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USP44 is dispensable for normal hematopoietic stem cell function, lymphocyte development, and B-cell-mediated immune response in a mouse model

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Ubiquitin-specific protease 44 (USP44) is a nuclear protein with deubiquitinase (DUB) catalytic activity that has been implicated as an important regulator of cell cycle progression, gene expression, and genomic stability. Dysregulation in the molecular machinery controlling cell proliferation, gene expression, and genomic stability in human or mouse is commonly linked to hematopoietic dysfunction, immunodeficiency, and cancer. We therefore set out to explore the role of USP44 in hematopoietic and immune systems through characterization of a *Usp44*-deficient mouse model. We report that USP44 is dispensable for the maintenance of hematopoietic stem cell numbers and function under homeostatic conditions, and also after irradiation or serial transplantation. USP44 is also not required for normal lymphocyte development. *Usp44*-deficient B cells show normal activation, proliferation, and immunoglobulin class switching in response to *in vitro* stimulation, and *Usp44*-deficient mice mount normal antibody response to immunization. We also tested the effects of USP44 deficiency on disease progression and survival in the Emu-myc model of mouse B-cell lymphoma and observed a trend toward earlier lethality of *Usp44*$^{-/-}$ Emu-myc mice; however, this did not reach statistical significance. Overall, we conclude that USP44 is dispensable for the normal physiology of hematopoietic and immune systems, and its functions in these systems are likely redundant with other USP family proteins.

Most cells of our blood and immune system are produced from hematopoietic stem cells (HSCs) in the bone marrow through the process of cell proliferation and differentiation, which is known as hematopoiesis. Immune response against infection or immunization involves multiple further cell proliferation, differentiation, and activation checkpoints. Dysregulation in the molecular mechanisms controlling cell cycle progression, gene expression, and genomic stability in hematopoietic and immune cells is commonly linked to bone marrow failure, immunodeficiency, and cancer.

Ubiquitin-specific protease 44 (USP44) is a nuclear protein with deubiquitinase (DUB) catalytic activity that comprises ZF-UBP and UCH domains [1,2]. USP44 is implicated as an important regulator of many cellular

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processes, including cell cycle progression, gene expression, and DNA repair.

USP44 acts to prevent premature activation of the anaphase promoting complex (APC), the ubiquitin ligase that triggers the separation of sister chromatids during mitosis. USP44 can deubiquitinate the CDC20 co-activating subunit of APC, thus preventing its dissociation from the mitotic checkpoint proteins MAD2, BUBR1, and BUB3 and inhibiting the activation of APC until all chromosomes are attached to the mitotic spindle [3]. USP44 is also implicated in the regulation of centrosome positioning and the organization of the mitotic spindle through its interactions with centrin [4].

USP44 is also an important regulator of gene expression and DNA repair via its DUB catalytic activities on histones H2B and H2A. Monoubiquitination of histone H2B at K120 by ubiquitin ligase RNF20 promotes gene expression and transcriptional elongation [5], whereas USP44 can reverse H2B-K120 ubiquitination and promote gene silencing [6]. Importantly, this USP44 activity was shown to be required for the normal differentiation of embryonic stem cells [6]. USP44 was also shown to localize to DNA damage foci, antagonize the RNF168-dependent polyubiquitination of histone H2A, and inhibit the recruitment of 53BP1 and other factors to the sites of DNA damage [7].

Both USP44 deficiency and overexpression predispose to errors in chromosome segregation, aneuploidy, and cancer [4,8]. *Usp44* knockout in mice results in increased susceptibility to lung adenomas [4], whereas in humans, *USP44* deletions or silencing are seen in lung and colorectal cancer [9,10]. In contrast, overexpression of *USP44* was observed in T-cell acute lymphoblastic leukemia [8], glioma [11], and gastric cancer [12].

