Hhex regulates Kit to promote radioresistance of self-renewing thymocytes in Lmo2-transgenic mice.

Running title: Hhex promotes radioresistance in a T-ALL model

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Abstract

Lmo2 is an oncogenic transcription factor that is frequently overexpressed in T-cell acute leukemias, in particular poor prognosis early T-cell precursor-like (ETP-) ALL. The primary effect of Lmo2 is to cause self-renewal of developing $CD4^{-}CD8^{-}$ (DN) T-cells in the thymus, leading to serially transplantable thymocytes that eventually give rise to leukemia. These self-renewing thymocytes are intrinsically radioresistant implying that they may be a source of leukemia relapse after therapy. The homeobox transcription factor, Hhex, is highly upregulated in Lmo2-transgenic thymocytes and can phenocopy Lmo2 in inducing thymocyte self-renewal, implying that Hhex may be a key component of the Lmo2-induced self-renewal program. To test this, we conditionally deleted Hhex in the thymi of Lmo2-transgenic mice. Surprisingly, this did not prevent accumulation of DN thymocytes, nor alter the rate of overt leukemia development. However, deletion of Hhex abolished the transplantation capacity of Lmo2-transgenic thymocytes and overcame their radioresistance. We found that Hhex regulates Kit expression in Lmo2-transgenic thymocytes and that abrogation of Kit signaling phenocopied loss of Hhex in abolishing the transplantation capacity and radioresistance of these cells. Thus targeting the Kit signaling pathway may facilitate the eradication of leukemia-initiating cells in immature T-cell leukemias in which it is expressed.

Introduction

Acute lymphoblastic leukemia (ALL) is the most common childhood cancer, with the T-cell subtype (T-ALL) comprising approximately 15% of all cases[1]. While cure rates for pediatric ALL have improved dramatically to a current event-free survival of over 80%, the prognosis remains poor for adult ALL patients and those with relapsed disease[1, 2]. T-ALL is associated with a worse prognosis than B-cell ALL, and there are certain subtypes for which prognosis is especially poor[2, 3]. Foremost among these is the recently described early T-cell precursor-like ALL (ETP-ALL) subtype[4, 5], which shows overlap with previously characterized LYL1-expressing or immature ALL[6-8]. This subtype, which accounts for approximately one in 8 T-ALL cases, is associated with rates of remission failure or relapse that are more than 4 times greater than non-ETP ALL cases[4, 9, 10]. Leukemia blasts in ETP-ALL are arrested at an early stage in T-cell development and share a similar transcriptional profile to normal early T-cell precursors (ETPs)[5, 8]. In addition, due to their developmental immaturity, ETP-ALLs are transcriptionally similar to hematopoietic stem cells (HSCs) and myeloid leukemias[5]. The mechanistic basis of ETP-ALL is distinct from that of mature T-ALL, being defined by frequent activation of the Ras signaling pathway along with mutations in other signaling and epigenetic pathways[5]. However, the molecular mechanisms that mediate therapeutic resistance of ETP-ALL are poorly understood.

A characteristic of ETP-ALL is the overexpression of stem cell associated transcription factors that are normally extinguished early during T-cell development. These include the T-cell oncogenes LMO2 and LYL1, which are co-expressed in ETP-ALL and interact to form a macromolecular transcriptional complex[5, 11]. LMO2 and LYL1 were originally identified as T-cell oncogenes due to their transcriptional activation in T-cell leukemia via chromosomal rearrangements[12, 13]. Notably, while these abnormalities are exclusively found in mature T-ALL cases, high expression of LMO2 and LYL1 is significantly associated with ETP-ALL, implying other mechanisms of overexpression[5, 6, 8, 11]. One such mechanism has recently been identified in

that both LMO2 and LYL1 can be directly upregulated by the key immature T-ALL oncogene MEF2C[8].

LMO2 is a member of the LIM-domain only class of transcription factors that lack DNA binding ability but interact with members of the basic-helix-loop helix (bHLH) family to form transcriptional regulatory complexes[14, 15]. To study leukemogenesis caused by LMO2, the CD2-Lmo2 transgenic model has been used[16, 17]. In this model, thymus-specific expression of Lmo2 results in the development of T-cell leukemia with a long latency (approximately 10 months). We have shown that the primary effect of LMO2 is to cause self-renewal of developing T-cells in the thymus, giving rise to serially transplantable, stem-like T-cells which accumulate at the DN3 stage of T-cell development[17]. Interestingly, these self-renewing thymocytes exhibit a gene expression profile that is strikingly similar to human ETP-ALL, including upregulation of the Lmo2-binding bHLH cofactor Lyl1[11, 17]. Moreover, these cells are remarkably therapeutically resistant, being able to survive high doses of radiotherapy despite elimination of over 99% of thymocytes[17]. This resistance implies that these self-renewing thymocytes may survive leukemia therapy and contribute to disease relapse [14, 18].

