The differentiation of several T- and B-cell effector programs in the immune system is directed by signature transcription factors that induce rapid epigenetic remodelling. Here we report that promyelocytic leukaemia zinc finger (PLZF), the BTB-zinc finger (BTB-ZF) transcription factor directing the innate-like effector program of natural killer T-cell thymocytes14, is prominently associated with cullin 3 (CUL3), an E3 ubiquitin ligase previously shown to use BTB domain-containing proteins as adaptors for substrate binding15,16. PLZF translocates CUL3 to the nucleus, where the two proteins are associated within a chromatin-modifying complex. Furthermore, PLZF expression results in selective ubiquitination changes of several components of this complex. CUL3 was also found associated with the BTB-ZF transcription factor BCL6, which directs the germinal-centre B cell and follicular T-helper cell programs. Conditional CUL3 deletion in mice demonstrated an essential role for CUL3 in the development of PLZF- and BCL6-dependent lineages. We conclude that distinct lineage-specific BTB-ZF transcription factors recruit CUL3 to alter the ubiquitination pattern of their associated chromatin-modifying complex. We propose that this new function is essential to direct the differentiation of several T- and B-cell effector programs, and may also be involved in the oncogenic role of PLZF and BCL6 in leukaemias and lymphomas17,18.

To investigate the molecular mechanisms that PLZF uses to regulate the innate-like natural killer T (NKT)-cell thymocyte differentiation program during development, we examined its protein-interaction partners. NKT-cell thymocytes were purified from Vα14-Jα18-transgenic mice and, after immunoprecipitation with an anti-PLZF antibody, associated proteins were submitted to mass spectrometry analysis (Table 1, columns 1, and Supplementary Fig. 1). A major group was composed of nuclear proteins involved in binding and modifying chromatin, including HDAC1 and DNMT1, which were previously reported to be ‘adaptors’ for CUL3-mediated ubiquitination by binding both CUL3 and its substrates19,20. Reciprocal immunoprecipitation of CUL3-associated proteins pulled down PLZF as a major protein along with an overlapping set of proteins (Table 1, column 2, and Supplementary Fig. 1). Furthermore, confocal microscopic analysis of NKT-cell thymocytes demonstrated colocalization of the two proteins in a speckled nuclear pattern (Fig. 1a, top).

By contrast, in the major lineage of CD4 T cells, CUL3 was mainly found in the cytosol with only a faint presence in nuclear speckles (Fig. 1a, middle). However, after expression of a CD4 promoter-driven PLZF transgene, which induces developmental acquisition of the NKT-cell lineage effector program1–3, CUL3 was mostly in the nucleus, colocalizing with PLZF in nuclear speckles (Fig. 1a, bottom). A similar binding and transport of CUL3 from the cytoplasm to the nucleus was previously demonstrated after cotransfection with the nuclear BTB-domain-containing protein speckle-type POZ protein (SPOP) in HeLa cells17. Mass spectrometric analysis of anti-PLZF and anti-CUL3 immunoprecipitates from PLZF-transgenic thymocytes identified a similar set of proteins as that from NKT-cell thymocytes (Table 1, columns 3 and 4, and Supplementary Fig. 2), including other known partners of PLZF such as NCOR and SIN3A (ref. 9). Western blot analyses confirmed that a fraction of PLZF coprecipitated with CUL3, and that chromatin-binding and -modifying proteins such as HDAC1, SATB1 and lamin B1 were associated with the PLZF–CUL3 complex (Fig. 1b). The specificity of the interaction between PLZF and CUL3 was further tested using in vitro translated proteins, and shown to depend on the CUL3 residues Leu 52 and Glu 55 (Supplementary Table 1 | PLZF–CUL3 interactions

| Ubiquitination | NKT | Anti-PLZF IP | Anti-CUL3 IP | PLZF-transgenic | Anti-PLZF IP | Anti-CUL3 IP |
|----------------|-----|-------------|-------------|----------------|-------------|-------------|
| CUL3 | PLZF | H4 | H1.3 | CUL3, CUL4B, CAND1 | PLZF, BTBD11 | H4 | H1.5, H1.2 |
| LMNB1, DNMT1, SATB1, HDAC1, HP1BP3 | LMNB1, DNMT1, BAZ1B, EZH2, HP1BP3 | TOP2A, TOP2A, EEF2 | TOP2A, EEF2 | H2A.1 |
| H2A.1, H2B.1 | TOP2A, EEF2 | NUP155 | NUP155 | H2A.1 |
| H2B.1 | NUP153, NUP214, NUP98, NUP88 | TOP2A, TOP2A, TOP1, NUP205 |