Given the reported roles of USP44 in cell proliferation, differentiation, DNA repair, and carcinogenesis, and the expression of *Usp44* gene in hematopoietic and immune cells [13], we hypothesized that USP44 may be essential for these physiologic systems. We therefore conducted the first in-depth characterization of hematopoietic and immune functions of *Usp44*-knockout mice. Interestingly, other nuclear DUBs targeting ubiquitinated histones, such as USP3, USP16, and MYSM1, were recently reported to have essential roles in regulation of hematopoietic and immune functions [14-18]. We report that the loss of USP44 in mice is dispensable for the maintenance of HSC function, for lymphocyte development, and for B-cell-mediated immune responses. Loss of *Usp44* also did not have a significant effect on the onset and progression of Emu-myc mouse B-cell lymphoma.

Methods

Mouse line and allele validation

The *Usp44*$^{tm1b(EUCOMM)Hmgu}$ mouse line, also known as *Usp44*$^{-/-}$, was generated by the Mouse Genetics Program of the Wellcome Trust Sanger Institute for the International Mouse Phenotyping Consortium (IMPC) [19,20] and carries deletion of *Usp44* exon *ENSMUSE00001396196*. The mouse line is therefore derived independently from the previously described *Usp44*$^{tm1.2Pjgl}$ strain [4], but it carries deletion of the same exon and therefore is expected to be functionally equivalent. The deleted exon encodes the first 475 out of 711 amino acids of USP44 protein, including the catalytic site. Information on the *Usp44*$^{-/-}$ allele structure and its validation conducted by the IMPC mouse production pipeline is available online at: www.infrafrontier.eu/search?keyword=Usp44; www.mousephenotype.org/about-ikmc/targeting-strategies; and https://mpi2.github.io/IKMC-knowledgebase/ikmc/screens_and_quality_control/wtsi_mouse_clinic_quality_control_tests__mice. Allele structure was additionally validated in this study through extensive *Usp44* locus genotyping, sequencing, and quantitative reverse transcription polymerase chain reaction (qRT-PCR) analyses (Supplementary Figure E1 and Supplementary Tables E1 and E2, online only, available at www.exphem.org). *Usp44*$^{-/-}$ mice were crossed with the B6.Cg-Tg(IghMyc)22Bri/J mouse model of B-cell lymphoma (Emu-myc, JAX: 002728) [21]. All lines were maintained on the C57BL/6 background under specific pathogen-free conditions. All procedures were in accordance with the guidelines of the Canadian Council on Animal Care and the animal use protocol approved by the McGill University Animal Care Committee.

Whole-body irradiation

Mice were irradiated with a single dose of 6 or 7 Gy in an RS2000 irradiator (Rad Source) and their survival was monitored.

Bone marrow transplantation

Recipient mice were lethally irradiated with two doses of 4.5 Gy in an RS2000 irradiator (Rad Source) and injected intravenously with 2 million donor bone marrow cells. For the primary transplantation, the donor cells consisted of a 1:1 mixture of CD45.1$^+$ and CD45.2$^+$ bone marrow cells, with the CD45.2$^+$ cells derived either from the control *Usp44*$^{+/+}$ or the knockout *Usp44*$^{-/-}$ mice. The primary recipients were analyzed at >20 weeks after reconstitution, and the experiment was repeated twice with at least four recipient mice per group per experiment. For the secondary transplantation, 2 million donor bone marrow cells harvested from the primary recipients were transplanted into new groups of lethally irradiated recipient mice and these secondary recipients were analyzed after further >20 weeks.

Flow cytometry

Flow cytometry was performed as previously described [17,22]. A list of antibodies is provided in the supplementary materials (online only, available at www.exphem.org).

Immunization

Mouse immunizations, followed by ELISA measurements of antigen-specific serum antibody titers, were performed as described by Förster et al. [17], with full protocols provided

in the supplementary materials (online only, available at www.exphem.org).

In vitro B-cell stimulation assays

The assays were performed as described by Förster et al. [17], with full protocols provided in the supplementary materials (online only, available at www.exphem.org).

Statistical analyses

Statistical comparisons were performed with Prism version 7.01 software (GraphPad Software) using the Student *t* test for two groups, ANOVA with Bonferroni post hoc test for multiple comparisons and Kaplan–Meier regression analysis for survival data.

