Conflict of interest
The authors declare no conflict of interest.

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References
Arellano-Rodrigo, E., Alvarez-Larran, A., Reverter, J.C., Villamor, N., Colomer, D. & Cervantes, F. (2006) Increased platelet and leukocyte activation as contributing mechanisms for thrombosis in essential thrombocythemia and correlation with the JAK2 mutational status. Haematologica, 91, 169–175.
Finazzi, G., Carobbio, A., Guglielmelli, P., Cavallo, C., Salmoiraghi, S., Vannucchi, A.M., Cazzola, M., Passamonti, F., Rambaldi, A. & Barbui, T. (2014) Calreticulin mutation does not modify the IPSET score for predicting the risk of thrombosis among 1150 patients with essential thrombocythemia. Blood, 124, 2611–2612.
Finazzi, M.C., Carobbio, A., Cervantes, F., Isola, L.M., Vannucchi, A.M., Guglielmelli, P., Rambaldi, A., Finazzi, G., Barosi, G. & Barbui, T. (2015) CALR mutation, MPL mutation and triple negative identify patients with the lowest vascular risk in primary myelofibrosis. Leukemia, 29, 1209–1210.
Lozano, M.L., Gonzalez-Concejo, R., Corral, J., Rivera, J., Iniesta, J.A., Martinez, C. & Vicente, V. (2001) Polymorphisms of P-selectin glycoprotein ligand-1 are associated with neutrophil-platelet adhesion and with ischaemic cerebrovascular disease. British Journal of Haematology, 115, 969–976.
Passamonti, F., Thieke, J., Girodon, F., Rumi, E., Carobbio, A., Gisslinger, H., Kvasnicka, H.M., Ruggeri, M., Randi, M.L., Gangat, N., Vannucchi, A.M., Gianatti, A., Gisslinger, B., Mullauer, L., Rodeghiero, F., d’Amore, E.S., Bertorzi, L., Hanson, C.A., Boveri, E., Marino, F., Maffioli, M., Caramazza, D., Antonioili, E., Carrai, V., Buxhofer-Ausch, V., Pascutto, C., Cazzola, M., Barbui, T. & Tefferi, A. (2012) A prognostic model to predict survival in 867 World Health Organization-defined essential thrombocythemia at diagnosis: a study by the International Working Group on Myelofibrosis Research and Treatment. Blood, 120, 1197–1201.
Rotunno, G., Mannarelli, C., Guglielmelli, P., Pacilli, A., Pancrazzi, A., Pieri, L., Fanelli, T., Bosi, A. & Vannucchi, A.M. (2014) Impact of calreticulin mutations on clinical and hematological phenotype and outcome in essential thrombocythemia. Blood, 123, 1552–1555.
Rumi, E., Pietra, D., Ferretti, V., Klampfl, T., Harutyunyan, A.S., Milesevic, J.D., Them, N.C., Berg, T., Elena, C., Casetti, I.C., Milanese, C., Sant’antonio, E., Bellini, M., Fugazza, E., Renna, M.C., Boveri, E., Astori, C., Pascutto, C., Kravolics, R. & Cazzola, M. (2014) JAK2 or CALR mutation status defines subtypes of essential thrombocythemia with substantially different clinical course and outcomes. Blood, 123, 1544–1551.
Solé, G., Bernal-Visente, A., Anton, A.L., Torregrosa, J.M., Caparros-Perez, E., Sanchez-Serrano, I., Martinez-Perez, A., Sanchez-Vega, B., Vicente, V. & Ferrer-Marin, F. (2015) The JAK2 46/1 haplotype does not predispose to CALR-mutated myeloproliferative neoplasms. Annals of Hematology, 94, 789–794.
Tefferi, A., Wassie, E.A., Lasbo, T.L., Finke, C., Belachew, A.A., Ketterling, R.P., Hanson, C.A., Pardanani, A., Gangat, N. & Wolanskyj, A.P. (2014) Calreticulin mutations and long-term survival in essential thrombocythemia. Leukemia, 28, 2300–2303.
Vardiman, J.W., Bruning, R.D. & Harris, N.L. (2001) WHO histological classification of chronic myeloproliferative diseases. In: World Health Organization Classification of Tumors: Tumours of the Haematopoietic and Lymphoid Tissues (ed. by E.S. Jaffe, H.N. Stein & J.W. Vardiman), pp. 17–44. International Agency for Research on Cancer (IARC) Press, Lyon, France.

The AKT1 isoform plays a dominant role in the survival and chemoresistance of chronic lymphocytic leukaemia cells

The pathophysiology of chronic lymphocytic leukaemia (CLL) is characterised by a dynamic equilibrium of resting and proliferative tumour cells. While CLL cells in the peripheral blood are mostly G0-arrested, those residing in lymphoid organs have an activated signature due to supportive signals from diverse immune and stromal cell types (Herishanu et al, 2011). The clinical success of novel small molecule inhibitors targeting Bruton tyrosine kinase, such as ibrutinib, and phosphatidylinositol-3 kinase (PI3K), such as Idelalisib, strengthens the idea that signals downstream of the B cell receptor are critical for CLL development and progression. In this context, the protein kinase C (PKC) and PI3K pathways are indisputable chief players (for review see Woyach et al, 2012). We and others have shown that downstream of PI3K and PKC-beta, the serine/threonine kinase AKT, also known as protein kinase B (PKB), regulates

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various signalling cascades involved in survival (Hofbauer et al, 2010; Zhuang et al, 2010). AKT is encoded by three distinct genes, namely AKT1, AKT2 and AKT3 (also termed PKB-alpha, PKB-beta, PKB-gamma, respectively). AKT1 and AKT2 are ubiquitously expressed whereas AKT3 is mainly expressed in testes and brain (Yang et al, 2003). In this study, we aimed to gain insight into isoform-specific expression of AKT, and the contribution of AKT1 and AKT2 to stromal and activated T cell-mediated survival and chemoresistance in CLL.

Peripheral blood samples from CLL patients were collected after informed consent was obtained in accordance with the Declaration of Helsinki and under the ethical approval of the Ethics Commission of the Province of Salzburg (415-E/1287/4-2011, 415-E/1287/8-2011).

First, analysing basal AKT isoform expression in unstimulated purified CLL cells, we observed increased AKT2 mRNA and AKT2 protein expression as compared to AKT1 (Fig 1A and B). As we had previously noted increased AKT phosphorylation in CLL stromal cell co-cultures (Hofbauer et al, 2010), we evaluated AKT phosphorylation kinetics and isoform contribution to this phenomenon. AKT was rapidly phosphorylated at serine 473 (pS473), a phosphorylation site crucial for full AKT activation (Sarbassov et al, 2005), and remained in the activated form for at least 24 h (Fig 1C). To assess the relative activation of AKT1 versus AKT2 in CLL cells co-cultured with primary stromal cells, we analysed the phosphorylation at Ser473 (AKT1) and Ser474 (AKT2). We observed robust stromal cell-induced AKT activation of both isoforms in CLL cells cultured in direct cell-cell contact with stromal cells (Fig 1Ci), but not in CLL cells separated from the stromal layer by a transwell insert (Fig 1Di). AKT activation was associated with increased cell viability (Fig 1Dii).

Next, we treated co-cultured CLL cells with several AKT inhibitors. As there is no AKT1-specific inhibitor available, we used the pan-AKT inhibitors MK2206 and AiX, and the AKT2-selective inhibitor, Akti-2. The specificities of these inhibitors were confirmed by an AKT isoform-specific pull-down and subsequent in vitro kinase assay to detect phosphorylation of the AKT substrate GSK3A/B after MK2206 or Akti-2 treatment of the Epstein-Barr virus-positive CLL patient-derived MEC1 cells (Fig 1E). Applying the inhibitors to primary CLL cells co-cultured with stromal cells indicated that the selective inhibition of AKT2 did not decrease cell viability, whereas pan-AKT inhibition resulted in significantly decreased survival (Fig 1F), suggesting a dominance of AKT1 or a cooperation of both isoforms in maintaining cell vitality. Genetic manipulations in primary CLL cells are hard to achieve, therefore, to address these alternatives, we used a siRNA approach to target AKT1 or AKT2 in MEC1 cells. Successful and similar knockdown efficiencies were achieved in both experimental settings (Fig 1G). However, the transient knockdown of AKT1, but not AKT2, resulted in loss of cell viability, establishing AKT1 as the dominant AKT isoform (Fig 1H). Consistently, simultaneous knockdown of both isoforms did not further reduce viability compared to the single AKT1 knockdown (Fig 1H).

Chronic lymphocytic leukaemia cells co-cultured with activated T cells are rapidly activated, allowing us to mimic in vitro at least part of the proliferative processes that take place in lymph nodes (Assalber et al, 2013). Under these co-culture conditions, we observed significant transcriptional upregulation of both AKT1 and AKT2 within 24 h (Fig 2A), which was accompanied by phosphorylation of both isoforms on the protein level (Fig 2B).

We next studied AKT isoform activation in the context of the oncogene STAT3, a factor of clinical significance to CLL, which has been described to directly interact with the AKT1 promoter (Park et al, 2005; Hazan-Halevy et al, 2010). Activation of CLL cells by T cells resulted in pronounced STAT3 signalling (pY(705)-STAT3), which could be antagonised by treatment with the STAT3 inhibitor S3I-201 (Fig 2C). STAT3 inhibition significantly decreased AKT1 mRNA expression (Fig 2D), indicating an interaction of these pathways upon CLL cell activation. Notably, relative AKT isoform transcription and protein expression in activated CLL cells was not altered upon treatment with the novel small molecule inhibitors Ibrutinib or Idelalisib (data not shown).

These observations prompted us to investigate potential synergistic effects of AKT- or STAT3-inhibition with conventional drugs used in the treatment of CLL. We recently observed that T cell activated-CLL cells gain resistance towards fludarabine (Hofbauer et al, 2014). Consistently, CLL cells co-cultured with activated T cells remained viable for up to 48 h even in the presence of fludarabine. However, exposure to the pan-AKT or STAT3 inhibitor induced CLL cell apoptosis within 24 h (data not shown), indicating that inhibition of AKT or STAT3 is able to overcome CLL activation-induced protection. Notably, after 48 h of culture, CLL cells exposed to the STAT3 inhibitor underwent strong apoptosis irrespective of the presence of fludarabine. Pan-AKT inhibition, but importantly not AKT2 inhibition, resulted in decreased cell viability levels, which were significantly pronounced when combined with fludarabine (Fig 2E).

Taken together, our results indicate a dominant role of AKT1 in microenvironment-mediated CLL survival and chemoresistance. CLL patients could particularly benefit from targeting the predominant AKT1 isoform, which may also increase the response rate towards classical chemotherapeutics.

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Fig 1. AKT1 is the dominant AKT isoform in stromal cell mediated chronic lymphocytic leukaemia (CLL) cell survival. (A) AKT1 and AKT2 mRNA expression in CD19-positive selected CLL cells (n = 10) were measured by quantitative real-time polymerase chain reaction (RT-PCR) using Taqman Gene Expression Assays relative to RPS18 rRNA expression. (B) AKT1 and AKT2 protein expression in purified CLL cells was determined by Western blot, analysed densitometrically and compared to the housekeeping gene PPIA (n = 10). (C i) CLL cells were cultured for the indicated time points or (C ii) for 24 h on stromal cells (SC) and AKT phosphorylation was determined by Western blot. (D i) AKT phosphorylation and (D ii) CLL cell survival was measured by fluorescence-activated cell sorting (FACS) analysis upon co-culture of CLL cells with primary SC, either separated by membrane filter with 0.4 μm pore size (transwell), or in direct cell-cell-contact (n = 6). Annexin-V and 7-aminoactinomycin D double negative cells were considered viable. (E) Upon an AKT isoform-specific protein G-coupled bead-based pull-down assay, AKT isoform expression and GSK3A/B phosphorylation in MEC1 cells was analysed by Western blot. (F) Flow cytometric determination of cell viability of SC co-cultured CLL cells after 48 h of pan-AKT (5 μmol/l of MK2206 or AIX) or AKT2 isoform specific (Akti-2, 5 μmol/l) inhibition (n = 5). (G) siRNA-mediated AKT isoform-specific knockdown in MEC1 cells was achieved using the Nucleofector® Technology (Lonza, Basel, Switzerland), and knockdown efficiency was determined by RT-PCR. (H) Following knockdown, MEC1 cell viability was assessed after 48 h of culture by FACS analysis and compared to the non-targeting control (n = 4). All panels: dark horizontal lines represent the median, with the box representing the 25th and 75th percentiles, the whiskers the smallest and largest value. Statistical analysis was performed using GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA, USA). All data were tested for normal distribution. Analysis of variance (ANOVA) and Tukey post tests were performed for normally distributed data. For non-normally distributed data, the Friedmann and Dunns test was used. *P < 0.05; **P < 0.01; ***P < 0.001; n.s., non significant.
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Author contributions

SWH, PWK, JHP, SP, DA performed research; SWH, PWK, TNH designed research and interpreted data; SWH, PWK, SP, JFP, DA analysed data; RG contributed reagents and analytical tools and interpreted data; SWH, PWK and TNH wrote the manuscript. All authors were involved in critical discussion.

Conflict of interest

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Fig 2. AKT1 phosphorylation and regulation by STAT3 upon T cell-mediated activation results in fludarabine resistance. Peripheral blood mononuclear cells from chronic lymphocytic leukaemia (CLL) patients containing >5% T cells were activated by addition of anti-CD3/CD28 beads (CD3/CD28), harvested, and purified at the indicated time points. (A) AKT1 (left panel) and AKT2 (right panel) mRNA levels were determined by real-time polymerase chain reaction (RT-PCR) as described before and compared to the untreated control (n = 10). (B) The activation state of AKT isoforms was analysed by Western blot. (C) The activation state of STAT3 (pY(705)) in resting or activated CLL cells in the absence or presence of the STAT3 inhibitor S3I-201 (50 μmol/l) was determined by Western blot. (D) AKT1 mRNA expression in activated CLL cells upon STAT3 inhibition was determined by RT-PCR (n = 4) (results presented as bars depict the mean ± standard deviation values, data normalised to resting, untreated control). (E) The viability of activated, fludarabine-resistant CLL cells was measured after 48 h and compared to the treatment with the single agents fludarabine (10 μmol/l), the pan-AKT inhibitor MK2206 (5 μmol/l), the AKT2 specific inhibitor Akti-2 (5 μmol/l), the STAT3 inhibitor S3I-201 (30 μmol/l) or the indicated combinations (n = 6; dark horizontal lines represent the median, with the box representing the 25th and 75th percentiles, and the whiskers the smallest and largest value). Statistical analysis was performed using GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA, USA). All data were tested for normal distribution, and t-test or analysis of variance (ANOVA) and Tukey post test were performed for normally distributed data. For non-normally distributed data, the Wilcoxon signed rank test or the Friedman and Dunn test were used. *P < 0·05; **P < 0·01; ***P < 0·001.

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Small nucleolar RNA expression profiles refine the prognostic impact of IGHV mutational status on treatment-free survival in chronic lymphocytic leukaemia

Chronic B-cell lymphocytic leukaemia (CLL) is generally an indolent disease, with most patients surviving years without treatment although some progress more rapidly. Several markers, such as IGHV mutational status (Hamblin et al., 1999), cytogenetic abnormalities (Döhner et al., 2000) and recurrent gene mutations (Wang et al., 2011; Jeromin et al., 2014), have helped to better stratify patient risk, but few are routinely used by clinicians and they are still relatively unreliable, reflecting the clinical and physiopathological heterogeneity of the disease. Thus there remains room for improvement and new molecular markers. Our understanding of the various roles of small nucleolar RNAs (snoRNAs) in cancer is continually increasing more than two-thirds of previously described snoRNAs could discriminate between CLL prognostic subgroups in an exploration set of 58 treatment-naïve CLL and five normal B-cells. Unsupervised hierarchical clustering showed that patients did not cluster together when considering criteria such as IGHV mutational status, Binet stage, age, gender, karyotype, fluorescence in situ hybridization and NOTCH1/TP53/SF3B1 mutation, but instead were scattered along the dendrogram (Fig S1). Supervised analysis also did not find a snoRNA signature specific to the conventional clinico-biological parameters, suggesting that snoRNA expression profiles were not associated with the aforementioned factors impacting on CLL outcome. The apparent contradiction between our results and those reported by Ronchetti et al (2013) could be explained by the differences between the two

