A DNA-binding mutant of TAL1 cooperates with LMO2 to cause T cell leukemia in mice

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Abstract

The most common translocation in childhood T-cell acute lymphoblastic leukemia (T-ALL) involves the *LMO2* locus, resulting in ectopic expression of the *LMO2* gene in human thymocytes. The *LMO2* gene was also activated in patients with X-linked Severe Combined Immune Deficiency treated with gene therapy because of retroviral insertion in the *LMO2* locus. The *LMO2* insertions predisposed these children to T-ALL, yet how LMO2 contributes to T cell transformation remains unclear. The LIM (Lin 11, Isl-1, Mec-3) domain containing LMO2 protein regulates erythropoiesis as part of a large transcriptional complex consisting of LMO2, TAL1, E47, GATA1 and LDB1 that recognizes bipartite E-box-GATA1 sites on target genes. Similarly, a TAL1/E47/LMO2/LDB1 complex is observed in human T-ALL and Tal1 and Lmo2 expression in mice results in disease acceleration. To address the mechanism(s) of Tal1/Lmo2 synergy in leukemia, we generated *Lmo2* transgenic mice and mated them with mice that express wild-type Tal1 or a DNA-binding mutant of TAL1. *Tal1/Lmo2* and *MutTAL1/Lmo2* bitransgenic mice exhibit perturbations in thymocyte development due to reduced E47/HEB transcriptional activity and develop leukemia with identical kinetics. These data demonstrate that the DNA-binding activity of Tal1 is not required to cooperate with Lmo2 to cause leukemia in mice and suggest that Lmo2 may cooperate with Tal1 to interfere with E47/HEB function(s).

Introduction

LMO2 was first implicated in leukemogenesis in 1992 when found associated with a t(11;14)(p13;q11) and t(7;11)(q34;p13) chromosomal translocations (Boehm et al., 1990; Garcia *et al.*, 1991). The LMO1 and LMO2 proteins are found expressed in human T-cell acute lymphoblastic leukemia (T-ALL) and LMO proteins are co-expressed in approximately 80% of TAL1 + human T-ALL patients (Rabbitts, 1994; Wadman *et al.*, 1994; Ferrando *et al.*, 2002). Interest in LMO2-mediated leukemogenesis was reignited in 2002, when children in two X-linked Severe Combined Immune Deficiency gene therapy trials developed T-ALL because of retroviral insertion in the LMO2 locus (Hacein-Bey-

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Conflict of interest
The authors declare no conflict of interest.

Abina *et al.*, 2003a, b). Two leukemic patients exhibited evidence of TAL1 activation and three patients developed NOTCH1 mutations (Hacein-Bey-Abina *et al.*, 2008; Howe *et al.*, 2008). Studies in mouse T-ALL models revealed that Tal1 and Lmo proteins cooperate to cause leukemia in mice (Wadman *et al.*, 1994; Larson *et al.*, 1996; Aplan *et al.*, 1997), but precisely how LMO proteins contribute to leukemogenesis remains unclear.

LMO2 is part of the 'LIM only' family of proteins that are thought to serve as bridging factors in transcriptional complexes (Wadman *et al.*, 1997). LIM-domains are cysteine-rich zinc-binding domains that are structurally similar to DNA-binding GATA finger domains; however, currently there is no evidence to suggest that LMO proteins bind DNA. LMOs are thought to mediate protein:protein interactions through their LIM domains and evidence suggests that LMOs act as transcriptional co-regulators. There are four human LMO proteins (LMO1-4) and all four LMO proteins have been associated with oncogenesis (Fisch *et al.*, 1992; Visvader *et al.*, 2001; Aoyama *et al.*, 2005).

LMO2 is a nuclear protein that in erythroid cells forms a multi-protein complex, which includes TAL1, E47, GATA-1 and LDB1 (Valge-Archer *et al.*, 1994). These proteins form a functional transcriptional complex and recognize a bipartite DNA sequence consisting of an E-box and a GATA site separated by one helix turn (Wadman *et al.*, 1997). The Tal1/E47/Lmo2/Ldb1/GATA-1 complex regulates the expression of genes important in erythroid or megakaryocytic differentiation, including *p4.2*, glycophorin A, *c-kit*, p21^{CIP} and the transcription factors *Gfi-1b* and *eklf* (Krosl *et al.*, 1998; Lecuyer *et al.*, 2002; Xu *et al.*, 2003; Lahlil *et al.*, 2004; Goardon *et al.*, 2006). Lmo2 is thought to bridge the TAL1/E47 heterodimer to GATA-1, and by mechanisms not fully understood, enhance transcription (Xu *et al.*, 2003). Based on their roles in regulating gene expression during hematopoiesis, Tal1 and Lmo2 have been thought to contribute to leukemia by transactivating the expression of genes such as retinaldehyde dehydrogenase, the transmembrane protein TALLA1, the receptor tyrosine phosphatase IA2, cyclin D2, heat shock cognate 73 and a bub-like gene (Ono *et al.*, 1997; Davenport *et al.*, 2000).

An alternative model posits that in leukemic cells the Tal1/Lmo2 complex interferes with the transcriptional activity of E47/HEB, by sequestering E47 and HEB proteins and/or by recruiting co-repressors to E-box regulated loci (Herblot *et al.*, 2000; O'Neil *et al.*, 2004). The E47/HEB complex directs the expression of several genes essential for proper lymphoid development including the preTα chain of the pre-T cell receptor (pre-TCR), the Rag1 and Rag2 recombinases, CD3, CD4, CD5 and the T cell receptor α/β genes (Greenbaum and Zhuang, 2002). A HEB or E47 deficiency disrupts thymocyte development and E47-deficient mice are predisposed to the development of T cell leukemia (Bain *et al.*, 1997; Yan *et al.*, 1997). Our published work provides genetic evidence to support the inhibition model of TAL1-mediated leukemogenesis. We demonstrate that a DNA-binding mutant form of TAL1 (R188G;R189G) (referred to here as mutTAL1) is able to induce T-ALL-like disease in mice and T cell leukemogenesis is accelerated when Tal1 is expressed on an E2A or HEB heterozygous background (O'Neil *et al.*, 2001, 2004). Moreover, the functions of Tal1 in early hematopoietic development also do not appear to require the DNA-binding properties of Tal1 (Schlaeger *et al.*, 2004). More recently, the ETO-2 repressor has been identified as a novel component of the TAL1/LMO2 complex in erythroid cells (Goardon *et al.*, 2006). Thus, Tal1/E47 or HEB/Lmo2 complexes may also actively repress the expression of E-box containing genes during normal erythroid differentiation and in leukemia.

To determine the molecular basis of the observed Tal1/Lmo2 synergy and to distinguish between the transcriptional activation and inhibition models, we asked whether the DNA-binding properties of Tal1 were required to collaborate with Lmo2 to cause T cell leukemia in mice. To test this model, we generated bitransgenic mice co-expressing Lmo2 with wild-

type Tal1 or with a DNA-binding TAL1 mutant. We observed disease acceleration in both the *Tal1/Lmo2* and *mutTAL1/Lmo2* transgenic mouse lines, indicating that the DNA-binding properties of TAL1 are not required to cooperate with Lmo2 to cause disease in mice. Moreover, thymocyte development was inhibited in pre-leukemic *Tal1/Lmo2* and *mutTAL1/Lmo2* mice because of reduced expression of E47/HEB-regulated genes important for thymocyte differentiation. These data indicate that Lmo2 may accelerate Tal1-mediated leukemogenesis by enhancing the sequestration and/or inhibition of the E47/HEB heterodimer.

Results and discussion

Generation of transgenic mice expressing a DNA-binding mutant of TAL1 and Lmo2

To determine how Lmo2 contributes to TAL1-mediated leukemogenesis and to understand the basis for the TAL1 and LMO oncogene cooperativity, we asked whether the DNA-binding properties of TAL1 were required to cooperate with Lmo2 to cause disease in mice. To express both oncogenes in developing mouse thymocytes, we generated transgenic mouse lines where the mouse Lmo2 complementary DNA was expressed under the control of the proximal *lck* promoter (Figure 1a). The 3′ untranslated regions of this construct contain introns, exons and the poly A addition site of the human growth hormone gene (Abraham *et al.*, 1991). The prox *lck Lmo2* construct was microinjected into the pronuclei of fertilized FVB/N oocytes. A total of 16 founder mice were identified initially by Southern blot analysis and two founder lines (Fo5 and 19) with variable expression levels were expanded for further study (Figure 1b).

The Lmo2 transgenic mouse lines were mated with transgenic mice expressing wild-type Tal1 or a DNA-binding mutant of TAL1. The DNA-binding mutant mice express a form of TAL1 where the contact residues of the basic DNA-binding domain, arginine 188 and 189 have been mutated to glycines (designated R188G;R189G) (Hsu *et al.*, 1994). This mutant form of TAL1 heterodimerizes with E47 or HEB proteins but fails to stably bind E-box binding sequences *in vitro* and *in vivo* (Hsu *et al.*, 1994; O'Neil *et al.*, 2001). Like Tal1 transgenic mice, expression of a TAL1 DNA-binding mutant perturbs thymocyte development and approximately half of the mice develop a T-ALL-like disease (O'Neil *et al.*, 2001).

To determine whether the DNA-binding activities of TAL1 were required to cooperate with Lmo2 to cause disease in mice, we generated a cohort of 15 *Tal1/Lmo2* and 11 *mutTAL1/Lmo2* bitransgenic mice and monitored the animals for development of disease. The *Lck-Lmo2* transgenic lines developed disease following a long latency and cooperated with Tal1 and the DNA-binding mutant to accelerate leukemogenesis in mice (P<0.0001) (Figure 1c). Surprisingly, *Tal1/Lmo2* and *mutTAL1/Lmo2* transgenic mouse lines developed disease with nearly identical kinetics (Figure 1c), in spite of the fact that the DNA-binding mutant *TAL1* transgenic mice develop disease more rapidly than wild-type *Tal1* transgenic mice (O'Neil *et al.*, 2001). Approximately 30% of *Tal1* transgenic mice develop leukemia after a long latency, whereas *mutTAL1* mice develop disease with a median survival of 215 days (Kelliher *et al.*, 1996; O'Neil *et al.*, 2001). In contrast, when Lmo2 is co-expressed with Tal1 or mutTAL1, 100% of the mice develop T-ALL-like disease with a median survival of approximately 100 days. No significant difference was observed when *Tal1/Lmo2* and *mutTAL1/Lmo2* survival curves were compared (P<0.581). Histopathologic examination of tumors isolated from the bitransgenic mice revealed a range of tumor immunophenotypes (Table 1), with tumors predominantly containing DN and DP or CD8SP cells observed in both *Tal1/Lmo2* and *mutTAL1/Lmo2* mice (Table 1). This study reveals that Tal1/Lmo2 synergy does not require the DNA-binding properties of TAL1, suggesting that Lmo2 does

not contribute to leukemia by enhancing or altering the transcriptional activity of the Tal1/ E47 or HEB heterodimer.

Thymocyte developmental arrest in Tal1/Lmo2 and mutTAL1/Lmo2 mice

We have shown that thymic expression of Tal1 or the DNA-binding mutant results in a decrease in overall thymocyte cellularity because of fewer DP and CD8-positive thymocytes (Kelliher *et al.*, 1996; O'Neil *et al.*, 2001). Expression of Lmo2 under the control of the proximal *Lck* promoter appeared to have no detectable effects on DP thymocyte development (Figure 2b), however, Lmo2 expression decreased thymocyte cellularity and skewed DN development; although no increase in the absolute numbers of DN thymocytes was observed (Figures 2a and c). Consistent with published reports (Larson *et al.*, 1996), co-expression of Tal1 and Lmo2 reduces thymic cellularity and blocks DP thymocyte development, resulting in increases in the relative and absolute number of DN thymocyte precursors in preleukemic *Tal1/Lmo2* mice (Figures 2b and d). DP thymocyte development was consistently more severely affected in *Tal1/Lmo2* mice derived from Lmo2 founder Fo19 compared with Fo5 (Figure 2b).

To determine whether the thymocyte developmental block reflects alterations in Tal1-mediated gene expression, we examined thymocyte development in *mutTAL1/ Lmo2* pre-leukemic mice. We found thymocyte cellularity futher reduced in *mutTAL1/Lmo2* mice compared with mice that express the TAL1 DNA-binding mutant only (Figures 2a). Overall cellularity was similarly reduced in all the *Tal1/Lmo2* and *mutTAL1/Lmo2* mice examined. Similar to the *Tal1/Lmo2* mice, DP thymocyte development was consistently more severely affected in *mutTAL1/Lmo2* mice derived from Fo19 compared with founder Fo5 (Figure 2b). Importantly, the DNA-binding TAL1 mutant cooperated with Lmo2 to induce similar effects on thymocyte development as wild-type TAL1 (Figure 2).

Expression of Lmo2 with Tal1 or mutTAL1 also altered DN thymocyte development, resulting in arrest at the DN3-DN4 precursor stage (Figure 2c and d). In contrast, an E2A or HEB deficiency results in an arrest of DN thymocyte development at the DN1 and DN3 stages, respectively (Bain *et al.*, 1997; Greenbaum and Zhuang, 2002). These studies demonstrate that Lmo2 does not require the DNA-binding properties of TAL1 to perturb thymocyte development. The block in thymocyte development induced by either form of Tal1 is not complete, as DP and SP thymocytes are detected (Figure 2c). Moreover, immunophenotyping of *Tal1/Lmo2* and *mutTAL1/ Lmo2* tumors revealed that tumors from both bitransgenic lines were often heterogeneous, consisting of DN and DP and/or CD8 SP cells (Table 1). For example, although the primary *Tal1/Lmo2* tumor 5338 is clonal, it contains DN, DP and CD8-SP cells, suggesting that differentiation proceeds at some level after leukemic transformation.

The expression of E47/HEB-regulated genes is decreased in both Tal1/Lmo2 and mutTAL1/ Lmo2 mice

To understand how Lmo2 can cooperate with a DNA-binding mutant of TAL1 and interfere with thymocyte development, we examined the expression of E47/HEB target genes known to regulate thymocyte differentiation. Previously, we found the expression of PreTa, Rag1/2, CD3 CD4, T cell receptor α/β and CD5 repressed in the presence of the Tal1 oncogene (O'Neil *et al.*, 2004). The expression of these thymocyte differentiation genes was further decreased in *Tal1/E2A* +/− or *HEB* +/− mice, indicating that Tal1 alters thymocyte development by interfering with the transcriptional activities of the E47/HEB heterodimer (O'Neil *et al.*, 2004). Using real-time PCR, we quantified the expression of the immunoglobulin and T cell receptor gene recombinases Rag1 and Rag2 and the pre-Tα chain of the pre-TCR. We found the expression of Rag1 and Rag2 significantly decreased in

Tal1 and *mutTAL1* preleukemic thymocytes (Figure 3). Slight decrease in Rag1/2 expression were also detected in the preleukemic *Lck-Lmo2* transgenic thymus, although no gross perturbations in the thymocyte developmental profile were observed. Co-expression of Tal1 or its DNA-binding mutant with Lmo2 resulted in consistent further decreases in Rag1 and Rag2 expression (Figure 3). Similarly, we found preTα expression further repressed in preleukemic *Tal1/Lmo2* or *mutTAL1/Lmo2* thymocytes than in thymocytes expressing Tal1, mutTAL1 or Lmo2 alone (data not shown). The reduced expression of these E47/HEB-regulated genes does not reflect changes in the relative percentage of DN versus DP stage thymocytes, as significant decrease in Rag1/2 and PreTα gene expression were observed in *Tal1* and *mutTAL1* animals, where the thymocyte developmental profile is only modestly altered (Figure 2b). These data reveal that in the presence of Tal1 and importantly, a DNA-binding mutant of TAL1, Lmo2 expression results in further reductions in E47/HEB transcriptional activity. These findings suggest that Lmo2 binding to Tal1 or mutTAL1/E47 or HEB heterodimers may stabilize the Tal1/E47 or HEB complex, resulting in more effective E47/HEB sequestration.

The TAL1 DNA-binding mutant is impaired in its ability to repress CD4 expression

Although we have demonstrated that mouse leukemic cells expressing a TAL1 DNA-binding mutant fail to bind E-box containing oligonucleotides (O'Neil *et al.*, 2001), whether the TAL1 DNA-binding mutant binds native promoters or enhancers remains untested. Moreover, whether the DNA-binding mutant is recruited to regulatory regions through its incorporation into a mutTal1-E47(HEB)-Lmo2-Ldb1-Gata3 transcriptional complex is also unclear. We have shown that Tal1 actively represses CD4 expression in preleukemic mouse thymocytes by recruiting the msin3a corepressor (O'Neil *et al.*, 2004). To determine whether mutant TAL1 binds the CD4 regulatory region and influences CD4 expression, we quantified CD4 messenger RNA levels in leukemic cell lines isolated from *Tal1/Lmo2* and *mutTAL1/Lmo2* mice. We found CD4 messenger RNA levels repressed on average 39-fold in *Tal1/Lmo2* leukemic cells compared with those expressing the DNA-binding mutant (Figure 4a). These data suggest that the DNA-binding mutant may be impaired in its ability to stably bind E-box sequences and influence CD4 expression. Using chromatin immunoprecipitation, we analyzed Tal1 and mutTAL1 binding to the CD4 enhancer. Tal1, Heb and msin3a recruitment to the CD4 enhancer was observed in the *Tal1/Lmo2* leukemic cell lines. Although Heb binding to the CD4 enhancer was detected in the *mutTAL1/Lmo2* leukemic cells, decreased mutTAL1 binding was observed with both human TAL1 antibodies used for immunoprecipitation (Figure 4b). The decrease in mutTAL1 binding was accompanied by a reduction in msin3a recruitment, consistent with published studies demonstrating that Tal1 directly binds msin3a (Huang and Brandt, 2000). Thus, Tal1 and mutTAL1 bind and sequester E47 and HEB proteins, whereas active CD4 repression requires the DNA-binding properties of Tal1.

Collectively, these studies suggest that Lmo2 binding to Tal1 stabilizes the Tal1/E47 or HEB interaction. Consistent with this idea, the binding affinities for the Tal1/E47 heterodimer have been measured *in vitro* and shown to significantly increase in the presence of Lmo2 (KA of $\sim4 \times 10^7$ versus KA of $\sim1 \times 10^8$) (Ryan *et al.*, 2008). Therefore, Lmo2 binding appears to increase the affinity of Tal1 for E47 or HEB and this interaction is greater than the affinity of Lmo2 for its LIM domain binding partner, Ldb1 (KA of 5×10^7) (Ryan *et al.*, 2008). These estimates predict that E protein sequestration may be favored in a setting where Tal1 and Lmo2 are both expressed.

Our findings have therapeutic implications and suggest that interfering with Tal1/E47 or Tal1/Lmo2 binding may increase E47/HEB levels and release Tal1/E47 hetero-dimers from GATA3 or other transcriptional complexes. Disruption of these complexes may stimulate leukemic cell differentiation and/or induce apoptosis of leukemic cells. However, disrupting

the Tal1/E47 or HEB protein interface with small molecules may not be feasible. In some instances, only a minor part of the protein dimer interfaces contribute to the affinity between proteins (Clackson and Wells, 1995; Kussie *et al.*, 1996; Wells, 1996; Wells and de Vos, 1996). Targeting these regions may be sufficient to inhibit protein:protein interactions. Small molecules have in fact been identified that interfere with the Myc/Max bHLH/LZ interaction and these molecules have been shown to interfere with c-Myc-induced transformation *in vitro* (Berg *et al*, 2002). Alternatively, interfering with Lmo2 binding to Tal1 may be sufficient to induce apoptosis of T-ALL cells. Retroviral expression of an anti-Lmo2 single chain Fv antibody fragment or an Lmo2 aptamer have been shown to reduce leukemic growth in transplanted mice (Nam *et al*, 2008), suggesting that interfering with LMO2 protein interactions may be an effective therapeutic strategy for some relapsed T-ALL patients.

Materials and methods

Generation of transgenic mice

Murine *Lmo2* complementary DNA was cloned into p1017, a plasmid containing the proximal *lck* promoter and the human growth hormone splice and poly adenylation addition sites. The plasmid DNA was sequenced and digested with SpeI for microinjection into the FVB/N pronuclei. Transgenic founders were identified by Southern blotting and mated with FVB/N mice. Proximal *lck-Tal1* and *mutTAL1* transgenic lines have been previously described (Kelliher *et al.*, 1996; O'Neil *et al.*, 2001).

Cell culture and flow cytometry

Mouse T-ALL tumors were minced into a single-cell suspension using frosted slides and cultured in RPMI with 10% fetal bovine serum, l% glutamine, penicillin/streptomycin and 50 mM b-mercaptoethanol at 37°C under 5% CO_2. Media was replaced once/week and once the culture appeared confluent, cells were expanded to generate cell lines. Cytospin preparations of the cultures were stained with Wright/Giemsa to confirm their lymphoblastoid morphology. Mouse leukemic cell lines were immunophenotyped by staining with antibodies to hematopoietic lineage markers as described below. Thymi from preleukemic mice were gently disrupted with frosted glass slides in order to produce single cell suspensions. The thymocytes were washed with PBS and stained with fluorescent-labeled antibodies and subjected to flow cytometry at the fluorescence-activated cell sorter facility at the University of Massachusetts Medical Center. For double negative analysis, cells were stained with antibodies for the lineage antibodies, and the lineage-negative cells were stained with CD44-APC and CD25-Fitc. Antibodies used in flow cytometry included CD3-PE, CD4-PE, CD4-Cy5-PE, CD8-FITC, CD8-Cy5-PE, CD25-Fitc, CD44-APC and lineage markers (BD Pharmingen, San Diego, CA, USA). Data were analyzed using FlowJo software (Treestar, Inc., Ashland, OR, USA). For tumor immunophenotyping, primary tumors cells were stained with either PE or FITC-conjugated anti-mouse Ly-2 (CD8), L3T4 (CD4), CD25 and CD3 antibodies (BD Pharmingen), and analyzed by flow cytometry.

Quantitative PCR

RNA was extracted from primary thymocytes using Trizol. complementary DNA was synthesized using the Superscript firststrand synthesis system (Invitrogen, Carlsbad, CA, USA). *Rag1*, *Rag2* and *pre-Tα* expression was assayed using primers as described in references (Hsu *et al.*, 2003; Huang *et al.*, 2003). To determine target-gene expression levels, complementary DNA was serially diluted and quantified using the SYBR green kit (Qiagen, Valencia, CA, USA) gene-specific primers and *β-actin*specific primers using relative quantification analysis. The copy number for the gene of interest was normalized to the copy number for *β-actin*.

Chromatin immunoprecipitation

To determine whether mutTAL1 bound the regulatory regions of the mouse CD4 gene, chromatin immunoprecipitation was performed essentially as described in (Sharma *et al.*, 2006). The following antibodies were used for immunoprecipitation: mouse Tal1 (generously provided by Richard Baer, Columbia University), human mutTAL1 (Millipore BTL73), TAL1 (Santa Cruz C-21, Santa Cruz, CA, USA), msin3a (Santa Cruz K-20), and HEB (Santa Cruz A-20). Rabbit IgG (Santa Cruz) was used as the negative control. The following primer sets were used to amplify the regulatory regions of the CD4 gene: Forward —5′-TTCTGAGCCCACC CTAAGATG-3′ and Reverse—5′-GTCTTTTTTCCAGAGC CCC-3′.

Acknowledgments

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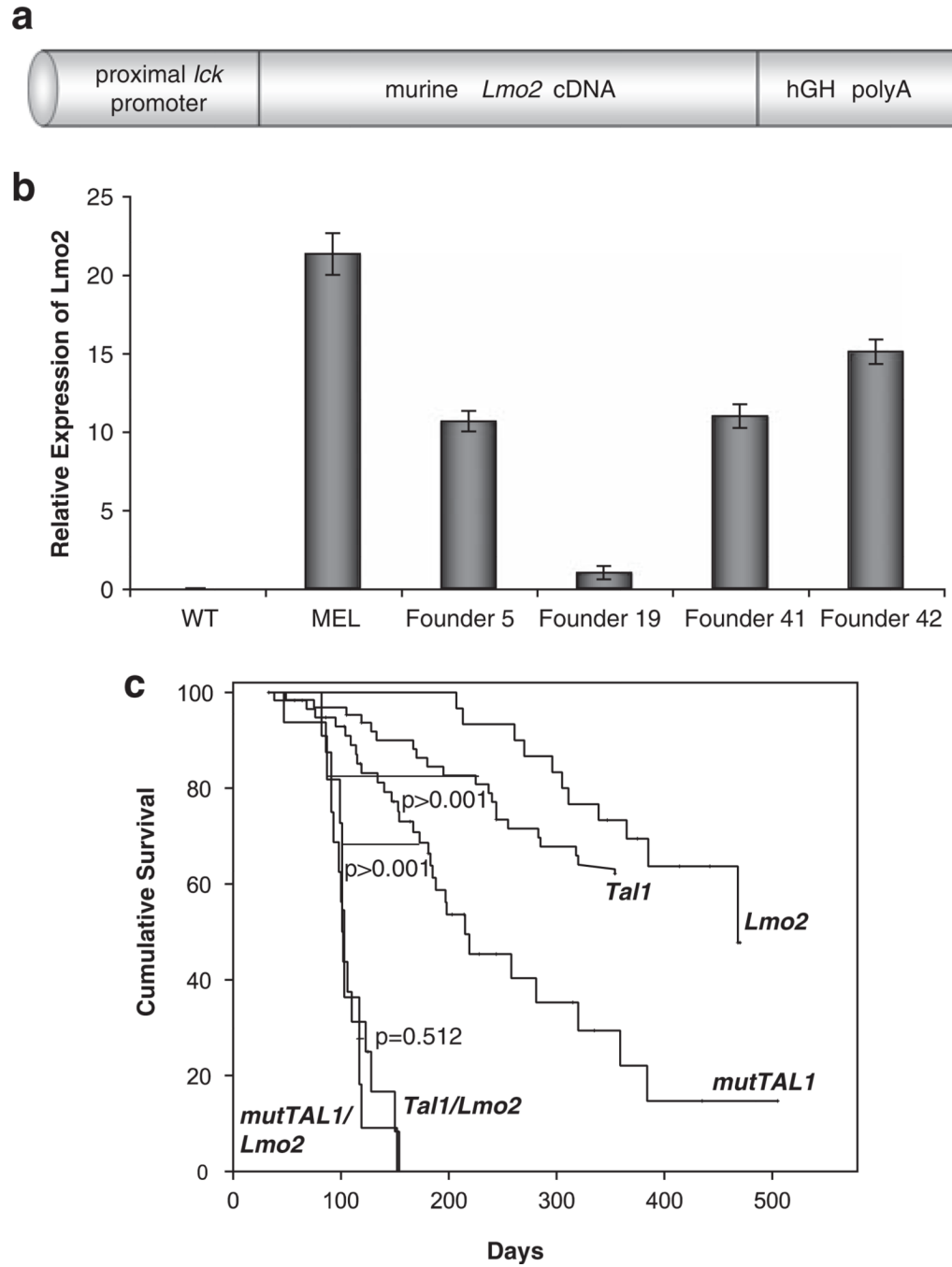
a

b

c

Figure 1.
The DNA-binding properties of TAL1 are not required to cooperate with Lmo2 to cause leukemia in mice. (**a**) Schematic representation of the prox *lck/Lmo2* transgene. The murine *Lmo2* cDNA is expressed under control of the proximal *lck* promoter and human growth hormone (hGH) splice and poly(A)+ addition sequences. (**b**) Expression of the *Lmo2* transgene. RNA was isolated from a murine erythroleukemic cell line (MEL) and from thymocytes isolated from wild-type (wt) or *Lmo2* founder mice designated F5, F19, F41 and F42. Lmo2 mRNA levels were determine using quantitative PCR with β-actin serving as an internal control. (**c**) Survival curves for the *Tal1, mutTAL1, Lmo2, Tal1/Lmo2 and*

mutTAL1/Lmo2 mice. Mice were monitored daily for evidence of disease, upon which the mice were sacrificed and a post-mortem examination performed. The cohort of *Tal1/Lmo2* mice consisted of 15 animals and the cohort of *mutTAL1/Lmo2* mice consisted of 11 animals. The *Lck-Lmo2* only cohort consisted of 26 mice. *Tal1* and *mutTAL1* survival curves have been published previously (Kelliher *et al.*, 1996; O'Neil *et al.*, 2001).
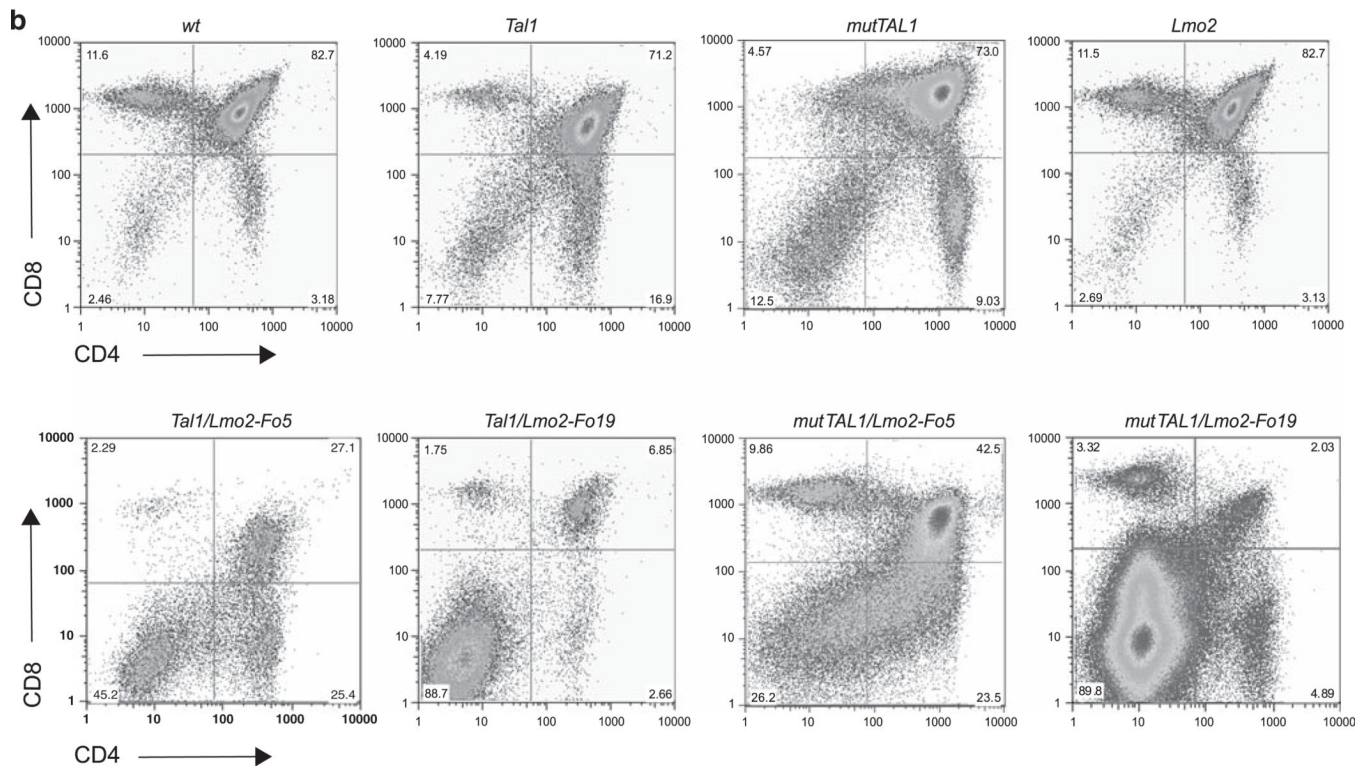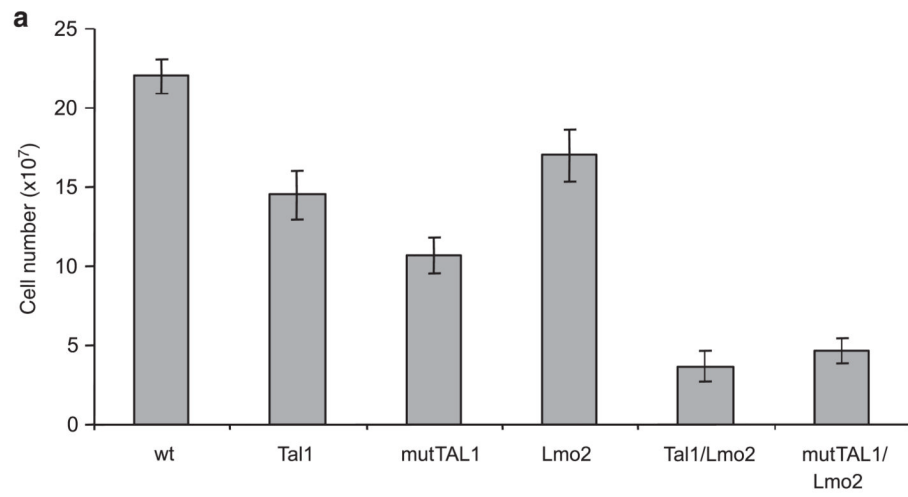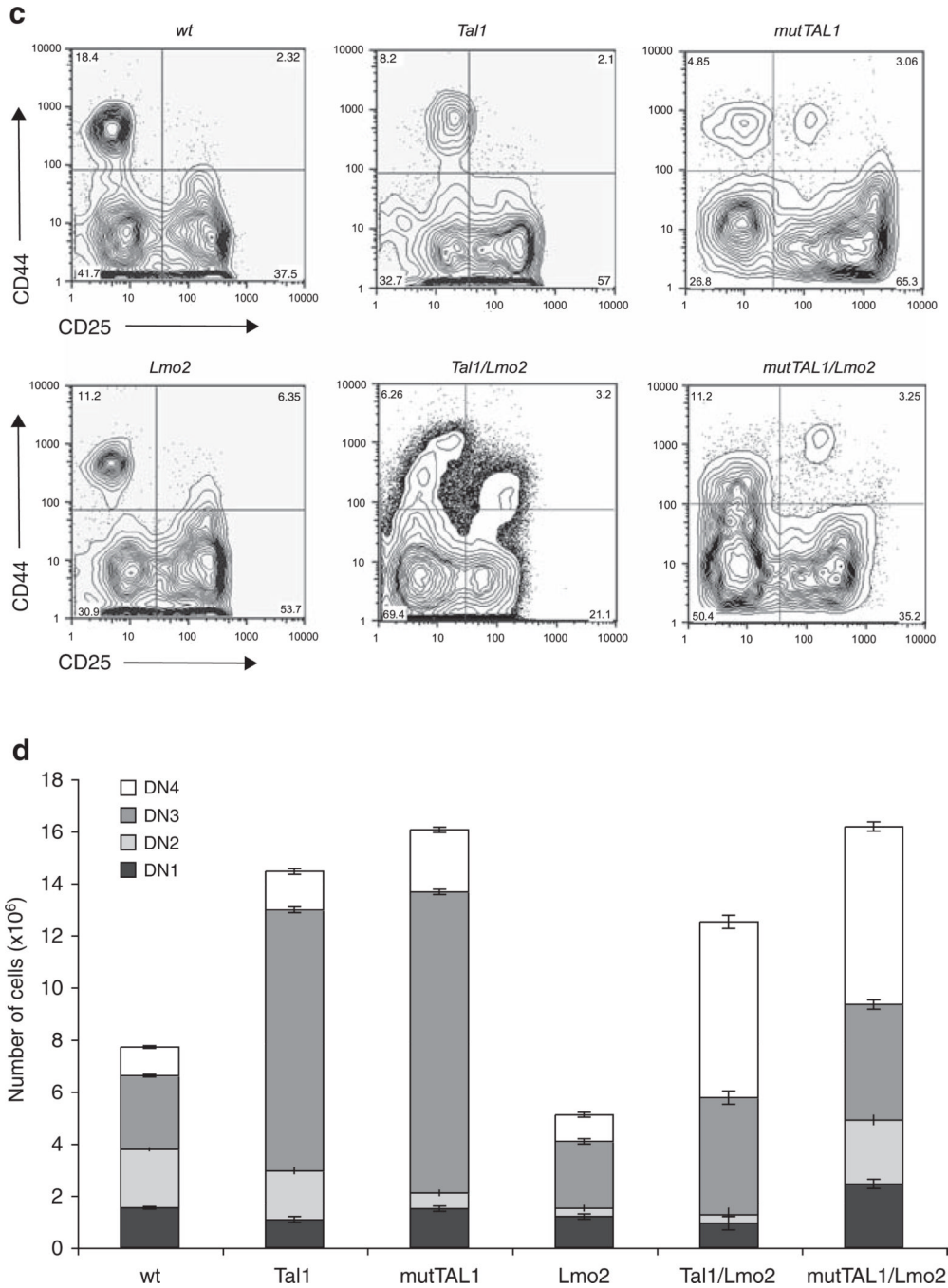
Figure 2.
A DNA-binding mutant of TAL1 cooperates with Lmo2 to perturb thymocyte development.
(a) Significant decreases in thymic cellularity are observed in *Tal1/Lmo2* and *mutTAL1/ Lmo2* bitransgenic mice. Total thymocyte numbers from wild-type, *Tal1, mutTAL1, Lmo2, Tal1/Lmo2* and *mutTAL1/Lmo2* transgenic mice. When compared with wild type, Lmo2 expression causes a decrease in thymic cellularity ($P = 8.0 \times 10^{-6}$) that is exacerbated when either *Tal1* or *mut-TAL1* is co-expressed ($P = 7.8 \times 10^{-15}$ and $P = 3.1 \times 10^{-6}$, respectively).
(b) Co-expression of *Lmo2* with either *Tal1* or *mutTAL1* perturbs thymocyte development. Thymocytes from 4-week old, preleukemic mice were stained with CD4-PE and CD8-FITC and analyzed by flow cytometry. A total of 14 *Lmo2*, 19 *Tal1/Lmo2* and 13 *mutTAL1/*

Lmo2 preleukemic mice were analyzed. Representative profiles are shown. (c) DN thymocyte development is altered when Lmo2 is co-expressed with either Tal1 or mutTAL1. Thymocytes from 4-week old mice were stained with antibodies for the lineage markers (CD4, CD8, CD3, B220, Mac1, Gr1 and Terr119) and the lineage-negative cells were isolated and stained with CD44-APC and CD25-FITC. A total of 14 *Lmo2*, 19 *Tal1/Lmo2* and 13 *mutTAL1/Lmo2* preleukemic mice were examined. Representative profiles are shown. (d) Increase in the absolute numbers of DN3 and DN4 thymic progenitors in preleukemic *Tal1* and *mutTAL1* transgenic mice. Thymocytes from wild-type ($n = 12$), *Tal1* ($n = 7$), *MutTAL1* ($n = 8$), *Lmo2* ($n = 15$), *Tal1/Lmo2* ($n = 11$) and *mutTAL1/Lmo2* ($n = 12$) mice were stained with CD4 and CD8 and lineage negative cells were stained with CD44 and CD25. Absolute numbers of DN1, DN2, DN3 and DN4 cells are indicated for each strain. A representative experiment is shown.
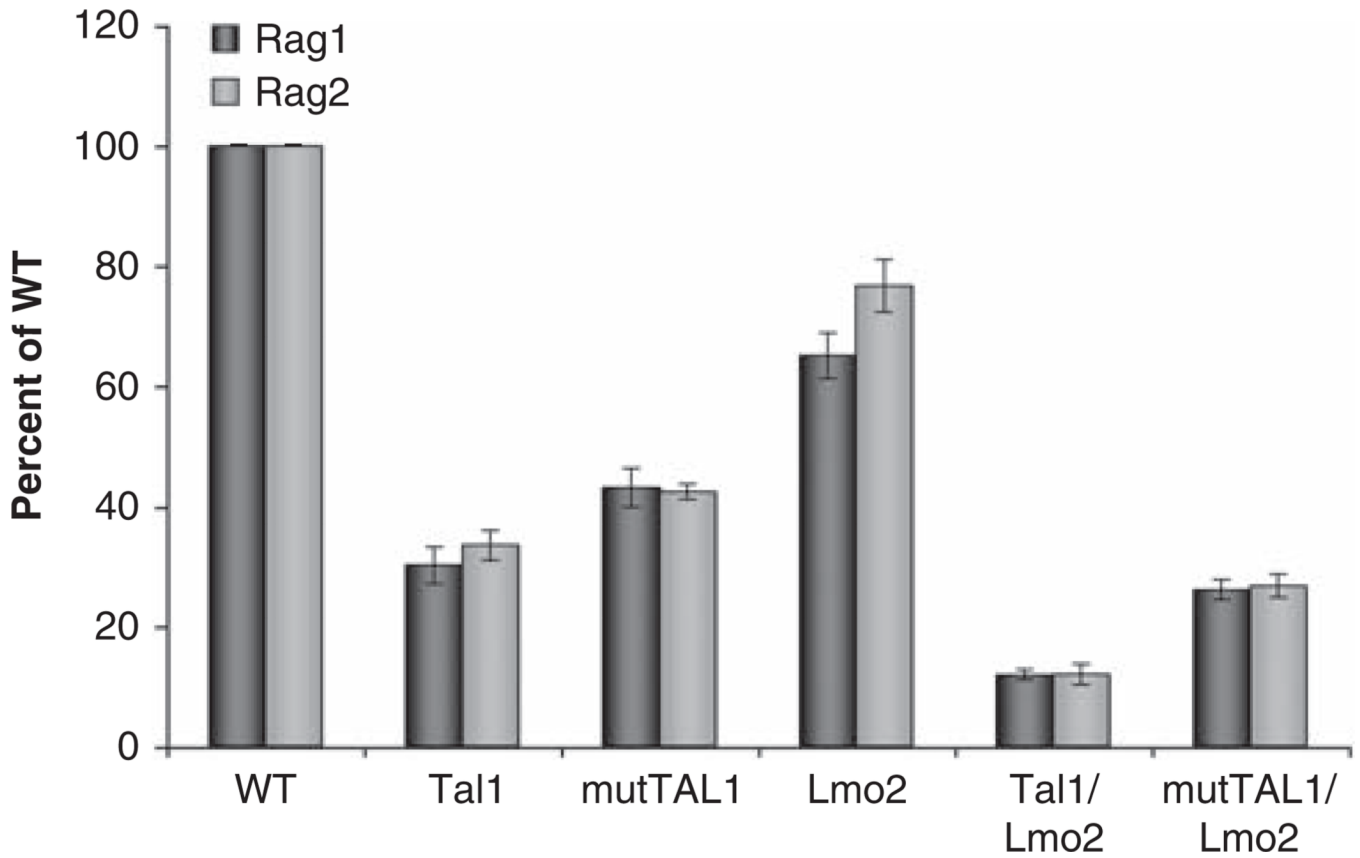
Figure 3.
Rag1 and Rag2 expression is further repressed in *Tal1* and *mut*-TAL1 transgenic thymocytes when *Lmo2* is co-expressed. Thymocytes were isolated from wild-type or preleukemic *Tal1, mutTAL1, Lmo2, mutTAL1/Lmo2* or *Tal/Lmo2* transgenic mice at 4–6 weeks of age. RNA was isolated and Rag1 and Rag2 expression levels quantified by qPCR. The copy number for the gene of interest was normalized to the copy number for β-*actin*. Data is represented as percent of wild-type control.
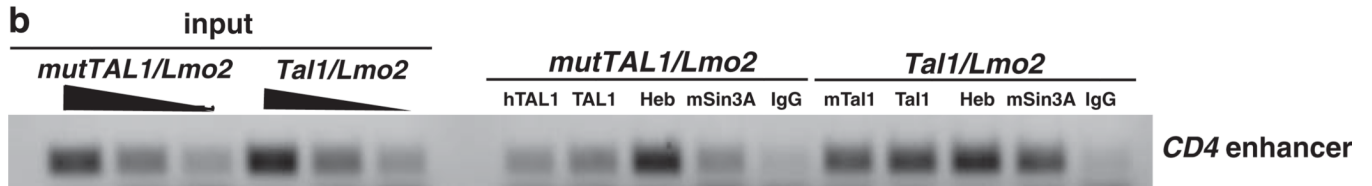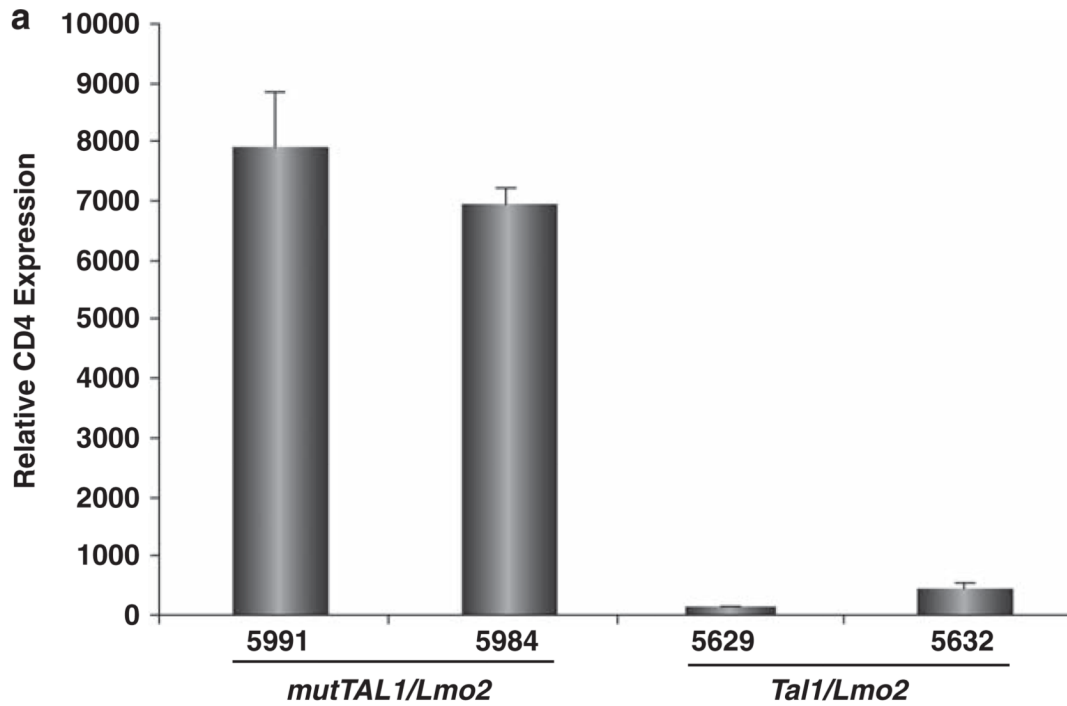
Figure 4.
The DNA-binding activity of TAL1 is required to stably bind and repress CD4. (**a**) The DNA-binding properties of TAL1 contribute to CD4 repression. RNA was isolated from *Tal1/Lmo2* and *mutTAL1/Lmo2* leukemic cell lines and CD4 expression levels quantified by qPCR. The copy number for CD4 was normalized to the copy number for β-actin. (**b**) The TAL1 DNA-binding mutant is impaired in its ability to bind the CD4 enhancer. Chromatin immunoprecipitation was performed on *mutTAL1/Lmo2* and *Tal1/Lmo2* leukemic cell lines using a rabbit IgG antibody or antibodies to HEB, mSin3a, mouse Tal1 (m258c), human TAL1 (Millipore, BTL73) or a TAL1 antibody that recognizes mouse and human TAL1 (Santa Cruz, C-21). Input-sheared DNA served as a positive control. PCR amplification was performed on the immunoprecipitated DNA with primers specific for the mouse CD4 enhancer region.

Table 1

Immunophenotype of *Tal1/Lmo2* and *mutTAL1/Lmo2* tumors

Animal number	Genotype	CD3	CD25	DN	CD4	CD8	DP	Phenotype	Survival (days)
5624	*Tal1/Lmo2*	31.6	55.7	27.9	0.1	71.2	0.8	DN, CD8-SP	102
5629	*Tal1/Lmo2*	71.3	0.4	30.9	0.2	68.1	0.7	DN, CD8lo	91
5632	*Tal1/Lmo2*	94.2	7.7	1.7	0.2	82.6	15.5	CD8-SP	91
5666	*Tal1/Lmo2*	95.1	95.3	3.3	2.0	2.1	92.6	DP	98
5354	*Tal1/Lmo2*	29.3	62.9	30.1	0.6	4.3	65	DN, DP	47
5338	*Tal1/Lmo2*	69.5	70.6	25.8	0.1	42.8	31.1	DN, CD8-SP, DP	86
5351	*Tal1/Lmo2*	23.7	97.9	0.3	0.1	0.5	99.1	DP	106
5313	*Tal1/Lmo2*	96.2	3.3	0.8	93.4	1.1	4.7	CD4-SP	154
5353	*Tal1/Lmo2*	27.5	4.6	9.7	80.1	0.4	9.8	CD4-SP	123
6540	*mutTal1/Lmo2*	52.7	13.8	53.7	8.2	10.9	27.1	DN, DP-lo	99
5984	*mutTal1/Lmo2*	50.0	95.5	51.0	1.0	39.5	8.5	DN, CD8-SP	101

Tumors were stained with antibodies to CD3, CD4, CD8 and CD25 and analyzed by flow cytometry.

The percentage of stained cells is indicated.