Results and Discussion

Usp44 *is dispensable for HSC function and radioresistance*

Usp44-deficient mice were viable, born in normal numbers, and had no obvious dysmorphology (Supplementary Figure E1 and Supplementary Table E3, online only, available at www.exphem.org), consistent with previous reports [4]. Hematopoietic and lymphoid organs were normal in size and cellularity (Supplementary Figure E2, online only, available at www.exphem.org). Mouse bone marrow was analyzed for the numbers of hematopoietic stem and progenitor cells, gated as $Lin^{-}cKit^{+}Sca1^{+}$ and subdivided into HSCs and multipotent progenitors (MPPs) based on CD150, CD48, CD34, and FLT3 marker expression. No differences were seen in the frequencies or absolute numbers of HSCs and MPPs between genotypes (Figures 1A–1C). Serial bone marrow transplantations were performed as a test of HSC function, comparing the capacity of $Usp44^{+/+}$ and $Usp44^{-/-}$ donor cells to engraft hematopoiesis in lethally irradiated recipients in direct competition with $CD45.1^{+}$ allotype-marked cells. No defects in $Usp44^{-/-}$ HSC function were observed, with normal $Usp44^{-/-}$ contribution to the myeloid cell lineage (Figure 1D) and to stem cell populations (Figures 1E–1F) in both primary and secondary recipients. As a further measure of stress hematopoiesis, the mice were challenged with whole-body irradiation (6–7 Gy) and no differences in survival were observed between the $Usp44^{+/+}$ and $Usp44^{-/-}$ genotypes (Figure 1G). We conclude that USP44 is dispensable for normal maintenance of HSC numbers and function under homeostatic conditions and after serial transplantation or irradiation.

Usp44 *is dispensable for lymphocyte development*

Detailed flow cytometry analysis of hematopoietic and lymphoid organs of $Usp44^{+/+}$, $Usp44^{+/-}$, and $Usp44^{-/-}$ mice was carried out to assess B- and T-cell development. $Usp44^{-/-}$ mice had normal frequencies and absolute numbers of all the major thymocyte populations, as well as mature CD4 T cells, CD8 T cells, and natural killer (NK) cells in peripheral lymphoid organs (Figures 2A and 2C–2E). B-cell development was also found to be normal (Figures 2B, 2F, and 2G). Lymphocyte development also progressed normally following transplantation of $Usp44^{-/-}$ bone marrow into lethally irradiated recipients in direct competition against wild-type $CD45.1^{+}$ cells, with normal reconstitution of $Usp44^{-/-}$ T cells, B cells, and NK cells in both primary and secondary recipients (Figure 2H).

Usp44 *is dispensable for B-cell-mediated immune response*

Given the high expression of *Usp44* in activated B cells [13], we further analyzed the responses of $Usp44^{-/-}$ B cells to *in vitro* stimulation and observed normal induction of activation markers and costimulatory molecules (Figures 3A and 3B), as well as normal cell proliferation, survival, and immunoglobulin class switching (Figures 3C–3G). $Usp44^{-/-}$ mice also produced normal titers of antigen-specific antibodies of diverse isotypes following immunization (Figure 3H). Overall, we conclude that *Usp44*-deficiency does not impair B-cell function and antibody-mediated immunity.

Effects of Usp44 *deficiency on B-cell lymphoma progression*

To study the effects of *Usp44*-loss on hematologic malignancy, $Usp44^{-/-}$ mice were crossed to the Eμ-myc mouse line [21], which is highly susceptible to B-cell lymphoma due to cMyc overexpression in B cells. Although there was some trend toward earlier lethality of the $Usp44^{-/-}$ Eμ-myc mice relative to the $Usp44^{+/+}$ Eμ-myc group, the combined comparison among the $Usp44^{-/-}$, $Usp44^{+/-}$, and $Usp44^{+/+}$ Eμ-myc genotypes did not reach statistical significance (Figure 3I).

