Signalling thresholds and negative B-cell selection in acute lymphoblastic leukaemia

Zhengshan Chen\textsuperscript{1,\*}, Seyedmehdi Shojaee\textsuperscript{1,\*}, Maike Buchner\textsuperscript{1}, Huimin Geng\textsuperscript{1}, Jae Woong Lee\textsuperscript{1}, Lars Klemm\textsuperscript{1}, Björn Titz\textsuperscript{2}, Thomas G. Graeber\textsuperscript{2}, Eugene Park\textsuperscript{1}, Ying Xim Tan\textsuperscript{1}, Anne Satterthwaite\textsuperscript{4}, Elisabeth Paitetta\textsuperscript{5}, Stephen P. Hunger\textsuperscript{6}, Cheryl L. Willman\textsuperscript{7}, Ari Melnick\textsuperscript{8}, Mignon L. Loh\textsuperscript{9}, Jae U. Jung\textsuperscript{10}, John E. Coligan\textsuperscript{11}, Silvia Bolland\textsuperscript{12}, Tak W. Mak\textsuperscript{13}, Andre Limander\textsuperscript{1,\*}, Hassan Jumaa\textsuperscript{15}, Michael Reth\textsuperscript{16}, Arthur Weiss\textsuperscript{3}, Clifford A. Lowell\textsuperscript{1} & Markus Münchens\textsuperscript{1}

B cells are selected for an intermediate level of B-cell antigen receptor (BCR) signalling strength: attenuation below minimum (for example, non-functional BCR)\textsuperscript{1} or hyperactivation above maximum (for example, self-reactive BCR)\textsuperscript{1,3} thresholds of signalling strength causes negative selection. In approximately 25\% of cases, acute lymphoblastic leukaemia (ALL) cells carry the oncogenic BCR-ABL1 tyrosine kinase (Philadelphia chromosome positive), which mimics constitutively active pre-BCR signalling\textsuperscript{1,4}. Current therapeutic approaches are largely focused on the development of more potent tyrosine kinase inhibitors to suppress oncogenic signalling below a minimum threshold for survival\textsuperscript{5}. We tested the hypothesis that targeted hyperactivation—above a maximum threshold—will engage a deleterional checkpoint for removal of self-reactive B cells and selectively kill ALL cells. Here we find, by testing various components of proximal pre-BCR signalling in mouse BCR-ABL1 cells, that an incremental increase of Syk tyrosine kinase activity was required and sufficient to induce cell death. Hyperactive Syk was functionally equivalent to acute activation of a self-reactive BCR on ALL cells. Despite oncogenic transformation, this basic mechanism of negative selection was still functional in ALL cells. Unlike normal pre-B cells, patient-derived ALL cells express the inhibitory receptors PECAM1, CD300A and LAIR1 at high levels. Genetic studies revealed that Pecami1, Cd300a and Lair1 are critical to calibrate oncogenic signalling strength through recruitment of the inhibitory phosphatases Ptpn6 (ref. 7) and Inpp5d (ref. 8). Using a novel small-molecule inhibitor of INPP5D (also known as SHIP1)\textsuperscript{7}, we demonstrated that pharmacological hyperactivation of SYK and engagement of negative B-cell selection represents a promising new strategy to overcome drug resistance in human ALL.

ALL represents the most frequent type of cancer in children and is frequent in adults as well. Although outcomes for patients with ALL have greatly improved over the past four decades, ALL driven by oncogenic tyrosine kinases (BCR-ABL1 in adults and other oncogenic fusion tyrosine kinases in childhood ALL)\textsuperscript{12,15} remains a clinical problem. Current tyrosine kinases (TKIs) have greatly improved over the past four decades, ALL driven by oncogenic tyrosine kinases (BCR-ABL1 in adults and other oncogenic fusion tyrosine kinases (Philadelphia chromosome positive), which mimics constitutively active pre-BCR signalling\textsuperscript{1,4}. Current therapeutic approaches are largely focused on the development of more potent tyrosine kinase inhibitors to suppress oncogenic signalling below a minimum threshold for survival\textsuperscript{5}. We tested the hypothesis that targeted hyperactivation—above a maximum threshold—will engage a deleterional checkpoint for removal of self-reactive B cells and selectively kill ALL cells. Here we find, by testing various components of proximal pre-BCR signalling in mouse BCR-ABL1 cells, that an incremental increase of Syk tyrosine kinase activity was required and sufficient to induce cell death. Hyperactive Syk was functionally equivalent to acute activation of a self-reactive BCR on ALL cells. Despite oncogenic transformation, this basic mechanism of negative selection was still functional in ALL cells. Unlike normal pre-B cells, patient-derived ALL cells express the inhibitory receptors PECAM1, CD300A and LAIR1 at high levels. Genetic studies revealed that Pecami1, Cd300a and Lair1 are critical to calibrate oncogenic signalling strength through recruitment of the inhibitory phosphatases Ptpn6 (ref. 7) and Inpp5d (ref. 8). Using a novel small-molecule inhibitor of INPP5D (also known as SHIP1)\textsuperscript{7}, we demonstrated that pharmacological hyperactivation of SYK and engagement of negative B-cell selection represents a promising new strategy to overcome drug resistance in human ALL based on the pharmacological hyperactivation of SYK. Pre-BCR signals are initiated from immunoreceptor tyrosine-based activation motifs (ITAMs) in the cytoplasmic tail of immunoglobulin (Ig) extracellular activation motifs (ITAMs) in the cytoplasmic tail of immunoglobulin (Ig)(\textsuperscript{79a}) and Ig\textbeta (CD79B) signalling chains\textsuperscript{7}, and are essential for survival and proliferation of normal pre-B cells. However, hyperactive signalling from a self-reactive pre-BCR, owing to the ubiquitous presence of self-antigen, induces negative selection and cell death\textsuperscript{2}. Here, we observed that Ph\textsuperscript{\textsuperscript{\textsuperscript{+}}} ALL cells consistently lack surface expression of ITAM-bearing Ig\textalpha and Ig\textbeta signalling chains (Extended Data Fig. 1a). Seemingly in contrast to compromised Ig\textalpha and Ig\textbeta expression, multiple components of proximal pre-BCR signalling were activated downstream of the BCR-ABL1 tyrosine kinase (Fig. 1a). These findings demonstrate that oncogenic BCR-ABL1 supplants ITAM-dependent signalling and mimics a constitutively active pre-BCR through engagement with its proximal signalling cascade. Besides BCR-ABL1, immaturity of BCR signalling was previously demonstrated for a number of viral oncoproteins including Epstein–Barr virus latent membrane protein 2A (LMP2A)\textsuperscript{13}. Reconstitution of Ig\textalpha expression induced strong tyrosine phosphorylation of proximal pre-BCR signalling molecules, followed by cell death (Extended Data Fig. 1b–d). Likewise, Ph\textsuperscript{\textsuperscript{+}} ALL cells from three patients were highly sensitive to reactivation of ITAM-dependent signalling (LMP2A\textsuperscript{13}; Extended Data Fig. 1e, f). Interestingly, activation of ITAM signalling was toxic in leukaemic but not in normal pre-B cells (Extended Data Fig. 1b). We therefore tested whether BCR-ABL1- and ITAM-dependent activation of proximal pre-BCR signalling are mutually exclusive because both engage the same pre-BCR-associated tyrosine kinases. Consequently, we repeated activation of ITAM signalling in the presence and absence of TKI treatment (imatinib; Fig. 1b). Seemingly paradoxically, treatment with imatinib, although designed to kill leukaemia cells, rescued BCR-ABL1 ALL cells in this experimental setting, and subsequent washout of imatinib reversed the protective effect (Fig. 1b). To pinpoint which aspect of proximal pre-BCR signalling is toxic to Ph\textsuperscript{\textsuperscript{+}} ALL cells, we used genetic systems for hyperactivation of Syk, Src and Btk. In contrast to Src and Btk, constitutively active Syk (Syk\textsuperscript{(Myv)}) induced rapid cell death (Fig. 1b and Extended Data Fig. 2a–d). Hyperactive Syk was synthetically lethal in combination with oncogenic BCR–ABL1, and cytotoxic effects were mitigated by TKI treatment (imatinib; Fig. 1b). Like BCR–ABL1, SYK kinase activity alone mimicked