As human ETP-ALL cases uniformly express high levels of both LMO2 and LYL1 [5-8], and show a gene expression profile that is strikingly similar to self-renewing thymocytes in the CD2-Lmo2 mouse model, it has been proposed that Lmo2 may be a driving oncogene of ETP-ALL [8, 19]. Accordingly, we have recently shown that LYL1 is essential both for leukemogenesis in the CD2-Lmo2 mouse model as well as for growth of human ETP-ALL cell lines[11]. Thus, the Lmo2/Lyl1 complex co-ordinates a gene expression program that is essential for self-renewal of Lmo2-transgenic thymocytes and subsequent leukaemogenesis, as well as for self-renewal of human ETP-ALL cells. These findings suggest that the CD2-Lmo2 transgenic mouse model may be useful in determining the molecular mechanisms leading to ETP-ALL, as well as understanding the unique therapeutic resistance of this leukemia subtype.

Among the most highly upregulated genes in both Lmo2-transgenic thymocytes and human ETP-ALL is the hematopoietically-expressed homeobox transcription factor, Hhex[11, 17, 20, 21]. We have previously shown that Hhex can phenocopy Lmo2 in inducing self-renewal when overexpressed[17]. Moreover, overexpression of Hhex in mice causes T-cell leukemia which is strikingly similar to that caused by Lmo2[22]. These results suggest that Hhex may be a key mediator of Lmo2-driven T-cell self-renewal and leukemia[8, 14, 19]. Here we report the results of Hhex deletion in the thymus of Lmo2-transgenic mice. Surprisingly, this did not affect the accumulation of DN T-cells in the thymi of Lmo2 transgenic mice, nor alter the rate of leukaemogenesis. However, loss of Hhex completely abrogated the transplantation capacity and therapeutic resistance of Lmo2-induced self-renewing thymocytes. This was accompanied by downregulation of the cytokine receptor Kit, and the effects of Hhex deletion could be phenocopied by loss of Kit signaling. Together, these results indicate that thymocyte self-renewal and radioresistance are separable in this model. Furthermore, they show that Kit signaling is specifically required for radioresistance of self-renewing thymocytes, implying that Kit may promote therapeutic resistance of T-cell leukemias in which it is expressed.

Materials and Methods

Mice, poly(I:C) treatment and tamoxifen treatment

The *CD2-Lmo2* transgenic[16], *Hhex*^{fl}[23], *Hhex*$^{-}$[24], Kit^{Wv} (white spotting variant)[25], *Mx1-Cre*[26], *Lck;Cre*[27], *ROSA26-YFP*[28] and Rosa26-Cre^{ERT229} mice have all been described previously. All mouse strains were on a C57BL/6 background. All mouse experiments were approved by the Walter and Eliza Hall Institute Animal Ethics Committee.

To induce expression of the *Mx1-Cre* allele, 2 month old mice were injected intraperitoneally three times at 48 hour intervals with 300μg of polyinosinic-polycytidylic acid (poly(I:C)) (sodium salt) (Sigma) dissolved in normal saline. To induce expression of Rosa26-Cre^{ERT2} allele mice were administered with 2 doses of tamoxifen (Sigma; 4.2mg/mouse) in peanut oil by oral gavage.

Flow cytometry

Antibodies used for flow cytometric analysis were purchased from eBiosciences (San Diego, CA) and BD Pharmingen (San Diego, CA) or produced by the WEHI monoclonal antibody production facility: CD45.1 (A20), CD45.2 (104), CD4 (GK 1.5), CD8 (53-6.7), CD25 (PC61.5), CD44 (IM7), Kit (ACK4), CD93 (AA4.1), B220 (RA3-6B2), Mac1 (M1/70), Gr-1 (RB6-8C5) and Ter119 (Ly76) and used as conjugates to FITC, PE, PerCP-Cy5.5, PE-Cy7, APC and Alexa-fluor 700 flurochromes. Data was acquired on FACSCaliber or LSR II flow cytometers (BD Pharmingen) and sorting performed on a FACSAria II flow cytometer (BD Pharmingen). Fluorescence intensities were determined by dividing the Mean Fluorescence Intensity (MFI) attributed to binding of fluorescently labeled Kit or CD93 antibodies by the MFI attributed to the binding of fluorescently labeled isotype control antibody.

Cell cycle analysis

Cell cycle analysis of thymocytes was performed using a method modified from that described by Barbier *et al.*[30]. Briefly, thymocytes were harvested and stained with antibodies specific for lineage

markers (CD4, CD8, Mac1, Gr1, B220 and Ter119), CD45.2, CD25 and CD44, then fixed and permeablised using Cytofix/Cytoperm (BD Pharmingen). Cells were then stained with a FITC-conjugated antibody specific for Ki67 (BD Biosciences; clone B56) and 10μg/ml DAPI, then the cell cycle status of thymocyte subsets was determined by flow cytometry.