USP44 was previously implicated as an important regulator of cell cycle progression, gene expression, and DNA repair using studies *in vitro* and in cell lines [3,6,7]. Nevertheless, normal viability of $Usp44^{-/-}$ mice reported here and previously [4] suggests that USP44 functions may be redundant with other USP family proteins or may be cell-type restricted. Based on in-depth phenotypic analyses, we conclude that USP44 is dispensable for HSC function under homeostatic and stress conditions, for lymphocyte development, and also for B-cell activation, immunoglobulin class switching, and antibody-mediated immune response. *Usp44* loss also did not have significant effects on Eμ-myc B-cell lymphoma onset and progression. This is consistent with previous reports showing that aged $Usp44^{-/-}$ mice have an elevated incidence of lung adenoma, but only a mild increase in lymphomas [4].

USP44 functions in other physiologic systems beyond hematopoiesis merit further investigation. Indeed, the

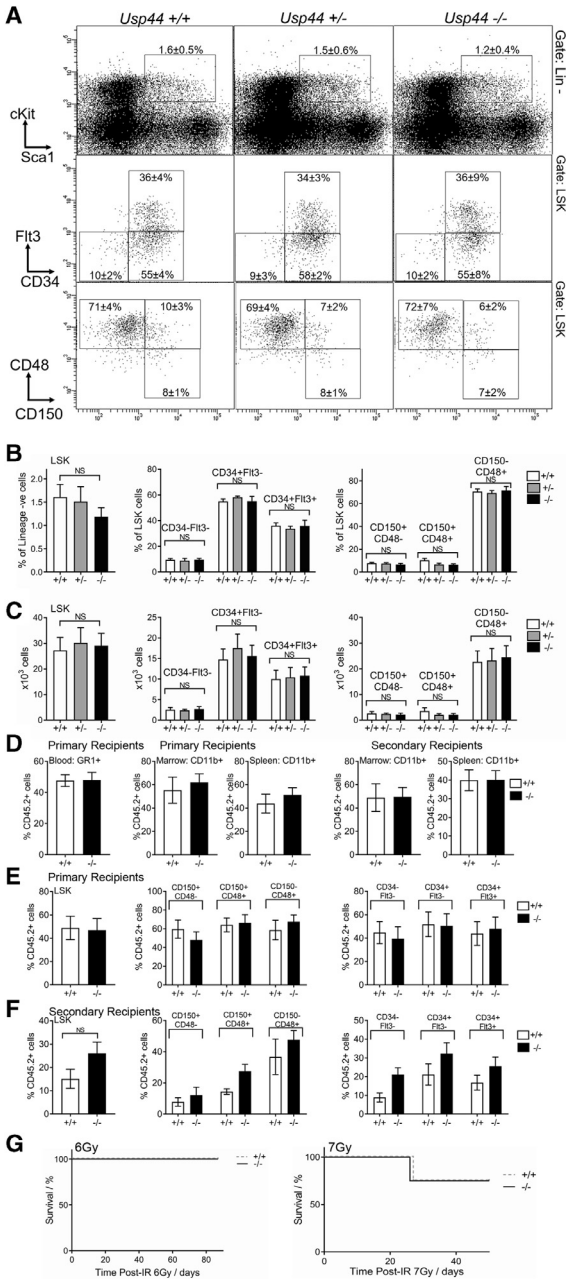

Figure 1. Loss of *Usp44* does not impair HSC function. **(A–C)** Flow cytometry analysis of HSCs and MPPs in the bone marrow of $Usp44^{+/+}$, $Usp44^{+/-}$, and $Usp44^{-/-}$ mice. **(A)** Representative flow cytometry dot plots. Hematopoietic stem and progenitor cells were gated as $Lin^{-}cKit^{+}Sca1^{+}$ (LSK, top panel), followed by either CD34/Flt3 or CD150/CD48 to separate the subpopulations. HSCs were gated as LSK $CD34^{-}Flt3^{-}$ (middle panel) or LSK $CD150^{+}CD48^{-}$ (bottom panel). **(B,C)** Numerical comparisons of the **(B)** frequencies and **(C)** absolute numbers of LSKs, MPPs, and HSCs in the bone marrow of the mice. **(D–F)** Analysis of $Usp44^{-/-}$ HSC function using serial bone marrow transplantation. For the primary transplantation, two cohorts of lethally irradiated recipient mice were injected intravenously with a 1:1 mixture of $CD45.1^{+}$ and $CD45.2^{+}$ donor bone marrow cells, with the $CD45.2^{+}$ cells derived either from the control $Usp44^{+/+}$ or the test $Usp44^{-/-}$ mice. For secondary transplantations, bone marrow cells harvested from the primary recipients were injected into secondary cohorts of lethally irradiated recipients. All recipients were analyzed at >20 weeks after reconstitution, comparing the contribution of control $Usp44^{+/+}$ $CD45.2^{+}$ and test $Usp44^{-/-}$ $CD45.2^{+}$ HSCs with the different hematopoietic cell types and lineages. **(G)** Mouse survival following total body irradiation at the dose of 6 Gy (left panel) or 7 Gy (right panel), comparing the radioresistance of $Usp44^{+/+}$ and $Usp44^{-/-}$ mouse bone marrow. Data are from four or five mice per group per experiment and were reproduced in two independent experiments, with the exception of secondary bone marrow transplantation, which was carried out in a single experiment. Bars represent mean ± SEM; statistical analysis was done using the Student *t* test for two groups, ANOVA with Bonferroni post hoc test for multiple comparisons and Kaplan–Meier regression analysis and log–rank test for survival data; no significant differences were found. NS=nonsignificant.

