LETTERS

Conversion of mature B cells into T cells by dedifferentiation to uncommitted progenitors

César Cobaleda[1]†, Wolfram Jochum[2] & Meinrad Busslinger[1]

Lineage commitment and differentiation to a mature cell type are considered to be unidirectional and irreversible processes under physiological conditions[1]. The commitment of haematopoietic progenitors to the B-cell lineage[2,3] and their development to mature B lymphocytes[4,5] critically depend on the transcription factor encoded by the paired box gene 5 (*Pax5*). Here we show that conditional *Pax5* deletion in mice allowed mature B cells from peripheral lymphoid organs to dedifferentiate *in vivo* back to early uncommitted progenitors in the bone marrow, which rescued T lymphopoiesis in the thymus of T-cell-deficient mice. These B-cell-derived T lymphocytes carried not only immunoglobulin heavy- and light-chain gene rearrangements but also participated as functional T cells in immune reactions. Mice lacking *Pax5* in mature B cells also developed aggressive lymphomas, which were identified by their gene expression profile as progenitor cell tumours. Hence, the complete loss of Pax5 in late B cells could initiate lymphoma development and uncovered an extraordinary plasticity of mature peripheral B cells despite their advanced differentiation stage.

The B-cell-specific *Cd19-cre* gene[6] efficiently deletes the floxed (fl) *Pax5* allele only in B cells of peripheral lymphoid organs, which facilitated the analysis of Pax5 function in mature B cells of young *Cd19-cre Pax5*$^{fl/-}$ mice[4]. In an ageing mouse colony, half of the *Cd19-cre Pax5*$^{fl/-}$ mice died within eight months, unlike the *Cd19-cre Pax5*$^{fl/+}$ mice retaining a functional *Pax5* allele (Fig. 1a). All diseased *Cd19-cre Pax5*$^{fl/-}$ mice developed aggressive lymphoma resulting in splenomegaly and massively enlarged lymph nodes (Fig. 1b, c) owing to diffuse infiltration by lymphoma cells, which were also found in many other organs (Supplementary Table 1), including the lung, liver, kidney and thymus (Fig. 1d–g). The lymphoma cells could readily be established in culture and rapidly developed into secondary lymphomas after transplantation into syngeneic mice (Supplementary Table 1). Spectral karyotype analysis identified two distinct chromosomal translocations in two lymphomas but a normal diploid karyotype in seven additional tumours, indicating that a specific translocation or high genomic instability is not required for lymphoma development in *Cd19-cre Pax5*$^{fl/-}$ mice (Supplementary Fig. 1). *Cd19-cre Pax5*$^{fl/-}$ *Eμ-bcl2* mice died after a shorter latency period, demonstrating that Bcl2-mediated cell survival cooperates with Pax5 loss in lymphoma development (Fig. 1a).

Flow cytometric analysis revealed large blastic *Cd19-cre Pax5*$^{fl/-}$ lymphoma cells compared to small quiescent B cells in control *Cd19-cre Pax5*$^{fl/+}$ lymph nodes (Fig. 1h). The tumour cells failed to express the B-cell surface proteins CD19, CD21, CD22, CD23, CD40, CD72, immunoglobulin (Ig)D and major histocompatibility complex class II (Fig. 1h and Supplementary Table 2), all of which are also downregulated on conditional Pax5 loss in mature B cells[4]. Consistent with a B-lymphoid origin, all lymphoma cells expressed B220 and the immunoglobulin Igμ protein (which was part of the pre-BCR) in the absence of Igκ and Igλ expression (Fig. 1h and Supplementary Table 2). In addition, the tumour cells expressed the early markers CD93 and CD25 together with the signalling receptors interleukin (IL)-7Rα, c-Kit and Flt3, which are normally present on lymphocyte progenitors (Fig. 1h). Because *Flt3* is a repressed Pax5 target gene[7,8], its expression indicated that the *Cd19-cre Pax5*$^{fl/-}$ lymphomas may correspond to Pax5-deficient progenitor cell tumours. Complementary DNA microarray analysis together with *Pax5* genotyping confirmed this hypothesis, because the lymphoma cells were indistinguishable from *Pax5*$^{-/-}$ pro-B cells with respect to the transcription of Pax5-repressed and Pax5-activated genes (Supplementary Fig. 2a, b).

To investigate the stage-specific origin of the *Cd19-cre Pax5*$^{fl/-}$ tumours, we analysed their *Igh*, *Igk* and *Igl* rearrangements by polymerase chain reaction (PCR) (Fig. 1i), Southern blotting (Supplementary Fig. 3), sequencing of individual PCR fragments (Supplementary Fig. 2c) and PCR with reverse transcription (RT–PCR) of rearranged immunoglobulin transcripts (Supplementary Fig. 4). All tumours contained a single, in-frame rearranged and expressed *Igh* allele (predominantly containing a *VHJ558*, also known as *Igh-VJ558*, gene), indicating that the tumours were of clonal origin. Most tumours also carried an in-frame rearranged *Igk* or *Igl* allele. It is unlikely that these rearrangements arose in pro-B cells undergoing *Pax5* deletion, because Pax5-deficient pro-B cells are unable to generate *VHJ558–DJH* and *Vκ–Jκ* rearrangements[9,10]. Instead, late pre-B cells correspond to the first developmental stage carrying functional immunoglobulin heavy- and light-chain gene rearrangements. We therefore conclude that the progenitor cell lymphomas in *Cd19-cre Pax5*$^{fl/-}$ mice must originate at least from late pre-B cells or, more probably, from immature or mature B cells.