Genomic DNA extraction and PCR

Fractionated thymocytes were lysed in DirectPCR (Viagen Biotech; Los Angeles, CA) lysis buffer containing Proteinase K (Sigma) at 55°C overnight to extract genomic DNA. PCR was performed with 500nM primers (Hhex Del; GAACTAAATTAAGAGGCTGC, WA924; AGACGCACCACCATCAATTT, WA927; GGTGGGGAGAGGTATTTCTGA) to detect floxed (fl), wild-type (WT) and Cre-deleted null (Δ) and Hhex alleles.

Transplantation studies

Thymocyte and bone marrow (BM) transplantation assays were performed as described previously[17]. Briefly, for thymocyte transplantation, C57BL/6-Ly5.1 congenic recipient mice were irradiated with 6.5 Gy from a ^{60}Co source. Mice were then injected via the tail vein with thymocytes derived from one quarter of a thymus. For tumor transplantations, recipient C57BL/6-Ly5.1 mice were irradiated as above and injected with 100,000 cells derived from Lmo2 induced thymomas via the tail vein.

Results

Hhex is dispensable for the Lmo2-induced T cell differentiation block

Previous studies demonstrated that Hhex is overexpressed in Lmo2-transgenic thymocytes and can phenocopy Lmo2 in inducing thymocyte self-renewal and T-cell leukemia[17, 22]. We thus sought to assess the specific role of Hhex in the development of Lmo2-induced T cell leukemia. To delete Hhex specifically in the T cell lineage, we bred CD2-Lmo2 transgenic (Tg) mice with mice bearing a conditionally targeted ($Hhex^{fl}$) and knockout ($Hhex^{-}$) alleles of Hhex, along with the Lck-Cre transgene, to generate T-cell-specific Hhex-deleted (CD2-Lmo2;Lck-Cre;$Hhex^{-/\Delta}$: hereafter known as Lmo2;$Hhex^{-/\Delta}$) and heterozygous (CD2-Lmo2;Lck-Cre;$Hhex^{+/\Delta}$: hereafter known as Lmo2;$Hhex^{+/\Delta}$) mice. Deletion of Hhex in non-Lmo2 transgenic mice had no effect on T cell development, indicating that Hhex expression is not required beyond the DN2 stage at which Lck-Cre is expressed (data not shown)[31].

Leukemia in Lmo2-transgenic mice is preceded by a pre-leukemic phase consisting of blocked T-cell differentiation and thymocyte self-renewal at the DN3 stage resulting in reduced thymic size and an accumulation of immature T-cells [17]. Analysis of thymocytes from 7-8 week old mice revealed a reduction in cellularity and accumulation of DN thymocytes at the DN3 stage in Lmo2;$Hhex^{-/\Delta}$ and Lmo2;$Hhex^{+/\Delta}$ thymi, to a comparable degree to Lmo2-transgenic mice, indicating that Hhex is not required for the Lmo2-induced T-cell developmental block in vivo (Figure 1A-D). Consistent with the onset of Lck promoter activity in late DN2 stage thymocytes[31], PCR analysis showed partial deletion of the $Hhex^{fl}$ allele in DN2 thymocytes, but complete deletion of the $Hhex^{fl}$ allele from the DN3 stage, at which point thymocyte self-renewal occurs in this model (Figure 1E)[17]. Thus Hhex is dispensable for the accumulation of DN3 thymocytes in Lmo2-transgenic mice.

Hhex is required for transplantation and radioresistance of Lmo2 transgenic thymocytes.

We have previously shown that the primary effect of Lmo2 is to cause self-renewal of thymocytes from a young age resulting in self-renewing DN3 thymocytes that engraft irradiated

recipient thymi long-term and retain differentiation capacity[17]. To assess the role of Hhex in the long-term engraftment capacity of Lmo2 transgenic thymocytes, thymocytes from pre-leukemic (8 week old) Lmo2;Hhex$^{-/\Delta}$ mice were injected cells into sublethally irradiated congenic (Ly5.1) recipients. 4 weeks later, Lmo2 transgenic thymocytes lacking a single Hhex allele (Lmo2;Hhex$^{+/\Delta}$) showed long-term engraftment, indicative of self-renewal capacity (Figure 2A). Strikingly, however, loss of both Hhex alleles (Lmo2;Hhex$^{-/\Delta}$) completely abolished the transplantation capacity of Lmo2 transgenic thymocytes (Figure 2A). Thus Hhex is required for the long-term engraftment capacity of Lmo2-transgenic thymocytes.