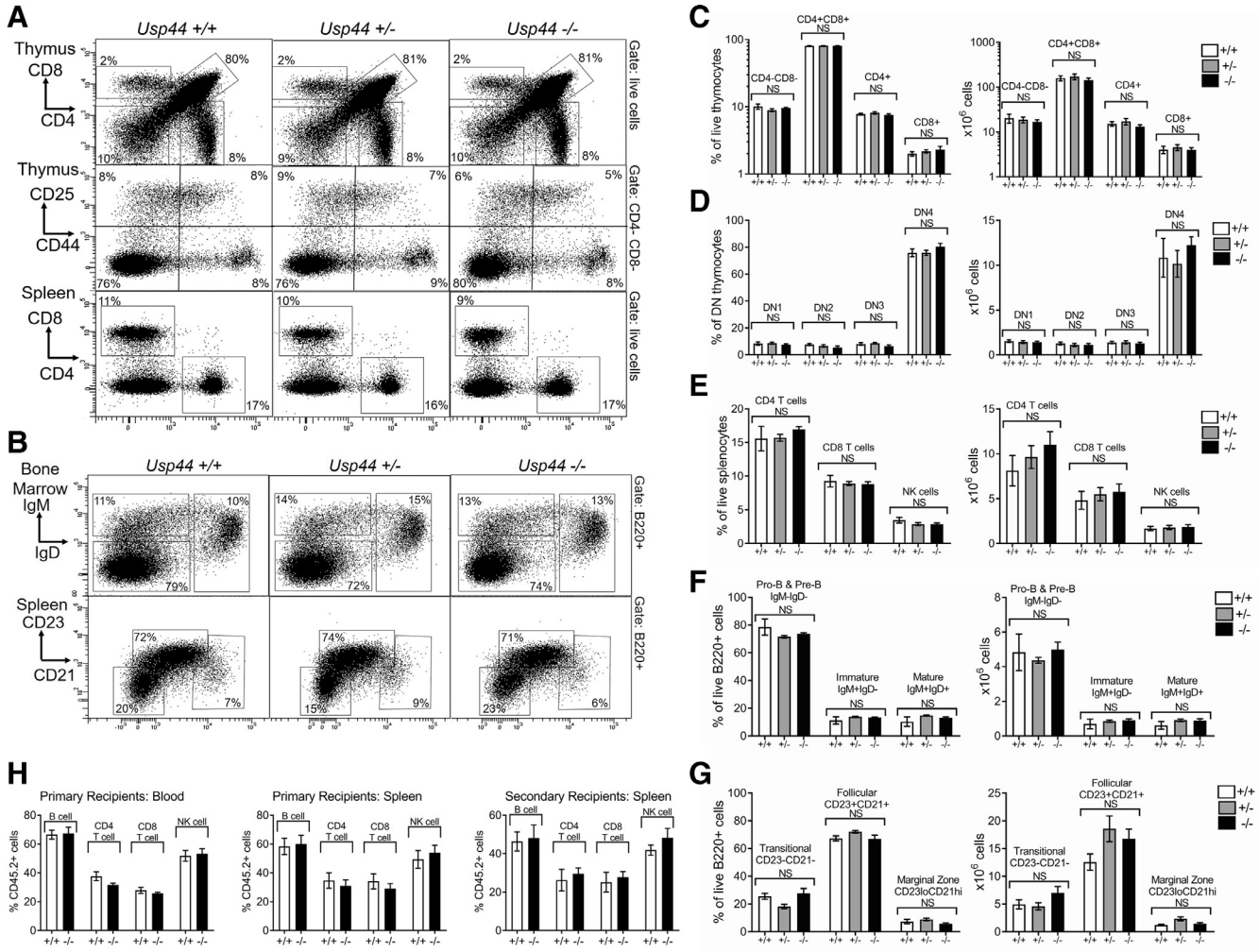

Figure 2. Loss of USP44 does not impair lymphocyte development. **(A–G)** Flow cytometry analysis of T- and B-cell development in the thymus, bone marrow, and spleen of *Usp44*$^{+/+}$, *Usp44*$^{+/-}$, and *Usp44*$^{-/-}$ mice. **(A)** Representative flow cytometry dot plots of the thymus and spleen showing CD4$^-$CD8$^-$ double-negative (DN), CD4$^+$CD8$^+$ double-positive (DP), and CD4$^+$CD8$^-$ and CD4$^-$CD8$^+$ single-positive cells, as well as CD4$^-$CD8$^-$CD44$^+$CD25$^-$ DN1, CD4$^-$CD8$^-$CD44$^+$CD25$^+$ DN2, CD4$^-$CD8$^-$CD44$^-$CD25$^+$ DN3, and CD4$^-$CD8$^-$CD44$^-$CD25$^-$ DN4 cells. **(B)** Representative flow cytometry dot plots of the bone marrow and spleen showing B220$^+$IgM$^-$IgD$^-$ pro- and pre-B cells, B220$^+$IgM$^+$IgD$^-$ immature B cells, and B220$^+$IgM$^+$IgD$^+$ mature B cells in the bone marrow (top panel), as well as B220$^+$CD23$^-$CD21$^-$ transitional, B220$^+$CD23$^+$CD21$^+$ follicular, and B220$^+$CD23^{lo}CD21^{hi} marginal zone B cells in the spleen. **(C–G)** Numerical comparisons of the frequencies and absolute numbers of **(C,D)** thymocytes, **(E)** splenic T cells and NK cells, **(F)** bone marrow B-cell precursors, and **(G)** splenic B-cell populations in *Usp44*$^{+/+}$, *Usp44*$^{+/-}$, and *Usp44*$^{-/-}$ mice. **(H)** Reconstitution of B cells, T cells, and NK cells in the blood and spleen by donor *Usp44*$^{+/+}$ or *Usp44*$^{-/-}$ HSCs following competitive bone marrow transplantation. For primary transplantation, two cohorts of lethally irradiated recipient mice were injected intravenously with a 1:1 mixture of CD45.1$^+$ and CD45.2$^+$ donor bone marrow cells, with the CD45.2$^+$ cells derived either from the control *Usp44*$^{+/+}$ or the test *Usp44*$^{-/-}$ mice. For secondary transplantation, bone marrow harvested from the primary recipients was injected into secondary cohorts of lethally irradiated recipients. All recipients were analyzed at >20 weeks after reconstitution, comparing the contribution of control *Usp44*$^{+/+}$ CD45.2$^+$ and test *Usp44*$^{-/-}$ CD45.2$^+$ cells to lymphocyte populations. Data are from four or five mice per group per experiment and were reproduced in two independent experiments, with the exception of secondary bone marrow transplantation, which was carried out in a single experiment. Bars represent mean ± SEM; statistical analysis was done using the Student *t* test for two groups or ANOVA with Bonferroni post hoc test for multiple comparisons; no significant differences were found. NS=nonsignificant.