Mass spectrometric analysis of proteins immunoprecipitated by anti-PLZF and anti-CUL3 antibodies from indicated thymocyte populations (data from two to three independent experiments). Other proteins that did not belong to the indicated categories are shown with the complete data sets in Supplementary Figs 1 and 2. Analysis of Gene Ontogeny term enrichment demonstrates P values ranging from 10−3 to 10−5 for nuclear transcriptional and chromatin-organization proteins. IP, immunoprecipitation.

1Committee on Immunology, Department of Pathology, The Howard Hughes Medical Institute, University of Chicago, Chicago, Illinois 60637, USA. 2Department of Biology, Portland State University, Portland, Oregon 96207, USA.

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...at least three independent experiments. 20% of the input lysate before immunoprecipitation. Data are representative of immunoprecipitates (IP) from PLZF-transgenic thymocytes. 20% IN refers to which regulates DNMT1 through ubiquitylation25; H2A.1, which is identified in the complex. The former included CAND1, CUL3–PLZF complex identified in our previous immunoprecipitation experiments, or were well-known interaction partners of one or several proteins (Fig. 2a). Notably, 14 of these 48 proteins were either components of the CUL3–PLZF complex identified in our previous immunoprecipitation experiments, or were well-known interaction partners of one or several proteins identified in the complex. The former included CAND1, LMNB1, DNMT1, SATB1 and H1.2. The latter included UHRF1, which regulates DNMT1 through ubiquitylation26; H2A.1, which is regulated by CUL3 in SPOP–CUL3 complexes26; H2A.2, which is loaded by EP400 onto chromatin27; the deubiquitinase BRCC3 interacting with H2A, H2B, H2A.X and CAND1 (ref. 28); and the repressor ASXL1 interacting with CBX5 and EZH2 (refs 27, 29). These results are summarized in a protein-interaction diagram in Fig. 2a.

Increased ubiquitination of the key nuclear proteins SATB1 and lamin B1 was directly confirmed by immunoprecipitation and western blot analysis of transfected HeLa cells confirmed this association (Supplementary Fig. 4). Rapid changes in ubiquitination pattern have recently been reported in chromatin-remodelling situations and are thought to regulate gene expression28–30. By bringing CUL3 from the cytosol to chromatin-modifying complexes in the nucleus, PLZF might be expected to induce changes in ubiquitination. This was tested using an unbiased ubiquitination proteomics method comparing whole cell lysates of PLZF-transgenic and wild-type littermates. Independent experiments with different batches of mice identified 48 proteins showing concordant changes, most of which consisted of increased ubiquitination in PLZF-transgenic cells (Fig. 2a and Supplementary Fig. 5). Notably, 14 of these 48 proteins were either components of the CUL3–PLZF complex identified in our previous immunoprecipitation experiments, or were well-known interaction partners of one or several proteins identified in the complex. The former included CAND1, LMNB1, DNMT1, SATB1 and H1.2. The latter included UHRF1, which regulates DNMT1 through ubiquitylation; H2A.1, which is regulated by CUL3 in SPOP–CUL3 complexes; H2A.Z, which is loaded by EP400 onto chromatin; the deubiquitinase BRCC3 interacting with H2A, H2B, H2A.X and CAND1 (ref. 28); and the repressor ASXL1 interacting with CBX5 and EZH2 (refs 27, 29). These results are summarized in a protein-interaction diagram in Fig. 2a.