To demonstrate developmental plasticity of mature B cells in the absence of oncogenic events, we used an experimental strategy that relied on the isolation of highly purified *Pax5*-deleted mature B cells. *Pax5* deletion was achieved either *in vivo* with *Cd19-cre* or *in vitro* with the *CreED-30* transgene encoding a hormone-inducible Cre-oestrogen receptor fusion (Cre-ER) protein[11]. Before the isolation of mature B cells, the *CreED-30 Pax5*$^{fl/fl}$ *Eμ-bcl2 Ly5.2*$^{+}$ mice (used for *in vitro Pax5* deletion) and *Cd19-cre Pax5*$^{fl/-}$ *Eμ-bcl2 Ly5.2*$^{+}$ mice (used for *in vivo Pax5* deletion) were repeatedly injected with anti-IL-7Rα antibody to block B-cell development[12], thus eliminating contaminating precursor and immature B cells in peripheral lymphoid organs (Supplementary Fig. 5). Subsequently, Lin^{-}IgM^{+}IgD^{high} mature B cells were FACS-sorted with a purity of >99% from the spleen or lymph nodes of *CreED-30 Pax5*$^{fl/fl}$ *Eμ-bcl2 Ly5.2*$^{+}$ mice (Fig. 2a and Supplementary Fig. 6a). The isolated B cells were treated *in vitro* for 40 h with 4-hydroxytamoxifen (OHT) to delete the floxed *Pax5* allele before injection into *Rag2*$^{-/-}$ *Ly5.1*$^{+}$ mice (Fig. 2a). *In vivo*

[1]Research Institute of Molecular Pathology, Vienna Biocenter, Dr. Bohr-Gasse 7, A-1030 Vienna, Austria. [2]Department of Pathology, University Hospital, Schmelzbergstrasse 12, CH-8091 Zürich, Switzerland. †Present address: Universidad de Salamanca, Campus Miguel de Unamuno, 37007 Salamanca, Spain.

Pax5-deleted mature B cells were purified as Lin$^-$CD25$^+$IgM$^+$IgD$^-$ cells[4] from the lymph nodes of *Cd19-cre Pax5*$^{fl/-}$ *Eμ-bcl2 Ly5.2*$^+$ mice (Supplementary Fig. 6b) followed by transplantation into *Rag2*$^{-/-}$ *Ly5.1*$^+$ mice (Fig. 2a). The allogeneic markers Ly5.1 and Ly5.2 were used to discriminate haematopoietic cells of host (Ly5.1$^+$) and donor (Ly5.2$^+$) origin in the reconstituted mice.

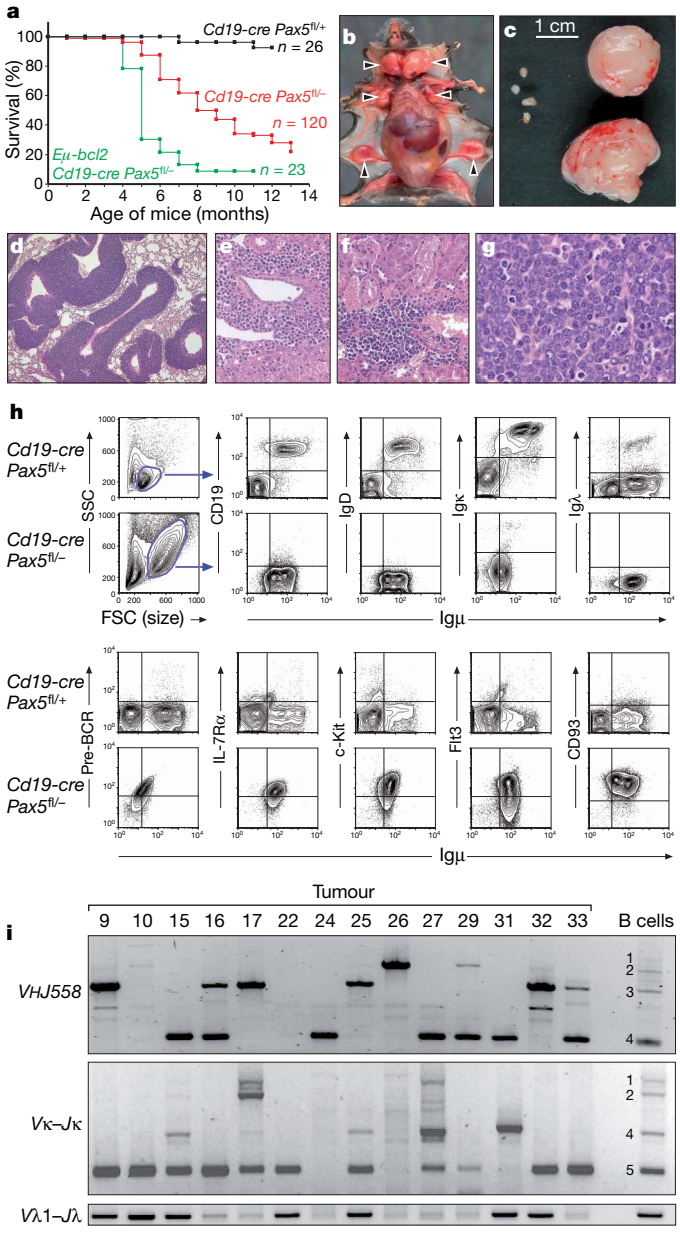

Figure 1 | Development of aggressive progenitor cell lymphomas on Pax5 loss in B lymphocytes. a, Kaplan–Meyer survival analysis of mice of the indicated genotypes. The number (*n*) of mice analysed is shown. **b**, Enlarged lymph nodes (arrowheads) in a *Cd19-cre Pax5*$^{fl/-}$ tumour mouse. **c**, Size comparison of lymph nodes from a *Cd19-cre Pax5*$^{fl/-}$ tumour mouse (right) and a control littermate (left). **d**–**g**, Eosin–haematoxylin-stained sections of the lung (**d**), liver (**e**), kidney (**f**) and thymus (**g**) of a *Cd19-cre Pax5*$^{fl/-}$ tumour mouse. The infiltration patterns of all tumour mice are shown in Supplementary Table 1. **h**, Flow cytometric analysis of lymph node cells of a *Cd19-cre Pax5*$^{fl/-}$ tumour mouse and a control *Cd19-cre Pax5*$^{fl/+}$ littermate. Cells within the forward and side scatter (FSC/SSC) gates (blue) were analysed. The entire immunophenotype of *Cd19-cre Pax5*$^{fl/-}$ tumour cells is summarized in Supplementary Table 2. **i**, PCR analysis of *VHJ558–DJH*, *Vκ–Jκ* and *Vλ1–Jλ* rearrangements in tumour-infiltrated lymph nodes of *Cd19-cre Pax5*$^{fl/-}$ mice. Rearrangements to all *JH* and *Jκ* segments (numbered) were detected in splenic B cells. The detailed characterization of the different immunoglobulin gene rearrangements by cloning and sequencing is summarized in Supplementary Fig. 2c.