References

Asslaber, D., Grossinger, E.M., Girbl, T., Hofbauer, S.W., Eglo, A., Weiss, L., Greil, R. & Hartmann, T.N. (2013) Mimicking the microenvironment in chronic lymphocytic leukaemia – where does the journey go? British Journal of Haematology, 160, 711–714.

Hazan-Halevy, I., Harris, D., Liu, Z., Liu, J., Li, P., Chen, X., Shanker, S., Ferrajoli, A., Keating, M.J. & Estrov, Z. (2010) STAT3 is constitutively phosphorylated on serine 727 residues, binds DNA, and activates transcription in CLL cells. Blood, 115, 2852–2863.

Herishanu, Y., Perez-Galan, P., Liu, D., Biancotto, A., Pittaluga, S., Vire, B., Gibellini, F., Njuguna, N., Lee, E., Stennett, L., Raghavachari, N., Liu, P., McCoy, J.P., Raffeld, M., Steiler-Stevenson, M., Yuan, C., Sherry, R., Arthur, D.C., Maric, I., White, T., Marti, G.E., Munson, P., Wilson, W.H. & Westner, A. (2011) The lymph node microenvironment promotes B-cell receptor signaling, NF-kappaB activation, and tumor proliferation in chronic lymphocytic leukemia. Blood, 117, 563–574.

Hofbauer, S.W., Pinon, J.D., Bracht, G., Haginger, L., Wang, W., Johrer, K., Tinhofer, I., Hartmann, T.N. & Greil, R. (2010) Modifying akt signaling in B-cell chronic lymphocytic leukemia cells. Cancer Research, 70, 7336–7344.

Hofbauer, S.W., Krenn, P.W., Ganghammer, S., Asslaber, D., Pichler, U., Oberascher, K., Herschler, R., Wallner, M., Kerschbaum, H., Greil, R. & Hartmann, T.N. (2014) Tiam1/Rac1 signals contribute to the proliferation and chemoresistance, but not motility, of chronic lymphocytic leukaemia cells. Blood, 123, 2181–2188.

Park, S., Kim, D., Kaneko, S., Szewczyk, K.M., Nicoula, S.V., Yu, H., Jove, R. & Cheng, J.Q. (2005) Molecular cloning and characterization of the human AKT1 promoter uncovers its up-regulation by the Src/Stat3 pathway. Journal of Biological Chemistry, 280, 38932–38941.

Sarbasosov, D.D., Guerlin, D.A., Ali, S.M. & Sabatini, D.M. (2005) Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. Science, 307, 1098–1101.

Woyach, J.A., Johnson, A.J. & Byrd, J.C. (2012) The B-cell receptor signaling pathway as a therapeutic target in CLL. Blood, 120, 1175–1184.

Yang, Z.Z., Tschopp, O., Hemmings-Mieszczak, M., Feng, J., Brodieck, D., Perentes, E. & Hemmings, B.A. (2003) Protein kinase B alpha/Akt1 regulates placental development and fetal growth. The Journal of Biological Chemistry, 278, 32124–32131.

Zhuang, J., Hawkins, S.F., Glenn, M.A., Lin, K., Johnson, G.G., Carter, A., Cawley, J.C. & Pettitt, A.R. (2010) Akt is activated in chronic lymphocytic leukaemia cells and delivers a pro-survival signal: the therapeutic potential of Akt inhibition. Haematologica, 95, 110–118.