Aberrant expression of aldehyde dehydrogenase 1A (ALDH1A) subfamily genes in acute lymphoblastic leukaemia is a common feature of T-lineage tumours

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Summary

The class 1A aldehyde dehydrogenase (ALDH1A) subfamily of genes encode enzymes that function at the apex of the retinoic acid (RA) signalling pathway. We detected aberrant expression of *ALDH1A* genes, particularly *ALDH1A2*, in a majority (72%) of primary paediatric T cell acute lymphoblastic leukaemia (T-ALL) specimens. *ALDH1A* expression was almost exclusive to T-lineage, but not B-lineage, ALL. To determine whether ALDH1A expression may have relevance to T-ALL cell growth and survival, the effect of inhibiting ALDH1A function was measured on a panel of human ALL cell lines. This revealed that T-ALL proliferation had a higher sensitivity to modulation of ALDH1A activity and RA signalling as compared to ALL cell lines of B-lineage.

Consistent with these findings, the genes most highly correlated with *ALDH1A2* expression were involved in cell proliferation and apoptosis. Evidence that such genes may be targets of regulation via RA signalling initiated by ALDH1A activity was provided by the *TNFRSF10B* gene, encoding the apoptotic death receptor TNFRSF10B (also termed TRAIL-R2), which negatively correlated with *ALDH1A2* and showed elevated transcription following treatment of T-ALL cell lines with the ALDH1A inhibitor citral (3,7-dimethyl-2,6-octadienal). These data indicate that ALDH1A expression is a common event in T-ALL and supports a role for these enzymes in the pathobiology of this disease.

Keywords: acute lymphoblastic leukaemia; aldehyde dehydrogenase; retinoic acid; cell proliferation; T cell

T cell acute lymphoblastic leukaemia (T-ALL) is an aggressive malignancy that frequently presents with unfavourable clinical features, such as high leucocyte counts, mediastinal enlargement and infiltration of the central nervous system (Uckun *et al*, 1998). This disease arises from progenitor T cells that have circumvented their normal dependence on the thymic microenvironment in order to undergo clonal expansion. Survival rates for T-ALL have improved, but only through the use of intensified multi-agent therapies with consequent side effects, and overall outcome remains inferior compared to the more common, and biologically distinct, B-lineage ALL (Hunger *et al*, 2012).

A better understanding of the molecular pathogenesis of T-ALL offers the prospect of developing improved treatment strategies. Two major groups of oncogenes, the TLX homeobox genes and the *TAL1* (*SCL*)/*LMO* (bHLH/LIM-only) genes, which have mutually exclusive expression patterns, are deregulated in a majority of T-ALL tumours (Ferrando *et al*, 2002). These encode transcription factors and are thought to represent critical first-hit events, resulting in the emergence of pre-leukaemic T cells that may subsequently develop into overt leukaemia following the acquisition of additional genetic mutations, such as those that activate *NOTCH1* or delete *CDKN2A* (Weng *et al*,2004;

Hebert *et al*, 1994). Recent evidence suggests that aberrantly expressed oncogenes in T-ALL cause leukaemic transformation by promoting thymocyte proliferation, survival and/or differentiation arrest (McCormack *et al*, 2010; Dadi *et al*, 2012). However, a full account of the mechanisms that mediate T cell transformation remains to be achieved.

The class 1A aldehyde dehydrogenase (ALDH1A) subfamily of genes, responsible for cellular retinoic acid (RA) synthesis (Molotkov & Duester, 2003) have provided a possible link between the distinct molecular T-ALL subtypes, having been identified as being downstream of both TLX and TAL1/LMO proteins. Family member *ALDH1A1*, which is not expressed in normal thymocytes, is transcriptionally regulated by TLX1 in multiple cell types including T cells (Greene *et al*, 1998; Rice *et al*, 2008), while the highly related *ALDH1A2* is a target gene of TAL1/LMO proteins in T-ALL (Ono *et al*, 1998; Palomero *et al*, 2006; Sanda *et al*, 2012). ALDH1A enzymes (also known as retinal dehydrogenases [RALDHs]) are of interest because the product of their activity, the hormone/morphogen RA, is an essential regulator of diverse biological processes via activation of nuclear receptors. Crucially, this includes haemopoiesis where synthesis of RA by ALDH1A enzymes has been implicated in the regulation of haemopoietic stem cell (HSC) development, differentiation and renewal (Chute *et al*,2006; Muramoto *et al*, 2010; Ma *et al*, 2010; Chanda *et al*, 2013; Ghiaur *et al*, 2013). Thus, high ALDH1A activity is a prominent feature of HSCs (Armstrong *et al*, 2004), being associated with enhanced repopulating ability and resistance to cytotoxic drugs, such as cyclophosphamide (Colvin *et al*, 1988), and exploited practically for their purification (Hess *et al*, 2004). Moreover, in haemopoietic progenitors ALDH1A expression appears to have a role in regulating the polarity of differentiation towards a myeloid rather than a lymphoid cell fate (Storms *et al*, 2005; Rice *et al*, 2008), an effect mirrored by the administration of RA alone (Purton *et al*, 1999; Leung & Verfaillie, 2005). Significantly, RA is known to promote the proliferation (Dillehay *et al*, 1989; Ertesvag *et al*, 2002; Engedal *et al*, 2006) and survival (Engedal *et al*, 2004) of human T cells, while inhibiting thymocyte differentiation (Zhou *et al*, 2008). This is in contrast to many other cell types, including B cells (Naderi & Blomhoff, 1999), where RA primarily appears to act as an anti-proliferative and pro-differentiation agent.