Eight weeks after transplantation, Ly5.2$^+$c-Kit$^+$B220$^+$ pro-B cells of donor origin could be detected in the bone marrow of the 19 mice analysed, regardless of whether these mice were injected with *in vitro* or *in vivo Pax5*-deleted mature B cells (Fig. 2b). These Ly5.2$^+$c-Kit$^+$B220$^+$ pro-B cells were identified as Pax5-deficient progenitors, because they did not express the Pax5 target gene *Cd19* (ref. 13) (Fig. 2b). Mature splenic B cells purified from control *Pax5*$^{fl/fl}$ *Eμ-bcl2 Ly5.2*$^+$ mice (lacking *CreED-30*) or *CreED-30 Pax5*$^{fl/fl}$ *Ly5.2*$^+$ mice (lacking *Eμ-bcl2*) failed to give rise to Ly5.2$^+$ pro-B cells in seven and four injected *Rag2*$^{-/-}$ *Ly5.1*$^+$ recipients, respectively (data not shown). Hence, mature B cells require *Pax5* inactivation as well as prolonged cell survival to be able to dedifferentiate to progenitor cells *in vivo*. The requirement of transgenic Bcl2 expression is consistent with a slow dedifferentiation process, because *in vitro Pax5*-deleted B cells labelled with carboxyfluorescein succinimidyl ester could still be detected as CD19$^+$IgM$^+$IgD$^+$c-Kit$^-$ B lymphocytes in the spleen of *Rag2*$^{-/-}$ *Ly5.1*$^+$ mice one week after injection (Supplementary Fig. 7).

The Ly5.2$^+$c-Kit$^+$B220$^+$CD19$^-$ pro-B cells isolated from the bone marrow of reconstituted mice could be cultured under lymphoid conditions as uncommitted progenitors that were able to differentiate *in vitro* into macrophages after IL-7 withdrawal and addition of macrophage-colony-stimulating factor (Supplementary Fig. 8a–c). Eight weeks after transplantation of *Pax5*-deleted B cells, myeloid Ly5.2$^+$Mac1$^+$Gr1$^-$ cells of donor origin could also be detected in the spleen of four of the 12 mice analysed (Supplementary Fig. 8d), in agreement with the inefficient *in vivo* myeloid differentiation of *Pax5*$^{-/-}$ pro-B cells[14].

In contrast, all *Rag2*$^{-/-}$ *Ly5.1*$^+$ mice showed robust reconstitution of T-cell development after transplantation with *in vitro* or *in vivo Pax5*-deleted B cells (Fig. 2b and Supplementary Fig. 9), consistent with the fact that Pax5-deficient progenitors from the bone marrow efficiently seed the thymus to initiate T lymphopoiesis[3]. Within 2–3 months, donor-derived Ly5.2$^+$ T cells restored the thymic cellularity to one-third of that of wild-type mice (Fig. 3c), revealed a normal distribution of CD4$^-$CD8$^-$ (double-negative), CD4$^+$CD8$^+$ (double-positive), CD4$^+$ (single-positive) and CD8$^+$ (single-positive) T cells (Fig. 2b and Supplementary Fig. 9) and carried polyclonal T-cell receptor-β gene (*Tcrb*) rearrangements like normal T cells (Fig. 3a, b and Supplementary Fig. 10). PCR analysis of *Igh*, *Igk* and *Igl* rearrangements confirmed the B-cell origin of the Ly5.2$^+$ thymocytes (Fig. 3a, b). In contrast to wild-type T cells, the sorted Ly5.2$^+$ double-positive thymocytes carried oligoclonal *VH–DJH* rearrangements of distinct *Igh VH* gene families in addition to *Vκ–Jκ* and *Vλ–Jλ* rearrangements (Fig. 3a, b and Supplementary Table 3).

PCR analysis revealed a similar oligoclonality of the immunoglobulin gene rearrangements in Ly5.2$^+$ pro-B cells from the bone marrow of reconstituted mice (Supplementary Fig. 11), which further identified the dedifferentiation of *Pax5*-deleted mature B cells to uncommitted progenitors as the rate-limiting step. Serial transplantation experiments demonstrated that the Ly5.2$^+$ pro-B cells isolated from a primary reconstituted *Rag2*$^{-/-}$ *Ly5.1*$^+$ mouse homed back to the bone marrow and reconstituted T-cell development in the thymus and spleen after injection into secondary *Rag2*$^{-/-}$ *cγ*$^{-/-}$ recipients (Supplementary Fig. 12). These serial transplantation data and the normal developmental progression of T lymphopoiesis in reconstituted *Rag2*$^{-/-}$ mice rule out transdifferentiation as a mechanism for the conversion of mature B cells into T cells. Instead, the loss of Pax5 allows mature B cells to develop into T cells in the thymus by dedifferentiation to an uncommitted progenitor cell stage in the bone marrow.

Double-positive thymocytes of reconstituted *Rag2*$^{-/-}$ mice exhibited a normal proliferation response and cytokine production on appropriate stimulation (Supplementary Fig. 13). To study the *in vivo* immune response of B-cell-derived T cells, we used *Tcra*$^{-/-}$ mice[15] as recipients for the injection of *in vitro Pax5*-deleted mature

B cells (Fig. 4a). Eight weeks after transplantation, the reconstituted *Tcra*$^{-/-}$ mice were immunized with sheep red blood cells, and the germinal-centre reaction was investigated by flow cytometric and immunohistochemical analyses of the spleen ten days after immunization (Fig. 4b, c). In contrast to the lymphopenic *Rag2*$^{-/-}$ mice, the *Tcra*$^{-/-}$ mice have normal B-cell compartments and an almost normal thymic cellularity, because they only fail to develop CD4 and CD8 single-positive T cells[15]. Owing to the absence of TCR-β^+ T cells in the spleen, the *Tcra*$^{-/-}$ mice are unable to generate germinal centres and PNA$^+$Fas$^+$ germinal-centre B cells in response to immunization (Fig. 4b, c). In contrast, the spleen of the reconstituted *Tcra*$^{-/-}$ mice contained polyclonal TCR-β^+ T cells (Fig. 4b) carrying wild-type *Tcra* and deleted *Pax5* alleles (Supplementary Fig. 14). These donor-derived T cells efficiently induced the development of germinal centres and PNA$^+$Fas$^+$ germinal-centre B cells (Fig. 4b, c), indicating that the B-cell-derived T cells are fully functional.