Our previous studies demonstrated that Lmo2-transgenic self-renewing thymocytes are highly radioresistant with the ability to survive high doses of γ-irradiation, despite killing of the vast majority (>99%) of thymocytes. As such, when Lmo2-transgenic mice are used as recipients in bone-marrow (BM) transplantation assays, wild-type bone marrow cells can engraft the BM but fail to engraft the thymus of Lmo2-transgenic mice due to competition from surviving self-renewing thymocytes[17]. To assess whether loss of Hhex affected the radioresistance of Lmo2-transgenic thymocytes, Lmo2;Hhex$^{-/\Delta}$ mice were irradiated (6.5 Gy), then injected with congenic (Ly5.1) BM. As expected, 4 weeks after transplant, donor BM repopulated the BM and thymus of irradiated wild-type recipient mice but was completely unable to repopulate the thymus of irradiated Lmo2-transgenic mice (Figure 2B, 2C). Lmo2-transgenic recipient thymi contained a prominent population of DN3 thymocytes (Representative analysis; Figure 2C), suggesting that this is the population that survives radiation and repopulates the thymus. In contrast, we found that deletion of Hhex almost completely abrogated the radio-resistance of Lmo2 transgenic thymocytes, with the thymi of 7 of 8 recipient Lmo2;Hhex$^{-/\Delta}$ mice comprised almost entirely of donor-derived thymocytes (Figure 2B). Thus Hhex is required for radioresistance of Lmo2-transgenic thymocytes.

It has been reported using an alternative CD2-Lmo2 mouse model that Lmo2-expressing DN thymocytes are more quiescent than WT DN thymocytes [32], an observation which may explain why Lmo2-transgenic thymocytes are inherently radio-resistant in our model. Cell cycle analysis showed

that Lmo2-transgenic DN2 and DN4 populations were significantly more quiescent (G_0) than in wild-type thymocytes (Figure 2D), whilst the cell cycle status of DN1 and DN3 thymocytes was similar. Interestingly, the cell cycle status of Lmo2-transgenic thymocytes was not altered in the absence of Hhex (Figure 2D). Thus, Hhex regulates radioresistance of Lmo2-transgenic thymocytes via mechanisms other than enhanced cell cycling.

Next, we examined the survival of Lmo2-transgenic thymocytes following irradiation. We found no significant difference in the amount of surviving thymocytes (including DN3 thymocytes) post-irradiation, suggesting that Lmo2-transgenic thymocytes are not intrinsically radioresistant (Supplementary Figure 1A-B). Moreover, sorted Lmo2-transgenic thymocytes showed poor survival in vitro, both with and without irradiation (Supplementary Figure 2A). To assess irradiation-induced DNA damage in these cells, we quantitated γH2AX positive cells following irradiation. We found significantly reduced DNA damage in Lmo2-transgenic DN3 thymocytes (Supplementary Figure 2B), which was largely overcome in Lmo2;Hhex$^{-/\Delta}$ thymocytes. Thus Lmo2-transgenic thymocytes are resistant to radiation-induced DNA damage, which may assist them to survive long-term and recolonize the thymus, and this resistance requires Hhex expression.

Hhex is dispensable for development of overt leukemia in Lmo2-transgenic mice.

To determine the requirement of Hhex for Lmo2-induced leukemia, we monitored cohorts of Lmo2;Hhex$^{-/\Delta}$ and Lmo2;Hhex$^{+/\Delta}$ mice long term. Surprisingly, we found no difference in the rate of leukemia development in Lmo2;Hhex$^{-/\Delta}$ and Lmo2;Hhex$^{+/\Delta}$ mice (Figure 3A). PCR analysis confirmed Hhex deletion in all thymomas that arose in Lmo2;Hhex$^{-/\Delta}$ mice (eg. Figure 3A, inset). Consistent with our previous observations in Lmo2-transgenic mice, thymomas from Lmo2;Hhex$^{-/\Delta}$ and Lmo2;Hhex$^{+/\Delta}$ mice consisted either of mature CD4$^+$CD8$^+$ (DP) T cells or a mixture of DP and DN T-cells (Figure 3B and Table 1). Thus Hhex is dispensable for the development of Lmo2-induced T-cell leukemia in vivo.

Previous studies demonstrated that in overt Lmo2-induced T-cell leukemias, DN T cells, but not DP T cells, are bona fide cancer stem cells (CSCs) with the capacity to transplant leukemia[11, 33, 34]. To assess whether loss of Hhex affects the leukemia-propagating capacity of Lmo2-induced CSCs, we injected cells from a panel of Lmo2;Hhex$^{-/\Delta}$ thymomas into sublethally irradiated Ly5.1 mice. The proportion of Lmo2;Hhex$^{-/\Delta}$ tumors with transplantation capacity (3 of 5) was similar to that of control Lmo2;Hhex$^{+/\Delta}$ tumors (2 of 6; Table 1). Moreover the immunophenotype of secondary Lmo2;Hhex$^{-/\Delta}$ tumors was enriched for DN cells (Figure 3C), suggesting that the phenotype of CSCs is the same as found previously in Lmo2-transgenic tumors[11]. Thus whilst Hhex is essential for the transplantation capacity of Lmo2 transgenic pre-leukemic thymocytes, overt CSC transplantation in this model does not require Hhex.

