bp. Postswitch transcripts were amplified using the following primer pairs: (γ3) ImF and CgR, 323 bp and (γ1) ImF and CgR, 353 bp; germine and postswitch transcripts were amplified by 30 or 35 cycles of PCR (19). The following PCR primers were used: ImF: 5′-CTCTGGCGCAAGATGCTCATC-3′; IgF: 5′-TGGCCACAGTGAGCTCGAGA-3′; Ig3: 5′-GGGCTTCCAGATCTTGAGG-3′; CmR: 5′-GGAGAAGATCTTCGAGA-3′; CgR: 5′-CTTCCAGGGAGATCAGTTGA-3′; and CgR: 5′-GGATCAACCTTCCAGTAC-3′.

**Western blot analysis**

Protein extracts from tissues and cultured cells were prepared with lysis buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1% Nonidet P-40, 1 mM EDTA, 20 mM β-glycerol phosphate, protease inhibitors [Roche], and phosphatase inhibitors [Santa Cruz]). Nuclear and cytoplasmic extracts were obtained using the NE-PER kit (Pierce; cat. no. 88833), according to the manufacturer’s protocol. A total of 20 μg total protein lysates or 10 μg cytoplasmic/nuclear extracts was used for Western blot analysis. Membranes were incubated with AID (cat. no. 4975), pAKT (cat. no. 4058), pFOXO1/3a (cat. no. 9464), FOXO1 (cat. no. 9462), FOXO3a (cat. no. 9467), β-actin (cat. no. 4970L) (all from Cell Signaling), UNG (ProSci; cat. no. 3859), HSP90 (Stress Marq; cat. no. SPC-104C), or Histone H1 (Santa Cruz; cat. no. SC-8030) Abs.

**Retroviral transduction of B Cells**

Retroviruses expressing AID-GFP or GFP were prepared by adapting previously published methods (34) in the following manner: Plate cells were transduced with pMys-mAID-ires-EFGP or pMys-ires-EFGP plasmid by Lipofectamine 2000 (Invitrogen), and recombinant retrovirus was collected 24–36 h after transfection. The CD43+ resting B cells isolated from spleens of WT or KI mice were reactivated for 24 h with 0.5 μg/ml anti-CD180 and transduced twice with retroviruses by spin inoculation (650 × g for 90 min). Cells were cultured for an additional 3 d with 50 μg/ml LPS, 20 ng/ml IL-4, and 0.5 μg/ml anti-CD180 before being analyzed for IgG1 cell surface expression by FACS.

**Results**

**GC formation is affected by mTOR expression**

To evaluate the effects of reduced mTOR on GC formation, mTOR KI mice were either primed with NP-CGG or primed and subsequently boosted with intact heat-killed Pn14, as described previously (9, 29). GCs were evaluated 14 and 21 d postpriming with NP-CGG and Ph14, respectively, using immunohistochemical (IHC) staining and analysis of cell surface markers by flow cytometry. The average number of GCs in spleens of KI mice was 3–5-fold lower than in WT spleens (Fig. 1A, Supplemental Fig. 2A). Furthermore, both the percentage of GC cells within the splenic section and the composite GC area relative to the total area of B cell lymphoid follicles were reduced disproportionately in KI mice (Fig. 1A, Supplemental Fig. 2A). Consistent with the IHC findings, flow analyses showed that the frequency of specific GC B cells (B220+, IgDlow, GL7+, Fas+ and B220+, IgDlow, CD38+,
Fas+) was lower in spleens and LNs of KI mice compared with WT mice (Fig. 1B, Supplemental Fig. 2B). In contrast, the splenic and LN populations of CD11c+, CD21/35+ cells were similar between WT and KI mice that had received a prime/boost of Pn14 (Supplemental Fig. 3A). In addition, CD3+ T cells were abundant in the lymphoid follicles of both WT and KI mice (Supplemental Fig. 3B).