Ectopic expression of lineage-specific transcription factors can convert one cell type into another, as documented by the *in vitro* transdifferentiation of B lymphocytes into macrophages in response to retroviral expression of the CCAAT/enhancer-binding protein-α (ref. 16). Moreover, forced expression of the transcription factors Oct3/4, Sox2, c-Myc and Klf4 is sufficient to transdifferentiate mouse fibroblasts *in vitro* into germline-competent embryonic stem cells[17,18]. In contrast to these gain-of-function experiments, we have demonstrated that the loss of a single transcription factor, Pax5, allows mature B cells to dedifferentiate *in vivo* to uncommitted haematopoietic progenitors and to develop into progenitor cell lymphomas. *Pax5* inactivation also induces the dedifferentiation of committed pro-B cells, which have entered the B-cell lineage for only one or two days[19]. In marked contrast, the mature B cells passed several B-cell developmental checkpoints, migrated to peripheral lymphoid organs, are quiescent and, owing to their longevity, are three to five months old[20,21]. Despite their advanced differentiation stage, mature B cells retain an extraordinary developmental plasticity, as revealed by their dedifferentiation potential on Pax5 loss. Under physiological conditions, *Pax5* and its transcription programme are downregulated during terminal differentiation of mature B cells to plasma cells[7,22], which is initiated in response to antigen stimulation of the BCR[23]. Hence, the loss of Pax5 in the context of strong BCR signalling results in forward differentiation of mature B cells to plasma cells, whereas *Pax5* inactivation in the absence of BCR signalling initiates the reversal of differentiation to uncommitted progenitors.

Chromosomal translocations implicated *PAX5* as an oncogene in the generation of human acute lymphoblastic leukaemias and non-Hodgkin's lymphomas[5]. A genome-wide analysis revealed monoallelic loss or point mutations of *PAX5* in 32% of B-progenitor acute lymphoblastic leukaemias[24]. These genetic lesions result in haploinsufficiency, suggesting that heterozygous *PAX5* mutations contribute to leukaemogenesis[24]. Our results indicate, however, that the inactivation of one *Pax5* allele in the absence of other oncogenic lesions is not sufficient to induce tumour development in heterozygous *Cd19-cre Pax5*$^{fl/+}$ mice. Instead, the complete loss of Pax5 in B cells leads to an aggressive progenitor cell lymphoma. The Pax5-deficient progenitor cell state probably contributes to lymphomagenesis, because *Pax5*$^{-/-}$ pro-B cells retain an extensive *in vivo* self-renewal potential[25] similar to stem cells[1] and cancer stem cells[26]. Our loss-of-function experiments thus identified *Pax5* as a tumour suppressor gene of the