\textsuperscript{1}Department of Laboratory Medicine, University of California, San Francisco, California 94143, USA.
\textsuperscript{2}Crump Institute for Molecular Imaging, Department of Molecular and Medical Pharmacology, University of California, Los Angeles, California 90095, USA.
\textsuperscript{3}Rosalind Russell-Ephraim P. Engleman Medical Research Center for Arthritis, Division of Rheumatology, Department of Medicine, Howard Hughes Medical Institute, University of California, San Francisco, California 94143, USA.
\textsuperscript{4}Department of Internal Medicine, University of Texas Southwestern Medical Center, Dallas, Texas 75390, USA.
\textsuperscript{5}Department of Medicine, Albert Einstein College of Medicine, Bronx, New York 10466, USA.
\textsuperscript{6}Division of Pediatric Oncology and Center for Childhood Cancer Research, Children’s Hospital of Philadelphia, Philadelphia, Philadelphia 19104, USA.
\textsuperscript{7}University of New Mexico Cancer Center, Albuquerque, New Mexico 87102, USA.
\textsuperscript{8}Departments of Medicine and Pharmacology, Weill Cornell Medical College, New York, New York 10065, USA.
\textsuperscript{9}Pediatic Hematology-Oncology, University of California, San Francisco, California 94143, USA.
\textsuperscript{10}Department of Molecular Microbiology and Immunology, University of Southern California, Los Angeles, California 90033, USA.
\textsuperscript{11}Receptor Cell Biology Section, Laboratory of Immunogenetics, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Rockville, Maryland 20852, USA.
\textsuperscript{12}Autoimmunity and Functional Genomics Section, Laboratory of Immunogenetics, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Rockville, Maryland 20852, USA.
\textsuperscript{13}The Campbell Family Institute for Breast Cancer Research, University Health Network, 620 University Avenue, Toronto, Ontario M5G 2M9, Canada.
\textsuperscript{14}Department of Anatomy, University of California, San Francisco, California 94143, USA.
\textsuperscript{15}Institute of Immunology, University of Heidelberg, Im Neuenheimer Feld 280, 69120 Heidelberg, Germany.

\textsuperscript{\*}These authors contributed equally to this work.

© 2015 Macmillan Publishers Limited. All rights reserved
constitutively active pre-BCR signalling and was sufficient to transform mouse pro-B cells (Extended Data Fig. 2e). Interestingly, BCR–ABL1 kinase activity induced phosphorylation of SYK at interdomain B (Fig. 1a), which relieves the autoinhibitory conformation of SYK<sup>4</sup>. To study the specific function of SYK interdomain B (Y348 and Y352) tyrosines in BCR–ABL1 ALL cells, we tested loss (Y→F) and phosphomimetic gain (Y→E) of function mutants of SYK. Empty vectors, kinase–dead SYK(K402A) and wild-type SYK were used as controls (Fig. 1c). In the absence of constitutive membrane localization, wild-type SYK had only minor toxic effects on ALL cells. Interestingly, however, the expression of SYK carrying phosphomimetic mutations of interdomain B tyrosines (Y348F/Y352E, Y348E/Y352F, K402A) or an empty vector and relative changes of transduced (GFP<sup>+</sup>) cells were monitored by flow cytometry. Data are presented as means ± standard deviation (s.d.) from three independent experiments (b, c).

To determine whether high expression levels of ITIM-bearing receptors influence the course of human ALL, we segregated patients from two clinical trials (the Children’s Oncology Group (COG) P9906 study and the Eastern Cooperative Oncology Group (ECOG) 2993 study) into two groups on the basis of whether they had higher or lower than median expression levels of PECA1, CD300A and LAIR1 at the time of diagnosis. Higher than median expression levels of ITIM receptors on ALL cells at the time of diagnosis predicted shorter overall and relapse-free survival (Extended Data Fig. 4a–e). These findings identify ITIM-bearing inhibitory receptors as a novel biomarker with potential use in risk stratification of children and adults with ALL.

To measure the functional consequences of ITIM-receptor deletion, pre-B cells from the bone marrow of Pacem<sup>1</sup>−/− and Cd300a<sup>−/−</sup>, as well as Lαιr<sup>β<sub>0</sub></sup> mice and wild-type controls were propagated in the presence of interleukin (IL)-7 or transformed with BCR–ABL1 to model human Ph<sup>+</sup> ALL. Lαιr<sup>β<sub>0</sub></sup> ALL cells were retrovirally transduced with 4-hydroxytamoxifen (4-OHT)-inducible Cre. Loss of ITIM receptors had no significant effects on the proliferation and survival of normal pre-B cells (Extended Data Fig. 5a). In contrast, in the absence of ITIM-bearing receptors, pre-B ALL cells underwent cellular senescence and cell cycle arrest and failed to form colonies (Fig. 2a and Extended Data Fig. 5a, b) in parallel with activation of cell cycle checkpoint molecules and increased levels of cytoplasmic reactive oxygen species (ROS; Extended Data Fig. 4g, h). Importantly, inducible Cre-mediated ablation of Lαιr1 surface expression (Extended Data Fig. 4f) resulted in massive hyperactivation of SYK (Y352), Src kinases (Y416) and Erk (T202/Y204; Fig. 2b), which promotes negative selection of autoreactive B-cell clones during early B-cell development<sup>17</sup>. In agreement with these findings, Cre-mediated deletion of Lαιr1 caused rapid cell death in vitro, remission of leukaemia in vivo and significantly prolonged survival of transplant-recipient mice (P = 0.0003, log-rank test; Fig. 2c, d and Extended Data Fig. 5c).