Reduced mTOR impairs IgG Ab-affinity maturation

KI and WT mice were given priming doses of either NP-CGG or prime/boost doses of heat-killed Pn14 to assess their ability to mount humoral responses to Ags. Primary and secondary Ag-specific Ab titers were measured against NP or PspA, as described (9, 29, respectively). In these experiments, anti-NP and anti-PspA IgG Ab titers were lower in KI mice 14 and 21 d postreceipt of priming doses (Fig. 1C, Supplemental Fig. 2C, 2D). More specifically, the diminished capacity for generating PspA-specific IgG-isotype responses remained low a week after the boost immunization with S. pneumoniae in which only higher-affinity Abs remained bound to PspA-coated microtiter plates after washing with 6.5 M urea. mTOR KI mice did not produce detectable amounts of high-affinity IgG to PspA until after the boost, whereas WT mice had readily measurable amounts as early as 1 wk following the initial immunization (Supplemental Fig. 2E). Although the boost induced some high-affinity Abs in KI mice, the degree of affinity maturation continued to lag behind that of WT mice (Supplemental Fig. 2E). Thus, mTOR can regulate Ab maturation to antigenic challenge in response to either intact or haptenated Ags.

mTOR hypomorphic mice are more sensitive to live S. pneumoniae challenge

Protection against S. pneumoniae relies not only on innate immune responses, it also requires an intact adaptive response (36). To determine whether mTOR plays a role in mediating infection and to assess whether impaired Ab responses in mTOR KI mice might be biologically relevant, mice were challenged with a low dose (10^6 CFU) of live, encapsulated Pn14 and were rechallenged after 14 d with a high dose (10^8 CFU) and followed for 15 d. Ab titers to PspA 14 d after the initial challenge, but before the high-dose

FIGURE 1. Constitutive reductions in mTOR impair GC formation and decrease anti-NP Abs in response to NP-CGG. KI and WT (n = 5/group) mice were immunized i.p. with NP-CGG in Rehydragel. Spleens were collected on day 14 for IHC staining and FACS analysis. (A) Splenic sections were stained with B220 or PNA. The numbers of GCs (arrows indicate GC) and the area of PNA staining were evaluated from scans of spleen sections stained with B220 or PNA using color deconvolution analysis software (Aperio Technologies). (B) The cells from spleens and LNs were stained with B220, GL7, Fas, CD38, and IgD Abs. There were fewer GC B cells in KI mice than in WT mice. Data are mean ± SEM. (C) Sera from KI and WT mice immunized with NP-CGG were collected on day 14 for measurement of Ag-specific IgM and IgG isotype Ab titers. Data are mean ± SEM. (D) The relative affinities of anti-NP Abs were determined using an ELISA with BSA coupled to NP at different ratios: NP4-BSA and NP14-BSA. *p < 0.01.
challenge, were considerably lower in mTOR KI mice than in WT mice (Fig. 2A). At 15 h postchallenge with $10^8$ CFU, only 1 of 9 KI mice was bacteremia-free compared with 5 of 10 WT mice; for the mice with detectable bacteremia at that time point, the average titer was slightly higher in KI mice (Fig. 2B). All of the WT mice survived the high-dose challenge, whereas only 60% of the KI mice survived (Fig. 2C).

Constitutive reductions in mTOR impair SHM

The lack of high-affinity Ab responses in mTOR KI mice led us to hypothesize that the frequency of SHM, a driver of Ab diversity, may be reduced in KI GC B cells. To address this question, SHM frequency was measured in mice immunized with NP-CGG. We analyzed the IgH JH4-intronic sequence downstream of the rearranged VDJ region in GC B cells (B220⁺ GL7⁺ Fas⁺) sorted from splenocytes of NP-CGG–immunized KI and WT mice. The frequency of SHM and pattern of point mutations were analyzed using SHMTool (32). The frequency of SHM was decreased in GC B cells from KI mice compared with WT littermates (Fig. 2, Table I). The nonunique mutation frequency/total sequences (per 100 bp) was 0.52 in WT JH4 introns and 0.32 in KI JH4 introns ($p < 0.001$, Table I). These findings held true for the total number of unique point mutations as well (Table I). Overall, the reduction in mutation frequency was due primarily to a paucity of mutated sequences in mTOR KI mice (Fig. 2D); 37% of sequences from KI splenic GC B cells were not mutated, whereas only 17% of sequences from WT cells fell in this category, representing a >2-fold decrease in mutated JH4 sequences in KI B cells (Fig. 2D). Furthermore, although 6% of WT splenic GC B cell sequences had six mutations, none of the KI JH4 intron sequences had as many mutations (Fig. 2D).

The pattern of SHM point mutations was skewed in GC B cells from KI mice compared with WT mice (Table II). The percentage of transversions from G to T or A to C was decreased for KI GC B cells ($p < 0.05$). Of the unique mutations, there were fewer G-T and C-A (12.5%) or T-G and A-C (15.4%) transversions in KI B cells compared with WT cells (26.1 and 25%, respectively) (Table II). Additionally, KI B cells had more (59.4%) transitions from G to A and C to T compared with WT cells (43.5%) (Table II). Overall, there were fewer transversions and an increase in transitions seen in the mTOR-deficient mice.

**mTOR KI mice have fewer mutations in Sμ sequences and defective Ig class switching to IgG1 ex vivo**

The inability of mTOR KI mice to mount high-affinity IgG responses led us to ask whether their B cells were impaired in their ability to undergo CSR. Class switching occurs by intrachromosomal deletion recombination within switch regions upstream...
of each IgH C region and initiates in the Sµ segment (37). Frequent AID-dependent mutations occur at Sµ sequences during CSR (38–40). Ex vivo–activated (LPS + IL-4) CD43<sup>−</sup> resting B cells from mTOR KI mice experienced a lower frequency of mutation (0.7 × 10<sup>−5</sup>) in their IgSµ region than did WT mice (1.4 × 10<sup>−5</sup>) (Fig. 2E, Table III).

The lower mutation rates seen in Sµ sequences from KI mice suggested that the ability of KI B cells to undergo CSR may also be impaired. The capability of KI mice to undergo CSR was determined by ex vivo stimulation of CD43<sup>−</sup> resting B cells, as described (41). Compared with WT cells, fewer KI B cells stimulated with LPS and IL-4 expressed IgG1 (Fig. 3A), suggesting that the decreases in IgG Ab production may be due to defective CSR in mTOR KI B cells.

To further evaluate deficits in IgG switching in KI mice, the germline (preswitch) and postswitch transcripts for IgG<sub>1</sub> and IgG<sub>3</sub> were measured by PCR (protocol adapted from Ref. 19). These analyses confirmed that KI mice had no change in levels of germline µ, γ1, and γ3 transcripts but had lower levels of post-switch γ1 and γ3 transcripts (Supplemental Fig. 4C, 4D).

Table I. Somatic mutations in J<sub>H4</sub> intronic sequences (403 bp) from splenic GC B cells of WT or KI mice with NP-CGG challenge

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<th>WT (NP-CGG)</th>
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<td>153</td>
<td></td>
</tr>
<tr>
<td>Mutation frequency/total sequences (per 100 bp)</td>
<td>0.32</td>
<td>0.52</td>
<td>0.0001</td>
</tr>
<tr>
<td>Mutation frequency/mutated sequences (per 100 bp)</td>
<td>0.50</td>
<td>0.62</td>
<td>0.0264</td>
</tr>
<tr>
<td>Total number of unique point mutations</td>
<td>71</td>
<td>102</td>
<td></td>
</tr>
<tr>
<td>Mutation frequency/total sequences (per 100 bp)</td>
<td>0.25</td>
<td>0.35</td>
<td>0.0257</td>
</tr>
<tr>
<td>Mutation frequency/mutated sequences (per 100 bp)</td>
<td>0.40</td>
<td>0.43</td>
<td>0.7227</td>
</tr>
</tbody>
</table>

<sup>a</sup><sup>χ²</sup> test, KI versus WT.

Independent evaluation of the role of mTOR in CSR was gained by treating resting B cells from WT mice with rapamycin, which preferentially inhibits mTORC1 and inhibits mTORC2 usually only after long-term treatment and in a cell type–specific manner (42). IgG<sub>1</sub> switching (Fig. 3C) was impaired in WT activated CD43<sup>−</sup> splenic B cells treated with low-dose rapamycin (0.1 nM), which had modest effects on cell viability and proliferation until the fifth cell division where KI cells were diminished with respect to WT (Fig. 3D), cell cycle arrest (Fig. 3E), or apoptosis (Fig. 3F). Together, these findings confirm that mTOR can affect CSR through its effect on cell division/proliferation, but they also suggest a possible role independent of proliferation.

Table II. Pattern of nucleotide changes in J<sub>H4</sub> intronic sequence from splenic GC B cells of WT or KI mice with NP-CGG challenge

<table>
<thead>
<tr>
<th></th>
<th>Within G/C</th>
<th>Within A/T</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Trans/Transv</td>
<td></td>
</tr>
<tr>
<td></td>
<td>C/T</td>
<td>C/A</td>
</tr>
<tr>
<td>Nonunique mutation counts</td>
<td>48.9:51.1</td>
<td>53.3:46.7</td>
</tr>
<tr>
<td>WT (NP-CGG)</td>
<td>47.1:52.9</td>
<td>47.6:52.4**</td>
</tr>
<tr>
<td>Unique mutation counts</td>
<td>45.1:54.9</td>
<td>59.2:40.8</td>
</tr>
<tr>
<td>WT (NP-CGG)</td>
<td>45.1:54.9</td>
<td>45.1:54.9**</td>
</tr>
</tbody>
</table>

<sup>a</sup><sup>p</sup> < 0.05, **<sup>p</sup> < 0.01, <sup>χ²</sup> test.

trans, Transition; transv, transversion.
transfer studies (45, 46) showed that constitutively active AKT in B cells can inactivate forkhead transcription factor FOXO1, resulting in concomitant decreases in AID and suppression of CSR; in addition, phosphorylation of AKT at Ser473 is required for phosphorylation of FOXO1/3a (47). In our earlier study (9), LPS-stimulated B cells from mTOR KI mice had increased levels of pAKT Ser473, and, in the current study, we showed that B cells from mTOR KI mice have increased levels of pAKT Ser473 and pFOXO1 Thr24, concomitant with lower levels of AID in response to LPS and IL-4 (Fig. 4B). Recent studies also showed that HSP90

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### Table III. Mutations in Sμ region (650 bp) from splenic CD43− resting B cells of WT and KI mice with LPS and IL-4 induction

<table>
<thead>
<tr>
<th></th>
<th>KI</th>
<th>WT</th>
<th>p Valuea</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of sequences</td>
<td>47</td>
<td>46</td>
<td></td>
</tr>
<tr>
<td>Total length sequenced (bp)</td>
<td>30,550</td>
<td>29,900</td>
<td></td>
</tr>
<tr>
<td>Unmutated sequences (n [%])</td>
<td>32/47 (68.1)</td>
<td>26/46 (56.5)</td>
<td></td>
</tr>
<tr>
<td>Number of deletions and insertions</td>
<td>1</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>Total number of nonunique point mutations</td>
<td>20</td>
<td>26</td>
<td></td>
</tr>
<tr>
<td>Mutation frequency/total sequences (per 100 bp)</td>
<td>0.07</td>
<td>0.14</td>
<td>0.0125</td>
</tr>
<tr>
<td>Mutation frequency/mutated sequences (per 100 bp)</td>
<td>0.22</td>
<td>0.32</td>
<td>0.1938</td>
</tr>
<tr>
<td>Total number of unique point mutations</td>
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<td>25</td>
<td></td>
</tr>
<tr>
<td>Mutation frequency/total sequences (per 100 bp)</td>
<td>0.06</td>
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<td>0.0073</td>
</tr>
<tr>
<td>Mutation frequency/mutated sequences (per 100 bp)</td>
<td>0.20</td>
<td>0.31</td>
<td>0.1285</td>
</tr>
</tbody>
</table>

*a* x² test, KI versus WT.

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**FIGURE 3.** CSR frequency in mTOR hypomorphs and rapamycin-treated WT cells. (A) CD43− resting B cells purified from spleens and LNs of KI and WT mice (age 5–12 wk) were stimulated with LPS and IL-4 for 72 h, and cells were stained with B220 and IgG1 Abs (n = 9 for LNs, n = 12 for spleens). (B) CD43− cells from spleens of KI and WT mice (age 8–10 wk) were stained with CFSE and then stimulated with LPS plus IL-4 (IgG1 induction) for 72 h. The cells were stained with IgG1 Ab (n = 6 samples) and analyzed with FACs and FlowJo software. The percentages of total cell number and IgG1+ cells in each division are presented. (C) Rapamycin decreases CSR in CD43− B cells from WT mice. CD43− resting B cells purified from spleens of WT mice were stimulated (≥72 h) with LPS and IL-4 to induce IgG1 switching over time (≥72 h). (C and D) Purified CD43− resting B cells were stained with CFSE and then stimulated with LPS and IL-4. (D) The cells were stained with IgG1 Ab. Cell proliferation was analyzed with CFSE staining, and the percentages of total cell number and IgG1+ cells in each division are presented. FACs analyses were performed with propidium iodide staining for cell cycle (E) or annexin V and 7-aminoactinomycin D staining for apoptosis (F) in CD43− resting B cells induced to switch to IgG1. Representative experiments are shown from two of three replicates (n = 3). Cells were analyzed with FACs and FlowJo software, and data are mean ± SEM. *p < 0.05.
can stabilize levels of AID in the cytoplasm and the nucleus (44). In contrast to cytoplasmic fractions, nuclear extracts from mTOR KI B cells stimulated with LPS and IL-4 had much lower levels of HSP90 and AID compared with WT B cells (Fig. 4C). Thus, lower AID protein levels in mTOR KI B cells may result from increases in phospho-AKT and phospho-FOXO, in addition to reductions in nuclear HSP90.

Increasing AID expression rescues IgG1 switching in mTOR KI B cells

AID expression levels are directly proportional to the extent of CSR and SHM (41, 48). To determine whether reintroduction of AID protein into mTOR hypomorphic KI cells could restore IgG1 switching, CD43{sup} resting B cells from WT and KI spleens were stimulated with LPS and IL-4 for 48 h. mRNA expression of AID, UNG, Polh, ExoI, MSH2, and MSH6 were measured by real-time PCR. The results are displayed as relative fold changes of mRNA expression in KI mice compared with WT mice, upon normalization by 18S RNA (mean ± SD, n = 3). (B) Western blots of AID signaling in CD43{sup} resting B cells from WT and KI spleens stimulated with LPS and IL-4 for 48 h. (C) Nuclear protein levels of HSP90 and its client protein, AID, are lower in CD43{sup} resting B cells from KI spleens stimulated with LPS and IL-4 for 48 h. The fold change was normalized to the WT protein level after normalization by β-actin. Western blots were repeated in at least two experiments. (D) CD43{sup} resting B cells isolated from spleens of WT or KI mice were infected with retroviruses expressing AID-GFP or GFP and stimulated with LPS and IL-4 for 72 h. CSR was measured by IgG1 cell surface expression. The percentage or ratio of IgG1{sup} cells from AID-GFP to GFP retrovirus infection was calculated in GFP{sup} cells. AID-KO mice were included as controls. Two independent experiments were in agreement.

Survival is increased in AID KO mice housed in filter-top cages and treated with antibiotics

AID KO mice are known to experience a 100-fold expansion of anaerobic flora in their small intestine (49), and we experienced difficulty in maintaining a healthy population of these mice in a conventional (non-SPF) colony. As a result, we monitored the overall survival of AID KO mice and their WT littermates, with and without metronidazole, housed in a conventional facility over the course of ~2 y (Supplemental Fig. 4D). WT mice lived longer (~780 d) than did AID KO mice (~240–360 d) (log-rank [Mantel–Cox] χ² = 19.1, p < 0.0001). Furthermore, AID KO mice that were given feed containing the antibiotic and antiprotozoal, metronidazole, and housed in autoclaved, filter-topped cages lived longer (~360 d) than did conventionally maintained AID KO mice (~240 d) (log-rank [Mantel–Cox] χ² = 6.51, p < 0.01).

B cell–specific mTOR KO mice are impaired in their GC responses

Because mTOR levels were constitutively reduced in all cell types from birth in the mTOR (KI) hypomorphs, we sought to determine whether mice deleted for mTOR specifically in their B cell lineage (mTOR KO mice; mTOR{supfl/fl}CD19{supCre/+}) (Supplemental Fig. 1A) would exhibit similar defects in GC formation and Ab production. We did not find differences in B cell subpopulations in the bone marrow (Supplemental Fig. 1D) or in the transitional T1 B cell
The age of the mice ranged from 8 to 12 wk (n increased levels of pAKTser473 and pFOXO1 Thr24 concomitant with mice, similar to their hypomorphic (KI) counterparts, have in-
6E). Furthermore, we confirmed that B cells from mTOR KO mice exhibited lower rates of CSR in ex vivo cultures (Fig. 6E). Resting B cells, stimulated with LPS and IL-4, from the mTOR KO mice exhibited lower rates of CSR in ex vivo cultures (Fig. 6E). Furthermore, we confirmed that B cells from mTOR KO mice, similar to their hypomorphic (KI) counterparts, have increased levels of pAktSer473 and pFOXO1Thr24 concomitant with lower levels of AID (Fig. 6F) in response to LPS and IL-4. Thus, these experiments confirm that mTOR deletion in CD19+ cells alone can negatively affect GC formation, Ab production, CSR, and establishment of B cell populations in the periphery.

Discussion

mTOR controls cell growth and is often dysregulated in several diseases, including cancer and immunosuppressive diseases (1, 2). Rapamycin analogs, specific inhibitors of mTOR kinase activity, have been used in the transplant setting to induce chronic immu-
se (3, 11). Long-term survivors of organ and bone marrow transplantation are at increased risk for infections, particularly from encapsulated bacteria, such as S. pneumoniae, which accounts for ~30% of community-acquired pneumonia and is known to be a common infection in immunosuppressed individuals (11–13).

We recently showed that constitutive reductions in mouse mTOR gene expression are associated with a partial block in B cell development in the bone marrow, altered percentages of various B cell popula-
tions in the spleen, and smaller spleens (9). We now report that B cell–specific reductions in mTOR impair GC formation, decrease the production of IgG isotypes in response to immuni-
and tional T2 and marginal zone (Fig. 5) B cell populations were lower in spleens of KO mice compared with WT mice. Reductions in B cell populations in LNs were also seen in mTOR KO mice (Fig. 5). Similar to results obtained in mTOR KI mice, fewer GCs, as assessed by IHC (Fig. 6A) and flow cytometry (Fig. 6B), formed in spleens of KO mice immunized with one dose of NP-CGG. These mTOR KO mice also produced fewer IgG isotypic Abs, with the exception of IgG2a (Fig. 6C), and fewer high-affinity anti-NP Abs (Fig. 6D) compared with their WT littermates. Resting B cells, stimulated with LPS and IL-4, from the mTOR KO mice exhibited lower rates of CSR in ex vivo cultures (Fig. 6E). Furthermore, we confirmed that B cells from mTOR KO mice, similar to their hypomorphic (KI) counterparts, have increased levels of pAKTser473 and pFOXO1Thr24 concomitant with lower levels of AID (Fig. 6F) in response to LPS and IL-4. Thus, these experiments confirm that mTOR deletion in CD19+ cells alone can negatively affect GC formation, Ab production, CSR, and establishment of B cell populations in the periphery.

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activated CD43+ B cells from KI mice, provide the link between mTOR expression and reductions in CSR/SHM and Ab affinity maturation, because AID is required for both CSR and SHM. In fact, CSR to IgG1 could be restored to WT levels in mTOR hypomorphic KI B cells following transduction with AID-GFP retrovirus.

Reductions in CSR/SHM and Ab affinity maturation ultimately affect Ab diversification, which can interfere with the ability of the immune system to respond to microbial challenge and recognize specific Ags. Indeed, under conventional conditions, the survival of AID KO mice was much lower than that of their WT littermates; prophylactic antibiotic treatment of AID KO mice increased their survival.

Our studies suggest that suppression of AID activity via rapamycin may contribute to the impairment of humoral immune responses and provide further rationale for potential immunization against microbial agents prior to immunosuppressive treatment. Immunizations have typically been given simultaneous with or after cessation of immunosuppressive drugs (50, 51). The improved survival of AID KO mice treated prophylactically with antibiotics suggests that the coadministration of antibiotics with rapamycin might decrease the risk for opportunistic streptococcal infections. In addition, these findings may have relevance in other settings, specifically the use of mTOR inhibitors as anticancer agents (2). In this situation, increased AID was shown to promote both tumor progression (41, 52–55) and drug resistance (56). Our studies confirmed that mTOR inhibition can downregulate AID as reported, perhaps by the phosphorylation of both AKT and FOXO proteins and the amount of nuclear HSP90 (43). Thus, fine-tuning mTOR inhibitors with appropriate dosing regimens may help to restore normal AID levels or activities needed for the establishment of GC reactions and genesis of high-affinity Abs while effectively regulating the AID mutator phenotype during tumor progression and/or drug resistance.

Acknowledgments
We thank Douglas Lowy, Crystal Mackall, Ron Gress, Dan Fowler, Howard Young, Glenn Merlino, Stuart Yuspa, Michael Potter, Richard Robinson, Val

FIGURE 6. mTOR KO in CD19+ B cells impairs GC formation and decreases anti-NP Ab response to NP-CGG. KO (n = 7) and WT (n = 5) mice were immunized i.p. with NP-CGG in Rehydragel. Spleens were collected on day 14 for IHC staining. (A) Splenic sections were stained with B220 and PNA. The numbers of GCs (arrows indicate GC) and the area of PNA staining were evaluated from scans of spleen sections stained with B220 or PNA using color deconvolution analysis software (Aperio Technologies). (B) FACS analysis of cells from spleens and LNs stained with B220, GL7, Fas, CD38, and IgD Abs. (C) Sera from KO and WT mice immunized with NP-CGG were collected on day 14 for measurement of Ag-specific IgM and IgG isotype Ab titers. Data are mean ± SEM. (D) The relative affinities of anti-NP Abs were determined using an ELISA with BSA coupled to NP at different ratios: NP23-BSA and NP45-BSA. (E) CD43+ resting B cells purified from spleens and LNs of KO and WT mice (age 8–10 wk) were stimulated with LPS and IL-4 for 72 h, and cells were stained with CD19 and IgG1 Abs (n = 6). The cells were analyzed with FACS and FlowJo software. Data are mean ± SEM. (F) CD43+ CD19+ resting B cells from WT and KO LNs were stimulated with LPS and IL-4 for 48 h. Western blot analyses of protein expression in KO mice relative to WT mice. AID expression levels were lower and pAKT Ser473 and pFOXO1 Thr24 levels were higher in KI or KO mice relative to WT mice. *p < 0.05, **p < 0.01.
Disclosures
The authors have no financial conflicts of interest.

References

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