Hhex regulates expression of Kit on Lmo2 transgenic thymocytes

In previous studies, we identified a gene expression pattern of Lmo2 transgenic thymocytes, that resembles hematopoietic stem cells (HSCs) and is similar to human ETP-ALL[11, 17]. Markers of this HSC-like transcriptional program include Kit, which is normally expressed on thymus seeding progenitors (TSP) and maintained until the DN2-DN3 transition [35]. During analysis of the thymi of Lmo2; Hhex$^{-/\Delta}$ mice, we noted that Kit was no longer upregulated on DN3 thymocytes (Figure 4A). In contrast, expression of another HSC marker, CD93 (also known as AA4.1), was maintained (Figure 4A). As Kit is required for transplantation of normal HSCs [36], we questioned whether it could be a critical downstream mediator of Hhex that is required for transplantation and radioresistance of Lmo2 transgenic thymocytes.

Firstly, we assessed the level of Kit on various thymocyte subsets from wild-type and Lmo2-transgenic mice by flow cytometry (Supplementary Figure 3). As expected, Kit expression was limited to the DN2-3 fraction of wild-type thymocytes. However, Kit was upregulated in Lmo2-transgenic mice, most extensively at the DN3 stage at which self-renewal occurs, before being downregulated (Supplementary Figure 3). Expression of CD93 followed a similar pattern

(Supplementary Figure 3). Moreover, Kit was expressed at high levels in thymocytes from aged (6 month old) Lmo2-transgenic mice, indicating that it is expressed in DN thymocytes that progressively accumulate with age in this model (Figure 4B). Despite Kit being expressed in only a minority of young Lmo2-transgenic thymocytes (Figure 4C), long-term engraftment potential resided in the Kit^+ fraction (Figure 4D). Thus Kit is a specific marker of self-renewing thymocytes in the Lmo2-transgenic mouse model.

To assess whether Kit is a target of Hhex in thymocytes, *in vitro* cultured DN thymocytes (grown on OP9-DL1 feeders) were transduced with retroviruses overexpressing Kit and Hhex. Whilst control DN thymocytes transduced with empty (MIG) retrovirus, or those overexpressing Kit, could differentiate to DP and $CD8^+$-SP cells, cells overexpressing Hhex exhibited arrested differentiation at the DN2 stage (Figure 4E), and showed abundant Kit expression, to a similar degree as that induced by retroviral Kit overexpression (Figure 4E). Thus Kit is a marker of Lmo2-induced self-renewing thymocytes that is regulated by Hhex.

Kit is required for transplantation and radioresistance of Lmo2 transgenic thymocytes

To directly assess the role of Kit in this model, Lmo2-transgenic were bred with White-spotted viable (Kit^{Wv}) mice, which have a dominant-negative mutation in the kinase domain of Kit, to generate Lmo2;$Kit^{Wv/+}$ (approximately 30% Kit activity) and Lmo2;$Kit^{Wv/Wv}$ (approximately 10% Kit activity) mice[25]. Thymi from young (non-Lmo2-transgenic) $Kit^{Wv/Wv}$ mice showed a loss of early T-cell precursors (ETPs (DN: $CD25^-/kit^+$)) and DN2 thymocytes (Figure 5A and Supplementary Figure 4), as has been shown previously[35]. However, Lmo2;$Kit^{Wv/Wv}$ mice showed an increase in the percentage of DN3 thymocytes with abnormally high levels of Kit, to a similar degree as in Kit wild-type Lmo2 transgenic mice (Figure 5A-B). Thus Lmo2 overexpression overcomes the loss of T-cell progenitors in $Kit^{Wv/Wv}$ mice and Kit signaling is dispensable for the Lmo2-induced T-cell differentiation block.

281 To test the requirement for Kit for transplantation of Lmo2 transgenic thymocytes,
282 thymocytes from Lmo2;$Kit^{Wv/Wv}$ mice were injected into irradiated recipients. Strikingly,
283 Lmo2;$Kit^{Wv/Wv}$ thymocytes were completely unable to engraft long-term in the thymus of recipient
284 mice, indicating that like Hhex, functional Kit signaling is required for long-term engraftment of
285 Lmo2 transgenic thymocytes (Figure 5C).

286 The loss of transplantation capacity but maintenance of a high proportion of DN thymocytes
287 in the thymi of Lmo2;$Kit^{Wv/Wv}$ mice suggested that Kit signaling is specifically required for
288 engraftment of Lmo2 transgenic thymocytes, but not their ability to self renew in vivo. To test this,
289 we used an in vivo cell lineage-tracing strategy that we have previously developed to assess
290 thymocyte self-renewal in the absence of transplantation[17]. This assay involves marking
291 hematopoietic progenitors in the bone marrow of ROSA26-EYFP reporter mice with YFP using the
292 inducible Mx-Cre allele. Progenitor migration to the thymus can subsequently be traced. We found
293 that regardless of Kit genotype, YFP^+ BM progenitors were unable to enter the thymus of Lmo2-
294 transgenic mice indicating presence of resident self-renewing thymocytes (Figure 6A-B). Thus Kit
295 signaling is not required for in vivo self-renewal of Lmo2 transgenic thymocytes, but is specifically
296 required for their engraftment capacity in transplantation experiments.