To gain insight into the importance of aberrant *ALDH1A* subfamily gene expression in T-ALL, we examined gene expression microarray data in primary tumours from 100 children with this disease compared to 55 children with B-lineage ALL. We found that *ALDH1A* genes, particularly *ALDH1A2*, were expressed at a high frequency specifically in T-ALL. The effect of blocking ALDH1A activity was then examined, which revealed that these enzymes selectively contribute to the growth and survival of ALL cells of T cell phenotype. These results implicate ectopic RA synthesis and subsequent modulation of gene expression via the retinoid signalling pathway as a contributing factor in T-ALL tumorigenesis.

Materials and Methods

Cell lines

The ALL cell lines PER-117, PER-255, PER-604, PER-377 and PER-495 have been described previously (Kees *et al*, 2003) and characterized cytogenetically and molecularly (Beesley *et al*, 2006; Hoffmann *et al*, 2006; Beesley *et al*, 2010). The CEM cell line was obtained from the Children's Cancer Institute Australia for Medical Research. Cell lines were cultured in a humidified atmosphere with 5% CO_2 in RPMI 1640 medium supplemented with 2 mmol/l L-glutamine, 10 nmol/l 2-mercaptoethanol and 10% heat-inactivated fetal calf serum (FCS; CSL, Melbourne, VIC, Australia). All PER cell lines were additionally supplemented with 1 mmol/l sodium pyruvate and non-essential amino acids. For PER-255, PER-604 and PER-377, 20% FCS was used. Cell lines were cultured in the absence of antibiotics and testing for mycoplasma was routinely performed by polymerase chain reaction (PCR).

ALDH assay

To determine cell line ALDH enzyme activity, 5×10^5 cells were incubated with Aldefluor reagent (StemCell Technologies, Vancouver, BC, Canada) at 37°C for 30 min in the presence or absence of the ALDH inhibitor diethylaminobenzaldehyde (DEAB, 10 μmol/l). Sytox staining (Life

Technologies, Carlsbad, CA, USA) was used to determine viability. After staining, cells were pelleted and resuspended in 500 μl Aldefluor assay buffer. The cell suspensions were analysed on an LSRII flow cytometer (BD Biosciences, San Jose, CA, USA). ALDH activity was determined by the net change in mean fluorescence intensity between DEAB-inhibited and uninhibited viable cells. All flow cytometric data were subsequently analysed using FlowJo software (TreeStar, Ashland, OR, USA).

Proliferation assay

Cells in exponential growth phase were counted by trypan blue exclusion and seeded in fresh media at a density of 0·5–1·5 × 10^6 cells/ml in the presence or absence of various concentrations of 3,7-dimethyl-2,6-octadienal (citral; Sigma Aldrich, St. Louis, MO, USA), or 75 μmol/l citral plus various concentrations of (E)-4-[2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2- naphthylenyl)-1 -propenyl] benzoic acid (TTNPB) or 4-[(E)-2-(7-heptoxy-4,4-dimethyl-1,1-dioxo-2,3- dihydrothiochromen-6-yl) prop-1-enyl] benzoic acid (Ro 41-5253; Enzo Life Sciences, Farmingdale, NY, USA) dissolved in dimethyl sulphoxide (DMSO), or DMSO alone, for 48 h. The final concentration of DMSO in the medium was 0·1%, which had no effect on cell viability. Testing was performed in triplicate using the following ranges: citral: 10–200 μmol/l, TTNPB: 0·6–12 μmol/l; Ro 41-5253: 2·5–50 μmol/l. Culture plates (96-well) were incubated at 37°C for 42 h prior to the addition of 10 μl of filter-sterilized 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; 5 mg/ml). Plates were then incubated for a further 6 h before addition of 100 μl acidified isopropyl alcohol solution to dissolve formazan crystals and measurement of absorbance at 595 nm on a microplate reader (Wallac Victor 2 Multi-label Counter 1420; Perkin Elmer, Waltham, MA, USA). Data represent the average of at least three independent experiments and were reported as an IC25/SC25 (concentration that inhibited or stimulated cell growth by 25%).

Microarray data analysis

Gene expression profiling of the primary patient cohort analysed in this study has been previously described (Cleaver *et al*, 2010) and comprised bone marrow specimens obtained at diagnosis from 50 T-ALL patients at either Princess Margaret Hospital, Perth, Australia or provided by the Children's

Oncology Group (COG). Expression data from an additional 50 T-ALL patients was obtained from two cohorts reported by Winter *et al* (2007) and normalized by robust multi-array analysis (RMA) as previously described (Cleaver *et al*, 2010). We corrected for batch effects across the data sets using ComBat (Johnson *et al*, 2007). Affymetrix Human Genome U133 Plus 2·0 expression microarray data from two normal human thymus glands were obtained from the NCBI GEO database (Edgar *et al*, 2002), accession numbers GSM175973 and GSM176262. Affymetrix Human Genome U133A expression data were also obtained for six ALL cell lines (Hoffmann *et al*, 2006; Beesley *et al*, 2010). We used hierarchical clustering with Euclidean distance and complete linkage in generating the heat maps. The probes used to represent each gene were 212224_at (*ALDH1A1*), 207016_s_at (*ALDH1A2*), 203180_at (*ALDH1A3*), 207179_at (*TLX1*), 206283_s_at (*TAL1*), 203750_s_at (*RARA*). The probes were selected for their presence in both microarray formats used, their reliability, signal strength and resolution.

Quantitative real-time PCR

Cells were cultured in duplicate for 16 h in the presence of 100 μmol/l citral, or control media containing vehicle only. Total RNA was extracted from cells by TRIzol lysis (Life Technologies), phenol extraction and ethanol precipitation. First strand cDNA was generated from 1 μg of total RNA using oligo-dT primer (Life Technologies) and Omniscript reverse transcriptase (QIAGEN, Hilden, Germany). *ALDH1A1*, *ALDH1A2* and *TNFRSF10B* (tumor necrosis factor receptor superfamily, member 10b, also termed TRAIL-2) expression was measured using TaqMan gene expression assays Hs00946916_m1, Hs00180254_m1 and Hs00366278_m1, respectively (Life Technologies) on one-hundredth of the cDNA. Reactions (20 μl) were performed in triplicate in 384-well plates on an ABI 7900HT Fast Real-Time PCR System (ABI, Grand Island, NY, USA). Gene expression was normalized using the TaqMan *RPL19* expression assay Hs02338565_gH.