Usp44$^{-/-}$ mouse strain is undergoing extensive phenotypic characterization of other physiologic systems with IMPC and the resulting data may serve as a foundation for further studies (www.mousephenotype.org/data/genes/MGI:3045318#section-associations). Possible functional redundancies between USP44 and other family members also need to be further explored. Phylogenetic analyses point to close homology of USP44 and USP49 [23–25] and the proteins also have many functional similarities; USP49 is also a nuclear protein [26] that can deubiquitinate histone H2B-K120ub [27], is recruited to DNA damage foci [28], and can interact with centrins [26]. Further data mining [29,30] and our qRT-PCR analyses indicate that *Usp49* is expressed in hematopoietic cells at similar

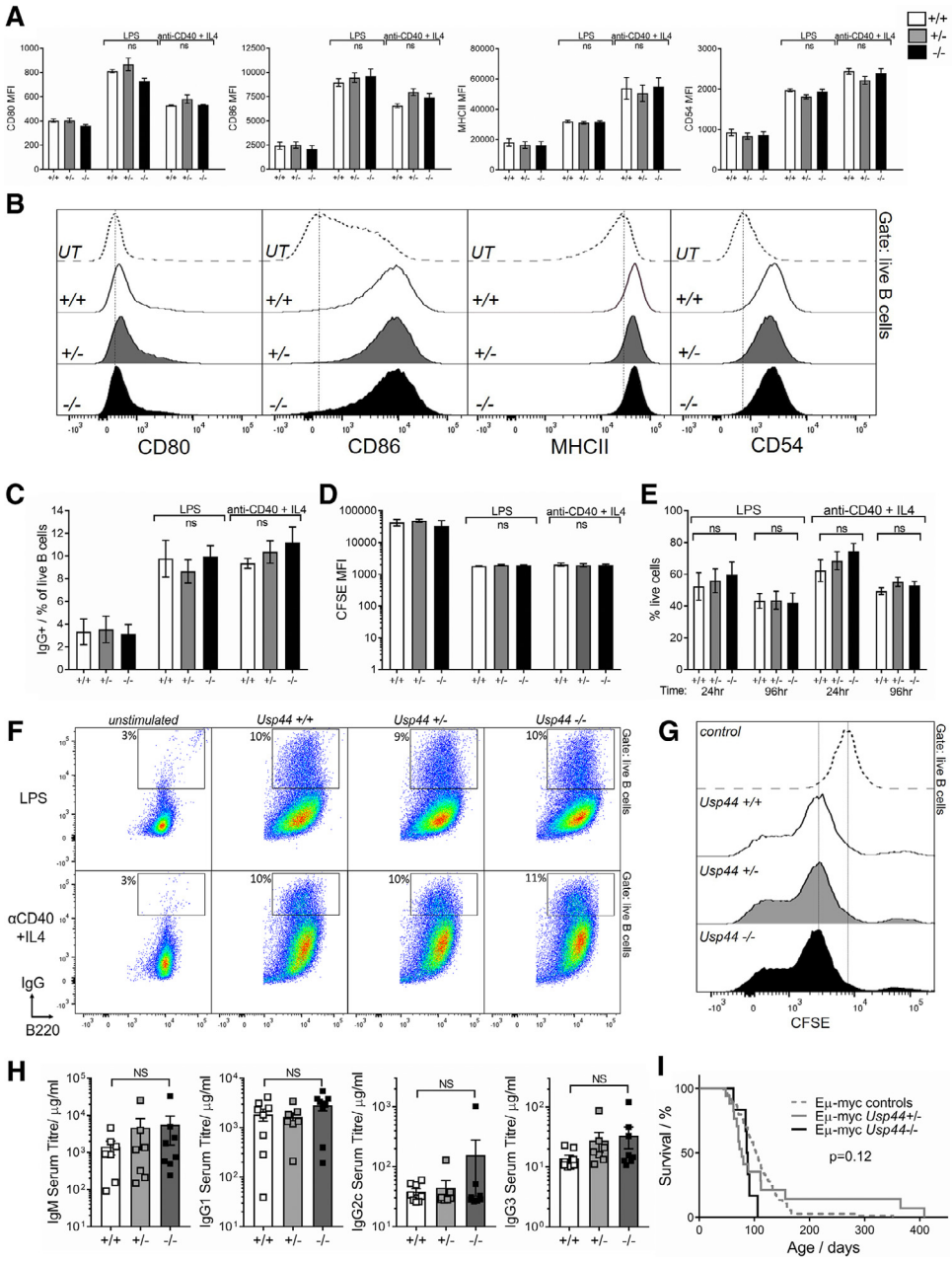

Figure 3. Loss of USP44 does not impair B-cell activation, immunoglobulin class switching, or antibody production. **(A–G)** Analyses of the activation, class switching, proliferation, and survival of *Usp44*$^{+/+}$, *Usp44*$^{+/-}$, and *Usp44*$^{-/-}$ B cells isolated through magnetic enrichment from mouse spleen, stimulated *in vitro* with lipopolysaccharide (LPS; 1 μg/mL) or anti-CD40 (2 μg/mL) + interleukin-4 (5 ng/mL), and analyzed by flow cytometry, gating on live B cells (B220^{+}). **(A,B)** Bar charts and representative histograms showing expression of CD80, CD86, MHCII, and CD54 activation markers at 24 hours of stimulation. MFI=mean fluorescence intensity. **(B)** CD80 and CD86 data are from LPS stimulation, whereas MHCII and CD54 data are from CD40 + IL4 stimulation. **(C)** Percentage of IgG^{+} cells in the cultures at 96 hours of stimulation as a measure of immunoglobulin class switching. **(D)** Analysis of carboxyfluorescein diacetate succinimidyl ester (CFSE) staining intensity of the cells at 96 hours of stimulation as a measure of cell proliferation. **(E)** Percentage of live cells in cultures at 24 and 96 hours of stimulation. **(F)** Representative flow cytometry dot plots showing IgG^{+} class-switched B cells. **(G)** Representative histograms showing CFSE staining intensity of the cells at 96 hours of LPS stimulation as a measure of cell proliferation. **(H)** Titers of antigen-specific antibodies of the IgM, IgG1, IgG2c, and IgG3 isotypes in the serum of *Usp44*$^{+/+}$, *Usp44*$^{+/-}$, and *Usp44*$^{-/-}$ mice at day 14 after subcutaneous immunization with R-phycoerythrin in Complete Freund's Adjuvant. Data are from four to eight mice per group per experiment and were reproduced in two independent experiments, except for cell proliferation and immunization assays, which were carried out in a single experiment. Bars represent mean ± SEM; statistical analyses use ANOVA with Bonferroni post hoc test; no significant differences were found. NS=nonsignificant. **(I)** Survival of Emu-myc *Usp44*$^{+/+}$ ($n = 80$), Emu-myc *Usp44*$^{+/-}$ ($n = 17$), and Emu-myc *Usp44*$^{-/-}$ ($n = 6$) mice; $p = 0.14$, log–rank test; $p = 0.12$, Gehan–Breslow–Wilcoxon test (GraphPad Prism 7.01).

or higher levels than *Usp44* (Supplementary Figure E3 and Supplementary Table E2, online only, available at www.exphem.org) and normal *Usp49* expression persists in *Usp44*-knockout (Supplementary Figure E3C, online only, available at www.exphem.org). Future studies should explore possible functional redundancies between USP44 and USP49 to determine whether USP49 may compensate for USP44 loss in hematopoiesis.

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Conflict of interest disclosure

The authors declare no competing financial interests.

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