297 We next assessed the impact of Kit loss-of-function on the radioresistance of Lmo2
298 transgenic thymocytes in BM transplantation experiments. Unlike Lmo2-transgenic mice, robust
299 engraftment of donor-derived cells was found in the thymi of Lmo2;$Kit^{Wv/Wv}$ mice, as well as in the
300 thymi of 2 out of 6 Lmo2;$Kit^{Wv/+}$ recipient mice (Figure 7A). Thus Lmo2 transgenic thymocytes
301 utilize Kit-dependent signaling pathways to enable radiation resistance.

302 To determine the requirement for Kit for Lmo2-induced leukaemogenesis, cohorts of
303 Lmo2;$Kit^{Wv/Wv}$ mice were monitored long-term. Interestingly, these mice developed leukemia at an
304 increased rate relative to CD2-Lmo2 and Lmo2;$Kit^{Wv/+}$ mice (Figure 7B). Thus, like Hhex, Kit is
305 required for transplantation and therapeutic resistance of Lmo2 transgenic thymocytes in this model

306 but is dispensable for their self-renewal and progression to leukemia. In addition, Kit signaling
307 suppresses Lmo2-driven leukemia in this model.
308
309 **Inducible deletion of Hhex in self-renewing Lmo2 thymocytes results in loss of Kit and**
310 **transplantation capacity**
311 Whilst the Lck-Cre transgene used in this study resulted in complete excision of the $Hhex^{fl}$
312 allele in the DN3 self-renewing thymocyte population (Figure 1E), it remained possible that a rare
313 self-renewing population existed in the thymi of these mice that retained non-deleted Hhex. To
314 address this, we created an Lmo2-transgenic mouse model in which Hhex is inducibly deleted using
315 the ubiquitously expressed tamoxifen-inducible Cre transgene ROSA26-Cre-ERT2[29]
316 (Supplementary Figure 5A). Thymocytes from these mice were transplanted into irradiated
317 recipients and 4 weeks later Hhex was deleted by tamoxifen induction of the Cre-ERT2 transgene. 3
318 weeks post-induction, Hhex was efficiently deleted in all T cell subsets (Supplementary Figure
319 5B,C). Nevertheless, large numbers of Lmo2-transgenic DN3 thymocytes remained (Supplementary
320 Figure 5D-F), demonstrating that Hhex is dispensable for in vivo self-renewal of these cells.
321 We next analyzed Kit expression and transplantation capacity of these Hhex-deleted Lmo2-
322 transgenic thymocytes. This showed reduced Kit expression (Supplementary Figure 5G) and an
323 almost complete loss of long-term transplantation capacity (Supplementary Figure 5H). These
324 results confirm that Hhex is dispensable for in vivo self-renewal of Lmo2-transgenic thymocytes
325 but required for Kit overexpression and transplantation capacity.
326

Discussion

Using refinements in treatment methodology and scheduling, treatment of T-ALL has improved spectacularly from 5-year survival rates of 9% in 1960 to over 80%[1]. However, the rates of improvement have slowed and therapy remains associated with long-term side-effects[37]. Moreover, certain high-risk subtypes exist that have poorer prognosis, highlighting the need for more specific therapeutics to reduce treatment toxicities and improve outcome. Here we have shown that in a mouse model of T-ALL, radioresistance is provided to self-renewing thymocytes via a signaling pathway involving Lmo2/Lyl1-mediated activation of a homeobox transcription factor, Hhex, and subsequent upregulation of the Stem Cell Factor receptor, Kit. These findings raise the possibility that targeting the Kit signaling pathway may sensitize T-ALL stem cells to conventional therapeutics and improve treatment outcomes.

Surprisingly, we found that Hhex was dispensable for the generation of leukemia by Lmo2. This was unexpected given our previous findings that Hhex is highly expressed in Lmo2 transgenic thymocytes and that overexpression of Hhex can phenocopy Lmo2 in causing thymocyte self-renewal and T-cell leukemia in mice[17]. Our findings indicate that whilst Hhex provides survival signals to Lmo2 transgenic thymocytes, their self-renewal is likely to be mediated by multiple, redundant pathways downstream of Lmo2/Lyl1, which remain to be defined.

In contrast to our results, a recent study has found delayed onset of T-cell leukemia in Lmo2-transgenic mice with conditional deletion of Hhex[38]. The differences between these findings and our own may relate to the different Cre transgenes used to delete Hhex in each model. Whereas our study used Lck-Cre to specifically delete Hhex in the thymus, Smith *et al*. used the Vav-Cre transgene to delete Hhex throughout the hematopoietic system. Thus the reduced leukemia seen in Hhex-deleted mice in this study may result from a loss of the cellular target of transformation for Lmo2 rather than a requirement for Hhex for Lmo2-induced thymocyte self-renewal. In keeping with this, we have found that loss of Hhex at the CLP stage results in a failure of commitment to the T-cell lineage (J. Jackson and M. McCormack, manuscript submitted).

