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LETTERS

# Role for Spi-C in the development of red pulp macrophages and splenic iron homeostasis

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**Tissue macrophages comprise a heterogeneous group of cell types differing in location, surface markers and function[1]. Red pulp macrophages are a distinct splenic subset involved in removing senescent red blood cells[2]. Transcription factors such as PU.1 (also known as Sfpi1) and C/EBPα (Cebpa) have general roles in myelomonocytic development[3,4], but the transcriptional basis for producing tissue macrophage subsets remains unknown. Here we show that Spi-C (encoded by *Spic*), a PU.1-related transcription factor, selectively controls the development of red pulp macrophages. Spi-C is highly expressed in red pulp macrophages, but not monocytes, dendritic cells or other tissue macrophages. *Spic*$^{-/-}$ mice have a cell-autonomous defect in the development of red pulp macrophages that is corrected by retroviral Spi-C expression in bone marrow cells, but have normal monocyte and other macrophage subsets. Red pulp macrophages highly express genes involved in capturing circulating haemoglobin and in iron regulation. *Spic*$^{-/-}$ mice show normal trapping of red blood cells in the spleen, but fail to phagocytose these red blood cells efficiently, and develop an iron overload localized selectively to splenic red pulp. Thus, Spi-C controls development of red pulp macrophages required for red blood cell recycling and iron homeostasis.**

Spi-C belongs to the Spi subfamily of Ets transcription factors, which includes PU.1 and Spi-B[5,6], and was initially reported to be expressed in B cells[5,7]. We compared expression of PU.1, Spi-B and Spi-C across a panel of immune cells such as B cells, bone marrow monocytes, dendritic cells and several types of resident tissue macrophages, including red pulp macrophages (RPMs; Fig. 1a, Supplementary Fig. 1). PU.1 was broadly expressed and Spi-B was predominantly restricted to B cells[8,9]. In contrast, Spi-C was highly expressed in RPMs, expressed at lower levels in B cells, and essentially absent in other cells. To test for a role of Spi-C in RPMs and B cells, we generated mice lacking *Spic* expression by gene targeting (Supplementary Fig. 2). Male and female *Spic*$^{-/-}$ mice are fertile and healthy, with a normal lifespan, but are born at a somewhat lower than expected Mendelian frequency (Supplementary Fig. 2). We also generated *Spic*$^{null/null}$ mice, in which the neomycin cassette was deleted from the targeted *Spic* allele (Supplementary Fig. 2).

RPMs have been defined as F4/80$^{hi}$CD68$^{+}$CD11b$^{lo/-}$ cells with strong autofluorescence[10,11]. Both *Spic*$^{-/-}$ mice and *Spic*$^{null/null}$ mice showed a phenotype characterized by the selective loss of RPMs (Fig. 1b and c, Supplementary Fig. 3a), but showed no abnormalities in the development of B cells, conventional dendritic cells, plasmacytoid dendritic cells (Supplementary Figs 3 and 4, and Supplementary Table 1) or T cells (Supplementary Fig. 5), and had normal serum immunoglobulin levels (Supplementary Fig. 4f). We confirmed the loss of RPMs by immunohistochemical analysis, finding an almost complete loss of F4/80$^{+}$ cells in the splenic red pulp (Fig. 2a). In contrast, SIGN-R1 (also known as CD209a)-expressing marginal zone macrophages and MOMA-1 (also known as Siglec1)-expressing metallophilic marginal zone macrophages were present normally in the spleens of *Spic*$^{-/-}$ mice (Fig. 2a). *Spic*$^{-/-}$ mice had normal F4/80$^{+}$ tissue macrophages in peritoneum and liver, normal macrophage and dendritic cell progenitors in bone marrow, and normal monocytes in bone marrow and blood (Supplementary Fig. 6).

Loss of RPMs could result either from a cell-autonomous defect or from the loss of a required extrinsic component such as a bone marrow or splenic stromal cell. To distinguish these possibilities, we generated haematopoietic chimaeras by transplanting bone marrow cells from CD45.2$^{+}$ *Spic*$^{+/+}$ or *Spic*$^{-/-}$ mice into irradiated congenic CD45.1$^{+}$

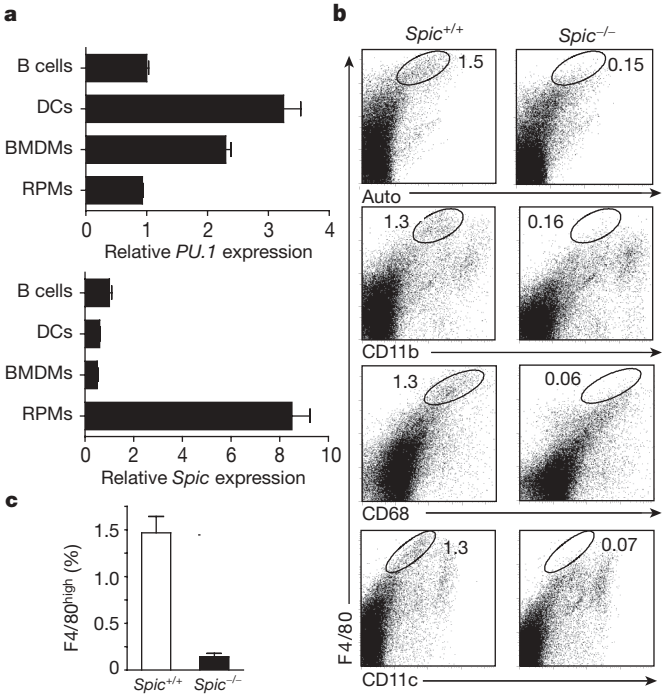


**Figure 1 | *Spic*$^{-/-}$ mice have a selective loss of red pulp macrophages.** **a**, *PU.1* and *Spic* expression was determined by quantitative polymerase chain reaction with reverse transcription (RT–PCR) in purified B cells, dendritic cells (DCs), bone-marrow-derived macrophages (BMDMs) and RPMs. Shown is the normalized messenger RNA expression relative to expression in B cells. **b**, *Spic*$^{+/+}$ and *Spic*$^{-/-}$ spleen cells were stained with antibodies to F4/80, CD11b, CD68 and CD11c and analysed by flow cytometry. Auto, autofluorescence. Numbers represent the percentage of cells in the indicated gate. **c**, Frequency of F4/80$^{hi}$ cells in spleen was determined as the mean (and s.d., $n = 7$) from total splenocytes as shown in **b**.

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recipients (Fig. 2b). In these chimaeras, F4/80$^+$ RPMs developed from *Spic*$^{+/+}$, but not from *Spic*$^{-/-}$, donor bone marrow (Fig. 2b). This defect was restricted to RPMs, because *Spic*$^{-/-}$ donor bone marrow generated normal B cells (Fig. 2b), CD11b$^+$ and CD11c$^+$ myeloid lineages, natural killer cells and T cells (Supplementary Fig. 6e). Development of F4/80$^+$ RPMs was restored when *Spic*$^{-/-}$ bone marrow was infected with a Spi-C-expressing retrovirus and allowed to develop in lethally irradiated hosts (Fig. 2c). These findings indicate that the loss of RPMs in *Spic*$^{-/-}$ mice results from an intrinsic haematopoietic defect.

Senescent red blood cells (RBCs) are normally phagocytosed by macrophages in spleen and liver, followed by degradation of their haemoglobin, and transport of the iron back into the circulation[12–14]. To study RBC clearance by splenic macrophages, we used the system of *Cd47*$^{-/-}$ RBCs[13]. We injected carboxy-fluorescein succinimidyl ester (CFSE)-labelled *Cd47*$^{-/-}$ RBCs into mice and examined their clearance and uptake by spleen and liver cells (Supplementary Fig. 7). The initial clearance of *Cd47*$^{-/-}$ RBCs from the circulation was similar between *Spic*$^{-/-}$ and *Spic*$^{+/+}$ mice (Supplementary Fig. 7a). However, we found differences in efficiency of phagocytosis of trapped RBCs by splenic macrophages between *Spic*$^{+/+}$ and *Spic*$^{-/-}$ mice. In *Spic*$^{+/+}$ mice, F4/80$^{hi}$ CD68$^+$ RPMs showed more than tenfold higher uptake of CFSE than F4/80$^{lo}$CD68$^+$ cells (Supplementary Fig. 7c, d). In *Spic*$^{-/-}$ mice, which lacked F4/80$^{hi}$CD68$^+$ macrophages (Supplementary Fig. 7c), the remaining F4/80$^{lo}$CD68$^+$ macrophages showed the low level of phagocytosis seen in *Spic*$^{+/+}$ mice (Supplementary Fig. 7d). Liver cells did not take up labelled *Cd47*$^{-/-}$ RBCs in either wild-type or *Spic*$^{-/-}$ mice (Supplementary Fig. 7e). These results suggest that the absence of F4/80$^{hi}$ RPMs in *Spic*$^{-/-}$ mice represents the loss of a functional macrophage subset that is normally responsible for efficient phagocytosis of RBCs in the spleen.

*Spic*$^{-/-}$ mice developed an age-dependent increase in the size and weight of the spleen not associated with increased numbers of splenocytes (Fig. 3a, b). Because mice with genetic defects in iron or haemoglobin metabolism can exhibit splenomegaly[15,16], we assessed the iron stores of *Spic*$^{-/-}$ mice. Serum and liver iron concentrations were normal in *Spic*$^{-/-}$ mice, but splenic tissue iron concentration was increased (Fig. 3c, d and Supplementary Fig. 8a, b). This iron accumulation was evident histologically in *Spic*$^{-/-}$ mice and largely confined to the red pulp (Fig. 3e and Supplementary Figs 8c and 9a), but was not observed in the liver or intestine (Supplementary Fig. 8d, and data not shown). *Spic*$^{-/-}$ mice did not exhibit abnormal erythroid parameters or morphology (Supplementary Table 2 and Supplementary Fig. 9c).

To identify potential Spi-C target genes, we compared global gene expression profiles of RPMs, alveolar and peritoneal macrophages, monocytes and dendritic cells (Fig. 4a). The haemoglobin scavenger receptor (*Cd163*)[17] and the iron transporter ferroportin 1 (*Slc40a1*)[18] were both highly expressed by RPMs relative to other macrophage subsets or B cells (Fig. 4a). Vascular cell adhesion molecule 1 (*Vcam1*) was also highly expressed in RPMs compared to peritoneal and alveolar macrophages (Fig. 4a), consistent with reported Vcam1 expression in the red pulp[19,20]. Vcam1 is most highly expressed in the red pulp of control spleens, but was virtually absent in *Spic*$^{-/-}$ spleens (Fig. 4b, Supplementary 10a, b). In *Spic*$^{+/+}$ spleens, Vcam1 was highly correlated with F4/80 expression (Supplementary Fig. 10a), both of which were substantially reduced in *Spic*$^{-/-}$ spleens. In contrast, other tissues did not show this coordinated expression of F4/80 and Vcam1 (Supplementary Fig. 10a). Peritoneal macrophages expressed high F4/80 without high Vcam1, and most F4/80$^+$ cells in lymph nodes expressed low levels of Vcam1 (Supplementary Fig. 10b). However, Vcam1 expression by B cells was reduced in *Spic*$^{-/-}$ mice compared to *Spic*$^{+/+}$ mice (Supplementary Fig. 10b). Bone marrow monocytes treated with M-CSF (colony stimulating factor 1, also known as Csf1)[21] induced both Spi-C and Vcam1 expression, whereas treatment with GM-CSF (also known as Csf2) caused much less induction of Spi-C and no induction of Vcam1 (Supplementary Fig. 10c). Consistently, *Spic*$^{-/-}$ bone marrow cells treated with M-CSF showed reduced levels of Vcam1 expression compared to wild-type cells (Supplementary Fig. 10d). Together, these results suggest the possibility that Spi-C may regulate the expression of Vcam1.

Reporter analysis and electrophoretic mobility shift assays (EMSAs) provide additional evidence that Spi-C regulates Vcam1 (Fig. 4c–e). PU.1 can augment FcγR2b promoter activity[22]. PU.1 and Spi-C each augment a Vcam1 reporter by a similar magnitude[22] (Fig. 4c). An Ets consensus element within the proximal Vcam1 promoter forms complexes with PU.1 and Spi-C in EMSAs (Fig. 4d), which are supershifted by anti-PU.1 or anti-Spi-C antisera, respectively (Fig. 4e). Formation of the Spi-C EMSA complex was specifically inhibited by an FcγR2b Ets probe and Vcam1 Ets probe, but not by a mutated Vcam1 Ets probe. Deletion of the Ets element within the Vcam1 promoter eliminated reporter augmentation by both PU.1 and Spi-C (Fig. 4c). To test whether Spi-C could induce

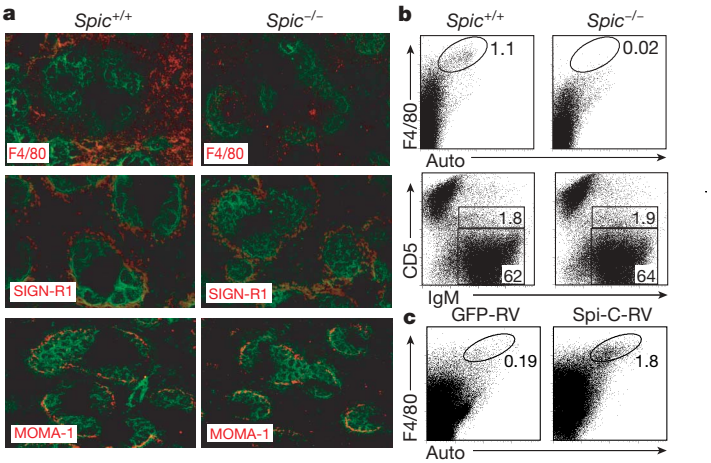


**Figure 2 | *Spic*$^{-/-}$ mice have a cell-autonomous defect in red pulp macrophages. a**, *Spic*$^{+/+}$ and *Spic*$^{-/-}$ spleens sections were stained for B220 (green) and F4/80, SIGN-R1 or MOMA-1 (red). **b**, Bone marrow cells from CD45.2$^+$ C57BL/6 *Spic*$^{+/+}$ or *Spic*$^{-/-}$ mice were transferred into irradiated CD45.1$^+$ B6.SJL mice. Splenocytes were stained for CD45.2, CD45.1, F4/80, CD5 and immunoglobulin (Ig)M. After 10 weeks, >97% of spleen cells were donor-derived (CD45.2$^+$CD45.1$^-$). Plots are gated on donor-derived cells. Numbers represent the percentage of donor-derived cells in the indicated gates. **c**, Bone marrow cells from CD45.2$^+$ C57BL/6 *Spic*$^{-/-}$ mice were infected with Spi-C-RV or control retrovirus (GFP-RV) and transferred into irradiated CD45.1$^+$ B6.SJL mice. After 6 weeks, spleen cells were stained for CD45.2, CD45.1 and F4/80. Plots are gated on donor-derived cells. Results are representative of four mice per group.

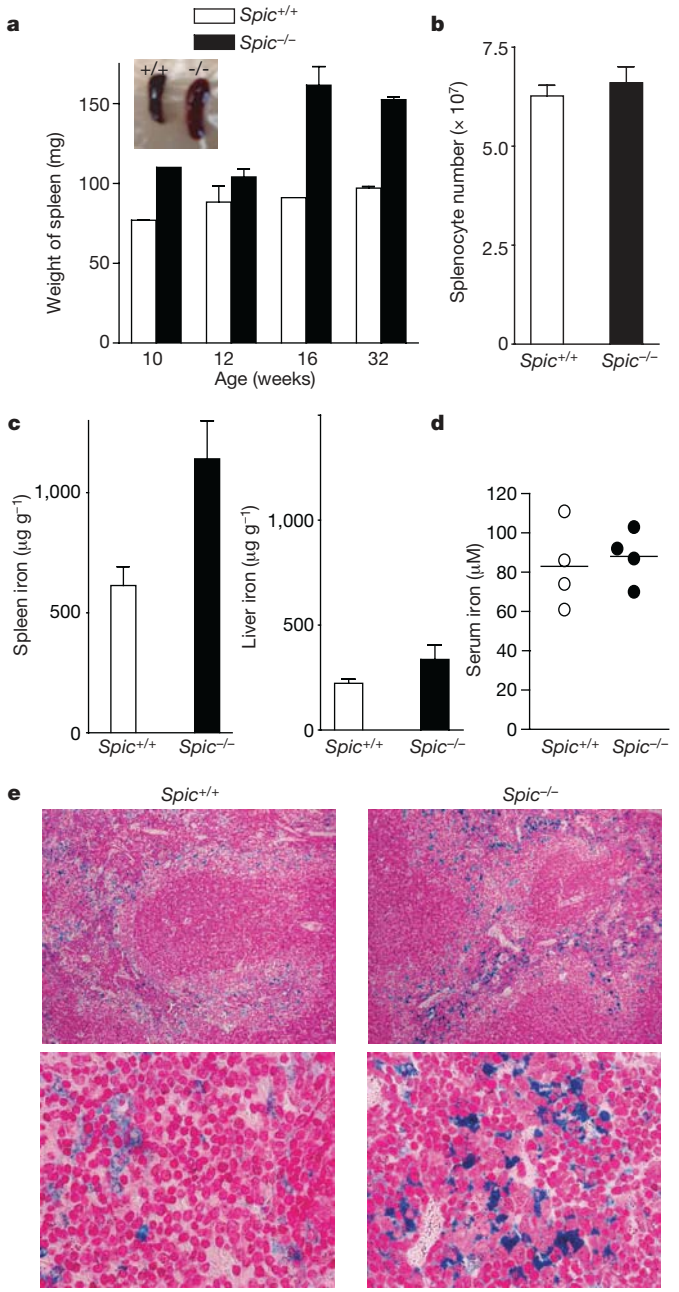


**Figure 3 | *Spic*$^{-/-}$ mice have increased splenic iron stores.** **a**, Spleens from *Spic*$^{+/+}$ and *Spic*$^{-/-}$ mice of the indicated age were weighed ($n = 3$ per time point). Representative spleens at 32 weeks are shown in the inset. **b**, Splenocytes from *Spic*$^{+/+}$ and *Spic*$^{-/-}$ mice were counted (mean and s.d., $n = 5$). **c**, Iron levels in spleen (left) and liver (right) of 129 SvEv background *Spic*$^{+/+}$ and *Spic*$^{-/-}$ male mice on a standard diet (mean and s.d., $n = 4$). **d**, Serum iron levels were determined for 32-week-old 129 SvEv background *Spic*$^{+/+}$ and *Spic*$^{-/-}$ male mice on a standard diet. **e**, Perl's Prussian blue stain for ferric iron in the spleens of 16-week old 129 SvEv *Spic*$^{+/+}$ and *Spic*$^{-/-}$ male mice.

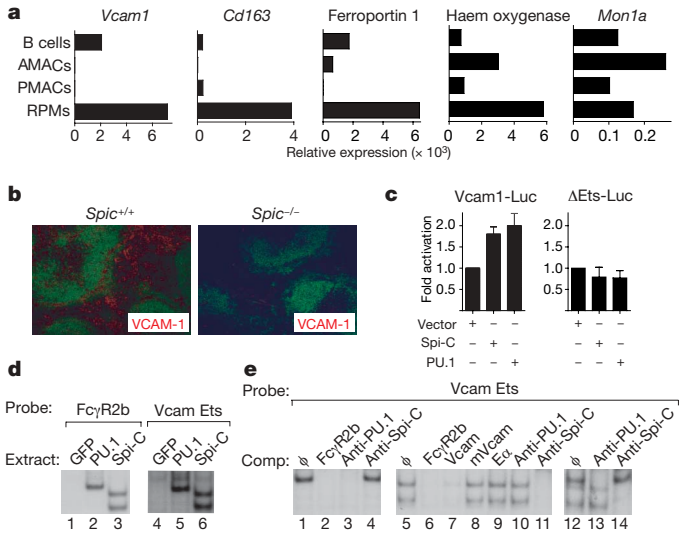


**Figure 4 | Spi-C regulates Vcam1 expression.** **a**, Normalized expression of *Vcam1*, *Cd163*, ferroportin I, haem oxygenase and *Mon1a* is shown for B cells, alveolar macrophages (AMACs), peritoneal macrophages (PMACs) and RPMs, assessed by expression microarrays. **b**, *Spic*$^{+/+}$ or *Spic*$^{-/-}$ spleen sections were stained for B220 (green) and Vcam1 (red). **c**, J774 cells were transfected with the Vcam1 reporter (Vcam1-Luc) or the ΔEts-Luc reporter, and with pEF4 (vector), Spi-C- or PU.1-expressing vectors. Cells were analysed for luciferase activity as described in the Supplementary Methods. Data are representative of four independent experiments (mean and s.d., $n = 3$). **d**, 293F/T cells were transiently transfected with GFP-RV (GFP), PU1-MIGR1 (PU.1) or Spi-C-RV (Spi-C), and whole-cell extracts were analysed for binding to the FcγR2b or the Vcam1 Ets probes. **e**, Extracts from PU.1-expressing cells (lanes 1–4), Spi-C-expressing cells (lanes 5–11) or mixtures of PU.1 and Spi-C extracts (lanes 12–14) were analysed for binding to the Vcam1 Ets probe. Shown are competitions (Comp) using unlabelled competitor oligonucleotides or supershifts using anti-sera against PU.1 or Spi-C as indicated. ϕ, no treatment; mVcam, mutated Vcam1 oligonucleotides.

Vcam1 in cell lines, we transfected the RAW264.7 macrophage cell line with Spi-C retrovirus (Spi-C-RV) or control retrovirus (Supplementary Fig. 10e). Control RAW264.7 cells do not express Spi-C or Vcam1, and treatment with M-CSF only weakly induced Vcam1. In Spi-C-expressing RAW264.7 cells, M-CSF strongly induced Vcam1, but not CD163, ferroportin, haem oxygenase or Mon1a. Thus, Vcam1 seems to be a target of Spi-C in macrophages.

Spi-C was initially identified as a PU.1-related transcription factor expressed in B cells, but its function was unknown[5,7]. An earlier report based on overexpression studies suggested Spi-C might regulate immunoglobulin E production[23]. Differential expression of Spi-C was suggested as evidence for independent ontogeny for splenic B cells and peritoneal B1a B cells[24]. We find *Spic*$^{-/-}$ mice to have normal B-cell development and antibody production, but do not exclude a subtle role for Spi-C in B-cell function. However, our evidence supports a critical role for Spi-C in the development of RPMs, and shows that this cell lineage is required for normal recycling of red blood cells and iron homeostasis in the spleen.

The known genetic defects of iron metabolism[14,25,26] frequently involve proteins that function as iron transporters/exporters (for example, ferroportin[18]), as receptors for iron-binding proteins (for example, transferrin receptor[27] and hemochtomatosis[28]), or to regulate the activity of these transporters or receptors (for example, hepcidin[29]). Such gene defects that directly alter iron transport can produce global changes in iron storage that may affect many tissues. In contrast, the transcription factor Spi-C is the first gene to affect iron metabolism by controlling the development of a particular cell lineage important for recycling red blood cells and their products. Future studies will be directed at analysis of the specific roles of transcriptional targets of Spi-C, such as Vcam1, in elaborating these functions of red pulp macrophages.

## METHODS SUMMARY

**Mice and reagents.** *Spic*$^{-/-}$ mice were generated by deleting the entire protein-coding regions exons 2–6 of the *Spic* locus. The neomycin resistance cassette was deleted by intercrossing *Spic*$^{+/-}$ mice with CMV-Cre deletor mice to produce *Spic*$^{null/null}$ mice, as described in Supplementary Methods.

**Histochemical analysis.** Six-micrometre frozen tissue sections were fixed in cold acetone and blocked with 5% bovine serum albumin (Roche). The monoclonal antibodies used were biotinylated antibody against F4/80 (Caltag), Vcam1 (eBioscience), MOMA-1 (BMA Biomedicals) and SIGN-R1 (ER-TR9, BMA

Biomedicals), Alexa488-conjugated antibody against B220 (BD PharMingen) and streptavadin-Alexa555 (Invitrogen). For Perl's Prussian blue stain, tissues were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.0), embedded in paraffin, and stained with Perl's Prussian blue and pararosaniline (Sigma).

**Adoptive transfer of bone marrow cells.** Recipient CD45.1$^+$ B6.SJL (Taconic) mice were lethally irradiated with 1,100 rads and injected intravenously with $10^7$ bone marrow cells obtained from CD45.2$^+$ F4 or F5 *Spic*$^{+/+}$ or *Spic*$^{-/-}$ mice. Donor-derived cells in spleen (CD45.2$^+$) were analysed 10 weeks after transfer by fluorescence-activated cell sorting.

**Measurement of iron content in spleen, liver and serum.** Age- and gender-matched mice were analysed for non-haem iron as described[30]. Livers and spleens were weighed and digested in 3 M hydrochloric acid/10% trichloroacetic acid, at 65 °C for 20 h. Ten microlitres of each acid extract was mixed with 0.5 ml of chromagen reagent. The absorbance at 535 nm was measured by a DU Series 500 spectrophotometer (Beckman), and compared to an iron standard treated identically. For serum measurements[30], 20 µl of serum was incubated with 20 µl of acid reagent for 5 min. Supernatant was mixed with 40 µl chromagen reagent, and absorbance at 535 nm was measured as described above.

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**Supplementary Information** is linked to the online version of the paper at www.nature.com/nature.

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**Author Contributions** M.K. designed experiments, analysed and interpreted results, and wrote the manuscript; W.I. sorted cells and did retrovirus experiments; B.T.E. contributed gene expression microarray data for mouse tissues; P.R.W. did B cell analysis; K.H. did T cell differentiation analysis; M.A.F. provided *Cd47*$^{-/-}$ mice; T.L.M. did EMSA; and K.M.M. directed the study and wrote the manuscript.

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