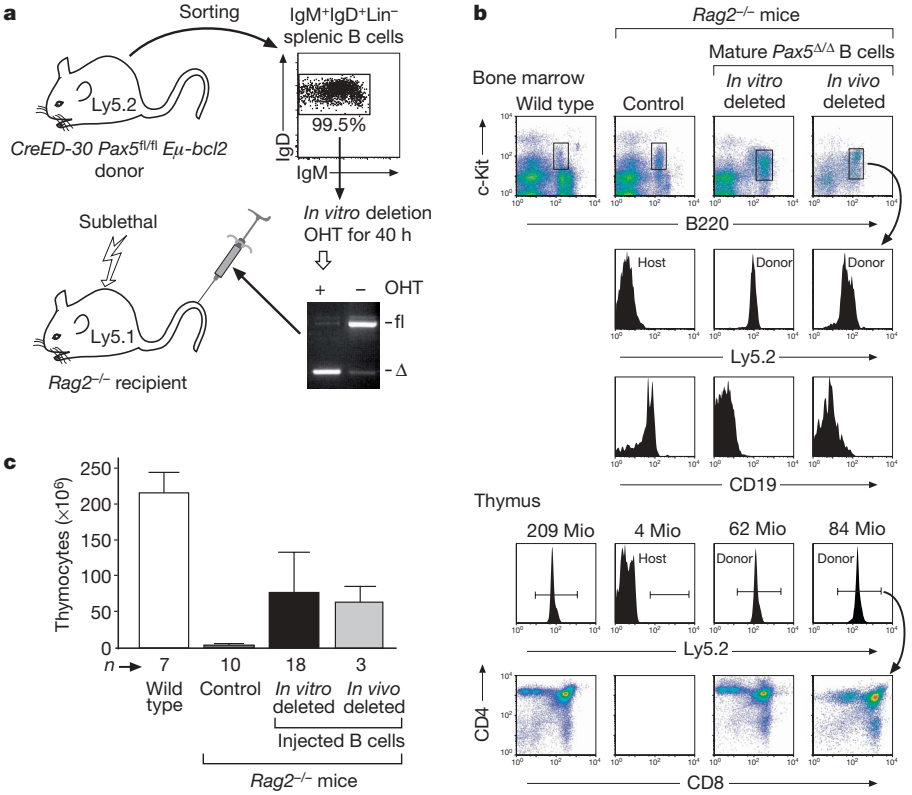

Figure 2 | T-cell reconstitution by *Pax5*-deleted mature B cells in *Rag2*$^{-/-}$ mice. a, Experimental strategy. IgM$^+$IgD$^+$Lin$^-$ B cells from the spleen or lymph nodes of *CreED-30 Pax5*$^{fl/fl}$ *Eμ-bcl2 Ly5.2*$^+$ mice were purified by FACS sorting (reanalysis shown) and incubated *in vitro* with OHT for 40 h before injection into sublethally irradiated *Rag2*$^{-/-}$ *Ly5.1*$^+$ mice. Deletion (Δ) of the floxed (fl) *Pax5* allele is shown. **b**, Flow cytometric analysis of the bone marrow and thymus of *Rag2*$^{-/-}$ *Ly5.1*$^+$ recipient mice eight weeks after transplantation of *in vitro* or *in vivo Pax5*-deleted mature B cells. Ly5.2 and CD19 expression is shown for the gated B220$^+$c-Kit$^+$ pro-B cells in the bone marrow (upper panels). The thymic cellularity and Ly5.2 expression of all thymocytes is shown together with the CD4 and CD8 expression pattern of gated Ly5.2$^+$ thymocytes (lower panels). The analysis of all reconstituted mice is shown in Supplementary Fig. 9. Mio, millions of cells. **c**, Restoration of thymic cellularity in reconstituted *Rag2*$^{-/-}$ mice. The average (+ s.d.) cellularity and number (*n*) of mice analysed are indicated.

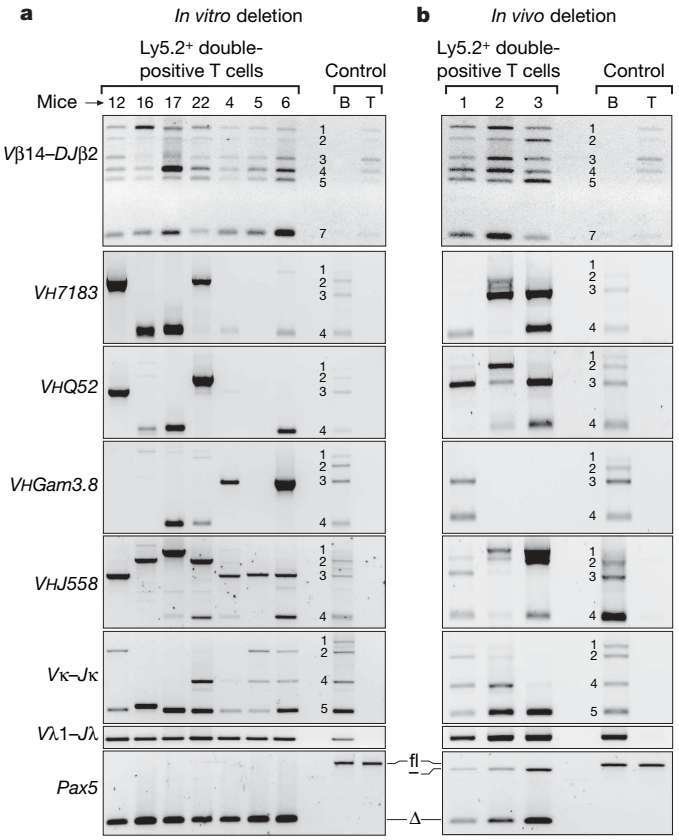

Figure 3 | Immunoglobulin gene rearrangements in thymocytes of reconstituted *Rag2*$^{-/-}$ mice. PCR detection of immunoglobulin gene rearrangements in Ly5.2$^+$CD4$^+$CD8$^+$ (double-positive) thymocytes, which were sorted 8–12 weeks after transplantation of *Rag2*$^{-/-}$ *Ly5.1*$^+$ recipient mice with *in vitro Pax5*-deleted mature B cells (**a**) or *in vivo Pax5*-deleted mature B cells (**b**). Oligoclonal *VH–DJH*, *Vκ–Jκ* and *Vλ1–Jλ* rearrangements were detected in Ly5.2$^+$ double-positive thymocytes in addition to polyclonal *Vβ14–Jβ2* rearrangements. B220$^+$ splenocytes (B) and double-positive thymocytes (T) from *Pax5*$^{fl/fl}$ mice were used as controls. Numbers indicate rearrangements involving different *Jβ2*, *JH* or *Jκ* segments. PCR genotyping revealed full conversion of the fl to the Δ *Pax5* allele in all Ly5.2$^+$ thymocytes. The *Pax5* mutant (–) germline allele is indicated. The *VHGam3.8*, *VHQ52* and *VH7183* genes are also known as *Igh-VGAM3.8*, *Igh-VQ52* and *Igh-V7183*, respectively.

B-lymphoid lineage. Hence, Pax5 can function as a tumour suppressor or oncoprotein in the generation of different lymphoid malignancies in analogy to a similar dual role of Notch1 (refs 27, 28) and c-Fos[29,30] in the development of distinct cancers.

METHODS SUMMARY

Mice. All mouse strains (see Methods) were maintained on the C57BL/6 background.

Tumour analysis. Organs were fixed in 4% formaldehyde followed by paraffin embedding, sectioning and staining with haematoxylin and eosin. Lymph node tumour cells were cultured with γ-irradiated stromal (ST2) cells, IL-7, Flt3L and stem cell factor before injection into syngeneic mice.

***In vitro Pax5*-deleted mature B cells.** *CreED-30 Pax5*$^{fl/fl}$ *Eμ-bcl2 Ly5.2*$^+$ mice were injected 5–7 times with the anti-IL-7Rα antibody A7R34, and Lin$^+$ non-B cells were removed from splenocytes or lymph node cells by magnetic cell sorting (MACS) after staining with lineage marker antibodies (CD3ε, CD4, CD8a, CD11c, CD49b, CD93, Gr1, c-Kit, Mac1, TCR-β, Ter119 and Thy1.2). After FACS sorting, the Lin$^-$IgM$^+$IgD^{high} B cells were incubated *in vitro* with the oestrogen analogue OHT to delete the floxed *Pax5* alleles by the OHT-induced Cre-ER activity (*CreED-30*).

***In vivo Pax5*-deleted mature B cells.** Lymph-node cells from anti-IL-7Rα antibody-treated *Cd19-cre Pax5*$^{fl/-}$ *Eμ-bcl2 Ly5.2*$^+$ mice were depleted of Lin$^+$ non-B cells with a similar lineage antibody cocktail before sorting of Pax5-deficient mature B cells as Lin$^-$CD25$^+$IgM$^+$IgD$^-$ cells[4].

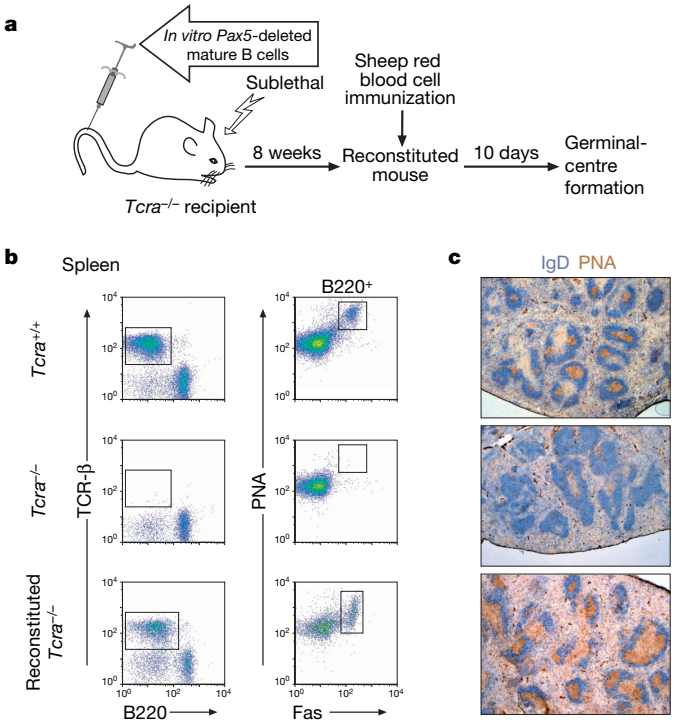

Figure 4 | Normal immune function of B-cell-derived T cells. **a**, Schematic diagram of the reconstitution and immunization of *Tcra*$^{-/-}$ mice with sheep red blood cells. **b**, Flow cytometric analysis of splenocytes from mice of the indicated genotypes ten days after immunization with sheep red blood cells. **c**, Formation of germinal centres in the same immunized mice shown in **b**. Cryosections of the spleen were stained with fluorescein isothiocyanate (FITC)–anti-IgD antibody (blue) and biotinylated peanut haemagglutinin (PNA, brown) followed by detection with an alkaline phosphatase-coupled anti-FITC antibody (visualized with Fast Blue) and horseradish peroxidase-conjugated streptavidin (visualized with diaminobenzidine), respectively.

B-cell transplantations. *In vitro Pax5*-deleted B cells ($2–4 \times 10^6$ cells per mouse) or *in vivo Pax5*-deleted B cells (5×10^5 cells per mouse) were intravenously injected into 8–12-week-old *Rag2*$^{-/-}$ *Ly5.1*$^+$ or *Tcra*$^{-/-}$ recipient mice that were γ-irradiated with a sublethal dose (4 Gy).

V(D)J recombination. *Igh*, *Igk*, *Igl* and *Tcrb* rearrangements were amplified by PCR using the primers listed in Supplementary Table 4. To characterize individual *V–(D)J* rearrangements, the PCR fragments were cloned, and at least ten clones corresponding to the same PCR fragment were sequenced.

Immunizations. Sheep red blood cells ($1–2 \times 10^8$ cells) were intraperitoneally injected into control or reconstituted *Tcra*$^{-/-}$ mice. Ten days later, the spleen was analysed by flow cytometry and immunohistochemistry[7].

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Author Information All microarray data have been deposited in the GEO repository at NCBI under accession numbers GSM210098, GSM210099, GSM210100, GSM210101, GSM215734, GSM215735 and GSM213736. Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Correspondence and requests for materials should be addressed to M.B. (busslinger@imp.ac.at).

METHODS

Mice. The following mice were maintained on the C57BL/6 background and genotyped as described: *Pax5*$^{+/-}$ (ref. 31), *Pax5*$^{fl/fl}$ (ref. 4), *Rag2*$^{-/-}$ (ref. 32), *Tcra*$^{-/-}$ (ref. 15), *Cd19*$^{cre/cre}$ (ref. 33), *CreED-30* (ref. 11) and *Eμ-bcl2-36* (ref. 34). Heterozygous *Cd19*$^{cre/+}$ mice are referred to as *Cd19-cre* mice in this manuscript. All mice were *Ly5.2*$^{+}$ except for the *Rag2*$^{-/-}$ *Ly5.1*$^{+}$ mice, which were obtained from Taconic (000461-M).

FACS sorting and analysis. FITC-, phycoerythrin (PE)- or allophycyanin (APC)-coupled antibodies to the following cell-surface proteins were used for flow cytometry: cell-surface protein B220 (antibody RA3-6B2), CD3ε (145-2C11), CD4 (H129.19), CD8a (53-6.7), CD11b (also known as Mac1, CD11b/Mac1) (M1/70), CD11c (HL3), CD19 (1D3), CD21 (7G6), CD22 (Cy34.1), CD23 (B3B4), CD25 (PC61), CD40 (FGK45.5 or 3/23), CD49b (DX5), CD72 (K10.6), CD90.2/Thy1.2 (53-2.1), CD93/AA4.1 (PB.493), CD95/Fas (Jo2), CD117/c-Kit (2B8), CD127/IL-7Rα (A7R34), CD135/Flt3 (A2F10.1), CD138 (281-2), F4/80 (CI:A3-1), Gr1 (RB6-8C5), IgD (1.19), Igκ (187.1), Igλ (R26-46), IgM (M41-42), Ly5.1 (A20), Ly5.2 (104), M-CSFR (macrophage-colony-stimulating factor receptor) (AFS98), MHCII (M5-114), pre-BCR (SL156), TCR-β (H57-597) and Ter119 (TER-119). FITC-labelled PNA (Vector Laboratories) was used for staining of germinal-centre B cells. Red blood cells were eliminated from splenocytes by lysis in 150 mM NH_4Cl and 10 mM $KHCO_3$ for 5 min at 22 °C. Antibody staining and washing of single-cell suspensions were done in phosphate buffered saline solution (PBS) containing 2% heat-inactivated fetal calf serum (FCS). Unspecific antibody binding was suppressed by preincubation of cells with CD16/CD32 Fc-block solution (BD Pharmingen). For all analyses, a forward and side scatter gate was used to include viable cells while excluding dead cells, debris and cell doublets. If possible, dead cells were also eliminated by staining with propidium iodide (1 μg ml^{-1}). A FACSAria cell sorter (Becton-Dickinson) was used to purify the different B- and T-cell types.

Anti-IL-7Rα antibody treatment. The purified anti-IL-7Rα antibody A7R34 (1 mg in 150 μl PBS) was injected into the tail vein of *CreED-30 Pax5*$^{fl/fl}$ *Eμ-bcl2 Ly5.2*$^{+}$ and *Cd19-cre Pax5*$^{fl/-}$ *Eμ-bcl2 Ly5.2*$^{+}$ mice at the age of 6–8 weeks. This injection was repeated every second day during 8–12 days (5–7 injections per mouse), which blocked B-cell development leading to the loss of pro-B, pre-B and immature B cells in the bone marrow and spleen (Supplementary Fig. 5). Mature B cells were sorted two days after the last injection.

***In vitro* deletion of *Pax5* in sorted mature B cells.** Single-cell suspensions of the spleen or lymph nodes from anti-IL-7Rα antibody-treated *CreED-30 Pax5*$^{fl/fl}$ *Eμ-bcl2 Ly5.2*$^{+}$ mice were stained with PE-coupled lineage marker antibodies (CD3ε, CD4, CD8a, CD11c, CD49b, CD93, Gr1, c-Kit, Mac1, TCR-β, Ter119 and Thy1.2), and the Lin^{+} non-B cells and traces of residual CD93^{+} B-cell precursors were eliminated by MACS with anti-PE beads (Miltenyi Biotec). Mature B cells were then FACS-sorted as Lin^{-}IgM^{+}IgD^{high} cells after staining with FITC–anti-IgM and APC–anti-IgD antibodies. Flow cytometric reanalysis of the sorted mature B cells revealed a purity of more than 99% (Fig. 2a and Supplementary Fig. 6a). The purified B cells were subsequently incubated for 40 h in Iscove's modified Dulbecco's medium supplemented with 10% FCS, 50 μM β-mercaptoethanol, 1 mM glutamine and 1 μM OHT (Sigma). Deletion of the floxed *Pax5* allele by the OHT-induced activity of the Cre-ER fusion protein (encoded by the *CreED-30* transgene) was verified by PCR genotyping[4] before transplantation of the *Pax5*-deleted mature B cells.

FACS sorting of *in vivo Pax5*-deleted mature B cells. Lymph node cells from anti-IL-7Rα antibody-treated *Cd19-cre Pax5*$^{fl/-}$ *Eμ-bcl2 Ly5.2*$^{+}$ mice were stained with biotinylated lineage marker antibodies (CD3ε, CD4, CD8a, CD11c, CD49b, Gr1, c-Kit, Mac1 and Ter119), and Lin^{+} non-B cells were depleted by magnetic cell sorting with streptavidin MACS beads (Miltenyi Biotec). Pax5-deficient mature B cells were sorted as Lin^{-}CD25^{+}IgM^{+}IgD^{-} cells[4] after staining with cychrome-anti-CD25, PE–anti-IgM and APC–anti-IgD antibodies in combination with PE–Cy7-streptavidin staining of the Lin^{+} non-B cells. Flow cytometric reanalysis of the sorted Pax5-deficient B cells indicated a purity of more than 95% (Supplementary Fig. 6b).

Transplantations of mature *Pax5*-deleted B cells. *In vitro Pax5*-deleted mature B cells were washed in PBS and injected into the tail vein of 8–12-week-old *Rag2*$^{-/-}$ *Ly5.1*$^{+}$ or *Tcra*$^{-/-}$ recipient mice (2–4 × 10^{6} cells per mouse) that had been γ-irradiated with a sublethal dose (4 Gy) one day previously. *In vivo Pax5*-deleted mature B cells were similarly injected into *Rag2*$^{-/-}$ *Ly5.1*$^{+}$ mice (5 × 10^{5} cells per mouse). The drinking water for all reconstituted mice contained 1.14 g l^{-1} neomycin.

Sorting of donor-derived pro-B and T cells. Dedifferentiated pro-B cells of donor origin were sorted from the bone marrow of transplanted *Rag2*$^{-/-}$ *Ly5.1*$^{+}$ mice as Ly5.2^{+}CD19^{-}B220^{+}c-Kit^{+} cells after staining with FITC–anti-Ly5.2, PE–anti-CD19, PE–Cy5-anti-B220 and APC–anti-cKit antibodies. Donor-derived double-positive thymocytes were sorted from the thymus of transplanted *Rag2*$^{-/-}$ *Ly5.1*$^{+}$ mice as Ly5.2^{+}CD4^{+}CD8a^{+} cells after staining with FITC–anti-Ly5.2, PE–anti-CD4 and APC–anti-CD8a antibodies.

V(D)J recombination analysis. Sorted cells as well as tumour cells were digested with proteinase K, and DNA was isolated by phenol extraction and ethanol precipitation. V(D)J rearrangements of the *Igh*, *Igk*, *Igl* and *Tcrb* loci were amplified by PCR using published primers[9,35–37] as well as newly designed Vβ gene primers, which are listed in Supplementary Table 4. PCR products were separated on agarose gels and visualized by ethidium bromide staining except for the data shown in Supplementary Fig. 10, which were obtained by Southern blotting using a germline PCR fragment (amplified with the Dβ2 and Jβ2 primers, Supplementary Table 4) as a DNA probe. To determine the DNA sequences of individual *V*–(*D*)*J* rearrangements, the PCR fragments were isolated from the agarose gel and cloned into the pGEM-Teasy vector (Promega); the DNA inserts of at least ten clones corresponding to the same PCR fragment were then sequenced.