Statistical analyses

Gene expression correlations with *ALDH1A* were determined using Pearson's correlation coefficient calculated using expression data of 102 samples (patient samples plus two normal thymus samples).

Associated *P*-values were corrected using the method of Benjamini and Hochberg (1995); *P*-values ≤0·05 were considered to be statistically significant. Nested analysis of variance (anova) was used to test for differences in IC25 values between T- and B-lineage ALL cell lines. The two-sample *t* test was used to assess differences in mRNA transcript levels determined by quantitative reverse transcription polymerase chain reaction (RT-PCR).

Results

ALDH1A genes are aberrantly expressed at high frequency in T-ALL

Genes encoding ALDH1A enzymes, whose main physiological function is cellular RA synthesis, are known to lie downstream of two oncogenic transcription factors aberrantly expressed in T-ALL, namely TLX1 in the case of *ALDH1A1* (Greene *et al*, 1998; Rice *et al*, 2008) and TAL1 (SCL) in the case of *ALDH1A2* (Ono *et al*, 1998; Palomero *et al*, 2006; Sanda *et al*, 2012). This prompted the hypothesis that transcription of *ALDH1A* genes may be a common feature of T-ALL. We therefore examined *ALDH1A* subfamily (*ALDH1A1*, *ALDH1A2 and ALDH1A3*) gene expression in 100 primary T-ALL tumour specimens using microarray data from two published datasets (Winter *et al*, 2007; Cleaver *et al*, 2010). Elevated levels of *ALDH1A1*, *ALDH1A2* and *ALDH1A3* mRNA expression were detected in 13%, 63% and 3% of T-ALL samples, respectively, and a total of 72% of the primary samples expressed at least one of these three genes (Fig 1A; Table 1). Thus, *ALDH1A* genes are expressed at a high frequency in T-ALL, with *ALDH1A2* expression being particularly prominent, both in terms of frequency and absolute levels of expression. *ALDH1A2* was highly overexpressed with a mean fold-change of 7·52 ($P = 0·007$) when microarray data were used to compare expression between the panel of 100 T-ALL tumours and two normal human thymus specimens. In contrast, examination of *ALDH1A2* expression using published microarray data from a cohort of 55 primary pre-B lineage ALL specimens (Hoffmann *et al*, 2008), revealed that it was not significantly expressed in any cases (Fig 1B). Overall, *ALDH1A1*, *ALDH1A2* and *ALDH1A3* mRNA transcripts were detected

in 4%, 0% and 0% of 55 pre-B ALL samples, respectively. Thus, aberrant expression of genes encoding ALDH1A enzymes is almost exclusively associated with ALL tumours of T cell lineage.

Consistent with previous observations (Ono *et al*, 1998), *TAL1*, which was expressed in 54% of tumours, showed significant correlation with that of *ALDH1A2* ($r = 0{\cdot}42$). Nevertheless, these data also indicate that additional mechanisms besides TLX1 and TAL1 are responsible for aberrant *ALDH1A1* and *ALDH1A2* expression in T-ALL. Interestingly, a high correlation coefficient of 0·60 was observed between*ALDH1A3* and *TAL2* gene expression. T-ALL tumours uniformly appeared to be competent for RA signalling, with all specimens expressing all retinoid receptor (*RAR/RXR*) genes, with the exception of *RXRG* (Table 1). *RARA* expression was particularly significant (Fig 1A), as it exhibited strong overexpression in the tumours as compared to the normal thymus samples with a mean fold-change of 6·1 ($P = 0{\cdot}004$).

T-all cell line proliferation is sensitive to inhibition of ALDH1A enzymes and RARA

The microarray results suggested that the RA pathway may be specifically relevant to T-ALL cell growth and/or survival. Consistent with this, RA has been documented to enhance normal human T cell proliferation (Dillehay *et al*, 1989; Ertesvag *et al*, 2002; Engedal *et al*, 2006). To extend these findings to the tumour setting, we assessed the functional importance of RA synthesizing ALDH1A enzymes, together with RA signalling via RARA, to the proliferation of T-ALL cell lines (PER-117, PER-255, PER-604, CEM), which were compared with those of B lineage (PER-377, PER-495). All six cell lines tested were negative by microarray for ALDH1A1 and ALDH1A3 mRNA expression, but expressed varying levels of ALDH1A2 transcripts, with the two TAL1-positive T-ALL lines (PER-604 and CEM) very strongly expressing *ALDH1A2* (Fig 2A). All of the cell lines expressed the retinoid receptor (*RAR/RXR*) genes, with the exception of *RXRG*. Thus, the cell lines closely mirrored the primary T-ALL tumours in terms of the frequency of *ALDH1A* and *RAR/RXR* gene expression. To test whether the expression of the ALDH1A transcripts was reflected at the protein level, we used the Aldefluor assay to detect intracellular ALDH1A enzymatic activity in each of the cell lines. As shown

in Fig 2B, enzyme activity correlated well with *ALDH1A2* mRNA expression levels, and was notably higher in the T-ALL cell lines overall, as compared to those of B lineage.