The surface receptors PECA1, CD300A and LAIR1 attenuate BCR–ABL1 signalling through ITIM-dependent recruitment and activation of inhibitory phosphatases (for example, PTPN6 (also known as SHP1), INPP5D<sub>8</sub> and PTPN11 (also known as SHP2))<sup>18</sup>. For this reason, we performed experiments to determine whether Lαιr1 contributes to activation of PTPn6 and Inpp5d. Consistent with a role of Lαιr1 in the recruitment and activation of PTPn6 and Inpp5d, activating tyrosine phosphorylation of PTPn6 (Y564) and Inpp5d (Y1020) was reduced by three- to fourfold upon inducible deletion of Lαιr1 (Extended Data Fig. 5d). In genetic rescue experiments, we demonstrated that intact ITIM motifs in the cytoplasmic tails of PTPn1 and Lαιr1 and Cd300a are critical for the survival of pre-B ALL cells: Pacem<sup>1</sup>−/−, Lαιr1−/− and Cd300a−/− pre-B cells were transduced with green fluorescent protein (GFP)-tagged vectors for reconstitution of PTPn1, Lαιr1 and Cd300a bearing either wild-type or mutant (Y→F/A) ITIM motifs or GFP empty vector controls, and then transformed by BCR–ABL1 (Fig. 2e–g). Reconstitution with wild-type-ITIM PTPn1, Lαιr1 and Cd300a rescued survival and proliferation, whereas reconstitution with receptors carrying tyrosine-mutant ITIMs had no effect (Fig. 2e–g).

The phosphatases PTPn6 (ref. 7), INPP5D<sub>8</sub> and PTPn11 (also known as SHP2)<sup>18</sup> can all bind to ITIM motifs. We determined their mechanistic contribution to calibration of oncogenic signalling in a genetic rescue experiment: Lαιr1<sup>β<sub>0</sub></sup> ALL cells were transduced with GFP-tagged expression vectors of constitutively active or phosphate-inactive forms of PTPn6, Inpp5d and PTPn11 (3a and Extended Data Fig. 5e). Expression of constitutively active Inpp5d or PTPn6, but not PTPn11, rescued cell death after Cre-mediated deletion of Lαιr1. Interestingly,
inducible deletion of \( \text{Ptpn6} \) or \( \text{Inpp5d} \) was sufficient to cause cell death and a sharp increase of cellular ROS levels in ALL cells (Fig. 3b, c and Extended Data Figs 6–e, 7a). Given that phosphatases are sensitive to reversible inactivation by cysteine oxidation of their active sites\(^8\), we tested whether deletion of one single phosphatase triggers an ROS-mediated chain reaction of phosphatase inactivation. Using antibodies against phosphatases in inactivated oxidized conformation, we found that deletion of either \( \text{Ptpn6} \) or \( \text{Inpp5d} \) caused widespread cysteine oxidation and inactivation of multiple other phosphatases (Extended Data Fig. 7b). Inducible ablation of \( \text{Ptpn6} \) or \( \text{Inpp5d} \) caused increased expression of Arf and p53 cell cycle checkpoint molecules, G0/1 cell cycle arrest and 15- to 40-fold reduced colony formation capacity (Fig. 3d, e). While inducible ablation of \( \text{Lair1} \) or \( \text{Ptpn6} \) resulted in strong hyperactivation of Syk (Y352; Fig. 3g, h), both \( \text{Ptpn6} \) and \( \text{INPP5D} \) attenuate ITAM-dependent pre-BCR signalling in normal pre-B cells\(^7,8\). Cre-mediated depletion of \( \text{Ptpn6} \) or \( \text{Inpp5d} \) significantly reduced pentenurance and extended the latency of the leukaemia (Fig. 3f; \( P < 0.0005 \), log-rank test). These findings reveal a novel and unexpected vulnerability and suggest that ITIM-bearing receptors and inhibitory phosphatases represent a novel class of therapeutic targets in pre-B ALL. Both \( \text{PTPN6} \) and \( \text{INPP5D} \) attenuate ITAM-dependent pre-BCR signalling in normal pre-B cells\(^8\). Cre-mediated deletion of \( \text{Ptpn6} \) or \( \text{Inpp5d} \) protein resulted in strong hyperactivation of Syk (Y352; Fig. 3g, h). While \( \text{PTPN6} \) directly dephosphorylates ITAMs and SYK\(^7\), \( \text{INPP5D} \) hydrolyses the membrane anchor PIP3 and thereby inhibits functional consequences in a mouse model for CML (Extended Data Figs 8 and 9). Consistent with these findings, \( \text{PTPN6} \) and \( \text{INPP5D} \) are highly expressed in patient-derived \( \text{Ph}^+ \) ALL (\( n = 5 \)) but barely detectable in CML cells (\( n = 5 \); Extended Data Fig. 6a). To test whether B-cell-inherent mechanisms of negative selection are still active and examine the underlying reason for the divergent behaviour of B-lineage and myeloid leukaemia, we engineered B-cell-lineage \( \text{Ph}^+ \) ALL cells with a doxycycline-inducible vector system for expression of \( \text{Cebpa}^\text{b2} \), which results in myeloid-lineage reprogramming (Extended Data Fig. 10a, b). \( \text{BCR-ABL1} \)-transformed pre-B ALL cells were transduced with GFP-tagged Cre and reprogrammed into myeloid-lineage leukaemia cells. While inducible ablation of \( \text{Lair1} \), \( \text{Ptpn6} \) or \( \text{Inpp5d} \) resulted in rapid cell death among B-lineage (CD19\(^+\) B220\(^-\) Mac1\(^-\)) ALL cells, myeloid-lineage reprogramming (CD19\(^+\) B220\(^-\) Mac1\(^+\)) rendered leukaemia cells resistant to the effects of inducible deletion (Extended Data Fig. 10c–e). These findings support a scenario in which \( \text{Ph}^+ \) ALL cells are subject to B-cell-specific negative selection against hyperactive Syk tyrosine kinase signalling emanating from a self-reactive BCR, or its oncogenic mimic BCR–ABL1. Inducible expression of \( \text{Cebpa} \) subverts B-cell lineage commitment and raises the threshold for tyrosine kinase hyperactivation to trigger cell death. In this context, it is interesting to note that multiple genetic lesions in human pre-B ALL target transcription factors that mediate B-cell lineage commitment, including IKZF1, PAX5 and EBF1 (ref. 22). Although their mechanistic role is not known, we propose that deletions of \( \text{IKZF1} \), \( \text{PAX5} \) and \( \text{EBF1} \), like downregulation of \( \text{PAX5} \) in the context of \( \text{Cebpa} \) expression, reduce the stringency of negative selection against hyperactive tyrosine kinase signalling.