Increased ubiquitination of the key nuclear proteins SATB1 and lamin B1 was directly confirmed by immunoprecipitation and western blot analysis of transfected 293T cells. In these experiments, SATB1 and lamin B1 were overexpressed because technical limitations made it impossible to determine the ubiquitination pattern of endogenous proteins (Fig. 2b). Furthermore, ubiquitination was shown to require the E2-binding domain of CUL3, supporting the conclusion that PLZF can directly ubiquitinate these PLZF-associated proteins in vivo. In confocal microscopy experiments, PLZF and CUL3 were colocalized at lamin B1-positive sites in the nuclear lamina and at SATB1 sites of the nuclear matrix in a typical cage-like pattern (Supplementary Fig. 6). Furthermore, CUL3 was present at PLZF-bound promoters shown by chromatin immunoprecipitation coupled to quantitative PCR (ChIP-qPCR) (Supplementary Fig. 7), suggesting that PLZF–CUL3 complexes associate at PLZF-binding promoters across the genome and in nuclear subcompartments involved in gene expression or repression.

To explore further the functional role of CUL3 in lymphocyte development and function, in which BTB-ZF transcription factors K-GG antibodies (UbiScan) from wild-type and PLZF-transgenic thymocytes showed differential ubiquitination of the proteins depicted in blue-filled ovals in a global diagram of PLZF- and CUL3-interaction partners, with enrichment for proteins involved in chromatin organization (P < 10–6). Peptides from two independent experiments are listed in Supplementary Fig. 5. Red lines link proteins identified by coprecipitation in this study; grey lines represent previously established protein interactions (Ingenuity Pathway).

By contrast, the number of NKT cells was massively decreased, with a modest but statistically significant twofold increase (Fig. 3a). However, the number of NKT cells was massively decreased, with a modest but statistically significant twofold increase (Fig. 3a). By contrast, the number of NKT cells was masssively decreased, with a sharp developmental block occurring at the CD24+CD4−NK1.1− stage 1, similar to the block described in mice lacking PLZF1,2 (Fig. 3b and Supplementary Fig. 8). These results support the importance of the PLZF–CUL3 interaction in NKT-cell development.

Intriguingly, in older mice lacking CUL3 in T cells, the spleen and lymph nodes became enlarged, the result of a net increase in B-cell numbers with spontaneous formation of germinal centres made of peanut agglutinin (PNA)− B cells (Fig. 3c, d). Whereas thymic CD4+CD8− T cells exhibited a normal phenotype, a population of splenic CD4 T cells expressing a PD1+ CCR5+ follicular T-helper
cell phenotype progressively accumulated in ageing mice, concomitantly with GL7⁺ Fas⁻ germinal-centre B cells (Fig. 3e). Consistent with these findings, immunohistological analysis demonstrated large germinal centres with penetration of the B-cell follicles by CD4 T cells (Fig. 3d). In radiation chimaeras reconstituted with a 3:1 mixture of wild-type and Cul3⁻/⁻/+ T cells, the CUL3-compartment showed absence of NK T cells and increased follicular CD4 T-helper cells, demonstrating the cell-intrinsic nature of these defects (Fig. 3f). As expected, germinal-centre B cells of both compartments were indiscriminately expanded. Other effector programs available to CD4 T cells, however, appeared unperturbed as Cul3-deficient CD4 T cells normally expanded and differentiated towards T-helper 1 (Th1), Th17 or Th17 effector cells in vitro (Supplementary Fig. 8).

Mice lacking CUL3 in B cells showed normal development of follicular B cells but exhibited a selective four- to fivefold reduction of marginal-zone B cells in the spleen and of B1 B cells in the peritoneum (Fig. 4a). These cell-intrinsic defects were considerably amplified in the competitive environment of mixed bone marrow chimaeras (Fig. 4b, c). Whereas the circulating levels of immunoglobulins were normal or modestly decreased (Fig. 4d) and the antibody response to the T-cell-independent antigen dinitrophenylated Ficoll (DNF-Ficoll) appeared conserved (Fig. 4e), T-cell-dependent B-cell responses exhibited various defects. We noted that the germinal-centre B cells that spontaneously develop in the mesenteric lymph nodes of unimmunized mice were reduced, particularly in competitive bone marrow chimaeras (Fig. 4f).