RT–PCR analysis of rearranged immunoglobulin gene transcripts. RNA was prepared from *in vitro* grown *Pax5*$^{Δ/-}$ tumour cell lines using the TRIzol reagent (GIBCO-BRL), and was then treated with DNase I (Promega) to remove contaminating DNA. Complementary DNA was synthesized with random hexamers and superscript II reverse transcriptase (Invitrogen). Rearranged immunoglobulin transcripts were amplified by PCR using the primers listed in Supplementary Table 5. The PCR products were separated on agarose gels and visualized by staining with ethidium bromide.

Microarray analysis. For microarray analysis, total RNA (3–5 μg) prepared from *Pax5*$^{+/+}$ pro-B cells was used to generate a Cy3-labelled cDNA probe by oligo-dT$_{15}$-primed reverse transcription in the presence of Cy3–dUTP. Likewise, total RNA from *Pax5*$^{-/-}$ pro-B cells, *Pax5*$^{-/-}$ *Rag2*$^{-/-}$ pro-B cells or *Pax5*$^{Δ/-}$ lymph node tumours were used to generate Cy5-labelled cDNA probes by reverse transcription in the presence of Cy5–dUTP. The Cy3- and Cy5-labelled probes were hybridized to the mouse 'lymphochip'[38], which was scanned using a GenePix 4000 Scanner (Axon Instruments Inc.). The scanned data were evaluated with the GenePix Pro 5 chip software.

Intracellular cytokine and Foxp3 staining. Thymocytes were resuspended in RPMI 1680 medium (containing 10% FCS and 1 mM glutamine) at a concentration of 4 × 10^{6} cells ml^{-1} and incubated for 6 h in the presence of PMA (20 ng ml^{-1}, Sigma), ionomycin (1 μM, Invitrogen) and brefeldin A (0.01%, BD PharMingen). The cells were then washed, incubated with CD16/CD32 Fc-block solution (PD PharMingen) and stained with FITC–anti-TCR-β, PE–Cy5-anti-CD4 and APC–anti-CD8a antibodies. After washing, the cells were fixed with 4% formaldehyde in PBS, permeabilized with the Perm/Wash solution (BD Biosciences) and stained with PE–anti-TNFα (MP6-XT22) or anti-IL-2 (JE56-5H4) antibodies before flow cytometric analysis. PE-labelled rat IgG1 (R3-34) and IgG2b (A95-1, BD PharMingen) were used as negative isotype controls. The expression of Foxp3 was similarly analysed by intracellular staining of thymocytes using the PE–anti-Foxp3 (FJK-16s) antibody of the PE–anti-mouse/rat Foxp3 staining kit from eBiosciences.

T-cell proliferation assay. An anti-CD3ε antibody (2C11) was used at a concentration of 100 μg ml^{-1} in PBS to coat 96-well plates (50 μl well^{-1}) for 90 min at 37 °C followed by two washes with PBS. Thymocytes, which were resuspended at 10^{6} cells ml^{-1} in RPMI 1680 medium (containing 10% FCS and 1 mM glutamine), were seeded into the coated 96-well plates (2 × 10^{5} cells well^{-1}), and RPMI 1680 culture medium containing 5 μg ml^{-1} of the anti-CD28 antibody 37.51 was added to the wells. Culture medium without antibody was added to control wells lacking any coating. The cells were incubated for 4–7 days, and their proliferation was measured by thymidine incorporation after ^{3}H-thymidine addition for the last 24 h.

Immunization and germinal-centre analysis. Sheep red blood cells were washed in PBS and resuspended at 1–2 × 10^{9} cells ml^{-1} followed by intraperitoneal injection of 100 μl into control mice or *Tcra*$^{-/-}$ mice, which had been transplanted with *in vitro Pax5*-deleted mature B cells eight-weeks before. Ten days after immunization, the spleens were isolated, embedded in OCT compound (Sakura) and snap-frozen on dry ice. Cryosections of the spleen were stained with a FITC–anti-IgD antibody (11-26c.2a, BD PharMingen) and biotinylated PNA (B-1075, Vector Laboratories). FITC–anti-IgD was detected with an alkaline-phosphatase-coupled anti-FITC antibody (Roche), which was visualized by incubation with Fast Blue (Sigma). Biotinylated PNA was detected with horseradish peroxidase-conjugated streptavidin (Zymed) followed by incubation with diaminobenzidine (DAB; Sigma).

***In vitro* culture of tumour and pro-B cells.** Transformed cells isolated from *Cd19-cre Pax5*$^{fl/-}$ lymph node tumours were cultured on γ-irradiated ST2 feeder cells in Iscove's modified Dulbecco's medium containing 2% heat-inactivated FCS, 0.03% (w/v) primatone RL (Quest International), 1 mM glutamine, 50 mM β-mercaptoethanol, 1% supernatant of IL-7-secreting J558L cells, 2.5% supernatant of Flt3L-producing SP2.0 cells and 2% supernatant of stem cell

factor-secreting CHO cells. Ly5.2^{+}c-Kit^{+}B220^{+}CD19^{-} pro-B cells were sorted from the bone marrow of *Rag2*$^{-/-}$ *Ly5.1*$^{+}$ mice transplanted with *in vitro Pax5*-deleted mature B cells and were grown under the same conditions described above for the tumour cells.

Spectral karyotype analysis. Exponentially growing *Pax5*$^{\Delta/-}$ tumour cells were incubated for 5 h with 50 nM colcemid (Karyo-Max, GIBCO-BRL) in the absence of ST2 feeder cells. Metaphase chromosome spreads, prepared according to standard protocols[39], were hybridized using a SkyPaint DNA kit (Applied Spectral Imaging) according to the manufacturer's instructions. Spectral images were recorded and analysed using an interferometer and software from Applied Spectral Imaging[40].

Histological analysis. Organs of moribund *Cd19-cre Pax5*$^{fl/-}$ mice were fixed at 4 °C overnight with 4% formaldehyde in PBS followed by paraffin embedding, sectioning and staining with haematoxylin and eosin.

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