A small-molecule inhibitor against \( \text{INPP5D} \), 3-\( \alpha \)-aminocholestanol (3AC)\(^9\) (Extended Data Fig. 10f) selectively inhibited enzymatic activity of \( \text{INPP5D} \) (half-maximum inhibitory concentration (IC\(_{50}\)) \( \sim 2.5 \) \( \mu \text{mol l}^{-1} \)) but not the related phosphatases \( \text{INPP5L1} \) (also known as SHIP2) and \( \text{PTEN} \) (IC\(_{50} > 20 \) \( \mu \text{mol l}^{-1} \))\(^9\). Treatment of patient-derived \( \text{Ph}^+ \) ALL cells with 3AC induced strong hyperactivation of Syk (Fig. 4a). In patient-derived myeloid CML samples, baseline levels of SYK activity were very low and not responsive to 3AC treatment (Extended Data Fig. 10g). Biochemical characterization of 3AC-mediated inhibition of
INPP5D in patient-derived Ph⁺ ALL cells revealed potent and transient hyperactivation of proximal pre-BCR signalling molecules (Fig. 4a). Treatment of patient-derived TKI-resistant Ph⁺ ALL cells with 3AC induced cell death within 4 days. Importantly, pre-treatment of Ph⁺ ALL cells with PRT largely protected Ph⁺ ALL cells against 3AC-induced cell death (Fig. 4b), demonstrating that hyperactivation of SYK is required for induction of cell death. Dose–response analyses revealed that 3AC is selectively toxic for patient-derived Ph⁺ ALL cells (IC₅₀ = 2.8 μM; n = 5) compared to mature B-cell lymphoma (n = 5; Extended Data Fig. 10h). We next studied drug responses in a panel of six cases of Ph⁺ ALL from patients who relapsed under TKI therapy, including three cases with global TKI resistance owing to the BCR–ABL1(T315I) mutation. As expected, treatment with imatinib had no effect in BCR–ABL1(T315I) cases (Extended Data Fig. 10i). In contrast, 3AC induced massive cell death (>95%) in all six cases of Ph⁺ ALL regardless of BCR–ABL1 mutation status (Extended Data Fig. 10i). Likewise, treatment of NOD/SCID transplant-recipient mice carrying TKI-resistant patient-derived (BCR–ABL1(T315I)) Ph⁺ ALL cells with 3AC significantly prolonged overall survival (P = 0.0002, log-rank test; Fig. 4c) and reduced leukaemia burden (Fig. 4d). While further studies are needed...
Figure 4 | Small-molecule inhibition of INPP5D induces hyperactivation of SYK and triggers a deletional checkpoint in pre-B ALL cells. a, Patient-derived Ph− ALL cells (BLQ5) were treated with 3AC (10 μmol l−1) for the times indicated and phosphorylation of SYK, SRC, BTK and PLC-γ2 were measured by western blot. Data are representative of three independent experiments. b, ALL cells were treated with vehicle, PRT (2.5 μmol l−1), 3AC (7.5 μmol l−1) alone, or pre-treated with PRT for 2 days, after which 3AC was added. Viability was monitored by flow cytometry. Error bars represent mean ± S.D. from three independent experiments. c, d, TKI-resistant patient-derived Ph− ALL cells (BLQ5) were labelled with firefly luciferase and injected into sublethally irradiated NOD/SCID mice, treated with either 3AC or vehicle (50 mg kg−1, daily intraperitoneal injection, n = 7 per group). Overall survival of recipient mice in the two groups was compared by Kaplan–Meier analysis. The manuscript was written by Z.C. and M.M. and contributed to by all authors. 

Received 10 September 2013; accepted 13 January 2015.

Published online 23 March 2015.

1. Lam, K. P., Kuhn, R. & Rajewsky, K. In vivo ablation of surface immunoglobulin on mature B cells by inducible gene targeting results in rapid cell death. Cell 90, 1073–1083 (1997).
2. Nemazee, D. & Burki, K. Clonal deletion of B lymphocytes in a transgenic mouse bearing anti-MHC class I antibody genes. Nature 337, 562–566 (1989).
3. Keenan, R. A. et al. Censoring of autoreactive B cell development by the pre-B cell receptor. Science 321, 696–699 (2008).
4. Klein, F. et al. The BCR-ABL1 kinase bypasses selection for the expression of a pre-B cell receptor in pre-B acute lymphoblastic leukemia cells. J. Exp. Med. 199, 673–685 (2004).
5. Feldhahn, N. et al. Mimicry of a constitutively active pre-B cell receptor in acute lymphoblastic leukemia cells. J. Exp. Med. 201, 1837–1852 (2005).
6. Cortes, J. E. et al. Ponatinib in refractory Philadelphia chromosome-positive leukemias. N. Engl. J. Med. 367, 2075–2088 (2012).
7. Pao, L. et al. B cell-specific deletion of protein-tyrosine phosphatase Shp1 promotes B-1a cell development and causes systemic autoimmunity. Immunity 27, 35–48 (2007).
8. O’Neill, S. K. et al. Monophosphorylation of CD79a and CD79b ITAM motifs initiates a SHIP-1 phosphatase-mediated inhibitory signaling cascade required for B cell anergy. Immunity 35, 746–756 (2011).
9. Brooks, R. et al. SHIP1 inhibition increases immunoregulatory capacity and triggers apoptosis of hematopoietic cancer cells. J. Immunol. 184, 3582–3589 (2010).
10. Roberts, K. G. et al. Targetable kinase-activating lesions in Ph-like acute lymphoblastic leukemia. N. Engl. J. Med. 371, 1005–1015 (2014).
11. Duy, C. et al. BCL6 enables Ph− acute lymphoblastic leukemia cells to survive BCR–ABL1 kinase inhibition. Nature 473, 384–388 (2011).
12. Reth, M. Antigen receptor tail clue. Nature 338, 383–384 (1989).
13. Anderson, L. J. & Longnacre, R. EBV LMP2A provides a surrogate pre-B cell receptor signal through constitutive activation of the ERK/MAPK pathway. J. Gen. Virol. 89, 1563–1568 (2008).
14. Kulathu, Y., Gröthe, G. & Reth, M. Autoinhibition and adapter function of Syk. Immunol. Rev. 232, 286–299 (2009).
15. Bolland, S. & Ravetch, J. V. Inhibitory pathways triggered by ITIM-containing receptors. Adv. Immunol. 72, 149–177 (1999).
16. Staub, E., Rosenthal, A. & Hinzmann, B. Systematic identification of immunoreceptor tyrosine-based inhibitory motifs in the human proteome. Cell. Signal. 16, 435–456 (2004).
17. Limmander, A. et al. STIM1, PKC-δ and RasGRP set a threshold for proapoptotic Erk signaling during B cell development. Nature Immunol. 12, 425–433 (2011).
18. Zhang, S. Q. et al. Shp2 regulates SRC family kinase activity and Ras/Erk activation by controlling Csk recruitment. Mol. Cell 13, 341–355 (2004).
19. Meng, T.-C., Fukada, T. & Tonks, N. K. Reversible oxidation and inactivation of protein tyrosine phosphatases in vivo. Mol. Cell 9, 387–399 (2002).
20. Bolland, S., Pearse, R. N., Kurosaki, T. & Ravetch, J. V. SHIP modulates immune receptor responses by regulating membrane association of Btk. Immunity 8, 503–516 (1998).
21. Xie, H., Ye, M., Feng, R. & Graf, T. Stepwise reprogramming of B cells into macrophages. Cell 117, 663–676 (2004).
22. Mullichan, C. G. et al. BCR-ABL1 lymphoblastic leukemia is characterized by the deletion of Ikaros. Nature 453, 110–114 (2008).

Supplementary Information

Online Content Methods, along with any additional Extended Data display items and Source Data, are available in the online version of the paper; references unique to these sections appear only in the online paper.

Acknowledgements We thank R. W. Hendriks for encouragement and critical discussions; A. Park and C. Lin and all the members of the Münchén laboratory, L. Tian and B. Scott for their support. This work is supported by grants from the National Institutes of Health/National Cancer Institute through R01CA137060, R01CA130932, R01CA169458, R01CA172558 and R01CA157644 (to M.M.), ECOG-ACRIN grants CA180820 and CA180794 (to E.P.), grants from the Leukemia and Lymphoma Society (to M.M.), the California Institute for Regenerative Medicine through TR02-1816 (M.M.), and the William Lawrence and Blanche Hughes Foundation. M.M. is a Scholar of The Leukemia and Lymphoma Society and a Senior Investigator of the Wellcome Trust. M.R. is supported by the DFG through EXC294, TRR130 and SFB746.