The germinal-centre responses observed after immunization against the T-cell-dependent antigens nitrophenyl 23-coupled chicken γ-globulin (CGG-NP23) and sheep red blood cell (SRBC) were markedly impaired, as assessed by immunohistochmical staining of PNA⁺ B cells (Fig. 4g, h). The antibody response to SRBCs was depressed, whereas the serum antibody response to highly polyvalent nitrophenyl appeared conserved (Fig. 4g, h). These defects are similar to those reported in BCL6-deficient mice⁶.

Our study suggests that CUL3 is an essential partner of key BTB-ZF transcription factors in the lymphoid lineage. The different effect of CUL3 on the follicular T-helper cell and the germinal-centre B-cell responses suggests that CUL3 regulates distinct components of these
two BCL6-driven programs. In addition, the defect in marginal zone B and B1 cells may hint at the existence of as yet unidentified BTB-ZF factors controlling these enigmatic populations.

Although proteomic analysis of ubiquitination was performed at the whole cell level, a considerable proportion of changes induced by PLZF expression was concentrated on the PLZF–CUL3-associated complex itself, including key nuclear matrix proteins such as SATB1 and lamin B1, which target specific DNA sequences for chromatin remodelling and gene regulation, respectively. The precise nature and role of these changes remain to be determined, but the emerging evidence of the importance of ubiquitination in chromatin regulation suggests that they specify key aspects of the transcriptional programs directed by these transcription factors. A similar function of CUL3 may regulate the oncogenic properties of PLZF and BCL6 in leukemias and lymphomas.

**METHODS SUMMARY**

Mass spectrometric analysis of PLZF- and CUL3-associated proteins. NKT cells (15 x 10^6) purified from the thymus of V β 14-Ja18 transgenic mice, or whole thymocytes (5 x 10^7–5 x 10^8) from PLZF transgenic mice were subjected to lysis and immunoprecipitation with anti-PLZF or anti-CUL3 antibodies, trypsin-digested and analysed by liquid chromatography–electrospray tandem mass spectrometry on a nano LTQ Orbitrap Hybrid FT mass spectrometer.

Western blot ubiquitination assay. 293T cells were lipofectamine-transfected with the plasmids pH-A-Ub (2 µg), pSATB1-Myc-His (2 µg), pLMNB1-GFP (2 µg), pPLZF-Flag (0.5 µg), pCUL3-Myc (2 µg) or pCUL3AC (2 µg) as indicated. After 24 h, 20 µM proteasome inhibitor MG132 was added and cells were incubated for another 4–6 h. Ubiquitin conjugates were immunoprecipitated with anti-HA antibody conjugated to agarose and analysed by immunoblotting with anti-SATB1 and anti-lamin B1.

Mass spectrometric analysis of ubiquitinated proteins. Total thymocytes (3 x 10^7) were submitted to lysis, trypsin digestion and immunoprecipitation with the anti-ubiquitin branch antibody (Ubiscan, Cell Signaling Technology). Eluted peptides were characterized by tandem mass spectrometry collected into an LTQ-Orbitrap Velos Hybrid mass spectrometer (Thermo). Changes in ubiquitinated peptide levels were measured between PLZF-transgenic and wild-type thymocytes. Fold-changes above 1.45 were considered if confirmed in two independent immunoprecipitation experiments using different batches of mice.

**Confocal microscopy.** Purified thymocytes or lymphocytes were attached to glass coverslips and gene regulation, respectively. The precise nature and role of specific nuclear matrix proteins such as SATB1 and lamin B1, which target specific DNA sequences for chromatin remodelling and gene regulation, respectively,11–14. The precise nature and role of these changes remain to be determined, but the emerging evidence of the importance of ubiquitination in chromatin regulation22–24 suggests that they specify key aspects of the transcriptional programs directed by these transcription factors. A similar function of CUL3 may regulate the oncogenic properties of PLZF and BCL6 in leukemias and lymphomas.

