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Lymphotoxin-b receptor in microenvironmental cells promotes the development of T-cell acute lymphoblastic leukaemia with cortical/mature immunophenotype

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Lymphotoxin-mediated activation of the lymphotoxin-β receptor (LTβR; *LTBR*) has been implicated in cancer, but its role in T-cell acute lymphoblastic leukaemia (T-ALL) has remained elusive. Here we show that the genes encoding lymphotoxin (LT)-α and LTβ (*LTA*, *LTB*) are expressed in T-ALL patient samples, mostly of the TAL/LMO molecular subtype, and in the TEL-JAK2 transgenic mouse model of cortical/mature T-ALL (*Lta*, *Ltb*). In these mice, expression of *Lta* and *Ltb* is elevated in early stage T-ALL. Surface $LT\alpha_1\beta_2$ protein is expressed in primary mouse T-ALL cells, but only in the absence of microenvironmental LTβR interaction. Indeed, surface LT expression is suppressed in leukaemic cells contacting *Ltbr*-expressing but not *Ltbr*-deficient stromal cells, both *in vitro* and *in vivo*, thus indicating that dynamic surface LT expression in leukaemic cells depends on interaction with its receptor. Supporting the notion that LT signalling plays a role in T-ALL, inactivation of *Ltbr* results in a significant delay in TEL-JAK2-induced leukaemia onset. Moreover, young asymptomatic TEL-JAK2;*Ltbr*$^{-/-}$ mice present markedly less leukaemic thymocytes than age-matched TEL-JAK2;*Ltbr*$^{+/+}$ mice and interference with LTβR function at this early stage delayed T-ALL development. We conclude that LT expression by T-ALL cells activates LTβR signalling in thymic stromal cells, thus promoting leukaemogenesis.

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T-cell acute lymphoblastic leukaemia (T-ALL) is an aggressive malignancy of thymocytes that affects mainly children and adolescents, is fatal without therapy, and is characterized by a number of cell-autonomous genetic and molecular alterations (Graux *et al*, 2006; Van Vlierberghe & Ferrando, 2012). Several cell-extrinsic factors involved in T-ALL have been studied *in vitro* or *in vivo*, including growth factors [e.g. interleukin 7 (IL7), IL18 and insulin-like growth factor 1 (IGF1)], chemokines, Notch ligands (DLL4) and adhesion molecules (e.g. ICAM1) (Winter *et al*, 2001; Buonamici *et al*, 2009; Medyouf *et al*, 2011; Silva *et al*, 2011; Mirandola *et al*, 2012; Uzan *et al*, 2014; Minuzzo *et al*, 2015). Furthermore, our group has found that expression of the NF-κB transcription factor RELB in non-haematopoietic stromal cells contributes to the development of T-ALL in a mouse model (dos Santos *et al*, 2008). RELB is a critical NF-κB transcription factor that is activated following engagement of tumour necrosis factor receptor (TNFR) superfamily members, thus hinting that TNFR-like signalling is implicated in T-ALL (dos Santos *et al*, 2010).

The lymphotoxin-beta receptor (LTβR; *LTBR*) is a member of the TNFR superfamily that is activated by either of two ligands, the lymphotoxin (LT) heterotrimer $LT\alpha_1\beta_2$ or LIGHT/TNFSF14 homotrimer (Ware, 2005; Remouchamps *et al*, 2011). Expression of $LT\alpha_1\beta_2$ is found at the surface of activated T, B and NK cells (Ware *et al*, 1992; Browning *et al*, 1993), while LIGHT protein is mainly expressed on activated T cells (Mauri *et al*, 1998). The LTβR receptor is constitutively expressed in stromal cells (e.g. fibroblasts, epithelial cells, and endothelial cells) of primary and secondary lymphoid organs and in myeloid lineage cells, but not expressed in T or B lymphocytes (Force *et al*, 1995; Murphy *et al*, 1998). LTβR activates both canonical and noncanonical NF-κB signalling pathways (Dejardin *et al*, 2002; Ganeff *et al*, 2011), thus inducing specific transcriptional programmes that include the expression of chemokines (e.g. CXCL13, CCL21 and CCL19) and adhesion molecules (ICAM1, VCAM1 and MAdCAM1) (Dejardin *et al*, 2002; Ganeff *et al*, 2011; Bénézech *et al*, 2012). Knock-out mouse models inactivating LTβR or downstream signalling proteins showed that this pathway is essential for the development of secondary lymphoid organs, and for the generation and maintenance of splenic and thymic microarchitecture (Remouchamps *et al*, 2011). In the thymus, the $LT\alpha_1\beta_2$-LTβR signalling axis is involved not only in the development and maintenance of the stromal cell network, but also in thymocyte development, which requires reciprocal interactions between thymocytes and stromal cells either by direct cell-to-cell contact or by soluble factor production (Fütterer *et al*, 1998; Klug *et al*, 1998; Boehm *et al*, 2003; Ware, 2005).

Despite early evidence for anti-tumour properties of the LTβR signalling pathway, several reports pinpointed a pro-oncogenic role for LT signalling in both solid and haematological malignancies (Wolf *et al*, 2010). In some settings, LTβR signalling is expressed in malignant cells and is activated by LT-expressing lymphocytes present in the tumour microenvironment (Haybaeck *et al*, 2009; Ammirante *et al*, 2010), while in others LTβR is expressed in specific tumour microenvironmental cells and is activated by either cancer or other stromal cells expressing LT (Rehm *et al*, 2011; Lau *et al*, 2014). mRNA and protein expression of LT has long been reported in T-ALL cell lines (Sung *et al*, 1988; Ware *et al*, 1992), but the expression in primary T-ALL as well as its physiological relevance in disease development has not yet been addressed.

Here, we report that T-ALL patient samples express the *LTA* and *LTB* genes, this expression being more pronounced in the TAL/LMO molecular subtype. Using the TEL-JAK2 transgenic mouse model of cortical/mature T-ALL, we document that the $LT\alpha_1\beta_2$ ligand is expressed at the surface of TEL-JAK2 leukaemic cells and that LTβR genetic inactivation leads to a significant delay in T-ALL development.

MATERIALS AND METHODS

Mice

EμSRα-TEL-JAK2 transgenic (TJ2-Tg) mice (Carron *et al*, 2000) were bred with *Ltbr* knockout mice (Fütterer *et al*, 1998), provided by Dr. Jorge Caamaño (University of Birmingham, Birmingham, UK), all on the C57BL/6 background. Mice were maintained in the specific pathogen-free CBMR/UAlg Animal Facility (Faro, Portugal) and the experimental procedures followed recommendations for the care and use of laboratory animals from the European Commission (Directive 2010/63/UE) and Portuguese authorities (Decreto-Lei n°113/2013). TJ2-Tg and

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recipient mice were monitored for leukaemia development and killed by CO_2 inhalation when manifesting signs of disease (i.e. dyspnoea, lethargy, enlarged lymph nodes or enlarged abdomen). For LT *in vivo* inhibition, TJ2-Tg mice were intraperitoneally injected with 100 µg of muLTβR-muIgG fusion protein (provided by Biogen Idec, Cambridge, MA, USA) or control ChromPure mouse IgG (Jackson Immunoresearch Laboratories, West Grove, PA, USA) for five consecutive weeks, starting at 5 weeks of age. For detection of malignant thymocytes in early stage T-ALL, TJ2-Tg mice were killed at 8 weeks of age, and the percentage of $CD8^+CD25^+$ cells in thymocyte suspensions was determined.

Human T-ALL and thymocyte samples

Primary T-ALL samples were obtained at diagnosis from bone marrow and/or peripheral blood with high leukaemia involvement (>85%), and enriched by density centrifugation over Ficoll-Paque (GE Healthcare, Little Chalfont, UK). Microarray analyses were performed on samples from 51 paediatric patients with newly diagnosed T-ALL accrued from 2000 to 2013 at Centro Infantil Boldrini, Campinas, Brazil. Most patients (*n* = 44) were treated according to the Brazilian GBTLI-99 Childhood ALL Treatment protocol (Brandalise *et al*, 2010). The use of patient samples was approved by the FCM/UNICAMP Research Ethics Committee (CAAE: 0014.0.144.146-08) and informed consent was obtained from parents. Thymic samples, obtained from children undergoing cardiac surgery, were gently minced in culture medium and subsequently subjected to density centrifugation. Informed consent and Institutional Review Board approval were obtained for all sample collections in accordance with the Declaration of Helsinki.

Microarray data sets and data analysis

Microarray data sets were obtained from the publicly available Gene Expression Omnibus database, including gene expression data from the Microarray Innovations In Leukaemia (MILE) study (GSE 13159) to evaluate the expression of *LTA* and *LTB* in subtypes of leukaemia compared to non-leukaemia and healthy bone marrow samples (Haferlach *et al*, 2010). To compare the expression profile of TJ2-Tg mouse T-ALL with that of different stages of mouse thymocyte differentiation, we obtained thymocyte gene expression data from the Immunological Genome Project (Heng & Painter, 2008) and expression data from TJ2-Tg leukaemic cells (Waibel *et al*, 2013) (GSE15907 and GSE51243, respectively). All datasets were pre-processed using Robust Multi-array Average as implemented in the R/Bioconductor environment (Gentleman *et al*, 2004). The $\log_2$ signal intensities of the annotated genes were obtained and the co-efficient variation for each gene was computed. We thus obtained the top 1% (*n* = 163) of genes that showed least co-efficient of variation. Further, we performed hierarchical clustering of samples using the complete linkage analysis method implemented in the TM4 software on the above mentioned mouse datasets. In addition to the clustering analysis we performed functional enrichment analysis of the 163-gene dataset using Gene Set Enrichment Analysis (GSEA) to identify enriched biological processes, molecular functions and pathways (Subramanian *et al*, 2005). The statistical significance of enrichment was estimated and all the functional categories and pathways with a false discovery rate of ≤0·05 were considered.

Gene expression microarray analysis of T-ALL patient samples

Total RNA was extracted from 51 paediatric T-ALL samples preserved in guanidine isothiocyanate solution using the Illustra RNAspin Mini RNA Isolation Kit (GE Healthcare). Total RNA from two human thymocyte samples was extracted with Trizol (Life Technologies, Carlsbad, CA, USA). RNA samples were processed with the WT Expression Kit (Ambion, Austin, TX, USA) and GeneChip WT Terminal Labeling and Controls Kit (Affymetrix, Santa Clara, CA, USA), and hybridized on Human Gene 1.0 ST Arrays (Affymetrix). Array files are publicly available through the Gene Expression Omnibus (GEO) database under the accession numbers GSE50999 and GSE66638. Expression values were obtained with the iterPLIER+16 algorithm in the Affymetrix Expression Console, and transformed to Z-score to a $\log_2$ scale. Molecular subtypes were classified by hierarchical clustering analysis (Pearson correlation and pairwise average-linkage; http://genepattern.broadinstitute.org/) according to the expression of genes previously associated with each subtype (Homminga *et al*, 2011). We used the Affymetrix Complex Match table to identify those probe sets in the original Human Genome U133 Plus 2.0 Array (Homminga *et al*, 2011) that were most related to probe sets available in the Human Gene 1.0 ST Array. Two out of the 51 T-ALL samples could not be classified and thus were excluded from analysis.

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Human cell lines

T-ALL cell lines DND41, PF382, P12 and SUPT1 were provided by Dr. Hind Medyouf and Dr. Andrew P. Weng (BC Cancer Agency, Vancouver, Canada). Jurkat and Nalm6 cell lines were available in-house (Barata *et al*, 2006). Leukaemic cell lines were cultured in complete RPMI medium (Lonza, Basel, Switzerland) supplemented with 10% heat-inactivated foetal bovine serum (PAA Laboratories, Linz, Austria), 2 mmol/l L-glutamine, 100 U/ml of penicillin and 100 μg/ml of streptomycin (Lonza) and maintained at 37°C under a humidified atmosphere with 5% CO_2. Cell lines were cultured at 1×10^6 cells/ml and treated for 10 h with either dimethyl sulfoxide (DMSO; control), 50 ng/ml phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich, St. Louis, MO, USA) and/or 10 μmol/l BMS-345541 (IKKi) (Calbiochem, Billerica, MA, USA) for both mRNA and cell surface protein detection.

Reverse transcription quantitative PCR (RT-qPCR)

Total RNA from mouse cells and cell lines was prepared using Trizol reagent (Life Technologies) and Direct-zol RNA miniprep (Zymo Research, Irvine, CA, USA), following the manufacturer's instructions. RNA samples were treated with DNase I (Thermo Fisher Scientific, Waltham, MA, USA) to remove remaining genomic DNA. Then, total RNA (1 μg) was reverse-transcribed using the First Strand cDNA Synthesis Kit (Thermo Fisher Scientific) and oligo$(dT)_{18}$ primers. Quantitative PCR reactions, containing 2 μl of 1:20 diluted cDNA in 20 μl, SsoFast EvaGreen Supermix (Bio-Rad, Hercules, CA, USA) and 300 nmol/l of murine or human gene-specific primers (see Table SI) were performed on a C1000 Thermal Cycler coupled to a CFX 96 Real-time PCR detection system (Bio-Rad). PCR results were analysed by Bio-Rad CFX Manager software, version 3.0, treated using the comparative C_T method ($2^{-\Delta\Delta C(T)}$ method), and the mean fold change in expression of the target gene was calculated in relation to reference gene expression (*Gapdh* for murine samples and *GAPDH* or *RNA18S5* rRNA for human samples). For quantification of *LTA* and *LTB* expression in the molecular subgroups of T-ALL, total RNA (1 μg) was reverse transcribed using the ImProm II Reverse Transcriptase enzyme (Promega, Madison, WI, USA) and random hexamers (25 μmol/l). PCR reactions were carried out in 15 μl containing 5 μl of 1:25 diluted cDNA, 7·5 μl Maxima Probe/ROX qPCR Master Mix (Thermo Fisher Scientific), 300 nmol/l of each primer and 200 nmol/l of TaqMan probe (sequences listed in Table SI). Amplification reactions were performed in a 7500 Fast Real-Time PCR System (Life Technologies). Expression values of *LTA* and *LTB* were calculated by the $2^{-\Delta\Delta C(T)}$ method using *ABL1* as endogenous normalizer and Jurkat cells as calibrator. Efficiencies for both the normalizer and target genes were 100% ±10%.

Primary murine leukaemic T cell isolation and *ex vivo* culture

Primary TJ2-Tg leukaemic cells were obtained as single cell suspensions from the thymus or spleen, after gentle dissociation and filtration through 70 μm nylon cell strainers. Bone marrow single cell suspensions were obtained by flushing phosphate-buffered saline (PBS) through tibiae and femora. For molecular analyses, primary cells were cultured in complete RPMI medium at 2×10^6 cells/ml and treated with either DMSO (control), 1 μg/ml actinomycin D (ActD), 50 μg/ml cycloheximide (CHX), 10 ng/ml PMA and 250 ng/ml ionomycin (PMA+Iono) (all from Sigma-Aldrich), 10 μmol/l Tetracyclic Pyridone 6 (InSolution Jak Inhibitor I, JAKi) or 10 μmol/l BMS-345541 (IKKi) (both from Calbiochem), as indicated.

Co-cultures

Ltbr$^{+/-}$ and *Ltbr*$^{-/-}$ mouse embryonic fibroblasts (MEFs) were prepared by standard procedures. MEFs and the MS5 murine bone marrow stromal cell line, provided by Dr. Françoise Pflumio (CEA, Fontenay-aux-Roses, France), were maintained in Dulbecco's modified Eagle medium (DMEM; Lonza) or α minimal essential medium (αMEM, Gibco, Grand Island, NY, USA), respectively, and supplemented with 10% FBS (PAA), 2 mmol/l L-glutamine, antibiotics, and 0·1 mmol/l 2-mercaptoethanol (Gibco). TJ2-Tg leukaemic cells (1×10^6 cells/ml) were co-cultured in triplicate with confluent MS5 cells or MEFs in complete RPMI medium. Co-cultured leukaemic cells in suspension (non-adherent) were collected for flow cytometry analysis. The remaining culture was washed twice with PBS and then incubated with enzyme-free cell dissociation buffer (Gibco) at 37°C. Dissociated leukaemic cells (adherent) were discriminated from MS5 or MEFs through flow cytometry detection of the Thy1.2 T-cell surface marker.

Flow cytometry and cell sorting

Single-cell suspensions were stained with fluorochrome-labelled antibodies and detected in a FACS Calibur flow cytometer (BD Biosciences, San Jose, CA, USA) or sorted using a FACSAria I (BD

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Biosciences), as indicated. Fluorescein isothiocyanate (FITC)-, R-phycoerythrin (PE)-, PE-cyanin 5 (PE-Cy5)-, or allophycocyanin (APC)-conjugated antibodies specific for CD3ε (145-2C11), CD25 (PC61), CD69 (H1.2F3), CD4 (GK1.5), CD8 (53-6.7), CD24 (M1/69), CD44 (IM7) and Thy1.2 (30-H12) (BioLegend, San Diego, CA USA) were used. Membrane-bound LTβR ligands were detected using the muLTβR-huIgG (LTβR-Fc) fusion protein, as previously described (Ansel *et al*, 2000). Intracellular LTβ was detected in paraformaldehyde-fixed saponin-permeabilized cells using a hamster anti-mouse LTβ (BBF6.BF12) monoclonal antibody (Browning *et al*, 1997). The LTβR-Fc fusion protein (and human IgG negative control) and the hamster anti-mouse LTβ (and anti-KLH Armenian hamster Ig Ha4/8 negative control), all kindly provided by Dr. Jeffrey L. Browning (Biogen Idec and Boston University School of Medicine), were visualized using PE-conjugated AffiniPure $F(ab')_2$ fragment donkey anti-human IgG (H+L) (Jackson ImmunoResearch Laboratories) and FITC-conjugated goat anti-hamster (Armenian) IgG (BioLegend) secondary antibodies, respectively. Nonviable cells were excluded from analyses by propidium iodide (Sigma-Aldrich) or 7-AAD (BioLegend) staining and appropriate gating. The data were analysed on CellQuest (BD Biosciences) software.

Statistics

Statistical analyses were performed using Prism 5 (GraphPad Software, La Jolla CA, USA). Statistical tests were used as indicated in figure legends. Kaplan–Meier survival curves were plotted and *P* values were calculated using the log-rank Mantel Cox test. A $P < 0{\cdot}05$ was considered statistically significant.

RESULTS

Lymphotoxin gene expression in T-ALL patient cells

Initial analysis of *LTA* and *LTB* transcripts and cell surface LT protein showed heterogeneous but detectable expression in several human T-ALL cell lines, but not in the Nalm6 B-cell precursor leukaemia cell line (Fig 1A, B). *LTA* and *LTB* mRNA and surface LT expression was upregulated by phorbol ester treatment and blocked by an IKK inhibitor in all T-ALL cell lines tested (Fig 1C–E). Together, these data confirmed previous reports obtained with other T-ALL cell lines (Sung *et al*, 1988; Ware *et al*, 1992) indicating that the LT genes and $LT\alpha_1\beta_2$ heterotrimers are expressed in human T-ALL cells in an NF-κB-dependent manner.

Analysis of a publically available large multicentre dataset of 2022 leukaemia patient and 74 non-malignant bone marrow (BM) samples (Haferlach *et al*, 2010) showed that *LTA* and *LTB* genes were significantly more expressed in T-ALL and other lymphoid malignancies than in myeloid malignancies or non-malignant BM (Fig 2A). Human thymocytes express LT (Wolf & Cohen, 1992), so we evaluated *LTA* and *LTB* expression in T-ALL paediatric patient samples (Table SII) as compared to age-matched human thymocytes. By doing so, we found *LTA* and *LTB* expression in normal thymocytes and T-ALL samples, albeit heterogeneously in the latter, some expressing levels that were higher, while others were lower than those found in thymocytes (Fig 2B). To determine whether LT gene expression was associated with particular molecular or oncogenic subgroups (Graux *et al*, 2006), we assessed *LTA* and *LTB* expression in a larger cohort of 49 primary T-ALL patients by microarray analysis followed by RT-qPCR validation in a subset of 33 patients (see Table SIII for the immunophenotypic and genetic characteristics of T-ALL diagnostic samples). Molecular subtypes were classified by hierarchical clustering analysis (HCA) according to the expression of genes previously associated with each subtype (Homminga *et al*, 2011). The *LTA* and *LTB* genes were expressed at variable levels in the different molecular subtypes, but expression of *LTA* and *LTB* was significantly higher in the TAL/LMO molecular subgroup than in the remaining samples (Fig 1C). RT-qPCR analyses corroborated that *LTA* and *LTB* were expressed at higher levels in the TAL/LMO T-ALL subgroup than in immature T-ALL (Fig 1D and Table SIII). Together, these data indicate that higher LT expression is associated with a particular subset of T-ALL patients.

LT expression in a mouse model of cortical/mature T-ALL

The TAL/LMO molecular subtype of T-ALL has been associated with αβ T-cell receptor (TCR) lineage differentiation, cell surface expression of αβTCR and CD3, and cortical/mature immunophenotype (Ferrando *et al*, 2002; Asnafi *et al*, 2004; Soulier *et al*, 2005; Graux *et al*, 2006). Moreover, TAL/LMO samples were reported to cluster preferentially with post-β-selection human thymocyte subsets, including immature CD4 single-positive (SP), CD4/CD8 double-positive (DP),

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and mature CD4 and CD8 SP cells (Soulier *et al*, 2005). To study the role of LT in T-ALL development, we used TEL-JAK2 transgenic mice (TJ2-Tg), a model of cortical/mature T-ALL that is characterized by the presence of CD4/CD8 DP and CD8 SP leukaemic cells, expression of surface αβTCR, CD3 and CD5, and responsiveness to TCR stimulation (Carron *et al*, 2000; dos Santos *et al*, 2007). Furthermore, unsupervised clustering analysis of four TJ2-Tg leukaemic samples and nine thymocyte subpopulations, based on the expression levels of 163 highly variable genes, revealed that the latter clustered with double negative stage 4 (DN4), CD8 ISP and DP thymocytes (Fig S1 and Table SIV). These results indicate that TJ2-Tg mouse leukaemias, like most TAL/LMO T-ALL cases, are malignancies of late cortical, post-β-selection thymocytes. We first assessed *Lta* and *Ltb* expression in primary TJ2-Tg leukaemic cells from diseased mice, and found these genes to be expressed on average at four- to five-fold higher levels than wild-type thymocytes (Fig 3A). Additionally, *Lta* and *Ltb* expression was higher in TJ2-Tg leukaemic cells than in sorted CD4 SP thymocytes, which are one of the main sources of LTβR ligands in the adult thymus (Fig 3B) (Boehm *et al*, 2003; Hikosaka *et al*, 2008). These results show that, similarly to human T-ALL samples, murine TEL-JAK2 leukaemic cells present high LT expression.

To study how LT expression is regulated in TJ2-Tg leukaemic cells, we inhibited the JAK/STAT and IKK/NF-κB signalling pathways, which were reported to be activated in these cells (Carron *et al*, 2000; dos Santos *et al*, 2008) and to induce *Lta* and *Ltb* expression in T lymphocytes (Shebzukhov & Kuprash, 2011). Treatment of leukaemic cells with a pan-JAK inhibitor downregulated *Lta* (and the control *Socs2* gene) but not *Ltb* mRNA expression (Fig 3C). Similarly to human T-ALL cell lines, mouse leukaemic cell treatment with an IKK kinase inhibitor markedly downregulated both *Lta*and *Ltb* mRNAs (and the control *Nfkb2* gene) (Fig 3D). As expected, the IKK inhibitor blocked *Lta*and *Ltb* induction by PMA/ionomycin in TJ2-Tg leukaemic cells (Fig S2). It is worth noting that short treatment with these inhibitors did not induce significant leukaemic cell death (data not shown). Together, these experiments indicate that JAK and IKK kinases are key regulators of lymphotoxin gene expression in human and mouse T-ALL cells.

In thymocytes, NF-κB transcription factors are activated by pre-TCR and αβTCR signalling (Moore *et al*, 1995; Aifantis *et al*, 2001). Given that *Lta* and *Ltb* expression is induced by TCR during TCR-mediated thymocyte positive selection (Irla *et al*, 2012), we reasoned that the pre-TCR or TCR could regulate LT gene expression in leukaemic cells. Quantification of *Lta* and *Ltb* mRNAs in TJ2-Tg;*Rag2*$^{-/-}$ leukaemic cells, which are pre-TCR and TCR-deficient (dos Santos *et al*, 2007), showed that *Ltb*, but not *Lta* expression was decreased, as compared to pre-TCR/TCR-proficient TJ2-Tg leukaemic cells (Fig 3E). These data indicate that TCR signalling induces *Ltb* gene expression in T-ALL cells.

LT$\alpha_1\beta_2$ heterotrimer surface expression on TEL-JAK2 T-ALL cells is downmodulated *in vivo* and depends on *de novo* protein synthesis

To detect LT surface protein on TJ2-Tg cells, we first treated cells with PMA and ionomycin. Consistent with mRNA expression results, we detected membrane-bound LTβR ligands at higher levels in TJ2-Tg leukaemic cells than in wild-type thymocytes (Fig S3). However, in untreated leukaemic cells, LT protein was detected only upon cell permeabilization (Fig 4A). Interestingly, upon *ex vivo* culture, primary leukaemic cells showed LTβR-Fc staining, peaking at around 24 h (Figs 4A, B and S4). Anti-LTβ monoclonal antibody (mAb) pre-incubation blocked completely the cell surface LTβR-Fc signal in *ex vivo*-cultured and in PMA/ionomycin-treated cells, indicating that only LT$\alpha_1\beta_2$ heterotrimers but not LIGHT were expressed at the cell surface (Figs 4A and S5A). Collectively, these results indicate that *ex vivo*-cultured or PMA/ionomycin-stimulated TJ2-Tg leukaemic cells express surface LT$\alpha_1\beta_2$ heterotrimers.

The observed LT cell surface expression following *ex vivo* culture and PMA/ionomycin stimulation of mouse leukaemic cells could result from a combination of *de novo* protein synthesis and protein subcellular relocalization. To assess the role of *de novo* protein synthesis, we blocked transcription and translation by treating TJ2-Tg leukaemic cells with actinomycin D or cycloheximide, respectively. Both compounds blocked LT$\alpha_1\beta_2$ cell surface induction upon either *ex vivo* culture or PMA/ionomycin stimulation (Fig S5A, B), indicating that sustained LT$\alpha_1\beta_2$surface expression in leukaemic cells depends on *de novo* mRNA and protein synthesis in both conditions. To verify whether *ex vivo* culturing also induced *Lta* and *Ltb* mRNA, we performed RT-qPCR analysis in leukaemic cells freshly collected or cultured *ex vivo*. *Ltb*, but not *Lta* mRNA expression increased following *ex vivo* culture (Fig S5C). Together, these results indicate that LT$\alpha_1\beta_2$ expression at the

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surface of *ex vivo*-cultured and stimulated leukaemic cells depends on LT gene transcription and continuous protein synthesis.

Microenvironmental LTβR downmodulates cell surface LT expression on TEL-JAK2 leukaemic T cells

Given that surface LT expression was detected on primary leukaemic cells only upon *ex vivo*culture, we sought to determine whether lack of LT expression *in vivo* depended upon interaction with LTβR expressed by stromal cells. To this end, we inoculated TJ2-Tg leukaemic cells in *Ltbr*$^{+/-}$ and *Ltbr*$^{-/-}$ mice and collected expanded cells for analysis 3–4 weeks later. Similarly to spontaneously generated leukaemic cells (Fig 4), leukaemic cells collected from *Ltbr*-proficient mouse organs (gated on $CD8^+CD25^+$ cells) did not display detectable $LT\alpha_1\beta_2$ surface expression (Fig 5A). In contrast and strikingly, $LT\alpha_1\beta_2$ surface expression was markedly sustained in leukaemic cells collected from different tissues (thymus, spleen, bone marrow and peripheral blood) of *Ltbr*$^{-/-}$ recipient mice (Figs 5A, B and S6). Similar results were obtained upon inoculation of TJ2-Tg;*Ltbr*$^{-/-}$ leukaemic cells in *Ltbr*$^{+/-}$ and *Ltbr*$^{-/-}$ mice (data not shown). These results indicate that $LT\alpha_1\beta_2$ expression on the TJ2-Tg leukaemic cell surface is downmodulated when these cells are localized within LTβR-expressing tissue microenvironments.

To investigate whether downmodulation of $LT\alpha_1\beta_2$ surface expression depends on direct cell contact, we co-cultured TJ2-Tg leukaemic cells with stromal cell lines. The bone marrow-derived MS5 stromal cell line, which expresses surface LTβR (Fig S7), was shown to sustain the survival of co-cultured TJ2-Tg leukaemic cells (Fig 5C). Upon co-culture, leukaemic cells adhering to the MS5 monolayer showed lower levels of surface $LT\alpha_1\beta_2$ than either non-adhering co-cultured leukaemic cells or leukaemic cells cultured in absence of MS5 cells (Fig 5D, E). Conditioned medium collected from co-cultured leukaemic cells with MS5 cells did not impair $LT\alpha_1\beta_2$upregulation in leukaemic cells cultured in the absence of MS5 cells (Fig S8), indicating that soluble factors produced in co-cultures are not sufficient for $LT\alpha_1\beta_2$ downmodulation. To determine whether interaction with LTβR was required for $LT\alpha_1\beta_2$ downmodulation, TJ2-Tg leukaemic cells were co-cultured with either *Ltbr*$^{-/-}$ or *Ltbr*$^{+/-}$ mouse embryonic fibroblasts (MEFs). Strikingly, *Ltbr*-deficient, but not *Ltbr*-proficient MEFs failed to downmodulate LT from the surface of adhering leukaemic cells (Fig 5F, G), thus indicating that this phenomenon is mediated by interaction with LTβR in microenvironmental cells. These results indicate therefore that direct contact between the leukaemic cell and LTβR-expressing stromal cells is required for surface $LT\alpha_1\beta_2$ downmodulation. Moreover, we conclude that the increased levels of leukaemic cell surface $LT\alpha_1\beta_2$ in *Ltbr*-deficient recipient mice is caused by defective LTβR expression in microenvironmental cells, and not by putative indirect cellular or systemic defects in *Ltbr*-deficient recipient mice.

LTβR inactivation delays leukaemogenesis in TEL-JAK2 transgenic mice

The above results indicate a potential $LT\alpha_1\beta_2$-LTβR functional interaction between leukaemic and stromal cells. To investigate whether LTβR activation is involved in T-cell leukaemogenesis, TJ2-Tg mice were bred with *Ltbr* knockout mice. Strikingly, we found that TJ2-Tg;*Ltbr*$^{-/-}$ mice developed T-ALL with longer latency than TJ2-Tg;*Ltbr*$^{+/-}$ mice (median survival of 23 weeks vs. 15 weeks) (Fig 6A). Despite the observed delay in leukaemogenesis, *Ltbr* gene inactivation did not impact on the leukaemic cell surface marker phenotype (CD3, CD4, CD8, CD24 and CD25 expression) characteristic of TJ2-Tg mice (dos Santos *et al*, 2007) (Fig S9), nor on endpoint tumour burden in lymphoid and visceral organs (Fig 6B), except for lymph nodes, which are absent in *Ltbr* knockout mice (Fütterer *et al*, 1998). Similar to leukaemic cells transplanted to *Ltbr*$^{-/-}$ mice, those collected from *Ltbr*-deficient, but not from *Ltbr*-proficient TJ2-Tg mice expressed surface $LT\alpha_1\beta_2$ (Fig 6C, D), thus confirming that $LT\alpha_1\beta_2$ surface expression in both normal and malignant T cells is only detectable in the absence of the cognate receptor (Boehm *et al*, 2003). Of note, the observed delay in leukaemogenesis in the absence of LTβR was not linked to putative T-cell defects, because LTβR-deficient mice aged 19–22 weeks, an age when most *Ltbr*-proficient TJ2-Tg mice have developed terminal disease, presented no major defects in cellularity and differentiation of thymocytes (Fig S10A–D), the targets of TJ2-Tg-induced transformation. Furthermore, inhibition of LT signalling through the administration of neutralizing LTβR-Fc fusion protein to young pre-leukaemic TJ2-Tg mice (with intact thymocyte and lymphoid organ differentiation) prolonged mouse survival (Fig 6E). Together, these results indicate that delayed leukaemogenesis in *Ltbr*-deficient mice could not be attributed to either T-cell defects or absence of lymph nodes and, more

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importantly, that LTβR signalling in non-malignant microenvironmental cells promotes leukaemogenesis.

LT signalling fosters the early stages of leukaemogenesis

To determine the role of LTβR signalling at early stages of disease, we analysed young (8-week-old) TJ2-Tg mice without signs of disease for the presence of aberrant $CD8^+CD25^+$ cells in organs commonly affected by T-ALL. $CD8^+CD25^+$ cells were consistently found, although at variable proportions, in the thymus of asymptomatic mice, but not to same extent in spleen, bone marrow and blood (Fig S11). Upon transplantation to nude mice, thymocyte suspensions including $CD8^+CD25^+$ cells generated secondary leukaemia (data not shown). These data confirm that the first malignantly transformed T cells in the TJ2-Tg mouse model arise in the thymus.

To investigate whether LT signalling participates in the early phases of T-ALL, we analysed LT gene expression in thymocytes from 8-week-old asymptomatic TJ2-Tg mice and non-transgenic littermates. Interestingly, the increased proportion of $CD8^+CD25^+$ malignant thymocytes observed in young TJ2-Tg mice (without thymic enlargement) was associated with increased *Lta*and *Ltb* expression (Figs 6F, G and S12A, B). More specifically, significantly higher levels of *Lta*and *Ltb* expression were found in samples where $CD8^+CD25^+$ cells accounted for more than 5% of thymocytes (Fig S12C). To investigate whether LT signalling promotes leukaemogenesis at the initial steps of disease, we detected malignant ($CD8^+CD25^+$) thymocytes in asymptomatic 8-week-old TJ2-Tg mice of different *Ltbr* genotypes. Interestingly, the proportion of malignant thymocytes was significantly reduced in *Ltbr*-deficient mice, as compared to *Ltbr*-proficient mice (Fig 6H). Together, these data indicate that increased LT gene expression occurs early in TJ2-Tg-induced leukaemogenesis, and that LTβR signalling at early stages accelerates disease progression.

DISCUSSION

Although LT expression in T-ALL cell lines has long been known, the present study disclosed a role for LT expression in T-ALL development. Indeed, by studying patient samples, cell lines and a transgenic mouse model, we found that T-ALL cells express lymphotoxin protein and mRNA, that leukaemic cell surface LT interacts with LTβR expressed in stromal cells, and that LTβR expression in thymic microenvironmental cells promotes T-ALL, especially at the early stages. LT signalling was shown to be involved in pro-oncogenic interactions between leukaemic and microenvironmental cells in mouse models of B-cell lymphoma and chronic lymphocytic leukaemia (Rehm *et al*, 2011; Heinig *et al*, 2014). Together with these findings and the observed elevated LT gene expression in lymphoid malignancies, our results further reinforce the notion that the LT-LTβR signalling axis is crucial for lymphoid cell leukaemogenesis.

In our study, LT genes were found to be more expressed in TAL/LMO T-ALL, a subtype associated with the cortical and mature stages of thymocyte differentiation (Ferrando *et al*, 2002; Asnafi *et al*, 2004; Soulier *et al*, 2005; Graux *et al*, 2006). Being considered a thymocyte malignancy, T-ALL is associated with expression of thymocyte differentiation markers. As such, LT gene expression in T-ALL may reflect either the differentiation stage of the cell of origin or the oncogenic process itself. Regardless of the mechanism involved, the presented data suggests that, at least in a subset of patients, T-ALL cells can interact with and modulate LTβR-expressing microenvironments through LT expression.

In agreement with previous reports (Browning et al, 1997), signalling pathway activation by phorbol ester and ionophore treatment induced LT gene transcription and surface $LT\alpha_1\beta_2$expression in both mouse primary T-ALL cells and human cell lines. By treating TJ2-Tg leukaemic cells and several T-ALL cell lines with pharmacological inhibitors, we verified that both basal and PMA/ionomycin-induced LT expression in mouse and human T-ALL was dependent on IKK/NF-κB activity, and that *Lta* expression in TJ2-Tg leukaemic cells was dependent on JAK kinase activity. NF-κB signalling in T cells can be activated by αβTCR or pre-TCR signalling (Moore *et al*, 1995; Aifantis *et al*, 2001; Cheng *et al*, 2011). Supporting the notion that these receptor complexes may upregulate LT genes in T-ALL, we observed that *Ltb* mRNA levels were reduced in TJ2-Tg leukaemic cells lacking pre-TCR/αβTCR (*Rag2*-deficient). Thus, our findings indicate that *Ltb* gene expression in T-ALL is induced by TCR or pre-TCR signalling in an NF-κB-dependent manner.

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Despite the high levels of *Lta* and *Ltb* expression in TJ2-Tg leukaemic cells, surface $LT\alpha_1\beta2$ was barely detectable in cells collected from LTβR-proficient mice, but were readily detected in leukaemic cells collected from LTβR-deficient mice, as previously reported for mature T cells (Boehm *et al*, 2003). These results thus suggest that upon LTβR activation on stromal cells, the surface levels of $LT\alpha_1\beta_2$ in thymocytes are downmodulated, possibly due to an autoregulatory mechanism to dampen LTβR activation. Supporting this hypothesis, $LT\alpha_1\beta_2$ heterotrimers were found to be upregulated at the leukaemic cell surface upon *ex vivo* culture in the absence of stromal cells. Surface LT downmodulation was directly linked to LTβR expression, as demonstrated by the detection of surface LT in leukaemic cells in direct contact with LTβR-deficient MEFs, but not in leukaemic cells in contact with LTβR-expressing MEF or MS5 stromal cells. Furthermore, surface LT downmodulation depended on direct contact with LTβR-expressing cells, since it was weak in co-cultured leukaemic cells that did not adhere to stromal cells and it was not induced by soluble factors from co-culture conditioned medium. Therefore, these data show that surface LT downmodulation depends on direct interaction with LTβR.

Our results indicate that LT signalling occurs in the initial stages of TEL-JAK2-induced murine T-ALL, which, similar to TAL/LMO cases in humans, presents features of cortical/mature T-cell leukaemia. Indeed, increased LT gene expression was detected in thymocytes from young asymptomatic TJ2-Tg mice. Furthermore, LTβR deficiency hampered the initial expansion of T-ALL cells in the thymus. Given that LTβR is expressed in thymic stromal cells, including epithelial cells and fibroblasts, but not thymocytes (Seach *et al*, 2008), our findings indicate that emerging LT-expressing leukaemic cells require LTβR-expressing thymic stromal cells for accelerated disease onset. LTβR-Fc-mediated blockade of LTβR signalling in pre-leukaemic TJ2-Tg mice indicated that early interference with this pathway delays leukaemogenesis. At the terminal phase of TJ2-Tg-induced T-ALL, we observed no significant differences in tumour burden, indicating that at later stages of disease progression, LTβR activation plays a less prominent role. This suggests that interaction of LT-expressing leukaemic cells with LTβR-expressing stromal cells plays a pro-leukaemogenic role when the emerging malignant cells are in small numbers but not after expansion and consequent reduced contact with the former cells.

The identity of the LTβR-expressing stromal cells and the mechanisms by which their activation promotes leukaemogenesis remain to be elucidated. Given that LTβR is known to activate the noncanonical NF-κB pathway (Ganeff *et al*, 2011) and RelB expression in stromal cells has been implicated in murine T-ALL (dos Santos *et al*, 2008), we postulate that $LT\alpha_1\beta_2$ expression in emerging malignant thymocytes activates an NF-κB-dependent transcriptional programme in thymic microenvironmental cells that promotes T-cell leukaemogenesis. Cytokines (e.g. BAFF/TNFSF13B) and chemokines (e.g. CCL5, CCL19, CCL21, CXCL12 and CXCL13) are possibly involved, because several of these are activated by LTβR in stromal cells (Dejardin *et al*, 2002; Seach *et al*, 2008). Interestingly, expression of *CXCR5*, the gene encoding the CXCL13 receptor, was found to correlate with *LTB* expression in our microarray analysis of T-ALL patients (data not shown), raising the question of whether CXCL13 induces LT in this disease. As shown in Fig S1, TJ2-Tg leukemic cells express several chemokine receptor genes, including *Ccr7* and *Ccr9*. CCL21, a CCR7 ligand, was found to be highly expressed in TJ2-Tg lymphomas (by RT-qPCR and immunohistolgical staining) but such expression was not affected by LTβR deficiency (data not shown). In addition, *Ccr7* deficiency in TJ2-Tg mice did not delay leukemogenesis (unpublished data), which indicates that the role of LTβR in leukemogenesis is not mediated by CCL19/CCL21/CCR7 signalling. The identity of the LTβR target proteins, chemokines or others, and whether LTβR-expressing cells from other organ microenvironments are also involved in interaction with human or murine T-ALL, merit further investigation in future studies.

In summary, the identification of molecules mediating crosstalk between T-ALL and microenvironmental cells is important to understand how T-ALL arises from thymocytes and to determine which microenvironmental signals play a role in the progression, maintenance and chemoresistance of this malignancy. Several studies have highlighted the role of stromal factors in T-ALL. Secreted molecules, such as IL7 and IL18, and membrane-bound Notch ligands were shown to promote T-ALL *in vitro* proliferation and progression in xenografted mouse spleen and bone marrow (Silva *et al*, 2011; Uzan *et al*, 2014; Minuzzo *et al*, 2015; Yin *et al*, 2015). Moreover, competitive access to growth signals from the thymic microenvironment has been shown to be important for T-ALL development (Martins *et al*, 2014). The present work further demonstrates

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the importance of the microenvironment in T-ALL and indicates that LT signalling is important in the early (thymic) stages of this malignancy. Future studies should address whether LT signalling is involved in later phases of T-ALL progression and whether it is a potential therapeutic target in combination with other agents.

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Author contribution

NRdS conceived the study; MTF, ED, NLA, JG, JTB, JAY and NRdS designed the research study; MTF, MNG, ABS, VP and ARR performed the laboratory work for this study; RKK performed bioinformatics and statistical analyses; SRB provided and performed patient clinical analyses; MTF, VP, NLA, JTB, JAY and NRdS analysed the data; JG contributed the TJ2-Tg mice for the study; MTF and NRdS drafted the manuscript; MTF, ED, NLA, JG, JTB, JAY and NRdS were involved in critical review, revision and approval of the final manuscript.

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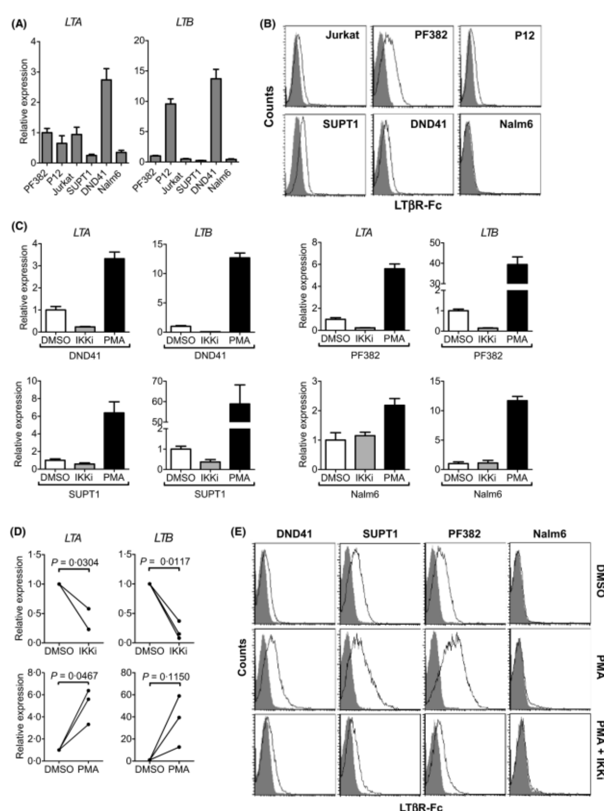

Figure 1 - T-cell acute lymphoblastic leukaemia (T-ALL) patient cell lines express lymphotoxin proteins in an NF-κB-dependent manner. (A) RT-qPCR quantification of LTA and LTB mRNA levels (relative to GAPDH expression) in the indicated cell lines (mean ± range of technical triplicates of one representative analysis of at least 2 performed for each cell line). (B) Cell surface immunostaining with LTβR-Fc (solid line) or secondary antibody alone (grey shading) on the indicated T-ALL cell lines and Nalm6 B-cell line. (C) RT-qPCR quantification of LTA and LTB (relative to GAPDH expression) in DND41, PF382, SUPT1 and Nalm6 cell lines treated for 10 h with either IKK inhibitor (IKKi), phorbol 12-myristate 13-acetate (PMA) or dimethyl sulfoxide (DMSO) (mean ± range of technical triplicates). (D) Statistical analyses of combined RT-qPCR data from three T-ALL cell line biological replicates (DND41, PF382, and SUPT1). P values determined by paired t-tests are indicated. (E) LTβR-Fc immunostaining of indicated cell lines treated for 10 h with PMA, PMA plus IKKi, or DMSO.

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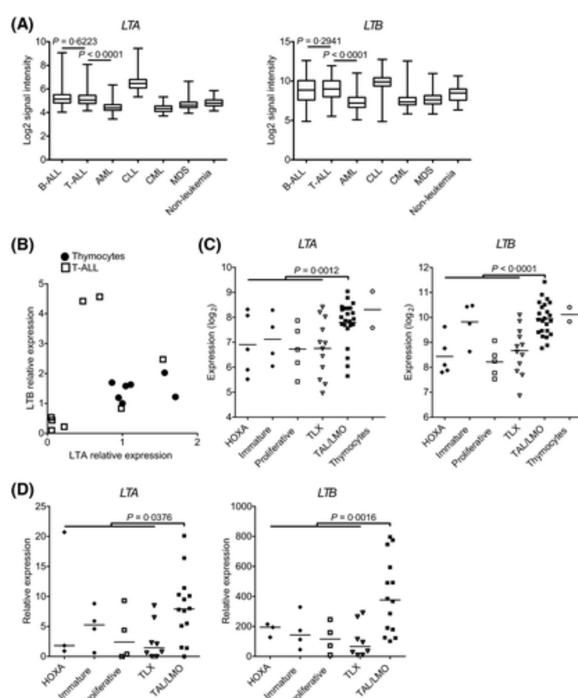

Figure 2 - T-ALL patient samples express lymphotoxin genes. (A) Microarray (GEO GSE 13159) analysis of LTA and LTB gene expression levels in several human samples: B-ALL, B-cell acute lymphoblastic leukaemia (n = 576); T-ALL, T-cell acute lymphoblastic leukaemia (n = 174); CLL, chronic lymphocytic leukaemia (n = 448); AML, acute myeloid leukaemia (n = 542); CML, chronic myeloid leukaemia (n = 76); MDS, myelodysplastic syndrome (n = 206); Non-leukaemia, non-leukaemia and healthy bone marrow samples (n = 74). Lines, bars and whiskers represent the median, quartiles and minimum and maximum values, respectively. P values were calculated by pairwise comparisons between B-ALL and T-ALL, and T-ALL and AML using the two-tailed unpaired Student's t test with or without Welch's correction to account for unequal variances when appropriate. See Table SV for further pairwise comparisons. (B) RT-qPCR quantification of LTA and LTB mRNA levels in human thymocytes (n = 7) and T-ALL patient samples (n = 8) (relative to RNA18S5 rRNA expression); mean of technical triplicates for each sample. (C) LTA and LTB gene expression, as determined by microarray analysis, in 49 T-ALL samples, classified according to molecular subtype [HOXA (n = 5), immature (n = 4), proliferative (n = 5), TLX (n = 12) and TAL/LMO (n = 23)], and 2 human thymocyte samples (bar represents mean expression). P values were calculated by comparing TAL/LMO and the other molecular subtypes combined (i.e. HOXA, immature, proliferative and TLX) using the two-tailed unpaired Student's t test. (D) RT-qPCR quantification of LTA and LTB (relative to ABL1 expression and calibrated to expression in Jurkat cells) in 33 T-ALL patient samples classified according to molecular subtype [HOXA (n = 3), immature (n = 4), proliferative (n = 4), TLX (n = 8) and TAL/LMO (n = 14)]. P values were calculated by comparison between TAL/LMO and the other molecular subtypes combined (i.e. HOXA, immature, proliferative and TLX) using the two-tailed unpaired Student's t test alone or with Welch's correction to account for unequal variances when appropriate.

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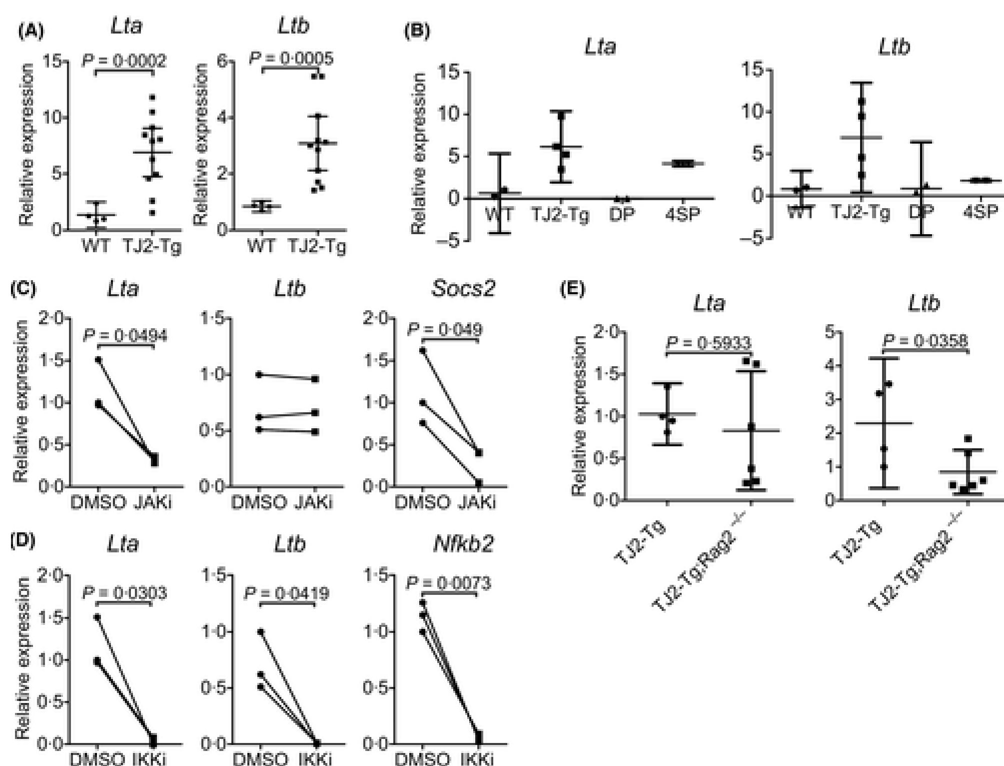

Figure 3 - Mouse T-ALL cells express lymphotoxin genes in an NF-κB-dependent manner. (A) RT-qPCR quantification of Lta and Ltb in leukaemic cells from independent TJ2-Tg mouse thymic lymphomas (n = 11) and wild-type thymocytes (WT; n = 4). Results are expressed as mean + 95% confidence interval (CI). P values determined by the two-tailed, unpaired t test with Welch's correction. (B) Two WT thymocyte and 4 TJ2-Tg leukaemic cell samples randomly selected from those tested in (A) were compared with sorted CD4+CD8+ double-positive (DP) and CD4+ single-positive (CD4 SP) thymocytes (from 2 independent cell sorting experiments). Results are expressed as mean + 95% CI. (C,D) Lta, Ltb, Socs2 and Nfkb2 mRNA expression were quantified by RT-qPCR in three independent TJ2-Tg leukaemic cell samples treated ex vivo for 10 h with either DMSO, JAKi or IKKi. Results are expressed as mean ± SEM. P values determined by two-tailed, paired t tests (E) RT-qPCR quantification of Lta and Ltb mRNA levels in Rag2-deficient TJ2-Tg leukaemic cells (TJ2-Tg;Rag2−/−; n = 6) and control leukaemic cells (TJ2-Tg; n = 4). Results are expressed as mean + 95% CI. P values determined by two-tailed, unpaired t tests with Welch's correction.

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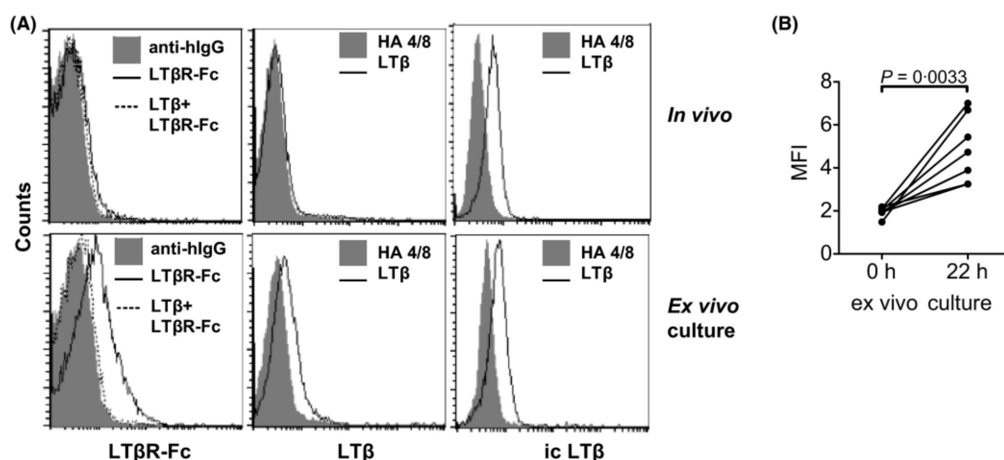

Figure 4 - Mouse T-ALL cells express LTα1β2 heterotrimers. (A) Flow cytometry immunostaining of LTβR ligands (black solid line) on representative TJ2-Tg leukaemic cells freshly collected (in vivo) or cultured ex vivo for 22 h with LTβR-Fc protein or anti-LTβ mAb on viable (left and middle panels) or fixed/permeabilized (ic) (right panels) cells. The secondary antibody alone or non-specific mAb were used as negative controls for LTβR-Fc and LTβ mAb, respectively (grey shading). LTβR-Fc immunostaining was also performed following anti-LTβ mAb pre-incubation (black dashed line). (B) LTβR-Fc staining mean fluorescence intensity (MFI) for seven independent leukaemic cell samples before (0 h) and after 22 h of ex vivo culture. P value determined by the two-tailed, paired t-test.

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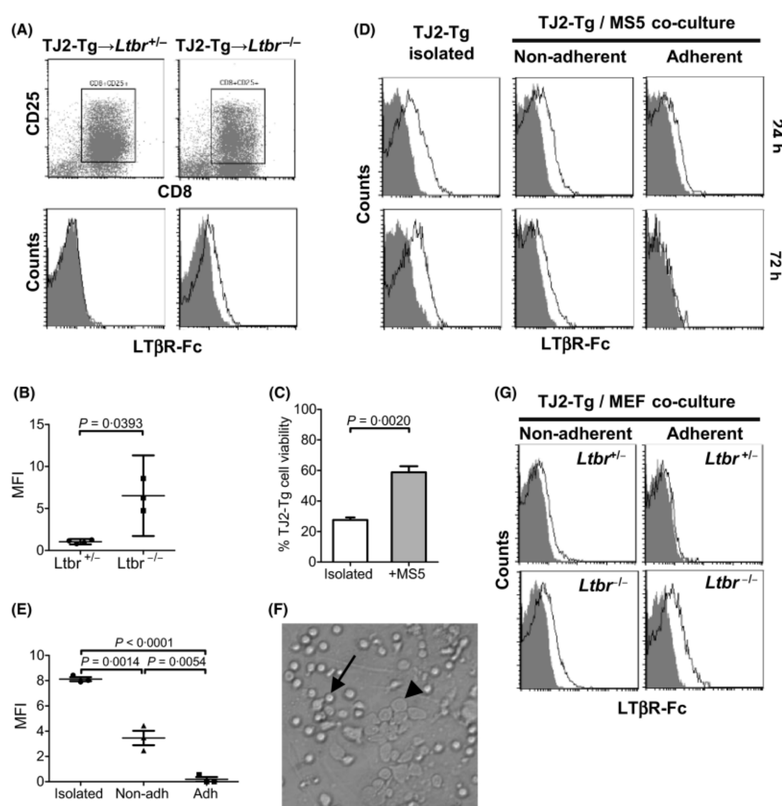

Figure 5 - $LT\alpha_1\beta_2$ heterotrimer expression on leukaemic T cells depends on LTβR expression in microenvironmental cells. (A) Cell surface immunostaining with LTβR-Fc (solid line) or secondary antibody alone (grey shading) of thymic $CD8^+CD25^+$ TJ2-Tg leukaemic cells collected 3 weeks post-inoculation of *Ltbr*$^{+/-}$ and *Ltbr*$^{-/-}$ mice. (B) LTβR-Fc mean fluorescence intensity (MFI) subtracted from control MFI of $CD8^+CD25^+$ cells collected from 3 to 4 transplanted mice of the indicated genotypes. One representative of three independent experiments is shown as the mean + 95% CI. *P* value determined by the two-tailed, unpaired *t* test with Welch′s correction. (C) TJ2-Tg leukaemic cell viability upon 3 days in culture medium alone (isolated) or in co-culture with MS5 cells. Representative results of three experiments are expressed as the mean ± SEM of technical triplicates. *P* value determined by the two-tailed, unpaired *t* test. (D) Cell surface LTβR-Fc immunostaining (solid line) of TJ2-Tg leukaemic cells cultured for the indicated times in culture medium alone or in co-culture with MS5 cells. (E) LTβR-Fc MFI subtracted from control MFI of leukaemic cells adhering (Adh) or non-adhering (Non-adh) to co-cultured MS5 cells, or cultured in isolation and collected at 72 h. Results from one experiment representative of three are expressed as the mean ± SEM of technical triplicates. *P* values determined by pairwise comparisons using two-tailed, unpaired *t* tests. (F) Phase contrast microscopy depicting TJ2-Tg leukaemic cells adhering (arrowhead) or non-adhering (arrow) to mouse embryonic fibroblasts (MEFs), after 24 h of co-culture. (G) Cell surface LTβR-Fc immunostaining of TJ2-Tg leukaemic cells co-cultured for 24 h with *Ltbr*$^{+/-}$ or *Ltbr*$^{-/-}$ MEFs. Leukaemic cells adherent or non-adherent to MS5 or MEF cells were gated on $Thy1.2^+$ cells.

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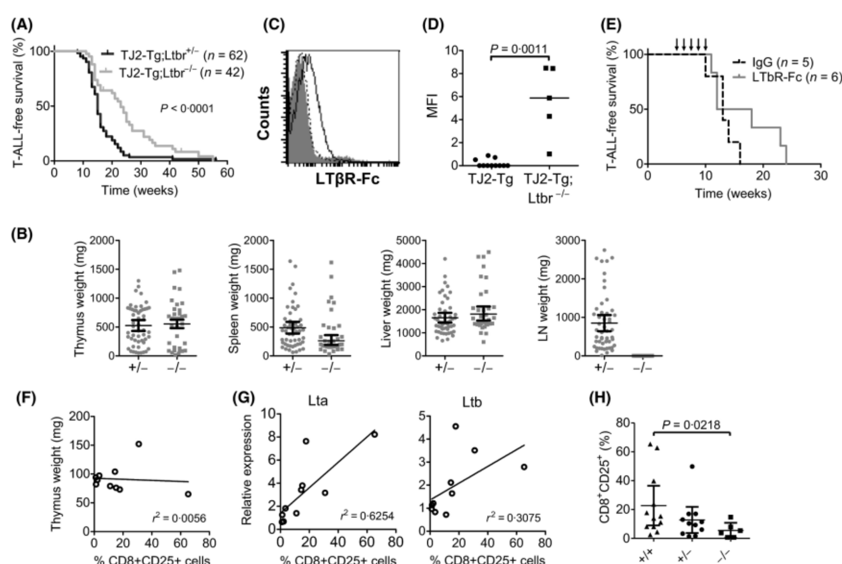

Figure 6 - LTβR inactivation delayed TEL-JAK2-induced leukaemogenesis. (A) Kaplan–Meier survival curves for cohorts of TJ2-Tg;*Ltbr*$^{+/-}$ and TJ2-Tg;*Ltbr*$^{-/-}$ mice. Tick marks represent leukaemia-free mice followed until the indicated age. (B) Weights of thymus, spleen, liver and lymph nodes (mandibular, axillary, sub-iliac and jejunal) collected from terminally ill TJ2-Tg;*Ltbr*$^{+/-}$ (n = 53) and TJ2-Tg;*Ltbr*$^{-/-}$ (n = 33) mice represented as the mean + 95% CI. (C) Flow cytometry immunostaining of representative thymic TJ2-Tg;*Ltbr*$^{-/-}$ leukaemic cells using LTβR-Fc (black solid line) or secondary antibody alone (grey shading). Where indicated, cells were pre-incubated with an anti-LTβ mAb (black dashed line). (D) LTβR-Fc mean fluorescence intensity (MFI) (median) of TJ2-Tg (n = 11) and TJ2-Tg;*Ltbr*$^{-/-}$ (n = 5) leukaemic cells subtracted from the MFI of cells stained with control antibody alone. P value determined by the Mann–Whitney test. (E) Kaplan–Meier survival curves (P = 0·27; log-rank test) for TJ2-Tg mice treated with intraperitoneal injections of LTβR-Fc or mouse polyclonal IgG once a week for 5 weeks, starting at 5 weeks of age (arrows). At 16 weeks all control-treated mice were dead while half of LTβR-Fc-treated mice were still alive (P = 0·06; χ^2 test) (F) Percentage of CD8$^+$CD25$^+$ thymocytes plotted with thymic weights. P = 0·4047; two-tailed Spearman test. (G) *Lta* and *Ltb* mRNA expression levels plotted with the percentage of CD8$^+$CD25$^+$thymocytes from 8-week-old asymptomatic TJ2-Tg mice (n = 10) (mean of triplicates for each sample). *Lta*, P = 0·0008; *Ltb*, P = 0·0174; two-tailed Spearman test. For (F) and (G) r^2 measures goodness-of-fit of linear regression. (H) Percentage of malignant thymocytes (as identified by CD8 and CD25 positivity) represented as the mean + 95% CI in thymi from 8-week-old TJ2-Tg mice. Pvalue determined by pairwise comparison between +/+ and −/− using the Mann–Whitney test.

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