Whilst dispensable for leukemogenesis, Hhex was found to be required for the transplantation capacity and radiation resistance of Lmo2-transgenic thymocytes. This indicates that self-renewal/leukemogenesis and transplantation/radioresistance are separable in this model, and that specific pathways might be targeted to specifically sensitize leukemic stem cells to conventional therapies. Using a candidate approach, we identified regulation of Kit by Hhex as a pathway downstream of Lmo2/Lyl1 that provides survival signals to self-renewing thymocytes in this model. Using a cell lineage tracing strategy, we showed that loss of Kit does not abrogate in vivo thymocyte self-renewal in Lmo2-transgenic mice, but rather that transplantation and radioresistance of these cells is lost. This highlights the superiority of lineage tracing strategies for examining in vivo self-renewal over transplantation-based approaches.

Interestingly, loss of Kit expression leads to more rapid development of leukemia in Lmo2-transgenic mice. A potential explanation for this finding is the loss of early thymocyte subsets (ETP and DN2 cells) in $Kit^{Wv/Wv}$ mice (Supplementary Figure 4), which may result in self-renewing Lmo2;$Kit^{Wv/Wv}$ DN3 thymocytes lacking competition from incoming BM progenitors. Indeed, it has recently been shown that cell competition from early thymocyte subsets acts to suppress tumorigenesis in thymus, with loss of DN2 progenitors promoting downstream self-renewal and leukemogenesis[39].

Whilst CSCs in overt T-cell leukaemias arising in the Lmo2-transgenic model retain an immature immunophenotype and ETP-like gene expression programme[11], Kit expression is usually lost (data not shown). This is in contrast to human ETP-ALL cases, which express high levels of LMO2 and co-express Kit is in the majority of cases[5, 8]. This, along with their immature developmental status, suggests that self-renewing, preleukemic Lmo2-transgenic thymocytes may represent a better model of ETP-ALL than overt leukemia in this model. Moreover, Treanor et al have recently shown that activated IL-7 receptor mutants induce ETP-like ALL in Arf-null thymocytes with abundant overexpression of Lmo2[40]. Moreover, Lmo2 was sufficient to induce ETP-like ALL in this model, 2 out of 4 of which expressed Kit. In these models, self-renewal

379 occurs at the DN2 stage[40, 41] in contrast to the Lmo2-transgenic model in which self-renewal occurs
380 at the DN3 stage[17]. The reasons for this discrepancy are presently unclear, but may relate to the
381 timing of expression of the CD2-Lmo2 transgene or evolution of the leukaemia-initiating cell in the
382 thymi of Lmo2-transgenic mice.

383 It remains to be determined whether secondary mutations in Lmo2-transgenic T-cell
384 leukemias activate pathways similar to Kit to provide survival signals to overt cancer stem cells.
385 One candidate for such a pathway would be the related cytokine receptor Flt3. It has been shown
386 that activating FLT3 mutations occur in T-ALL and are restricted to the immature/TCRγδ subtypes[5,
387 42, 43]. Moreover, in these cases the Kit and Flt3 signaling pathways are redundant, in that the
388 leukemic cells are frequently resistant to FLT3-inhibition yet sensitive to dual inhibition of FLT3
389 and KIT[43]. Thus Kit can provide survival signals to human immature T-ALL cases, as we have
390 shown here in a mouse model of ETP-ALL. The mouse model used herein may prove useful in
391 determining the survival pathways regulated by Kit in pro-T cells and designing therapeutic
392 strategies incorporating Kit inhibition.

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Authorship Contributions

M.P.M., B.J.S. designed research, performed research, analyzed data and wrote the manuscript, C.N. and R.A. performed research and analyzed data, C.W.B. designed research and provided critical reagents W.S.A designed research, analyzed data and wrote the manuscript.

Conflict of interest.

The authors declare no conflict of interest.

Supplementary information is available at Leukemia's website.