ALL cell line growth was determined by MTT assay after exposure to the ALDH1A inhibitor citral (Kikonyogo *et al*, 1999) for 48 h. All four T-ALL cell lines tested showed sensitivity to citral with IC25 (concentration causing 25% inhibition of cell number) values varying from 55 to 130 μmol/l (Fig 3A). Use of citral on the pre-B ALL cell lines, which had much lower ALDH1A activity, was significantly less effectual, with IC25 values of 160 and 250 μmol/l. Individually, the T-ALL line CEM was the most sensitive to ALDH1A inhibition, while the pre-B ALL PER-377 was the least sensitive. Of the T-ALL lines, PER-604 required the most citral to achieve 25% growth inhibition, which may reflect its very high level of ALDH1A activity. Reasoning that ALDH1A enzymes produce RA that signals via RA receptors, we next measured the effect of inhibiting both ALDH1A enzyme and RA receptor activity. Citral (at a constant concentration of 75 μmol/l) was therefore incubated with cells in combination with the RARA-selective antagonist Ro 41-5253. Similar to the results using citral alone, the T-ALL cell lines were found to be more sensitive than the pre-B ALL cell lines to inhibition of RA signalling via the RARA, with IC25 values for Ro 41-5253 being 8 to 48 μmol/l (T-lineage) compared with 60 and 85 μmol/l (B-lineage) (Fig 3B). Conversely, in the presence of 75 μmol/l citral (to inhibit endogenous RA production), the T-ALL cell lines all responded positively to the addition of the pan-RAR agonist TTNPB, with SC25 (concentration causing 25% increase in cell number) values varying from 2·5 to 14 μmol/l (Fig 3C). In sharp contrast, the B lineage cell lines did not benefit from pan-RAR stimulation by TTNPB, at least over the concentration range used, and thus SC25 values could not be derived. Together, these data indicate that T-ALL cells positively respond to signalling via retinoid receptors and that RA-synthesizing ALDH1A enzyme activity specifically contributes to cell growth in T-ALL.

Genes associated with cell proliferation, survival and differentiation correlate with ALDH1A expression in T-ALL tumours

We next sought to provide insight into the molecular basis by which aberrant expression of *ALDH1A* genes affect the proliferation and/or survival of T-ALL cells. One obvious mechanism by which ALDH1A enzyme activity could alter the cell phenotype is by activating nuclear RARs via production of RA to induce or repress the expression of downstream target genes. We therefore examined the transcriptional profile associated with *ALDH1A2* among the 100 primary human T-ALL specimens. Functional evaluation of the top 200-correlated probe sets from this analysis revealed a significant association between *ALDH1A2* expression and genes involved in cell proliferation, apoptosis and differentiation (Fig 4). We focused on the most highly correlated 27 genes, 10 of which showed a positive and 17 an inverse correlation with *ALDH1A2* (Table 2). As a proof of principle test to examine whether modulation of ALDH1A activity could affect the transcriptional level of correlated genes as putative downstream targets, we measured the mRNA levels of *TNFRSF10B* encoding TNFRSF10B (TRAIL-R2), which is associated with apoptosis and was strongly negatively correlated with ALDH1A2. This was achieved by real-time RT-PCR using RNA isolated from the four T-ALL cell lines (PER-117, PER-255, PER-604 and CEM) with and without treatment with 100 μmol/l citral. RT-PCR was also performed using primers and probes specific for *ALDH1A1* and *ALDH1A2*, as the promoters of these genes are reportedly subject to feedback regulation by the RA signalling pathway (Niederreither *et al*, 1997; Elizondo *et al*, 2000). *ALDH1A1* and *ALDH1A2*mRNA levels in the T-ALL cell lines approximately halved after ALDH1A inhibition whereas increases in *TNFRSF10B* mRNA levels of approximately two-fold were observed in three of the four T-ALL cell lines (Fig 5). These results indicate that ALDH1A activity in the T lineage leads to repression of the apoptotic death receptor gene *TNFRSF10B* as well as activation of *ALDH1A1* and *ALDH1A2*, possibly via a positive feedback loop.

Discussion

Understanding the molecular mechanisms by which T-ALL arises is essential in order to improve outcomes for patients. In this study, we have demonstrated that genes belonging to the aldehyde dehydrogenase 1A (*ALDH1A*) subfamily are aberrantly expressed in a large majority (close to three quarters) of childhood T-ALL tumours, but not in B-lineage ALL. *ALDH1A2* expression was by far the most commonly observed (in 63% of T-ALL tumours) and showed a good correlation with *TAL1* expression, consistent with previous studies indicating that TAL1/LMO transcriptional complexes are capable of regulating this gene (Ono *et al*, 1998). In an additional finding, *ALDH1A3* mRNA expression in a small number of tumours (3%) was highly correlated with that of the *TAL2* oncogene, which warrants further investigation. Complete correlations were not observed between TLX1 and ALDH1A1, between TAL1 and ALDH1A2 nor between TAL2 and ALDH1A3, indicating that distinct mechanisms underlie the dysregulation of the various *ALDH1A* genes in T-ALL.

The main biological role of the enzymes encoded by *ALDH1A* genes is to convert the aldehyde form of vitamin A (retinal) to its biologically active form, RA (Molotkov & Duester, 2003). Hence, ALDH1A enzymes are also known as retinal dehydrogenases (RALDHs). The tissue distribution and levels of RA are controlled by the activities of these enzymes, which have distinct roles in development and exhibit patterns of expression that are spatially and temporally regulated (Sládek, 2003). This study therefore implicates ectopic synthesis of RA in thymocytes as a common event in the pathogenesis of T-ALL. RA mediates its crucial effects on cellular growth and development by acting as a ligand for nuclear RA receptors (RARs) and retinoid X receptors (RXRs). Importantly, in all 100 T-ALL tumour specimens examined, all three *RAR* genes (*RARA, RARB* and *RARG*) and two of three *RXR* genes (*RXRA*, *RXRB*) were expressed. Moreover, the *RARA* gene was strongly upregulated in T-ALL (about 6-fold) as compared to normal thymus. Thus, T-ALLs appeared uniformly competent to respond to cellular production of RA and seemingly with heightened sensitivity via RA receptor alpha (RARA).

A functional role for RA synthesis by ALDH1A enzymes and signalling via RARA in T-ALL growth was confirmed by blocking ALDH1A and RARA activity using specific inhibitors. We demonstrated that the proliferation of T-ALL cell lines had a higher sensitivity than B-lineage cell lines to ALDH1A and RARA inhibition. Conversely, treatment with a pan-RAR agonist specifically stimulated proliferation of the T-ALL cell lines while no such stimulation occurred in the B-lineage lines within the concentration range used. These data are consistent with previous studies in humans and mice which have shown that RA is stimulatory to T lymphocyte growth (Dillehay *et al*, 1989; Jiang *et al*,1993; Ertesvag *et al*, 2002; Seguin-Devaux *et al*, 2005; Engedal *et al*, 2006) but inhibitory to the growth of B lymphocytes (Bosma & Sidell,1988; Fahlman *et al*, 1995; Naderi & Blomhoff, 1999), while promoting B cell differentiation (Chen *et al*, 2008). This provides a plausible explanation for why aberrant *ALDH1A* expression is almost completely restricted to ALL tumours of T cell phenotype. One mechanism proposed to explain the T cell growth-promoting effect of RA involved stimulation of interleukin 2 signalling (Jiang *et al*, 1993; Ertesvag*et al*, 2002; Engedal *et al*, 2006).

Consistent with this study, RA is also known to inhibit T cell apoptosis and block the negative selection of thymocytes. This has been demonstrated *in vitro* in thymocytes, T cell hybridomas, peripheral human T cells, as well as mouse thymocytes *in vivo* (Iwata *et al*, 1992; Bissonnette *et al*, 1995; Yang *et al*, 1995; Yagi *et al*, 1997; Szondy *et al*, 1998; Szegezdi *et al*, 2003; Engedal *et al*, 2004; Rasooly *et al*,2005). Interference by retinoids in thymocyte negative selection is reportedly due to activation of RARA leading to inhibition of both DNA binding by nur77 and synthesis of the pro-apoptotic protein BCL2L11 (BIM) (Szegezdi *et al*, 2003). Besides its roles in modulating T cell growth and survival, RA has been implicated as a negative regulator of thymocyte maturation (Meco *et al*, 1994; Napolitano *et al*, 1997; Zhou *et al*, 2008). In either mouse fetal thymic organ cultures or thymuses obtained from young children, administration of RA halted thymocyte differentiation at the CD4+CD8+ double-positive stage of development (Yagi *et al*, 1997; Zhou *et al*, 2008), the same stage at which TLX1 and TAL1 halt maturation in T-ALL (Ferrando *et al*, 2002).

Taken together, previous work implicates RA as a critical signal for regulating normal thymocyte growth and development *in vivo*. That ALDH1A enzymes functionally underlie this RA synthesis in the thymus has been demonstrated using mutant mice carrying a hypomorphic allele of the *Aldh1a2* gene. A major feature of these mice is thymic aplasia/hypoplasia resembling DiGeorge syndrome (Vermot *et al*, 2003). Given that normal T cells do not express ALDH1A enzymes, and thus are unlikely to synthesize appreciable amounts of RA endogenously, a key question is where RA is produced in the thymus. Evidence to date points to the thymic microenvironment, which supports thymocyte survival and proliferation, specifically thymic stromal cells, which reportedly express ALDH1A enzymes and synthesize retinoids (Kiss *et al*, 2008). Such a scenario, where functional cells require exogenous RA produced by accessory cells for their survival and development appears to be a common theme among a variety of physiological systems. For example, gonadal germ cell fate is dictated by RA produced by mesonephroi (Bowles *et al*, 2006) whereas astrocytes provide a regulated source of RA for the brain (Shearer*et al*, 2012). RA is also provided to peripheral T lymphocytes by dendritic cells and this has been shown to imprint gut-homing specificity by enhancing the expression of integrin alpha4beta7 and chemokine receptor CCR9, an effect suppressed by the ALDH1A inhibitor citral (Iwata *et al*, 2004). The regulation of haemopoietic stem cell (HSC) differentiation represents an interesting variation on this theme. In an inverse situation, HSCs are ALDH1A positive and produce their own RA, which primes their ability to undergo differentiation. However, the microenvironment, specifically bone marrow stromal cells, inactivate RA via CYP26B1 enzyme activity to control HSC fate and allow for HSC self-renewal (Ghiaur *et al*, 2013).

Our microarray analysis additionally provided an insight into the gene expression signature associated with aberrant ALDH1A expression in T-ALL. In accord with our experimental data, genes correlated with *ALDH1A2* (the most commonly and robustly expressed *ALDH1A*gene) were involved in cell proliferation, apoptosis and differentiation. This also fits with *ALDH1A2* being listed as one of the genomic classifiers that distinguish induction failure patients with T-ALL (Winter *et al*, 2007). Genes highly concordant with *ALDH1A2* included*NUDT11* (nudix [nucleoside diphosphate linked moiety X]-type motif 11), which is involved in a variety of cellular functions and is implicated in cancer

pathogenesis (Grisanzio *et al*, 2012), *BEX2* (brain expressed X-linked 2), which is linked to cell cycle regulation and apoptosis (Naderi *et al*, 2010) and whose product functionally interacts with LMO2 (Han *et al*, 2005), and *CDK5RAP3* (CDK5 kinase regulatory subunit-associated protein 3), which is associated with tumour metastasis (Mak *et al*, 2011). Genes showing an inverse correlation with*ALDH1A2* included *TNFSF10* (tumor necrosis factor [ligand] superfamily, member 10 encoding TNFSF10, also termed TRAIL), a TNF-related ligand that induces apoptosis especially in transformed cells (Wang & El-Deiry, 2003), *TNFRSF10B,* (encoding TNFRSF10B, also termed TRAIL-R2/death receptor 5), a receptor for TNFSF10 whose low expression in T-ALL has been previously noted (Akahane *et al*, 2010), and *SOCS2* (suppressor of cytokine signalling 2), a key regulator of cytokine and various hormone signalling pathways that has been implicated in carcinogenesis (Rico-Bautista *et al*, 2006). At least some of these genes may lie downstream of ALDH1A enzyme activity via RA activation of nuclear RARs. In particular, both *TNFSF10* and *TNFRSF10A* (*TRAIL-R1*) are known to be regulated by RA (Altucci *et al*, 2001; Dhandapani *et al*, 2011). Focusing on *TNFRSF10B*, we confirmed a functional link between ALDH1A activity and the repression of this gene in T-ALL, with ALDH1A inhibition resulting in an increase in *TNFRSF10B* mRNA levels. In parallel, ALDH1A enzyme inhibition led to a decrease in both *ALDH1A1* and *ALDH1A2* mRNA levels. This indicates that a positive feedback loop may be operational in T-ALL in which RA produced by ALDH1A activity leads to a chronic enhancement and stabilization of *ALDH1A* gene expression, reinforcing its oncogenic program. Given that hundreds of genes are regulated by RA, this program is likely to be complex and involve crosstalk with other pathways. For example, upregulation of *NOTCH1* and/or repression of *CDKN2A* gene expression by RA (Lin*et al*, 2003; You *et al*, 2000) could potentially synergize with activating mutations to *NOTCH1* and hemizygous loss of *CDKN2A*,respectively, in T cell tumourigenesis.

In conclusion, our data suggest a model of leukaemogenesis in which the ability of thymocytes to endogenously synthesize RA independent of thymic stromal cells (following acquisition of ALDH1A enzymatic activity e.g. downstream of TLX1 or TAL1) would lead to an unregulated expansion and arrested development of such cells within the thymus and promote malignancy. If substantiated, this

may lead to more specific therapies for T-ALL, on the basis that it should be possible to target susceptible retinoid signalling pathway components with small-molecule inhibitors. Attempts at treating T-ALL with RA itself have, not surprisingly, been unsuccessful. On the contrary, our data indicate that treatment with RA might actually promote the development of T cell malignancy, based on its thymocyte pro-growth and anti-differentiation properties. Indeed, multiple cases of secondary T-ALL following differentiation therapy using RA for acute promyelocytic leukaemia have been reported (Bee *et al*, 2004; Szotkowski *et al*, 2009; Maschan *et al*, 2011). Further studies are required to determine the extent to which aberrant retinoid signalling contributes to the T-ALL phenotype and whether deregulated *ALDH1A* gene expression is sufficient to initiate T cell leukaemogenesis.

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Authorship contributions

WKG and URK were the principal investigators and WKG takes primary responsibility for the paper; BL and MW performed the laboratory work for this study; DA and BL performed the bioinformatics and statistical analyses; WKG and URK co-ordinated the research and wrote the paper.

Conflict of interest

The authors report no potential conflicts of interest.

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Table 1. Percentage of primary T-ALL specimens (*n* = 100) expressing retinoic acid pathway gene transcripts.

Gene	% Specimens
ALDH1A1	13
ALDH1A2	63
ALDH1A3	3
Any *ALDH1A* gene[a]	72
RARA	100
RARB	100
RARG	100
RXRA	100
RXRB	100
RXRG	7

[a] ALDH1A1, ALDH1A2 or ALDH1A3.

Table 2. Genes most highly correlated with *ALDH1A2* in T-ALL tumours.

Gene	r^2	Gene function
(A) Positively correlated		
NUDT11	0·766	Phosphohydrolase/signal transduction
TMSB15A	0·761	Cytoskeleton organisation
RPL39L	0·700	Ribosomal protein
BEX2	0·694	Apoptosis/G1 cell cycle regulator/interacts with LMO2
CDK5RAP3	0·693	CDK5 regulatory subunit associated
LCK	0·672	Protein tyrosine kinase/TCR signalling from CD4 and CD8
IDH2	0·666	Oxidative respiration/energy production
METRN	0·656	Neuronal growth factor
RASGRP1	0·645	Ras signalling
CXADR	0·645	Cell adhesion
(B) Negatively correlated		
PLXND1	−0·751	Cell adhesion/intrathymic cell migration
CYSLTR1	−0·746	Leucotriene receptor
PLXNC1	−0·735	Cell adhesion
TNFRSF10B	−0·722	Death receptor/apoptosis
HSH2D	−0·713	Negative regulator of T cell activation
GALNT1	−0·707	Mucin-type O-glycosylation
KLF3	−0·706	Transcriptional regulator/repressor
LAT2	−0·694	Negative regulator of signalling
TNFSF10	−0·686	Apoptosis inducer/binds TNFRSF10B death receptor
DEPTOR	−0·685	Inhibitor of mTOR Signalling
PLXNB2	−0·683	Cell adhesion
UBR5	−0·677	Ubiquitin ligase/genome stability
GAB3	−0·668	Signal transduction
PKIG	−0·668	Protein kinase A inhibitor
PTPN12	−0·651	Tyrosine phosphatase
SOCS2	−0·633	Negative regulator of cytokine signalling
RGS1	−0·631	Negative regulator of G protein signalling

Figure 1. *ALDH1A* Gene Expression is a Common and Specific Feature of T-ALL. (A) Expression levels of *ALDH1A* genes and *RARA* compared to T-lineage acute lymphoblastic leukaemia (T-ALL) oncogenes *TLX1* and *TAL1* in 100 primary T-ALL specimens. Absolute expression levels (log_2) determined by microarray are shown by colour intensity with low levels in green, intermediate levels in black and high levels in red. Each column represents one tumour specimen. (B) Mean expression level (±standard error) of *ALDH1A2* in 100 T-ALL and 55 B-precursor (pre-B) ALL specimens as measured by microarray.

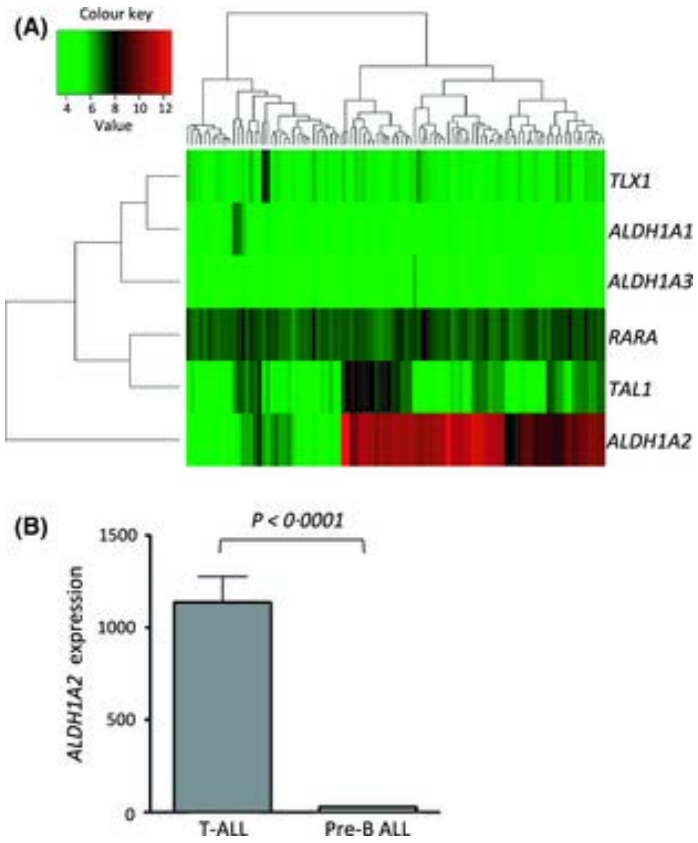

Figure 2. *ALDH1A* mRNA Expression Levels and Enzymatic Activity Correlate in T-ALL Cell Lines. (A) Expression levels of *ALDH1A* genes and *RARA*compared to T-ALL oncogenes *TLX1* and *TAL1* as determined by microarray in four T-ALL (PER-117, PER-255, PER-604, CEM) and two B lineage ALL (PER-377, PER-495) cell lines. (B) ALDH1A enzymatic activity measured by Aldefluor assay. The means and standard errors (SEM) of three independent experiments are shown.

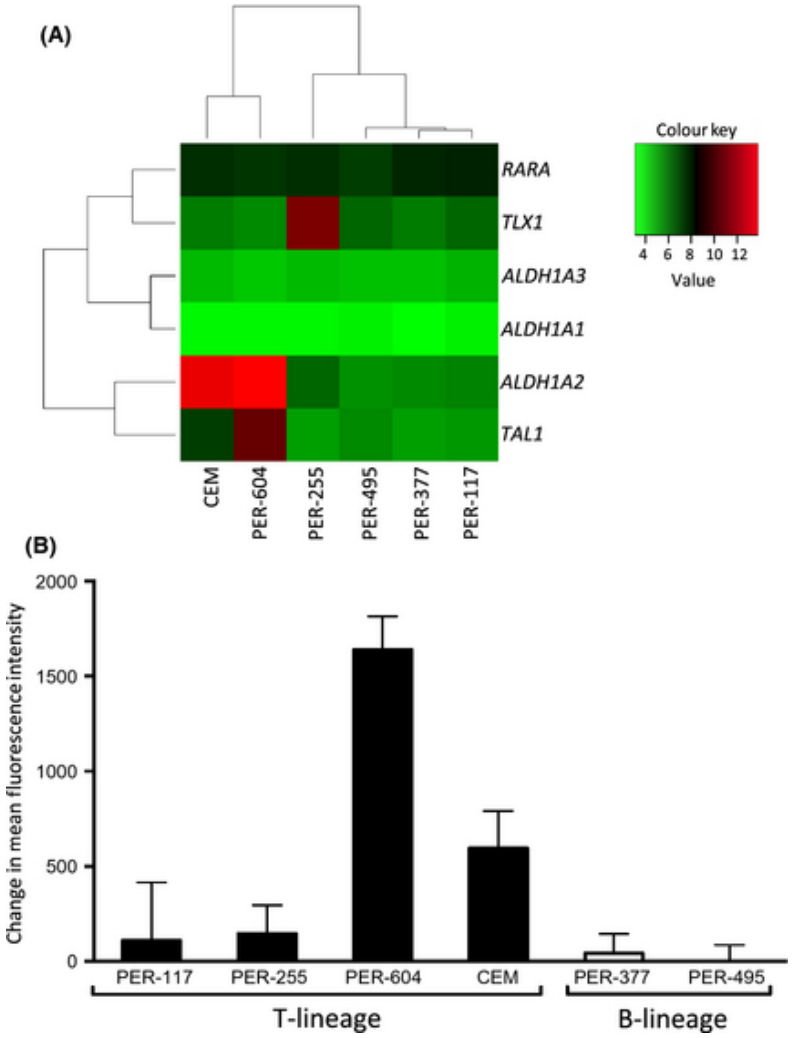

Figure 3. T-ALL Proliferation is Sensitive to Modulation of ALDH1A and RAR Activity. Sensitivity of human ALL cell lines to the ALDH1A inhibitor citral (3,7-dimethyl-2,6-octadienal) alone or in combination with the RARA selective inhibitor Ro 41-5253 (4-[(E)-2-(7-heptoxy-4,4-dimethyl-1,1-dioxo-2,3- dihydrothiochromen-6-yl) prop-1-enyl] benzoic acid) or pan-RAR agonist TTNPB ((E)-4-[2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2- naphthylenyl)-1 -propenyl] benzoic acid) following treatment for 48 h. IC25 or SC25 concentrations are shown. (A) Citral. (B) Citral (75 μmol/l) plus Ro 41-5253. (C) Citral (75 μmol/l; to inhibit endogenous retinoic acid production) plus TTNPB. An SC25 determination was unable to be achieved for the B-lineage cell lines within the tested range. Data represent the mean of three independent experiments performed in triplicate. Error bars show SEM.

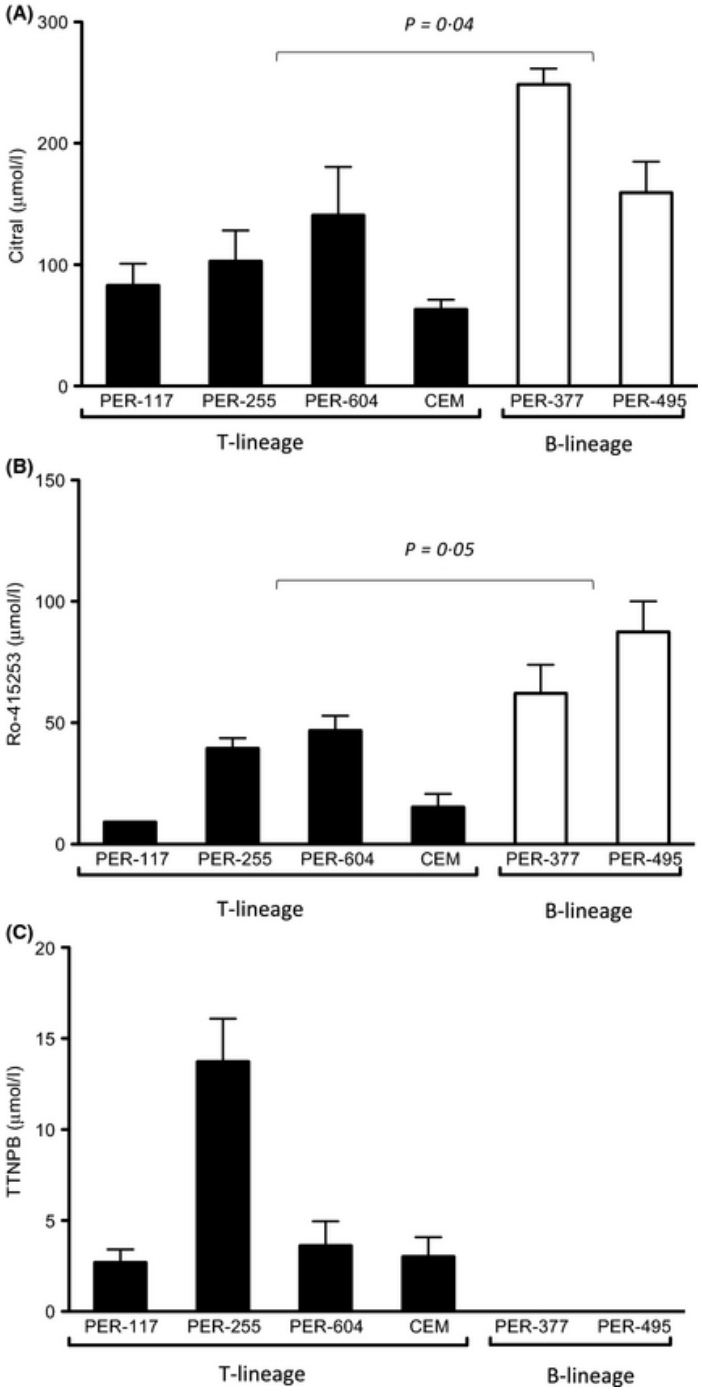

Figure 4. Functional Characterisation of the 200 Top-Ranked Probe Sets Correlating with *ALDH1A2* Expression in T-ALL. Shown are the main Gene Ontology (GO) biological process categories and the number of probe sets correlated with *ALDH1A2* expression.

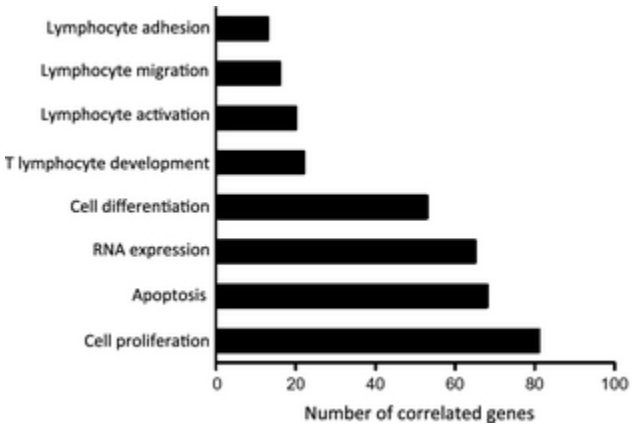

Figure 5. Modulation of Gene Expression in T-ALL Following ALDH1A Enzyme Inhibition. T-ALL cell lines were treated in duplicate with 100 μmol/l citral (3,7-dimethyl-2,6-octadienal) for 16 h followed by real-time reverse transcription polymerase chain reactionanalysis for *ALDH1A1*, *ALDH1A2* and*TNFRSF10B* gene expression. Each amplification reaction was conducted in triplicate. The change in mRNA levels in citral-treated cells compared with those treated with vehicle control are shown. Only significant *P*-values are displayed. Error bars show standard error of the mean.