Author Contributions Z.C. and M.M. designed experiments. M.M. conceived the study. Z.C. and M.M. performed experiments and interpreted data. H.G. and S.S. provided patient samples. B.T. and T.G.G. helped with gene expression analysis. P. value calculated by log-rank test (c) and leukemia burden was measured by luciferase imaging (d).

to optimize pharmacological targeting of this pathway, these experiments identify transient hyperactivation of SYK and engagement of negative B-cell selection as a powerful new strategy to overcome drug resistance in Ph− ALL.

When reading this paper, please note the following:

- The manuscript was written by Z.C. and M.M. and contributed to by all authors.
- Supplementary Information is available in the online version of the paper.
- Acknowledgements We thank R. W. Hendriks for encouragement and critical discussions; A. Park and C. Lin and all the members of the Münchén laboratory, L. Tian and B. Scott for their support. This work is supported by grants from the National Institutes of Health/National Cancer Institute through R01CA137060, R01CA130932, R01CA169458, R01CA172558 and R01CA157644 (to M.M.), ECOG-ACRIN grants CA180820 and CA180794 (to E.P.), grants from the Leukemia and Lymphoma Society (to M.M.), the California Institute for Regenerative Medicine through TR02-1816 (M.M.), and the William Lawrence and Blanche Hughes Foundation. M.M. is a Scholar of The Leukemia and Lymphoma Society and a Senior Investigator of the Wellcome Trust. M.R. is supported by the DFG through EXC294, TRR130 and SFB746.
- Author Contributions Z.C. and M.M. designed experiments. M.M. conceived the study. Z.C., S.S., M.B., J.W.L., A.L., H.J., M.R., A.W. and C.L. provided important reagents and mouse samples. E.P., S.P.H., C.L.W., A.M. and M.L.L. provided patient samples. B.T. and T.G.G. helped with gene expression analysis. The manuscript was written by Z.C. and M.M. and contributed to by all authors.
- Author Information Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to M.M. (markus.muschen@ucsf.edu).

©2015 Macmillan Publishers Limited. All rights reserved
METHODS

Patient samples and human cell lines. Patient samples (Supplementary Tables 1 and 2) were obtained with informed consent in compliance with Institutional Review Board regulations of the University of California San Francisco. Leukemia cells from bone marrow biopsy of patients with Ph1 or Ph2 ALL were xenografted into sublethally irradiated NOD/SCID mice via tail vein injection. After passages, leukemia cells were harvested and cultured on top of OP9 stroma cells with minimum essential medium (MEMx; Life Technologies) GlutaMAX without ribonucleotides and deoxyribonucleotides, supplemented with 20% FBS, 100 IU ml−1 penicillin, 100 μg ml−1 streptomycin and 1 mmol l−1 sodium pyruvate. The human cell lines (Supplementary Table 3) were cultured in RPMI-1640 (Life Technologies) with GlutaMAX containing 20% FBS, 100 IU ml−1 penicillin and 100 μg ml−1 streptomycin at 37 °C in a humidified incubator with 5% CO2. All of the human xenograft cells and were subjected to PCR.

Murine cell culture and BCR–ABL1 transduction. Bone marrow cells from constitutive or inducible knockout mice (for a list of genetic mouse models used in this study see Supplementary Table 4) were harvested and cultured in Iscove’s modified Dulbecco’s medium (IMDM; Invitrogen) with GlutaMAX containing 20% FBS, 50 μM 2-mercaptoethanol, 100 IU ml−1 penicillin, 100 μg ml−1 streptomycin in the presence of cytokines. For pre-B-cell culture, bone marrow cells were cultured in IMDM with 10 ng ml−1 recombinant mouse IL-7 (Peprotech) on OP9 stroma cells. For the ALL leukemia model, pre-B cells were retrovirally transduced by BCR–ABL1. ALL cells generated from inducible knockout mice were retrovirally transduced with ER12 or Cre-ER12 virus, and puromycin selection was performed. 4-OH-T was used to induce Cre-mediated gene deletion. For the CML-like leukemia model, the myeloid-restricted protocol described previously was used23, which generates CML-like cells. Briefly, bone marrow cells were cultured in IMDM with recombinant mouse IL-3 (10 ng ml−1), IL-6 (25 ng ml−1), Scf (50 ng ml−1), Pip-1 and then transduced by BCR–ABL1 retrovirus. Cytokines were removed after BCR–ABL1 transduction.

In vivo transplantation of leukemia cells. Murine pre-B ALL cells transduced by BCR–ABL1 were transduced with firefly luciferase retrovirus, selected with blastidin, and then transduced with ER12 or Cre-ER12 virus, selected with puromycin. 4-OH-T was used to induce Cre-mediated gene deletion for 24 h and 1×106 viable cells were injected into sublethally irradiated (250 cGy) NOD/SCID mice via the tail vein. For human leukemia cells, a lentiviral vector encoding firefly luciferase was used. Bioimaging of leukemia progression in mice was performed after transplantation with an in vivo IVIS 100 bioluminescence/ optical imaging system (Xenogen). Fifteen minutes before measuring the luminescence signal, d-luciferin (Promega) prepared in PBS was injected intraperitoneally at an amount of 2.5 mg for each mouse. General anaesthesia was induced by using 5% isoflurane and then transduced with ER12 or Cre-ER12 virus, selected with puromycin. 4-OH-T was used to induce Cre-mediated gene deletion. For the CML-like leukemia model, the myeloid-restricted protocol described previously was used23, which generates CML-like cells. Briefly, bone marrow cells were cultured in IMDM with recombinant mouse IL-3 (10 ng ml−1), IL-6 (25 ng ml−1), Scaf (50 ng ml−1), Pip-1 and then transduced by BCR–ABL1 retrovirus. Cytokines were removed after BCR–ABL1 transduction.

DNA extraction and genotyping. Genomic DNA was extracted from mouse cells with NucleoSpin Tissue kit (MACHEREY-NAGEL) and PCR was performed using Taq DNA polymerase (NEB). The primer sequences are provided in Supplementary Table 8.

Gene expression and clinical outcome data. Clinical outcome and gene expression microarray data were derived from the National Cancer Institute TARGET Data Matrix (http://catftp.ncbi.nih.gov/pub/dcc_target/ALL/Phase_I/Discovery/clinical/) of the Children’s Oncology Group (COG) Clinical Trial P9906 and from the ECOG Clinical Trial E2993. The end points of the clinical data include minimal residual disease (MRD) after 29 days of treatment (COG), overall survival (OS) and relapse-free survival (RFS) probability (COG and ECOG). Detailed information about the gene expression microarray data is provided in Supplementary Tables 9 and 10.