413 **References**

414 1. Pui CH, Evans WE. Treatment of acute lymphoblastic leukemia. *N Engl J Med* 2006 Jan 12;
415 **354**(2)**:** 166-178.
416
417 2. Pui CH, Mullighan CG, Evans WE, Relling MV. Pediatric acute lymphoblastic leukemia:
418 where are we going and how do we get there? *Blood* 2012 Aug 9; **120**(6)**:** 1165-1174.
419
420 3. Pieters R, Carroll WL. Biology and treatment of acute lymphoblastic leukemia. *Pediatr Clin*
421 *North Am* 2008 Feb; **55**(1)**:** 1-20, ix.
422
423 4. Coustan-Smith E, Mullighan CG, Onciu M, Behm FG, Raimondi SC, Pei D*, et al.* Early T-
424 cell precursor leukaemia: a subtype of very high-risk acute lymphoblastic leukaemia. *Lancet*
425 *Oncol* 2009 Feb; **10**(2)**:** 147-156.
426
427 5. Zhang J, Ding L, Holmfeldt L, Wu G, Heatley SL, Payne-Turner D*, et al.* The genetic basis
428 of early T-cell precursor acute lymphoblastic leukaemia. *Nature* 2012 Jan 12; **481**(7380)**:**
429 157-163.
430
431 6. Ferrando AA, Neuberg DS, Staunton J, Loh ML, Huard C, Raimondi SC*, et al.* Gene
432 expression signatures define novel oncogenic pathways in T cell acute lymphoblastic
433 leukemia. *Cancer Cell* 2002 Feb; **1**(1)**:** 75-87.
434
435 7. Soulier J, Clappier E, Cayuela JM, Regnault A, Garcia-Peydro M, Dombret H*, et al.* HOXA
436 genes are included in genetic and biologic networks defining human acute T-cell leukemia
437 (T-ALL). *Blood* 2005 Jul 1; **106**(1)**:** 274-286.
438

8. Homminga I, Pieters R, Langerak AW, de Rooi JJ, Stubbs A, Verstegen M*, et al.* Integrated transcript and genome analyses reveal NKX2-1 and MEF2C as potential oncogenes in T cell acute lymphoblastic leukemia. *Cancer Cell* 2011 Apr 12; **19**(4)**:** 484-497.

9. Inukai T, Kiyokawa N, Campana D, Coustan-Smith E, Kikuchi A, Kobayashi M*, et al.* Clinical significance of early T-cell precursor acute lymphoblastic leukaemia: results of the Tokyo Children's Cancer Study Group Study L99-15. *Br J Haematol* 2012 Feb; **156**(3)**:** 358-365.

10. Allen A, Sireci A, Colovai A, Pinkney K, Sulis M, Bhagat G*, et al.* Early T-cell precursor leukemia/lymphoma in adults and children. *Leuk Res* 2013 Sep; **37**(9)**:** 1027-1034.

11. McCormack MP, Shields BJ, Jackson JT, Nasa C, Shi W, Slater NJ*, et al.* Requirement for Lyl1 in a model of Lmo2-driven early T-cell precursor ALL. *Blood* 2013 Sep 19; **122**(12)**:** 2093-2103.

12. Boehm T, Foroni L, Kaneko Y, Perutz MF, Rabbitts TH. The rhombotin family of cysteine-rich LIM-domain oncogenes: distinct members are involved in T-cell translocations to human chromosomes 11p15 and 11p13. *Proc Natl Acad Sci U S A* 1991 May 15; **88**(10)**:** 4367-4371.

13. Baer R. TAL1, TAL2 and LYL1: a family of basic helix-loop-helix proteins implicated in T cell acute leukaemia. *Semin Cancer Biol* 1993 Dec; **4**(6)**:** 341-347.

14. Curtis DJ, McCormack MP. The molecular basis of Lmo2-induced T-cell acute lymphoblastic leukemia. *Clin Cancer Res* 2010 Dec 1; **16**(23)**:** 5618-5623.

15. Matthews JM, Lester K, Joseph S, Curtis DJ. LIM-domain-only proteins in cancer. *Nat Rev Cancer* 2013 Feb; **13**(2)**:** 111-122.

16. Larson RC, Fisch P, Larson TA, Lavenir I, Langford T, King G*, et al.* T cell tumours of disparate phenotype in mice transgenic for Rbtn-2. *Oncogene* 1994 Dec; **9**(12)**:** 3675-3681.

17. McCormack MP, Young LF, Vasudevan S, de Graaf CA, Codrington R, Rabbitts TH*, et al.* The Lmo2 oncogene initiates leukemia in mice by inducing thymocyte self-renewal. *Science* 2010 Feb 12; **327**(5967)**:** 879-883.

18. Hoang T. Of mice and men: how an oncogene transgresses the limits and predisposes to T cell acute lymphoblastic leukemia. *Sci Transl Med* 2010 Mar 3; **2**(21)**:** 21ps10.

19. Homminga I, Pieters R, Meijerink JP. NKL homeobox genes in leukemia. *Leukemia* 2012 Apr; **26**(4)**:** 572-581.

20. Crompton MR, Bartlett TJ, MacGregor AD, Manfioletti G, Buratti E, Giancotti V*, et al.* Identification of a novel vertebrate homeobox gene expressed in haematopoietic cells. *Nucleic Acids Res* 1992 Nov 11; **20**(21)**:** 5661-5667.

21. Bedford FK, Ashworth A, Enver T, Wiedemann LM. HEX: a novel homeobox gene expressed during haematopoiesis and conserved between mouse and human. *Nucleic Acids Res* 1993 Mar 11; **21**(5)**:** 1245-1249.

22. George A, Morse HC, 3rd, