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Although ribosomal proteins facilitate the ribosome's core function of translation, emerging evidence suggests that some ribosomal proteins are also capable of performing tissue-restricted functions either from within specialized ribosomes or from outside of the ribosome. In particular, we have previously demonstrated that germline ablation of the gene encoding ribosomal protein Rpl22 causes a selective and p53-dependent arrest of αβ T cell progenitors at the β-selection checkpoint. We have now identified a crucial role for Rpl22 during early B cell development. Germline ablation of Rpl22 results in a reduction in the absolute number of B-lineage progenitors in the bone marrow beginning at the pro–B cell stage. Although Rpl22-deficient pro–B cells are hyporesponsive to IL-7, a key cytokine required for early B cell development, the arrest of B cell development does not result from disrupted IL-7 signaling. Instead, p53 induction appears to be responsible for the developmental defects, as Rpl22 deficiency causes increased expression of p53 and activation of downstream p53 target genes, and p53 deficiency rescues the defect in B cell development in Rpl22-deficient mice. Interestingly, the requirement for Rpl22 in the B cell lineage appears to be developmentally restricted, because Rpl22-deficient splenic B cells proliferate normally in response to Ag receptor and Toll receptor stimuli and undergo normal class-switch recombination. These results indicate that Rpl22 performs a critical, developmentally restricted role in supporting early B cell development by preventing p53 induction. ***The Journal of Immunology*****, 2015, 194: 200–209.**

Adult B cell development initiates from a long-term, self-renewing hematopoietic stem cell (HSC) present in adult bone marrow. Commitment to the B cell lineage from the HSC is a tightly controlled process in which alternative lineage potential is gradually lost, whereas B cell identity is enforced (1). HSCs give rise to pro–B cells, which represent the first committed B-lineage progenitors to have lost differentiation potential for all other lineages (2). During the pro–B cell stage, rearrangement of the IgH chain locus is completed. Successful rearrangement of the IgH locus leads to the expression of cytoplasmic μ protein, which pairs with the surrogate L chains λ5 and VpreB and the signaling components Igα and Igβ to form the pre-BCR. Expression of the pre-BCR initiates differentiation to the large pre-B cell stage. Following two to five rounds of cellular division, large pre-B cells differentiate to the small pre-B cell stage and initiate rearrangement of the Ig L chain loci. Successful L chain rearrangement leads to expression of L chain protein, which pairs with the μ H chain to form membrane-bound IgM and initiates differentiation to the immature B cell stage. Immature B cells emigrate to the spleen, where they undergo three transitional B cell stages prior to entering the mature B cell pool (3). Three populations of mature B cells are present in the periphery (4). Follicular B cells are highly enriched within secondary lymphoid organs, whereas marginal zone B cells are localized to the marginal sinus of the spleen. B1 B cells, a third population of mature B cells, are abundant within the pleural and peritoneal cavities, but represent only a small proportion in the spleen.

Studies describing the molecular networks that govern the differentiation of uncommitted HSCs into mature B cells have primarily focused on key transcription factors and cytokine receptors that are responsible for this process. Differentiation of HSCs to the pro–B cell stage and commitment to the B cell lineage is dependent on the transcription factors PU.1, E2A, Ikaros, Ebf1, and Pax5 as well as the cytokine receptors Flt3 and IL-7R (5). IL-7 is also the crucial cytokine that mediates survival and proliferation during the pro–B cell stage by regulating expression of Mcl1 and cyclin D3 (6–9). Following successful rearrangement of the IgH locus, differentiation of pro–B cells to the small pre–B cell stage is dependent on a second network of transcription factors including Pax5, Foxo1, E2A, and Irf4/8, as well as the IL-7R and pre-BCR (10). Although there has been growing interest in the posttranscriptional mechanisms that control the immune response (11, 12), relatively little is known regarding posttranscriptional control of B cell development.

Ribosomal proteins are crucial components of cellular ribosomes that are required for the synthesis of proteins. Recent evidence, however, has demonstrated that ribosomal proteins have extraribosomal functions including regulation of translation by binding to specific target mRNAs (13–17). In addition, defects in ribosomal proteins have been observed in human diseases such as Diamond-Blackfan anemia and 5q-syndrome, which are characterized by defects in erythroid development (18). Defects in lymphocyte development upon mutation of ribosomal proteins, however, had not been previously shown. Recently, it has been demonstrated that deficiency in the ribosomal protein Rpl22 causes remarkably restricted developmental defects, disrupting

Immune Cell Development and Host Defense Program, Fox Chase Cancer Center, Philadelphia, PA 19111

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Address correspondence and reprint requests to Dr. David L. Wiest, Fox Chase Cancer Center, R364, 333 Cottman Avenue, Philadelphia, PA 19111-2497. E-mail address: David.Wiest@FCCC.edu

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Abbreviations used in this article: ALP, all-lymphoid progenitor; BLP, B cell–biased lymphoid progenitor; HSC, hematopoietic stem cell; PDCA, plasmacytoid dendritic cell Ag; PI, propidium iodide.

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αβ, but not γδ, T cell development (19). Rpl22 is a ubiquitously expressed RNA binding protein that is a component of the 60S ribosomal subunit but it is not required for global translation (19, 20). Surprisingly, germline ablation of Rpl22 does not affect the health or growth of the mice, but causes a specific and severe block in αβ T cell development at the β-selection checkpoint. The block at the DN3 stage in *Rpl22*$^{-/-}$ mice is a result of posttranscriptional induction of the p53 tumor suppressor, which transactivates its downstream targets Puma, p21, Bax, and Noxa, and these effectors are responsible for blocking αβ T cell development, because the developmental arrest is rescued by their elimination (19, 21). Notably, the regulation of p53 by Rpl22 is posttranscriptional, as there was no change in p53 mRNA in the absence of Rpl22. Although previous studies have suggested a role for Rpl22 during B cell development (19, 22), neither the stages affected nor the underlying mechanism was addressed.

We now demonstrate that Rpl22 is selectively required during early B cell development. Elimination of Rpl22 leads to a decrease in the pro–B, pre–B, and immature B cell subsets within the bone marrow and transitional B cell subsets in the spleen. Rpl22-deficient pro–B cells fail to survive or proliferate in response to IL-7, the crucial cytokine required for the pro–B cell stage. The arrest of B cell development does not result from disruption of IL-7 signaling, as IL-7Rα and the downstream targets of IL-7 signaling are not altered by Rpl22 deficiency. Instead, the arrest is due to increased p53 protein levels and increased expression of the p53 target genes Puma and p21 in Rpl22-deficient pro–B cells. Importantly, knockdown of p53 expression is able to rescue the ability of Rpl22-deficient pro–B cells to respond to IL-7, and p53 deficiency completely rescues the defects in B cell development in *Rpl22*$^{-/-}$ mice in vivo. Interestingly, in contrast to previous reports implicating Rpl22 in peripheral T cell function, the absence of Rpl22 does not affect peripheral B cell proliferation or activation, suggesting that Rpl22 loss produces distinct effects in B and T cells. These results demonstrate that Rpl22 is a crucial mediator of B cell development that regulates p53 at critical developmental checkpoints.

Materials and Methods

Mice

All mouse strains were housed in the Laboratory Animal Facility at Fox Chase, which is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, and handled in accordance with protocols approved by the Institutional Animal Care and Use Committee. *Rpl22*$^{-/-}$, *Rag1*$^{-/-}$, and *Tp53*$^{-/-}$ mice have been previously described (19, 23, 24).

Flow cytometry

Single-cell suspensions were prepared from bone marrow, spleen, and peritoneal fluid and stained, as indicated, with optimal amounts of the following fluorochrome-conjugated Abs: anti-CD5 (53-7.3), anti-CD8 (53-6.7), anti-CD43 (S7), anti-CD69 (H1.2F3), anti-CD86 (GL1), anti-IgG1 (X56), anti-IgG3 (R40-82), and anti-Ly6C/G (RB6-8C5) (BD Biosciences, San Jose, CA); anti-B220 (RA3-6B2) and anti-CD4 (GK1.5) (BioLegend, San Diego, CA); and anti-AA4.1 (AA4.1), anti-CD3ε (17A2), anti-CD11b (M1/70), anti-CD19 (1D3); anti-CD23 (B3B4); anti-CD44 (1M7), anti-CD127 (A7R34), anti-CD135 (A2F10), anti-IgM (ll/41), anti-Ly6D (49-H4), anti-NK1.1 (PK136), anti–plasmacytoid dendritic cell Ag (PDCA1; 927), and anti-Ter119 (TER-119) (eBioscience, San Diego, CA). For apoptosis and cell cycle analysis, cells were stained with Annexin V FITC (eBioscience) and DRAQ5 (eBioscience), respectively, per the manufacturer's protocol. Data were collected using the LSRII (BD Biosciences) and analyzed using FlowJo software (Tree Star, Ashland, OR). Cells were sorted using the FACSAria II (BD Biosciences).

Peripheral B cell stimulation

Splenic B cells were purified by negative selection using anti-CD43 magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's protocol. Cells were stimulated at 1×10^6 cells/ml in 96-well plates with 50 μg/ml LPS (Sigma-Aldrich, St. Louis, MO), 10 μg/ml polyclonal anti-IgM, F(ab′)$_2$ fragments (Jackson ImmunoResearch Laboratories, West Grove, PA), or 10 μg/ml anti-CD40 (HM40-3; BioLegend) plus 100 U/ml IL-4 (PeproTech, Rocky Hill, NJ). After 72 h, expansion was measured by flow cytometry using AccuCount 5.27-μm Blank Particles (Spherotech, Lake Forest, IL) according to the manufacturer's protocol.

Pro–B cell culture

Rag1$^{-/-}$ pro–B cells were purified using anti-CD19 magnetic beads (Miltenyi Biotec). Cells were cultured at 1×10^6 cells/ml in 96-well plates in the presence of 10 ng/ml IL-7 (PeproTech). After 24, 48, and 72 h, proliferation was measured by flow cytometry using AccuCount 5.27-μm Blank Particles (Spherotech). Q-VD-OPH (Sigma-Aldrich) was used at 100 μM. For transduction, *Rag1*$^{-/-}$ pro–B cells were cultured for 24 h prior to transduction with MLP or MLP-shp53 retroviral vectors, as previously described (19, 25).

Quantitative real-time PCR

Total RNA was isolated from cells using the RNeasy system (Qiagen, Valencia, CA). DNA was removed using on-column DNase treatment (Qiagen), and cDNA was synthesized using Superscript II reverse transcriptase and oligo-dT (Invitrogen, Carlsbad, CA). Quantitative real-time PCR was performed on the Prism 7700 thermocycler (Applied Biosystems, Carlsbad, CA) using TaqMan real-time PCR prime/probe sets specific for Mcl1/*Mcl1* (Mm00725832_s1), cyclin D3/*Ccnd3* (Mm01612362_m1), p53/*Trp53* (Mm01731287_m1), puma/*Bbc3* (Mm00519268_m1), p21/*Cdkn1a* (Mm01303209_m1), and GAPDH/*Gapdh* (Mm99999915_g1).

Immunoblot analysis

Cultured pro–B cells were lysed in RIPA buffer (20 mM HEPES [pH 7], 150 mM NaCl, 1% deoxycholate, 1% Nonidet P-40, 0.1% SDS, 1 mM Na_2VO_4, 2 mM EDTA, and a complete protease inhibitor mixture; Roche, Basel, Switzerland). Samples were resolved on NuPage Novex Bis-Tris gels (Invitrogen) and blotted with the following Abs: anti-p53 (IMX25; Leica Microsystems, Newcastle Upon Tyne, UK), anti-Rpl22 (19), and anti-GAPDH (6C5; Millipore, Billerica, MA).

Statistics

Statistical significance of alterations in cell populations or changes in gene expression in *Rpl22*$^{-/-}$ mice was assessed using the two-tailed Student *t* test.

Results

Rpl22 is dispensable for peripheral B cell proliferation and activation

Although previous evidence suggested that Rpl22 might play a role during B cell development (19, 22), neither the stages affected nor the mechanistic basis for the impairment of B cell development had been assessed. To begin to understand the role of Rpl22 during B cell development, we analyzed splenic B cells from *Rpl22*$^{+/+}$ and *Rpl22*$^{-/-}$ mice. As noted previously, there was an 85% decrease in the absolute number of CD4$^+$ and CD8$^+$ T cells in the spleens of *Rpl22*$^{-/-}$ mice compared with controls (Fig. 1A, Supplemental Fig. 1A). In addition, there was a somewhat milder reduction (50% decrease) in the absolute number of B cells in the spleens of *Rpl22*$^{-/-}$ mice (Fig. 1A, Supplemental Fig. 1A). Interestingly, when we subdivided splenic B cells into follicular or marginal zone B cells, we found that the reduction was restricted to follicular B cells, as they were reduced by 60% in *Rpl22*$^{-/-}$ mice compared with control mice, whereas the absolute number of marginal zone B cells was unaffected (Fig. 1B, Supplemental Fig. 1B). To determine if there was an alteration in B1 B cells in *Rpl22*$^{-/-}$ mice, peritoneal fluid from *Rpl22*$^{+/+}$ and *Rpl22*$^{-/-}$ mice was analyzed by flow cytometry. We found that B1 B cells (B220^{low}CD19$^+$), as well as the B1a subset (CD5$^+$IgM$^+$), in the peritoneal cavity were reduced by 50% in *Rpl22*$^{-/-}$ mice compared with wild-type controls (Fig. 1C). These results indicate that Rpl22 is required for the development and/or maintenance of peripheral B cell subsets.

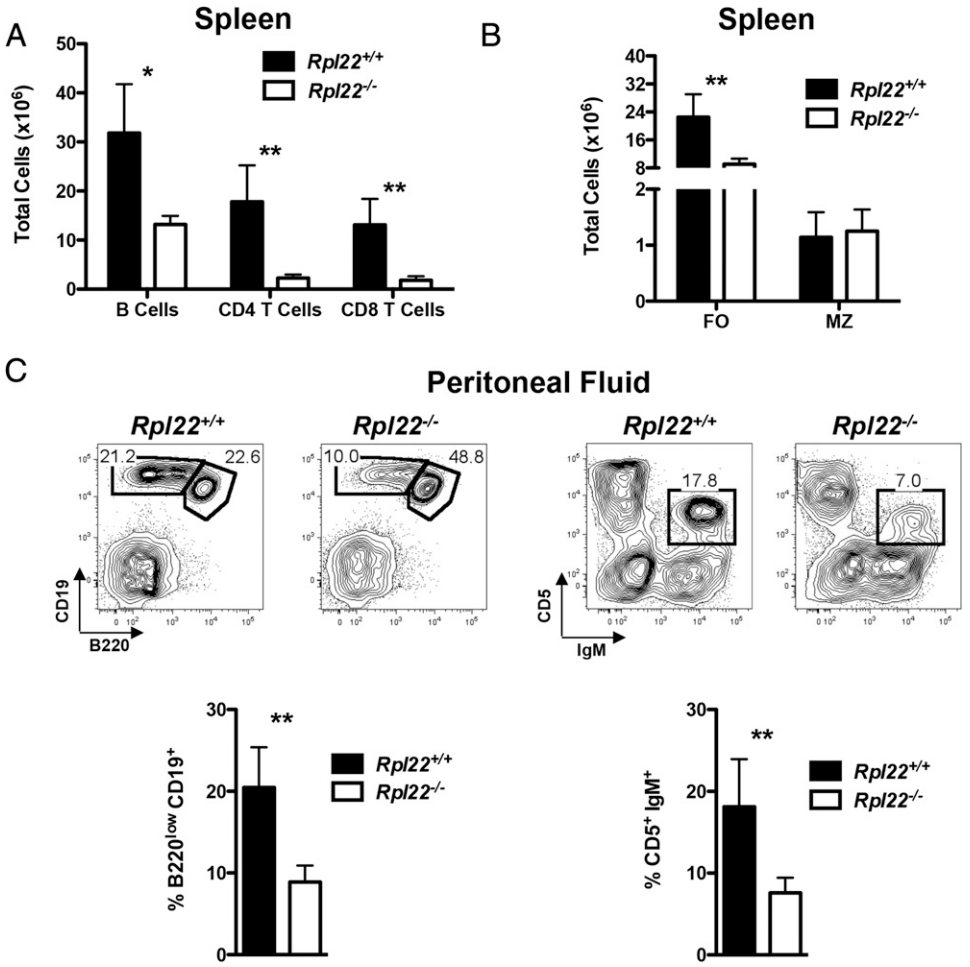

FIGURE 1. Reduction in peripheral B cell subsets in Rpl22-deficient mice. (**A**) Absolute number of B cells ($B220^+CD19^+$), $CD4^+$ T cells ($CD4^+CD8^-$), and $CD8^+$ T cells ($CD4^-CD8^+$) from the spleens of $Rpl22^{+/+}$ and $Rpl22^{-/-}$ mice. (**B**) Absolute number of follicular B cells (FO; $B220^+AA4.1^-IgM^+CD23^+$) and marginal zone B cells (MZ; $B220^+AA4.1^-IgM^{high}CD23^-$) in the spleens of $Rpl22^{+/+}$ and $Rpl22^{-/-}$ mice. (**C**) Peritoneal fluid from $Rpl22^{+/+}$ and $Rpl22^{-/-}$ mice was analyzed from surface expression of B220 and CD19 (*left panel*) to identify B1a and B1b cells ($B220^{low}CD19^+$) or CD5 and IgM (*right panel*) to identify $CD5^+$ IgM^+ B1a B cells. $n = 6$ per genotype. $^*p < 0.01$, $^{**}p < 0.005$.

Along with its critical role in αβ T cell development, we had previously found that Rpl22 played an important role in T cell function, as Rpl22-deficient peripheral $CD4^+$ and $CD8^+$ T cells undergo apoptosis following anti-CD3 stimulation (19). To determine if Rpl22 played a comparable role in the function of peripheral B cells, splenic B cells from $Rpl22^{+/+}$ and $Rpl22^{-/-}$ mice were isolated and stimulated in vitro with LPS, anti-IgM, or anti-CD40 plus IL-4 for 72 h. Surprisingly, there was no difference in the recovery of $Rpl22^{+/+}$ or $Rpl22^{-/-}$ B cells under any stimulation condition (Fig. 2A). There was also no difference in the upregulation of two activation markers, CD86 and CD69, in $Rpl22^{-/-}$ B cells compared with controls (Fig. 2B). To assess whether $Rpl22^{-/-}$ B cells were able to undergo class-switch recombination, these cells were cultured in the presence of LPS alone or with IL-4 for 72 h, and class-switch recombination was measured by surface expression of IgG1 or IgG3, respectively. Class-switch recombination was also not affected by Rpl22 loss, as the proportion of B cells that switched to IgG1 or IgG3 in response to LPS or LPS plus IL-4 was not altered by Rpl22 deficiency (Fig. 2C). These results suggest that, in contrast to its apparent role in peripheral T cell function, Rpl22 may not be required for peripheral B cell function.

Rpl22 is required for B cell development in the bone marrow

As there was no apparent defect in peripheral B cell function in the absence of Rpl22, we next sought to determine if the reduction in peripheral B cell numbers was a consequence of an earlier defect in B cell development. Common lymphoid progenitors are the first lymphoid progenitors present in the bone marrow, and recent reports have further subdivided common lymphoid progenitors into $Ly6D^-$ all-lymphoid progenitors (ALPs) and the more restricted $Ly6D^+PDCA1^-$ B cell–biased lymphoid progenitors (BLPs) (26, 27). To determine if there were any changes in the earliest stages of B cell development in the absence of Rpl22, bone marrow from $Rpl22^{+/+}$ and $Rpl22^{-/-}$ mice was harvested and analyzed by flow cytometry. There was no significant difference in the absolute number of ALPs or BLPs in $Rpl22^{-/-}$ bone marrow compared with controls (Fig. 3A, Supplemental Fig. 2A). BLPs differentiate to the $B220^+CD19^-$ pre-pro–B cell stage. The $B220^+CD19^-$ compartment also contains NK and plasmacytoid dendritic cells and pre-pro–B cells are further characterized as $NK1.1^-PDCA1^-AA4.1^+Ly6D^+$ (27, 28). Similar to ALPs and BLPs, flow cytometric analysis of $Rpl22^{+/+}$ and $Rpl22^{-/-}$ bone marrow revealed that Rpl22 deficiency did not alter the absolute number of pre-pro–B cells (Fig. 3A, Supplemental Fig. 2B). These results suggest that Rpl22 is dispensable for the earliest stages of B cell development in the bone marrow.

Because the earliest stages of B cell development were not altered by Rpl22 deficiency, we next sought to determine if the later stages following commitment to the B-lineage were altered in $Rpl22^{-/-}$ mice. To do so, we analyzed bone marrow from $Rpl22^{+/+}$ and $Rpl22^{-/-}$ mice using the gating strategy developed by Hardy and colleagues (29). Unlike ALPs, BLPs, and pre-pro–B cells, the loss of Rpl22 resulted in a statistically significant 40% reduction in the absolute number of pro–B cells (defined as $Ly6C^-$ $NK1.1^-$ $B220^+IgM^-CD43^+CD19^+$) (Fig. 3B, 3C). There was also a 60% decrease in the absolute number of pre-B cells (defined as $Ly6C^-$ $NK1.1^-B220^+IgM^-CD43^-$) and $Ly6C^-NK1.1^-B220^+IgM^+$ cells

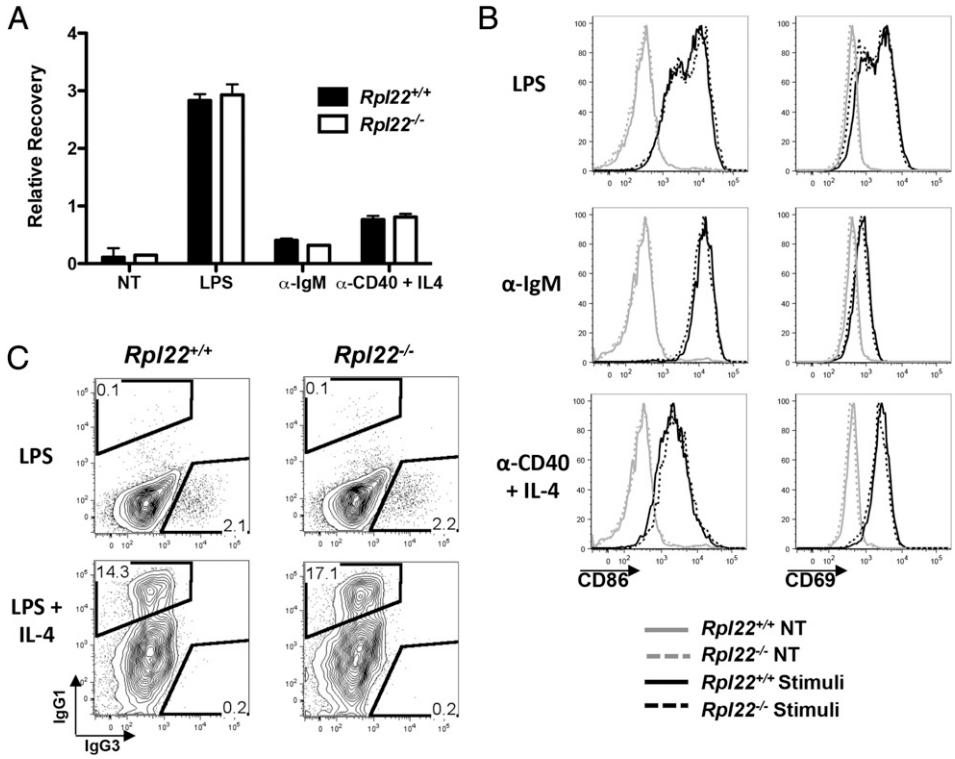

FIGURE 2. Rpl22 is not required for peripheral B cell proliferation and activation. (**A**) Relative recovery of purified splenic B cells from *Rpl22*$^{+/+}$ and *Rpl22*$^{-/-}$ mice cultured in the presence of LPS, anti-IgM, or anti-CD40 + IL-4 for 72 h. Total viable cells were determined by flow cytometry and normalized to the total number of cells plated. Results are representative of four independent experiments. (**B**) Surface expression of CD86 and CD69 on splenic B cells from *Rpl22*$^{+/+}$ and *Rpl22*$^{-/-}$ mice cultured in the presence of LPS, anti-IgM, or anti-CD40 + IL-4 for 72 h. Gray histograms represent splenic B cells cultured in the absence of stimulation, whereas the black histograms represent splenic B cells cultured in the presence of the indicated stimulation. Results are representative of two independent experiments. (**C**) Surface expression of IgG1 and IgG3 on splenic B cells from *Rpl22*$^{+/+}$ and *Rpl22*$^{-/-}$ mice cultured in the presence of LPS or LPS + IL-4. Results are representative of two independent experiments. NT, not treated.

(representing immature as well as mature recirculating B cells) in the bone marrow of *Rpl22*$^{-/-}$ mice (Fig. 3B, 3C). Examination of the splenic transitional B cell subsets by flow cytometry also revealed that Rpl22 deficiency reduced the absolute number of T1, T2, and T3 transitional B cell subsets by 80% (Fig. 3D, Supplemental Fig. 1B). These results demonstrate that Rpl22 is required for B cell development within the bone marrow.

Rpl22 is required for pro–B cells to respond to IL-7

We have now demonstrated that Rpl22 is required for the B cell development starting at the pro–B cell stage. Deficiencies within the pro–B cell compartment in the absence of Rpl22 could be due to rapid evacuation of cells that have successfully rearranged their IgH loci and differentiated into pre–B cells. To test this possibility, we blocked the ability of Rpl22-deficient pro–B cells to differentiate by rendering them Rag1 deficient, which prevents V(D)J recombination (23). Similar to Rag1-sufficient mice, *Rpl22*$^{-/-}$*Rag1*$^{-/-}$ mice had a decrease in the percentage (Fig. 4A) and absolute number (Fig. 4B) of pro–B cells compared with *Rpl22*$^{+/+}$*Rag1*$^{-/-}$ controls, demonstrating that Rpl22 is required for the maintenance of the pro–B cell compartment prior to the pre-BCR checkpoint.

IL-7 is the crucial cytokine required for B cell development that mediates survival, proliferation, and differentiation during the pro–B cell stage (30). Therefore, the decrease in pro–B cells caused by Rpl22 deficiency might result from an inability of these cells to respond to IL-7. To assess this possibility, CD19^{+} pro–B cells from *Rpl22*$^{+/+}$*Rag1*$^{-/-}$ and *Rpl22*$^{-/-}$*Rag1*$^{-/-}$ mice were purified and cultured for 72 h in the presence of IL-7. Although *Rpl22*$^{+/+}$*Rag1*$^{-/-}$ pro–B cells were able to proliferate in response to IL-7 and expanded 5-fold in culture over 72 h, *Rpl22*$^{-/-}$*Rag1*$^{-/-}$ pro–B cells were unable to expand in response to IL-7 in vitro (Fig. 4C). To determine the extent to which defects in proliferation and survival contributed to the inability of Rpl22-deficient pro–B cells to expand in response to IL-7, *Rpl22*$^{+/+}$*Rag1*$^{-/-}$ and *Rpl22*$^{-/-}$*Rag1*$^{-/-}$ pro–B cells were cultured for 48 h in the presence of IL-7 and analyzed by flow cytometry using Annexin V/propidium iodide (PI) staining to measure survival and DRAQ5 to measure proliferation. Rpl22-deficient pro–B cells had impaired survival in response to IL-7, as <30% of *Rpl22*$^{-/-}$*Rag1*$^{-/-}$ pro–B cells were viable (as identified as Annexin V^{-}PI^{-}) compared with >80% of *Rpl22*$^{+/+}$*Rag1*$^{-/-}$ pro–B cells (Fig. 4D). In addition, Rpl22 may also act to support the proliferation of pro–B cells in response to IL-7, as only 25% of *Rpl22*$^{-/-}$*Rag1*$^{-/-}$ pro–B cells were in cell cycle compared with ~40% of *Rpl22*$^{+/+}$*Rag1*$^{-/-}$ pro–B cells (Fig. 4D). Nevertheless, it is difficult to define a role for Rpl22 in the proliferation of pro–B cells in response to IL-7 that is distinct from the underlying survival defect. To rescue survival of Rpl22-deficient pro–B cells and identify a potential role for Rpl22 in the proliferation of pro–B cells, *Rpl22*$^{+/+}$*Rag1*$^{-/-}$ and *Rpl22*$^{-/-}$*Rag1*$^{-/-}$ pro–B cells were cultured in the presence of IL-7 as well as the pan-caspase inhibitor Q-VD-OPH. However, although Q-VD-OPH treatment of Rpl22-deficient pro–B cells substantially increased the percentage that were Annexin V^{-}PI^{-} (Fig. 4D; 62%) and overall recovery, they remained unable to expand in culture in response to IL-7 (Fig. 4E). Indeed, Q-VD-OPH treatment of Rpl22-deficient pro–B cells failed to rescue their cell-cycle defect, as the fraction of cells in S/G_2/M was unchanged (Fig. 4D). These

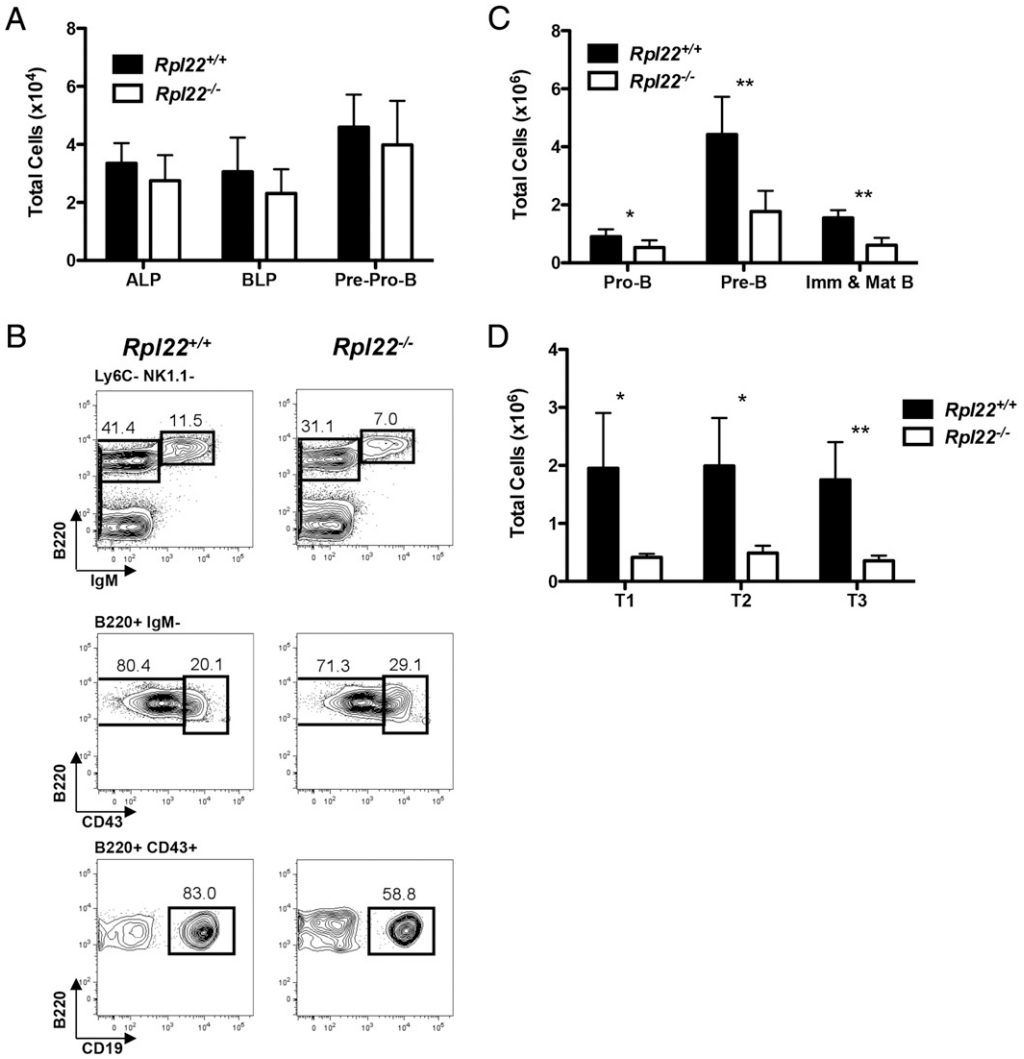

FIGURE 3. Reduced B-lineage progenitors in the bone marrow of *Rpl22*$^{-/-}$ mice. (**A**) Absolute number of ALPs (Lin [CD11b, CD3, Ter119, Gr1, NK1.1, B220, CD19]$^-$ CD135$^+$CD127$^+$PDCA1$^-$Ly6D$^-$), BLPs (Lin[CD11b, CD3, Ter119, Gr1, NK1.1, B220, CD19]$^-$CD135$^+$CD127$^+$PDCA1$^-$Ly6D$^+$), and pre-pro–B cells (Lin [CD11b, CD3, Ter119, Gr1, NK1.1, CD19]$^-$ B220$^+$AA4.1$^+$PDCA1$^-$Ly6D$^+$) from the bone marrow of *Rpl22*$^{+/+}$ and *Rpl22*$^{-/-}$ mice. (**B**) Bone marrow from *Rpl22*$^{+/+}$ and *Rpl22*$^{-/-}$ mice was analyzed for surface expression of Ly6C, NK1.1, B220, IgM, CD43, and CD19. Viable cells were identified as PI$^-$. The *top panels* represent B220 versus IgM after gating out Ly6C$^+$ and NK1.1$^+$ cells. The *middle panels* represent B220 versus CD43 expression on B220$^+$IgM$^-$ gated cells. The *bottom panels* represent B220 versus CD19 expression on B220$^+$CD43$^+$ gated cells. (**C**) Absolute number of pro–B cells, pre–B cells, and immature and mature B cells from the bone marrow of *Rpl22*$^{+/+}$ and *Rpl22*$^{-/-}$ mice. (**D**) Absolute number of T1 (B220$^+$ AA4.1$^+$IgM^{high}CD23$^-$), T2 (B220$^+$AA4.1$^+$IgM^{high}CD23$^+$), and T3 (B220$^+$AA4.1$^+$IgM^{low}CD23$^+$) transitional B cell subsets in the spleen of *Rpl22*$^{+/+}$ and *Rpl22*$^{-/-}$ mice. $n = 6$ per genotype. *$p < 0.05$, **$p < 0.005$.

results demonstrate that Rpl22 is required for both the survival and proliferation of pro–B cells in response to IL-7.

Rpl22-deficient pro–B cells have increased p53 expression

The inability of Rpl22-deficient pro–B cells to respond to IL-7 could result from the loss of cytokine receptor expression and/or impaired induction of crucial downstream mediators. However, surface expression of IL-7Rα on Rpl22-deficient pro–B cells was identical to that of control pro–B cells (Fig. 5A). Two key downstream mediators activated by IL-7 during the pro–B cell stage that are required for survival and proliferation are Mcl-1 and cyclin D3 (6, 8, 9). To determine if expression of Mcl1 or cyclin D3 in response to IL-7 signaling was repressed by Rpl22 deficiency, CD19$^+$ pro–B cells from *Rpl22*$^{+/+}$*Rag1*$^{-/-}$ and *Rpl22*$^{-/-}$*Rag1*$^{-/-}$ mice were isolated and cultured for 24 h in the presence of IL-7 and Q-VD-OPH. CD19$^+$ pro–B cells were then electronically sorted and Mcl1 and cyclin D3 mRNA levels were analyzed by quantitative real-time PCR. Expression of Mcl1 or cyclin D3 mRNA was not reduced by the absence of Rpl22 (Fig. 5B). These results suggest that the IL-7 signaling pathway is intact in Rpl22-deficient pro–B cells.

Because we have previously reported that Rpl22 regulates β-selection during αβ T cell development through a p53-dependent checkpoint (19), we next asked if Rpl22 regulated a similar p53-dependent checkpoint during B cell development. To measure p53 protein expression in Rpl22-deficient pro–B cells, CD19$^+$ pro–B cells were isolated from *Rpl22*$^{+/+}$*Rag1*$^{-/-}$ and *Rpl22*$^{-/-}$*Rag1*$^{-/-}$ mice and cultured with Q-VD-OPH and IL-7 for 24 h. CD19$^+$ pro–B cells were then electronically sorted, and p53 protein was measured by Western blot. As we had previously observed with DN3 thymocytes, Rpl22 deficiency also induced p53 protein in pro–B cells (Fig. 5C). Two key downstream targets of p53 are the proapoptotic factor Puma and the cell cycle inhibitor p21, and both of these factors are upregulated in Rpl22-deficient DN3 thymocytes

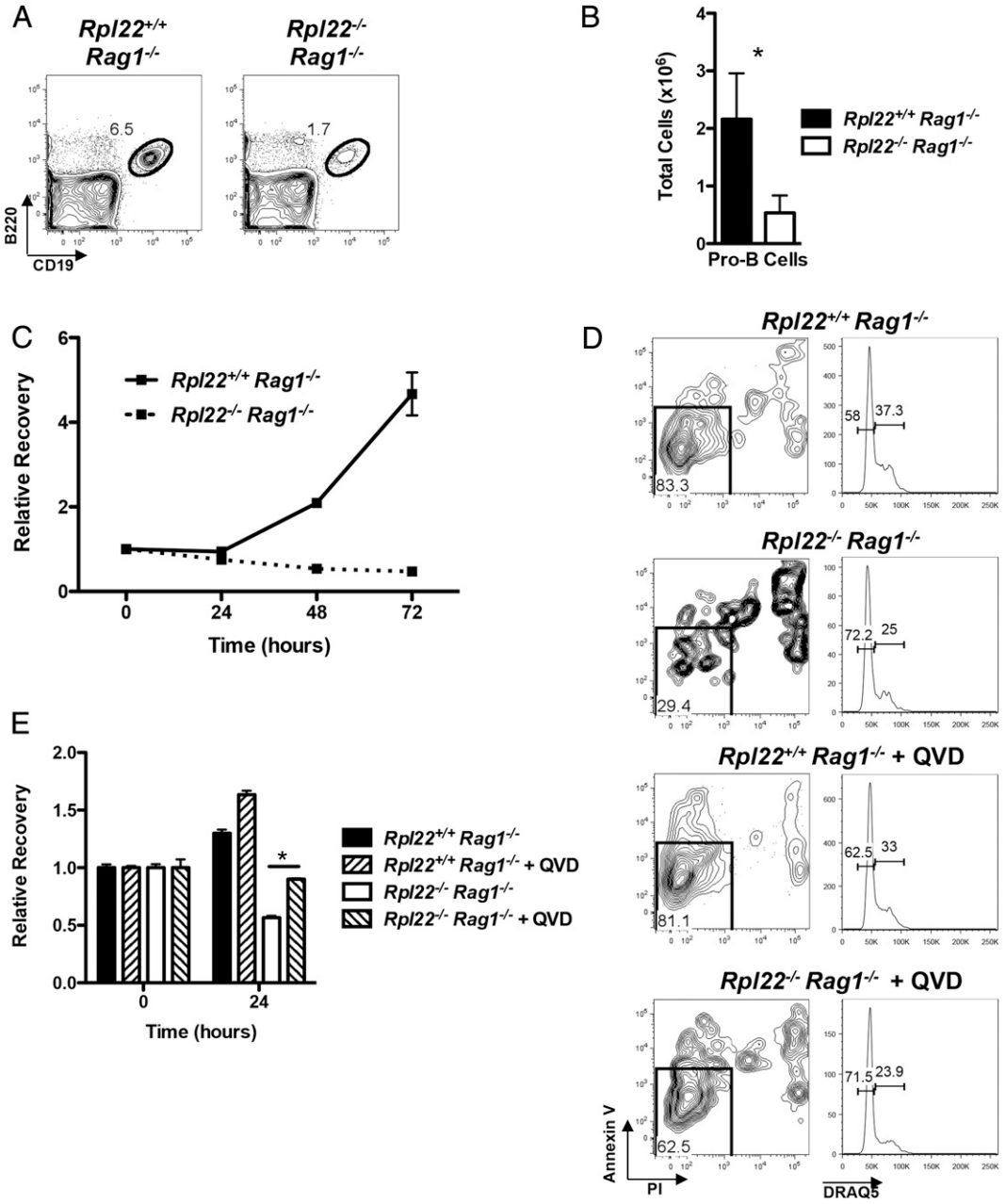

FIGURE 4. Rpl22-deficient pro–B cells are hyporesponsive to IL-7. (**A**) Bone marrow from $Rpl22^{+/+}Rag1^{-/-}$ and $Rpl22^{-/-}Rag1^{-/-}$ was analyzed for surface expression of B220 and CD19. Viable cells were identified as PI^-. $Rpl22^{+/+}Rag1^{-/-}$: 7.03 ± 1.87%; $Rpl22^{-/-}Rag1^{-/-}$: 2.60 ± 1.16%; $p < 0.005$. (**B**) Absolute number of pro–B cells ($B220^+CD19^+$) from the bone marrow of $Rpl22^{+/+}Rag1^{-/-}$ and $Rpl22^{-/-}Rag1^{-/-}$ mice. $n = 5$ per genotype; $*p < 0.05$. (**C**) Relative recovery of pro–B cells from $Rpl22^{+/+}Rag1^{-/-}$ and $Rpl22^{-/-}Rag1^{-/-}$ mice after 24, 48, and 72 h in liquid culture in the presence of IL-7. Total viable cells were determined by flow cytometry, and relative recovery was normalized to the total number of cells plated. Data are representative of five independent experiments. (**D**) Total Annexin V and PI staining (*left panel*) and DRAQ5 staining (*right panel*) of $Rpl22^{+/+}Rag2^{-/-}$ and $Rpl22^{-/-}Rag2^{-/-}$ pro–B cultured for 48 h in the presence of IL-7 (*top two panels*) or IL-7 + Q-VD-OPH (*bottom two panels*). Data are representative of two independent experiments. (**E**) Relative recovery of $Rpl22^{+/+}Rag1^{-/-}$ and $Rpl22^{-/-}Rag1^{-/-}$ pro–B cells cultured for 24 h in the presence of IL-7 and Q-VD-OPH. Total viable cells were determined by flow cytometry, and relative recovery was normalized to the total number of cells plated. Data are representative of four independent experiments. p values were calculated for triplicate measurements within each experiment. $*p < 0.005$.

(21). To determine if these two genes were also upregulated in Rpl22-deficient pro–B cells, Rpl22-deficient pro–B cells were cultured with Q-VD-OPH and IL-7 for 24 h, and p53, PUMA, and p21 mRNA was analyzed by quantitative real-time PCR. Similar to previous reports in DN3 thymocytes (19, 21), there was no increase in p53 mRNA in Rpl22-deficient pro–B cells compared with control pro–B cells (Fig. 5D), consistent with posttranscriptional control of p53 by Rpl22. Puma and p21 mRNAs, however, were increased 13-fold and 2-fold, respectively, in Rpl22-deficient pro–B cells. These results demonstrate that, similar to DN3 thymocytes, Rpl22 deficiency during the pro–B cell stage results in increased expression of p53 protein and its downstream targets that regulate growth and survival.

We next sought to determine whether the increase in p53 expression was responsible for the inability of Rpl22-deficient pro–B cells to respond to IL-7. To do so, we knocked down p53 expression by retroviral transduction of a vector encoding a p53 short hairpin RNA (19). $Rpl22^{+/+}Rag1^{-/-}$ and $Rpl22^{-/-}Rag1^{-/-}$

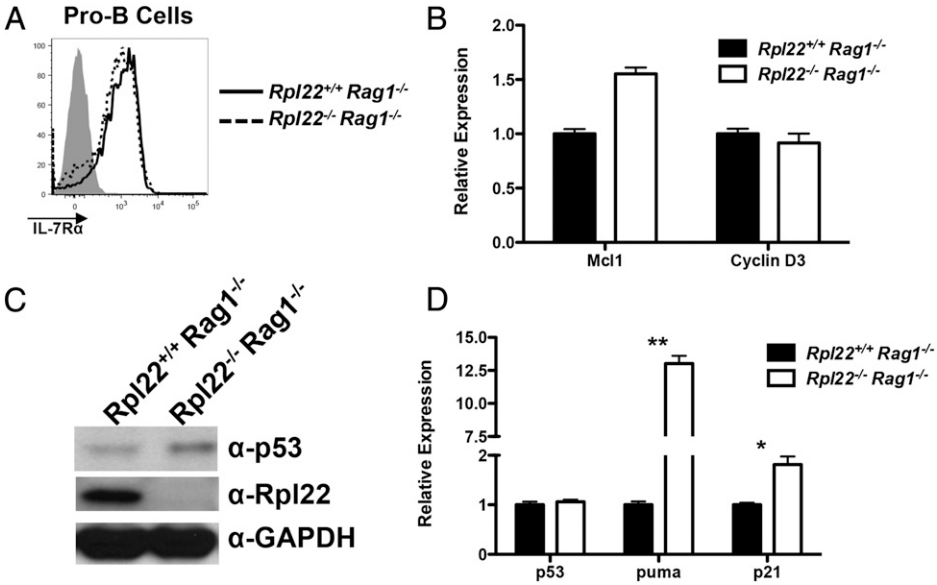

FIGURE 5. Increased p53 expression in Rpl22-deficient pro–B cells. (**A**) Surface expression of IL-7Rα on pro–B cells from $Rpl22^{+/+}Rag1^{-/-}$ and $Rpl22^{-/-}Rag1^{-/-}$ mice. Data are representative of three mice per genotype. (**B**) Quantitative real-time PCR analysis on sorted $Rpl22^{+/+}Rag1^{-/-}$ and $Rpl22^{-/-}Rag1^{-/-}$ pro–B cells cultured for 24 h in the presence of IL-7 and Q-VD-OPH. mRNA levels were normalized to the expression of GAPDH. Data are representative of two independent experiments. (**C**) Detergent extracts from sorted $Rpl22^{+/+}Rag1^{-/-}$ and $Rpl22^{-/-}Rag1^{-/-}$ pro–B cells cultured for 24 h in the presence of IL-7 and Q-VD-OPH were immunoblotted with anti-p53, Rpl22, or GAPDH Abs. Data are representative of two independent experiments. (**D**) Quantitative real-time PCR analysis on sorted $Rpl22^{+/+}Rag1^{-/-}$ and $Rpl22^{-/-}Rag1^{-/-}$ pro–B cells cultured for 24 h in the presence of IL-7 and Q-VD-OPH. mRNA levels were normalized to the expression of GAPDH. Data are representative of two independent experiments. p values were calculated for triplicate measurements within each experiment. $*p < 0.005$, $**p < 0.0005$.

pro–B cells were isolated and transduced with either the sh-p53-expressing retrovirus (MLP-shp53) or the empty vector control (MLP) and cultured in the presence of IL-7 for 72 h. Knockdown of p53 expression rescued the responsiveness of Rpl22-deficient pro–B cells to IL-7 relative to control MLP-transduced cells (Fig. 6A), demonstrating that increased p53 expression is responsible for the inability of Rpl22-deficient pro–B cells to respond to IL-7. To determine if p53 was responsible for the block in B cell development observed in $Rpl22^{-/-}$ mice in vivo, we crossed p53 knockout mice ($Tp53^{-/-}$) onto the $Rpl22^{-/-}$ background. Indeed, p53 deficiency completely rescued the absolute number of pro–B, pre–B, immature, and mature B cells in Rpl22-deficient mice (Fig. 6B, Supplemental Fig. 3A). In addition, p53 deficiency also completely rescued the absolute number of T1, T2, and T3 transitional B cell subsets and mature follicular B cells in the spleen (Fig. 6C, Supplemental Fig. 3B). Collectively, these results indicate that Rpl22 mediates a developmentally restricted function that regulates a crucial p53-dependent checkpoint during B cell development.

Discussion

Ribosomal proteins are key components of the ribosome, the cellular complex responsible for the translation of proteins. Recent reports, however, have demonstrated that several ribosomal proteins have specialized, tissue-restricted functions thought to be exerted either from within specialized ribosomes or outside of the ribosome in an extraribosomal manner (13). In particular, we have previously demonstrated that the ribosomal protein Rpl22 is not generally required for translation or life, but does perform a crucial and selective role in supporting traversal of the β-selection checkpoint by αβ T cell progenitors (19). Previous studies had also suggested that Rpl22 might also play a role in B cell development (19, 22), although the function of Rpl22 during B cell development was not examined. We have now demonstrated that Rpl22 is required during early B cell development in the bone marrow. Pro–B cells from Rpl22-deficient mice are unable to respond to IL-7, the crucial cytokine required during B cell development. Similar to T cell progenitors, Rpl22-deficient pro–B cells have increased protein expression of p53 as well as increased expression of downstream p53 target genes. Moreover, p53 deficiency is able to completely restore B cell development in the absence of Rpl22. These results demonstrate that Rpl22 is a crucial regulator of B cell development, but not peripheral B cell proliferation or activation, and that it does so through a p53-dependent checkpoint.

Ribosomal proteins play a critical role in supporting the structure and function of the ribosome. Consistent with their involvement in this essential task, mutations in ribosomal proteins can cause defects in ribosome biogenesis, leading to gross disruption of body morphology and embryonic lethality in zebrafish models (18, 31–33). Recently, however, mouse models haploinsufficient or deficient for several ribosomal proteins have been shown to exhibit tissue-specific defects, suggesting that some ribosomal proteins have tissue-restricted functions. For example, mice haploinsufficient for Rpl38 show unchanged global translation but exhibit tissue-specific patterning defects, especially in the axial skeleton, that correlate with elevated expression of *Rpl38* mRNA in these tissues in normal mice (17). Furthermore, we have recently reported that, despite Rpl22's ubiquitous expression, Rpl22 deficiency does not affect global protein synthesis or lead to embryonic lethality in mice or zebrafish (19, 34). Instead, we found that Rpl22 plays a crucial role in promoting traversal of the β-selection checkpoint during αβ T cell development, as well as in the survival of peripheral T cells following stimulation (19). We have now further demonstrated that Rpl22 deficiency also leads to a block in B cell development, starting at the pro–B cell stage, which results in a reduction in peripheral B cell numbers. Interestingly, although the number of follicular and B1 B cells was reduced in $Rpl22^{-/-}$ mice, the number of marginal zone B cells was unchanged. A relatively normal marginal zone B cell compartment is observed in several mouse

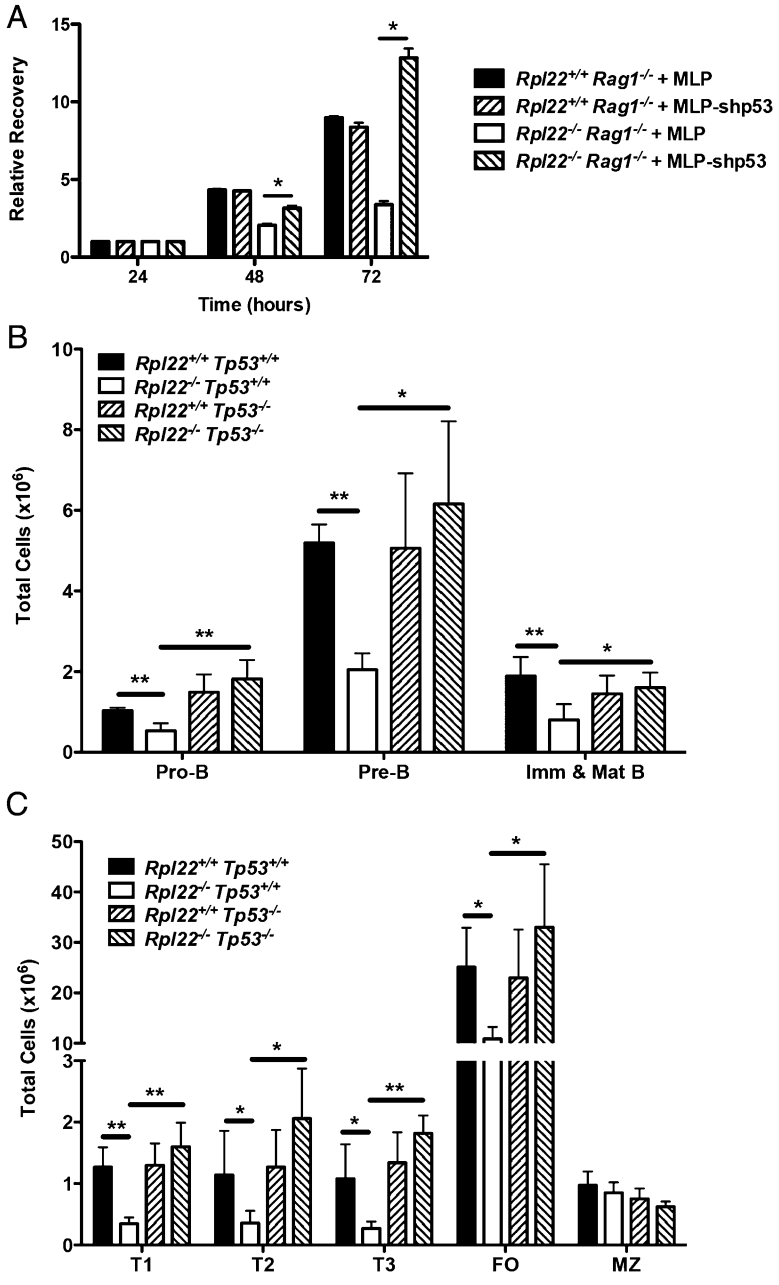

FIGURE 6. p53 deficiency rescues B cell development in the absence of Rpl22. (**A**) Relative recovery of $Rpl22^{+/+}Rag1^{-/-}$ and $Rpl22^{-/-}Rag1^{-/-}$ pro–B cells transduced with MLP or MLP-shp53 and cultured for 72 h in the presence of IL-7. Relative recovery was determined by normalization to the total number of GFP^+ cells present at 24 h posttransduction (designated 1.0). Data are representative of two independent experiments. *p* values were calculated for triplicate measurements within each experiment. $*p < 0.005$. (**B**) Absolute number of pro–B cells, pre–B cells, and immature and mature B cells (as defined in Fig. 3C) from the bone marrow of $Rpl22^{+/+}Tp53^{+/+}$, $Rpl22^{-/-}Tp53^{+/+}$, $Rpl22^{+/+}Tp53^{-/-}$, and $Rpl22^{-/-}Tp53^{-/-}$ mice. $n = 6$ mice per genotype. (**C**) Absolute number of T1, T2, T3, follicular (FO), and marginal zone (MZ) B cell subsets (as defined in Figs. 1B and 3D) from the spleens of $Rpl22^{+/+}Tp53^{+/+}$, $Rpl22^{-/-}Tp53^{+/+}$, $Rpl22^{+/+}Tp53^{-/-}$, and $Rpl22^{-/-}Tp53^{-/-}$ mice. $n = 6$ mice per genotype. $*p < 0.05$, $**p < 0.005$.

models in which the generation of peripheral B cells is impaired (35, 36), presumably due to a compensatory mechanism that diverts cells into this compartment to ensure that normal levels of natural Ab are maintained. Although our previous report described a potential role for Rpl22 in supporting the function of peripheral T cells (19), we have now determined that the functional deficit in $Rpl22^{-/-}$ peripheral T cells can be attributed to extensive homeostatic proliferation to compensate for the extreme T lymphopenia (data not shown), suggesting that Rpl22 function is dispensable in peripheral T cells. We report in this study that Rpl22 deficiency did not attenuate the ability of peripheral B cells to proliferate in response to mitogenic stimulation. It should be noted, however, that it is possible that Rpl22 does play a role in peripheral B cells that is masked in the periphery of $Rpl22^{-/-}$ mice by activation of a compensatory mechanism. Nevertheless, the simplest interpretation of these results is that Rpl22 function is selectively required in early B cell progenitors and dispensable in peripheral B cells. Why Rpl22 function is particularly crucial during the early stages of B and T cell development remains unclear. B and T cell progenitors undergo robust proliferation following successful rearrangements of the *IgH* and *TCRb* locus, which is rapidly triggered by signaling through the pre-BCR and pre-TCR, respectively, possibly due to their ability to self-oligomerize (37, 38). In contrast, following stimulation of peripheral B and T cells, there is a delay prior to the first cellular division, after which they rapidly divide (39, 40). Therefore, it is possible that differing proliferation kinetics play an important role in influencing the developmental restriction of the defects observed upon Rpl22 loss.

Although the Rpl22 deficiency impairs both B and T cell development, thereby causing reductions in peripheral B and T cell numbers, the defect was decidedly more pronounced in the T cell compartment, both during development in the thymus and in peripheral T cell populations (19). Why T cell development is more dependent on Rpl22 than B cell development is currently unknown. One possibility is that there is more robust proliferation associated with the β-selection checkpoint than the pre-BCR

checkpoint. Indeed, whereas T cell progenitors require at least four cell divisions prior to differentiation to the double-positive stage, B cell progenitors appear to undergo fewer cell divisions prior to their differentiation (41–43). Therefore, it remains possible that differing proliferation kinetics may also impact the extent of the defects observed in Rpl22-deficient mice, as well as their tissue specificity. Consistent with this possibility, whereas both B and T cell progenitors have enhanced p53 expression, the induction of p53 protein is less pronounced in B cell progenitors compared with T cell progenitors (19, 21), suggesting that the level of induction of the p53 pathway might be responsible for the different severities in the defects in early B and T cell development in the absence of Rpl22.

One of the common consequences of inactivation of a ribosomal protein is impaired ribosome biogenesis, which then activates the p53 pathway (31). Impaired ribosome biogenesis results in release of proteins from the nucleolus, including ribosomal proteins Rpl5, Rpl11, and Rpl23, which bind to MDM2 and inhibit its ability to induce degradation of p53 (44–46). Additionally, free Rpl26 directly increases p53 protein synthesis by binding to the 5′ untranslated region of p53 mRNA (47, 48). Although Rpl22 appears to be dispensable for ribosome biogenesis, we have observed an increase in p53 protein synthesis in Rpl22-deficient thymocytes, suggesting that Rpl22 acts to repress p53 protein synthesis, although the basis by which Rpl22 does so remains unclear (19, 21). Likewise, we also observe increased p53 protein levels in Rpl22-deficient pro–B cells with no alterations in the expression of p53 mRNA, suggesting that Rpl22 regulates p53 posttranscriptionally in both of these subsets. The p53 pathway is crucial during both the β-selection and pre-BCR checkpoints, and pre-TCR signaling is required to inactivate p53 to allow for differentiation to the double-positive stage (49–52). This suggests that one physiologic role of Rpl22 is to repress the p53 induction that normally accompanies traversal of the pre-BCR and β-selection checkpoints, respectively.

Although Rpl22 is not required for ribosome biogenesis or function and thus is unlikely inducing p53 through effects on either of these processes, it remains unclear how Rpl22 is able to regulate p53 protein expression. It is possible that Rpl22, like Rpl26, regulates p53 expression by directly binding to p53 mRNA. However, in contrast to Rpl26, which increases p53 translation, Rpl22 binding would be expected to inhibit translation. Rpl22 is an RNA-binding protein that recognizes a conserved stem-loop structure and has been shown to directly bind to EBER1 RNA, a latency RNA expressed by EBV, and the mRNA encoding Rpl22l1, a paralog of Rpl22 (22, 53). Other ribosomal proteins have been described to selectively bind subclasses of mRNA in a tissue-specific manner or in response to external signals. Rpl13a is released from the ribosome in response to IFN-γ and regulates the translation of GAIT element-containing mRNAs, whereas Rpl38 is required for efficient translation of a subset of Hox mRNAs in the neural tube during development (15–17). Nevertheless, because Rpl22 is ubiquitously expressed, direct and independent regulation of p53 by Rpl22 would be insufficient to explain the tissue restriction of p53 induction, because the loss of this function would be expected to cause widespread p53 induction. Accordingly, it is possible that other factors impinge on the requirement for Rpl22 to regulate p53 protein expression. p53 is activated in response to numerous stimuli, including the DNA damage and the unfolded protein response (54, 55). During B and T cell development, V(D)J recombination at the Ag receptor loci results in a DNA damage response, leading to the activation of p53 (49). However, we observed increased p53 expression in Rpl22-deficient Rag1-deficient pro–B cells, which are unable to undergo V(D)J recombination, arguing against activation of p53 downstream of this process. The unfolded protein response is active during the terminal differentiation of B cells into Ab-secreting cells, where a significant increase in the synthesis of Ab molecules occurs (56), and recent reports have demonstrated that the unfolded protein response is also active during early B and T cell development, presumably due to the robust proliferation of progenitors cells following successful Ag receptor rearrangements (57, 58). Therefore, it remains possible that activation of the unfolded protein response or some other p53-inducing pathway influences the susceptibility of particular tissues to p53 induction upon Rpl22 loss. Efforts are in progress to assess these possibilities.

In summary, these results demonstrate that Rpl22 plays a crucial role during B cell development. Although Rpl22 is required for B cell development within the bone marrow, it appears to be dispensable for peripheral B cell function. In the absence of Rpl22, pro–B cells have impaired survival and proliferation in response to IL-7. This hyporesponse is caused by enhanced activity of p53, although the mechanism by which Rpl22 controls p53 expression is unknown. Further studies to understand the direct targets of Rpl22 that are crucial for mediating Rpl22 function during B cell development should provide crucial insight into how Rpl22 mediates tissue-specific activities despite being globally expressed.

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Disclosures

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