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**Author Contributions** R.M. designed the research, performed experiments and analyzed data. M.P.S., S.T.S., A.M., M.G.C. and C.B.-V. performed experiments and analyzed data. J.D.S. designed the research and any associated references are available in the online version of the paper.

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METHODS

Mice. C57BL/6, B6.SJL-Ptprca Pep3b/Boy (CD45.1), Cd19-Cre (B6.129P2-Cd19im(cre)G8m) mice and Cd4-Cre (B6 Tg(cd4-cre)1Cwi) mice were obtained from Jackson Laboratories. B6.Cdul3pfag1fl/l (Addgene, plasmid 15380) lacking sequences 418–760 at the carboxy terminus and serving as a catalytically inactive mutant (CUL3AC); pcDNA3-1-SATB1-Myc-His (R. Grosschedl); pEGFP-C3-LMNBI (Y.-H. Fu); pfFlag-MV2-PLZF (in which PLZF was PCR-amplified from C57BL/6 thymic slices of SDS–PAGE gels stained with colloidal blue (NuPAGE, Invitrogen) were used. For the identification of coimmunoprecipitated proteins, Mass spectrometry.

PBS plus 0.05% Triton. Bound proteins were eluted by boiling for 5 min and anti-PLZF- or anti-CUL3-bound Protein A/G Sepharose (Invitrogen) beads. sepharose beads (Invitrogen) at 4°C for 30 min. Gel slices were washed with 0.1% BSA, 50 mM sodium deoxycholate, 0.1% SDS and 1 mM EDTA, pH 8.0. 10 μl of 10 μM TCEP (Tris(2-carboxyethyl)phosphine-HCl) at 37°C for 30 min. The proteins were alkylated by adding 100 μl 50 mM iodoacetamide and allowed to react in the dark at 20°C for 30 min. Gel slices were washed in water, then in acetonitrile and dried by SpeedVac for 30 min. Tryptsin digestion was carried out overnight at 37°C using sequencing grade modified trypsin (Promega) at a 1:50 enzyme to protein ratio in 50 mM ammonium bicarbonate, pH 7.5, and 20 mM CaCl2. Peptides were extracted from the gel pieces with 5% formic acid and dried by SpeedVac. The peptide samples were analysed by a liquid chromatography–electrospray tandem mass spectrometry (LC–MS/MS) on a Thermo LTQ Orbitrap Hybrid FT mass spectrometer. Spectra were then analysed with Mascot (Matrix Science, version 3, Mascot) and Sequest (ThermoFinnigan, version v27, rev. 11) set up to search the mus.musculus database. Peptide identifications were accepted if they could be established at greater than 90% probability and contained at least two unique identified peptides.

UbSiScan analysis. Thymocytes (3 × 106) were submitted to Cell Signaling Technology for UbSiScan analysis using the ubiquitin branch technology (1990 Cell Signaling Technology) following a method modified from ref. 32. Lysates were sonicated, cleared by centrifugation, reduced and carbamidomethylated. Total protein for each lysate was normalized before digestion. Lysates were digested with trypsin. Peptides were separated from non-peptide material by solid-phase extraction with Sep-Pak C18 classic cartridges (Waters). tryptic peptides were redisolved, and ubiquitinated peptides were isolated using slurries of the ubiquitin branch antibody. Peptides were eluted from antibody-resin into a total volume of 100 μl in 0.15% trifluoroacetic acid. Eluted peptides were concentrated with C18 spin tips immediately before LC–MS analysis. The samples run in duplicate to generate analytical replicates and increase the number of MS/MS identifications from each sample. Peptides were loaded directly onto a 10 cm × 75 μm PicoFrit capillary column packed with Magic C18 AQ reversed-phase resin. The column was developed with a 72-min linear gradient of acetonitrile in 0.125% formic acid delivered at 280 nl min⁻¹. Tandem mass spectra were collected with an LTQ-Orbitrap Velos hybrid mass spectrometer (Thermo), a top-20 method, a dynamic exclusion repeat count of 1 and a repeat duration of 30 s. Mass spectrometry spectra were collected in the Orbitrap component of the mass spectrometer. All MS/MS spectra were collected in the LTQ. MS/MS spectra were evaluated using SEQUEST 3G and the SORCERER 2 platform from Sage-N Research (v4.0, Milpitas CA). Peptide assignments were obtained using a 5% false positive discovery rate. Searches were performed against the mouse NCBI database updated on 9 June 2010. Cysteine carboxamidomethylation was specified as a static modification, oxidation of methionine residues was allowed, and ubiquitination was allowed on lysine residues. Each MS/MS spectrum arises from a parent ion observed during a survey mass spectrometry scan and can be linked to the intensity of that parent ion at its chromatographic apex, essentially measuring the abundance of the peptide in the sample. Parent ion intensities were extracted from the ion chromatogram file of each sample using proprietary software and are reported in the quantification tables. Changes in ubiquitinated peptide levels were measured by taking the ratio of raw intensities. Raw intensity values were used to calculate average values and raw ratios between samples. The raw ratios were normalized based on the median ratio found, and normalized raw ratios and fold-changes are reported.