Clinical trial parameters. Unpaired, two-tailed Student’s t-test was used to compare groups. OS or RFS probabilities were compared with the Kaplan–Meier method. Log-rank test (two-sided) was used to compare patient survival between different groups. R package ‘survival’ version 2.35-8 was used for the survival analysis. In survival analysis, patients with ALL in each clinical trial (COG P9906 or ECOG E2993) were divided into two groups based on whether their expression was above or below the median level of a probeset or a gene (that is, the average of multiple probe sets for a gene). For a multiple-gene predictor (that is, a set of genes, such as in ITAM (CD79A, CD79B, IGHM) and ITIM (PECAM1, LAIR1, CD300a)), the patients were split into four groups based on whether they had above or below the median expression levels of the sum of ITAM and the sum of ITIM gene expression levels: (1) ITAMHighITIMLow (≥ITAMmedian and <ITIMmedian), (2) ITAMHighITIMHigh (≥ITAMmedian and ≥ITIMmedian), (3) ITAMLowITIMLow (<ITAMmedian and <ITIMmedian), and (4) ITAMLowITIMHigh (<ITAMmedian and ≥ITIMmedian). Survival probabilities of the ITAMHighITIMLow versus ITAMLowITIMHigh groups in the multiple-gene survival analysis were compared.

23. Li, S., Iliara, R. L., Million, R. P., Daley, G. Q. & Van Elten, R. A. The P190, P210, and P230 forms of the BCR/ABL oncogene induce a similar chronic myeloid leukemia-like syndrome in mice but have different lymphoid leukemogenic activity. J. Exp. Med. 189, 1399–1412 (1999).
Extended Data Figure 1 | Reconstitution of ITAM signalling causes cell death in pre-B ALL. a, Flow cytometry staining for cell-surface Igα (CD79A) and Igβ (CD79B) was performed for patient-derived Ph+ ALL cases (n = 8) and B-cell leukaemia/lymphomas lacking oncogenic tyrosine kinases (n = 4). b, c, Normal mouse pre-B cells or BCR–ABL1-transformed pre-B ALL cells were retrovirally transduced with CD8–Igα–GFP or empty vector (GFP) controls (EV). Relative changes of transduced (GFP+) populations were monitored by flow cytometry. d, Tyrosine phosphorylation of Syk, Src/Lyn, Btk and Plc-γ2 was studied in BCR-ABL1 ALL cells that were transduced with Igα–GFP or GFP empty vector controls, using β-actin as loading control. Data (c, d) are representative of three independent experiments. e, Human Ph+ ALL cells were transduced with GFP-tagged vectors for LMP2A–ITAM or empty vector. Relative changes of transduced (GFP+) populations were monitored by flow cytometry (n = 3). f, LMP2A–ITAM or an empty vector was expressed in three cases of human Ph+ ALL cells and effects on LMP2A expression and phosphorylation of SYK, SRC, BTK and PLC-γ2 were measured by western blot (n = 3). g, BCR-ABL1-transformed ALL cells were transduced with GFP-tagged SykMyr or an empty vector, and these cells were treated with the SYK inhibitor PRT (2.5 μmol l⁻¹) or vehicle either 1 day before transduction (PRT-pre), or 1 day after transduction (PRT-post), or pre-treated, then washed out for 1 day after transduction, and treated again with PRT. The relative changes of transduced (GFP+) cells were monitored by flow cytometry. Error bars represent means ± s.d. from three independent experiments (b, e, g).
Extended Data Figure 2 | Reconstitution of proximal pre-BCR signalling in pre-B ALL cells. a, BCR-ABL1-transformed pre-B ALL cells were transduced with myristoylated (active) forms of Btk, Syk or empty vector controls (EV). Vectors were GFP-tagged and fractions of GFP + cells were monitored over time. b, Btk−/− BCR-ABL1-transformed pre-B ALL cells were transduced with myristoylated (active) Btk or empty vector (both tagged with GFP). Fractions of GFP + cells were monitored over time. c, Csk is a negative regulator of Src family kinase activity. Csk-AS transgenic mice express an analogue (3IB-PP1) sensitive form instead of endogenous Csk. For inducible activation of Src kinase activity, we transformed pre-B cells from Csk-AS transgenic mice with BCR-ABL1. Addition of 3IB-PP1 (10 μmol l−1) released Csk-mediated inhibition and induced increased phosphorylation of Src family kinases at Y416, but did not increase Syk Y352 phosphorylation (western blot). d, Pre-B cells from analogue-sensitive Csk-AS transgenic and wild-type mice were transformed with BCR-ABL1 and treated with 3IB-PP1. Cell viability in response to 3IB-PP1 treatment was monitored over time. Error bars (a, b, d) represent means ± s.d. from three independent experiments. e, Rag1−/− pro-B cells were expanded in the presence of 10 ng ml−1 Il-7 and transduced with an empty vector (GFP), constitutively active SYK (TEL-SYK–GFP), or a kinase-dead mutant of SYK (TEL-SYK(K402A)–GFP). Then Il-7 was removed from cell cultures and the effect of Il-7 removal on cell viability was studied. Rag1−/− pro-B cells transduced with empty vector or TEL-SYK(K402A)–GFP underwent apoptosis, whereas pro-B cells transduced with constitutively active SYK had acquired growth factor (Il-7) independence, whereas pro-B cells with empty vector and TEL-SYK(K402A)–GFP remained dependent on Il-7. Data (c, e) are representative of three independent experiments.
Extended Data Figure 3 | ITIM-bearing receptors are highly expressed on Ph¹⁺ ALL cells. a, Microarray data for 62 ITIM-bearing receptors are ranked based on the ratio of messenger RNA levels in Ph¹⁺ ALL compared to normal pre-B cells and mature B-cell lymphomas. b, Fluorescence-activated cell sorting (FACS) dot plots for double staining of PECAM1, CD300A, LAIR1 and BTLA with CD19 are shown for normal bone marrow pre-B cells (n = 1), Ph¹⁺ ALL cells (n = 8) and non-tyrosine-kinase-driven B-cell lymphoma (n = 4). c, Normal bone marrow mononucleated cells from bone marrow biopsies of healthy donors (n = 3), patient-derived Ph¹⁺ ALL (n = 11) and non-tyrosine-kinase-driven B-cell lymphoma (n = 11) were analysed by flow cytometry for surface expression of the ITIM-bearing inhibitory receptors PECAM1, CD300A, LAIR1, BTLA, CEACAM1, CD22, FCRL2. Additional staining for CD72 and LILRB5 did not show significant differences between Ph¹⁺ ALL cells and normal pre-B cells (data not shown). Statistical analysis of mean fluorescence intensities (MFIs) for normal pre-B cells (n = 3), Ph¹⁺ ALL (n = 11) and non-tyrosine-kinase-driven B-cell lymphoma (n = 11) showed significantly increased expression levels of PECAM1, LAIR1 and CD300A in Ph¹⁺ ALL compared to normal pre-B cells and non-tyrosine-kinase-driven B-cell lymphoma. P values were calculated using unpaired, two-tailed Student’s t-test.
Extended Data Figure 4 | Higher than median expression levels of ITIM-bearing inhibitory receptors predict poor outcomes in patients with pre-B ALL. a–c, mRNA levels for PECAM1, CD300A and LAIR1 were measured in 207 patients with paediatric ALL (COG P9906). PECAM1, CD300A and LAIR1 mRNA levels for ALL cells from 124 patients that had no detectable minimal residual disease (MRD negative; black) on day 29 in their bone marrow were compared to mRNA levels in 67 patients with positive MRD (red) at the time of bone marrow biopsy (day 29). On the basis of higher or lower than median expression levels of PECAM1, CD300A and LAIR1, patients were segregated into two groups (High, n = 104; Low, n = 103; plots in middle and right). Overall survival (OS; middle) and relapse-free survival (RFS; right) probabilities were estimated by Kaplan–Meier survival analyses. P values were calculated by Mann–Whitney–Wilcoxon test (left panels; MRD status) and log-rank test (middle and right panels; overall survival and relapse-free survival). d, e, ITAM-based agonists (CD79A, CD79B, IGHM) and ITIM-based inhibitors (PECAM1, CD300A, LAIR1) of pre-BCR signalling were combined into a six-gene outcome predictor based on ‘ITAM’ and ‘ITIM’ signatures and validated in two clinical trials for adults with Ph+ ALL (ECOG E2993) and children with ALL (COG P9906). P values were calculated by log-rank test. f, Lair1 deletion was confirmed by flow cytometry. g, Expression of checkpoint molecules Arf, p53, p21 and p27 was measured by western blot in the presence and absence of Pecam1 and Cd300a and upon inducible deletion of Lair1 in BCR-ABL1 pre-B ALL cells. h, Accumulation of ROS was measured by staining with 2′,7′-dichlorofluorescein diacetate (DCF) in BCR-ABL1 pre-B ALL cells (grey histograms for control; red for gene deletion). Data are representative of three independent experiments (f–h).
Extended Data Figure 5 | Consequences of genetic deletion of ITIM-bearing receptors in pre-B ALL cells. a, b, B-cell precursors from the bone marrow of Pecam1<sup>+/+</sup> and Cd300a<sup>+/+</sup> as well as Lair1<sup>fl/fl</sup> mice and wild-type controls were propagated with IL-7 and transduced with an empty vector control (EV; normal B-cell precursors) or transformed with BCR-ABL1 to model Ph<sup>+</sup> ALL. Lair1<sup>fl/fl</sup> pre-B and BCR-ABL1 leukaemia cells were transduced with 4-OHT-inducible retroviral Cre. Cell cycle progression of normal pre-B cells (EV) and BCR-ABL1 ALL cells was measured by BrdU staining (a). Propensity to cellular senescence was measured by staining of normal pre-B and BCR-ABL1 ALL cells for senescence-associated β-galactosidase (b). Numbers indicate percentage of cells in each cell cycle phase. Numbers indicate percentage of β-galactosidase-positive cells. c, Lair1<sup>fl/fl</sup> BCR-ABL1 ALL cells were transduced with 4-OHT-inducible Cre (Cre-ERT2) or an empty vector control (ERT2). Viability was measured by flow cytometry after 4-OHT treatment. d, Effects of inducible deletion of Lair1 on phosphorylation levels of Ptpn6 and Inpp5d were measured by western blot. Lair1<sup>fl/fl</sup> ALL cells were transduced with 4-OHT-inducible Cre. After antibiotic selection, ALL cells were transduced with a GFP-tagged empty vector control or GFP-tagged overexpression vectors for constitutively active forms of Ptpn6 (lacking autoinhibitory SH2 domain), Inpp5d (membrane-anchored by CD8) and Ptpn11 (constitutively active D61A mutation). Expression levels of Ptpn6, Inpp5d and Ptpn11 were measured by western blot using β-actin as loading control. The transduced cells were used for Cre-mediated deletion of Lair1 to determine if expression of constitutively active Ptpn6, Inpp5d and Ptpn11 can rescue leukaemia cell survival. Data (a, d, e) are representative of three independent experiments. Data (b, c) represent means ± s.d. from three independent experiments.
Extended Data Figure 6 | Inducible deletion of Ptpn6 or Inpp5d causes cell death in pre-B ALL cells. 

**a,** Protein levels of PTPN6 and INPP5D were measured by western blot in CD19<sup>+</sup> bone marrow pre-B cells from healthy donors (n = 3), patient-derived Ph<sup>+</sup> ALL (n = 8) and B-cell leukaemia/lymphoma (n = 4) lacking an oncogenic tyrosine kinase. Additional western blot analyses compared expression levels of PTPN6 and INPP5D in patient-derived Ph<sup>+</sup> ALL (n = 5) and patient-derived chronic phase CML cells (n = 5). 

**b, c,** Bone marrow cells were isolated from Ptpn6<sup>1fl/fl</sup> or Inpp5d<sup>fl/fl</sup> mice and pre-B cells were propagated with IL-7 (10 ng ml<sup>−1</sup>). Ptpn6<sup>1fl</sup> and Inpp5d<sup>fl/fl</sup> pre-B cells were then transformed with BCR-ABL1 retrovirus and subsequently transduced with 4-OHT-inducible Cre (Cre-ERT<sup>2</sup>) or an empty vector control (ERT<sup>2</sup>). Addition of 4-OHT induced nuclear translocation of Cre and Cre-mediated excision of Ptpn6<sup>1fl/fl</sup> (one allele) and Inpp5d<sup>fl/fl</sup> alleles as verified here by genomic PCR (b, left, for Ptpn6<sup>1fl/fl</sup>; c, left, for Inpp5d<sup>fl/fl</sup>). Near-complete deletion of the Ptpn6<sup>1fl/fl</sup> (one allele) and Inpp5d<sup>fl/fl</sup> floxed alleles was observed after 3 and 4 days, respectively, at the genomic level (left). Kinetics of protein depletion upon heterozygous deletion of Ptpn6 and homozygous deletion of Inpp5d (Inpp5d<sup>D70</sup>) was studied by western blot (right) using β-actin as loading control. 

**d, e,** Effects of Cre-mediated inducible deletion of Ptpn6 (d) or Inpp5d (e) on BCR-ABL1-transformed pre-B ALL cell viability were measured by flow cytometry at the times indicated. Numbers denote percentages of viable cells (determined by forward scatter (FSC) and propidium iodide (PI) uptake). Data are representative of three independent experiments (d, e).
Extended Data Figure 7 | Functional consequences of inducible Ptpn6 or Inpp5d deletion in pre-B ALL cells. a, The effects of deletion of Ptpn6 or Inpp5d on cellular ROS levels were measured by flow cytometry using DCF in BCR-ABL1 pre-B ALL cells (grey histograms for control; red for gene deletion). b, Whether ROS accumulation in response to deletion of Ptpn6 or Inpp5d results in wide-spread cysteine-oxidation and, hence, inactivation, of multiple other PTP active sites was determined by western blot using antibodies against oxidized PTP active sites. c, Protein levels of the checkpoint molecules Arf and p53 were measured by western blot in BCR-ABL1 ALL cells before (empty vector (EV)) and after (Cre) deletion of Ptpn6 and Inpp5d. Data are representative of three independent experiments (a–c). d, e, Functional readouts for inducible deletion of Ptpn6 and Inpp5d include measurement of proliferation (BrdU incorporation) (d) and colony formation capacity in methylcellulose (colony-forming unit (c.f.u.) assay) (e). BrdU assays (flow cytometry) and c.f.u. data (images from colonies on plates) are presented in Fig. 3d, e. Quantitative and statistical analysis for BrdU incorporation (d) and c.f.u. assays (e) are depicted here as bar charts. P values were calculated by unpaired, two-tailed Student’s t-test. Error bars (d, e) represent means ± s.d. from three independent experiments.
Extended Data Figure 8 | Deletion of the ITIM-bearing receptors Pecam1, Cd300a or Lair1 has no significant effects on myeloid CML-like cells.