Flow cytometry. CD4+ PBS57 tetramers were obtained from the NIH tetramer facility. Fluorochrome-labelled monoclonal antibodies (clones indicated in bracket) against CD4 (GK1.5, B2d) (53-67, 67°C; CRJ5 (H57-597), CD4 (M1/69, 67°C), CD25 (PC61), FOXP3 (FK-16), CD44 (IM7), NK1.1 (PKR1, 67°C, B20) (RA3-6B2), Cxcr5, P1D1 (29F.1A12), ICOS, CD80/86 (AbD Bioscience), CD80, 40.9 (eBioscience), CD3e, 24G2 (BD Biosciences), CD8, 53-1.7 (Dako), CD45.1 (A20), CD45.2 (104), CD4 (53-7.3), CD3 (29D, 67°C, 13D3, 13D4, 11H4, 11F2.3), and 8XCrl (GL3) were purchased from e-Bioscience, BD Biosciences or Biolegend. For FOXP3 intracellular flow cytometry, cells were fixed using the permeabilization and fixation buffer (Foxp3 staining buffer set) from eBioscience. Samples were analysed on an LSRII (Becton Dickinson), or sorted on a FACS Aria (Becton Dickinson) or MoFlo (Dako Cytomation). Data were analysed using FlowJo (Tree Star).

Conflusal microscopy. Purified NK-cell thymocytes or CD4 splenocytes were attached to slides (Superfrost plus microscope slides, Fisherbrand) by cytoospin, and fixed for 15 min with 4% paraformaldehyde in PBS for 10 min. Cells were permeabilized with 0.5% Triton in PBS for 10 min, washed and blocked with 10% donkey serum and 1% BSA for 1 h at room temperature before staining with anti-PLZF or anti-CUL3 for 2 h at room temperature in a humidifying chamber. After PBS washes, cells were stained with donkey anti-rabbit Alexa 488 (Invitrogen), donkey anti-goat Alexa 555 antibodies (Invitrogen) or donkey anti-mouse 647 (Invitrogen) for 30 min at room temperature. Cells were washed with PBS 0.005% Triton, then PBS, and mounted with prolong gold mounting solution (Invitrogen). Control staining included rabbit IgG and goat IgG followed by corresponding secondary antibody or secondary antibody alone. Images were captured with a U-MPlanapo 40×/1.3 Oil objective, a PIP-STEM-CW (365–490 nm) filter combination and an epi-illumination unit with 1014 oil and Olympus 1X81 laser scanning microscopes and were analysed with Image J software.

Immunoprecipitation and western blot detection of ubiquitinated proteins. 293T cells grown in 6-well plates were lipofectamine-transfected with plasmids pH-A-Ub (2 μg), pSATB1-Myc-His (2 μg), pLMNB1-GFP (2 μg), pPLZF-Flag (0.5 μg), pCUL3-Myc (2 μg) or pCUL3AC (2 μg) as indicated. The plasmid concentration was kept constant by adding pmxAGFP (Amaxa). After 24 h, 20 μM MG132 was added and cells were incubated for another 4–6 h. Cells were collected with gentle scraping and resuspended in 300 μl RIIPA buffer (25 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS and 1× protease inhibitor). Cells were lysed by sonication (Bioruptor, Diagenode) for 12 min, with 30 s on and 30 s off. Lysates were centrifuged at 13,800 g at 4°C for 10 min to remove cell debris. One-hundred microlitres of the lysate was diluted 1:4 with RIIPA buffer supplemented with 1× protease inhibitors, and incubated with 25 μl anti-HA–agarose (A2095 clone HA-7, Sigma) for 4 h. The beads were washed four times with RIIPA buffer and boiled for 5 min in 50 μl SDS–gel loading buffer containing 50 mM β-mercaptoethanol at 95°C. Samples were separated by SDS–PAGE and immunoblotted with antibodies against SATB1 or lamin B1.

Immunohistochemistry. For immunofluorescence studies, fixed OCT (Tissue-Tek)-embedded 5–μm sections of spleens were dehydrated overnight and stained with biotinylated rat anti-B220 (RA3-6B2 BD Biosciences) and Alexa-Fluor 488-conjugated rat anti-CD4 (RM4-5 Invitrogen) antibodies, followed by Cy3-streptavidin (Invitrogen) and visualized using a SP5 II microscope (Leica). Data were analysed using ImageJ (Bitplane) software.
For immunohistochemical studies, frozen OCT-embedded sections were dried overnight, fixed with ice-cold acetone and incubated with methanol and 0.3% hydrogen peroxide to neutralize endogenous peroxidase activity, and blocked with 5% rat serum, then streptavidin-biotin blocking (Vector Laboratories). Sections were stained with biotinylated PNA (Vector Laboratories), followed by Vectastain ABC-alkaline phosphatase kit (Vector Laboratories) and the Vector Red alkaline phosphatase substrate kit (Vector Laboratories). After treatment with serum and streptavidin-biotin block, sections were stained with biotinylated rat anti-B220 antibody (RA3-6B2 BD Biosciences), followed by reaction with the Vectastain ABC kit (Vector Laboratories) and ImmPACT SG peroxidase substrate kit (Vector Laboratories) according to manufacturer’s instructions. Sections were dehydrated, cleared with xylene and mounted using Permount solution (Fischer Scientific). Micrographs were taken with the FSX-100 microscope camera system (Olympus) and data were analysed using ImageJ (Bitplane) software.

**Generation of bone marrow chimaeras.** Six-to-eight-week-old B6 (CD45.1) mice were subjected to irradiation with 10 Gy using a γ-cell 40 irradiator with a caesium source and were injected intravenously 3–6 h later with $2 \times 10^7$–$5 \times 10^6$ bone marrow cells obtained from the femurs of donor mice. Bone marrow-reconstituted mice were analysed 8–10 weeks after irradiation.

**NKT cell enrichment.** NKT cells were labelled with allophycocyanin (APC)-conjugated CD1d-PBS57 tetramers, bound to anti-APC magnetic beads, and enriched on an MACS cell separator (Miltenyi Biotech) as described previously.

**Mouse immunizations.** Six-to-eight-week-old mice were immunized intraperitoneally with 100 µg of DNP-Ficoll (Biosearch Technologies) in PBS, 50 µg CGG-NP23 (Biosearch Technologies) mixed 1:1 with alum, or $2 \times 10^8$ SRBCs (Lampire Biological Laboratories). Mice injected with CGG-NP23 were boosted on day 21 with the same inoculum and killed on day 28. Levels of anti-DNP and anti-NP antibodies were determined by ELISA against BSA-DNP and BSA-NP23, respectively (Biosearch Technologies) and anti-SRBC antibodies were measured by FACS using an indirect isotype-specific immunofluorescence assay (eBiosciences, Southern Biotech).

**Statistical analysis.** Unpaired Student’s t-test was performed with Prism (Graph Pad Software). *P < 0.05; **P < 0.001; ***P < 0.0001.

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