a, b, Myeloid progenitor cells from the bone marrow of Pecam1−/− and Cd300a−/− mice as well as age-matched wild-type controls were propagated in the presence of Il-3, Il-6 and Scf and transformed with retroviral BCR-ABL1. After 7 days, outgrowth of myeloid-lineage CML-like leukaemia was observed. One-hundred-thousand Pecam1−/− and Cd300a−/− CML-like cells as well as wild-type controls were plated in methylcellulose. Colonies were counted two weeks later (a). P values were calculated by unpaired, two-tailed Student’s t-test (b). c–e, Myeloid progenitor cells from the bone marrow of Lair1fl/fl mice were propagated in the presence of Il-3, Il-6 and Scf and transformed with retroviral BCR-ABL1. After 7 days, outgrowth of myeloid-lineage CML-like leukaemia was observed and CML-like phenotype was verified by flow cytometry using antibodies against B220/CD19 (negative), Sca-1/c-Kit and CD13 (c). CML-like cells were transduced with 4-OHT-inducible Cre (Cre-ERT2) and empty vector controls (ERT2) and deletion of Lair1 was verified by measurement of Lair1 surface expression (d). After adding 4-OHT, cell viability of Lair1fl/fl CML cells carrying ERT2 or Cre-ERT2 was monitored over 9 days by flow cytometry and is plotted in e. Data (a, b, e) represent means ± s.d. from three independent experiments.
Extended Data Figure 9 | Deletion of Ptpn6 or Inpp5d specifically affects B-cell-lineage ALL cells but not normal pre-B cells, myeloid progenitors or myeloid leukaemia. a, b, Bone marrow mononuclear cells were isolated from Ptpn6<sup>1<sub>fl</sub></sup> and Inpp5d<sup>fl/fl</sup> mice. Myeloid progenitor cells were propagated with Il-6 (25 ng ml<sup>-1</sup>), Il-3 (10 ng ml<sup>-1</sup>) and Scf (50 ng ml<sup>-1</sup>) and propagated as common myeloid progenitor cells (CMPs) or transformed with BCR-ABL1 to induce myeloid CML-like leukaemia. Pre-B cells were expanded in the presence of Il-7 (10 ng ml<sup>-1</sup>) and either propagated as pre-B-cell cultures or transformed by BCR-ABL1 to induce Ph<sup>+</sup> ALL-like leukaemia. Lineage identity and >95% purity of cell populations was verified by flow cytometry. Ptpn6<sup>1<sub>fl</sub></sup> and Inpp5d<sup>fl/fl</sup> CMPs, pre-B cells, CML-like and Ph<sup>+</sup> ALL-like leukaemia cells were then transduced with 4-OHT-inducible Cre (Cre) or an empty vector control (EV). Addition of 4-OHT induced nuclear translocation of Cre and Cre-mediated excision of Ptpn6<sup>1<sub>fl</sub></sup> (one allele) (a) or Inpp5d<sup>fl/fl</sup> alleles (b). Effects of inducible deletion on cell viability were measured by flow cytometry at the times indicated. Error bars (a, b) represent means ± s.d. from three independent experiments.
The inhibitory receptor Lair1 and the phosphatases Ptpn6 and Inpp5d are specifically required by B-cell lineage leukaemia cells. a, b, B-cell lineage BCR-ABL1 ALL cells were engineered with a doxycycline-inducible vector system for expression of Cebpa, which results in downregulation of B-cell antigens and myeloid-lineage differentiation as measured by flow cytometry (a) and western blot (b). Data (a, b) are representative of three independent experiments. c–e, BCR-ABL1-driven Lair1\textsuperscript{fl/fl}, Ptpn6\textsuperscript{fl/fl} and Inpp5d\textsuperscript{fl/fl} B-cell lineage ALL cells (CD19\textsuperscript{+} Mac1\textsuperscript{−}) were reprogrammed into myeloid-lineage (CD19\textsuperscript{−} Mac1\textsuperscript{+}) leukaemia cells by addition of doxycycline. Cell cultures were then transduced with 4-OHT-inducible GFP-tagged Cre and viability was measured in B-cell (gated on CD19\textsuperscript{+} Mac1\textsuperscript{−}) and myeloid-lineage (gated on CD19\textsuperscript{−} Mac1\textsuperscript{+}) populations. f, Structure of the INPP5D small-molecule inhibitor 3AC. g, Patient-derived Ph\textsuperscript{+} ALL (n = 3) and chronic-phase CML cells (n = 3) were treated with 3AC (10 \textmu mol l\textsuperscript{−1}) for 15 min, and phosphorylation of SYK was measured by western blot, using β-actin as loading control. h, Dose–response curves are shown for five patient-derived cases of ALL (LAX2, LAX9, BLQ1, BLQ5 and PDX2, red curves) and five cases of B-cell leukaemia/lymphoma (lacking an oncogenic tyrosine kinase; KARPAS-422, MHH-PREB-1, JEKO-1, MN-60 and JIN-3, grey curves). i, Dose–response curves are shown for the treatment of six patient-derived cases of Ph\textsuperscript{+} ALL that have acquired global resistance to TKI treatment (LAX2, BLQ5, BLQ11) or partial resistance (ICN1, LAX9, PDX59). Dose–response curves for the TKI imatinib are shown in grey and for the INPP5D inhibitor 3AC in red (concentration plotted on same scale for both agents). Error bars (c–e, h–i) represent means ± s.d. from three independent experiments.
CORRIGENDUM

doi:10.1038/nature16997

Corrigendum: Signalling thresholds and negative B–cell selection in acute lymphoblastic leukaemia

Zhengshan Chen, Seyedmehdi Shojaee, Maike Buchner, Huimin Geng, Jae Woong Lee, Lars Klemm, Björn Titz, Thomas G. Graeber, Eugene Park, Ying Xim Tan, Anne Satterthwaite, Elisabeth Paietta, Stephen P. Hunger, Cheryl L. Willman, Ari Melnick, Mignon L. Loh, Jae U. Jung, John E. Coligan, Silvia Bolland, Tak W. Mak, Andre Linnander, Hassan Jumaa, Michael Reth, Arthur Weiss, Clifford A. Lowell & Markus Müschen

Nature 521, 357–361 (2015); doi:10.1038/nature14231

In Extended Data Fig. 3b of this Letter, 52 flow cytometry dot plots with double stainings for CD19 and ITIM-bearing receptors (PECAM1, LAIR1, CD300A and BTLA) were shown for 13 samples. The CD19-CD300A staining for sample ICN1 was inadvertently replaced with CD19-CD300A staining for sample PDX2. The Supplementary Information for this Corrigendum contains the corrected Extended Data Fig. 3b (showing the correct dot plot for sample ICN1). Our conclusions are not affected.

Supplementary Information is available in the online version of the Corrigendum.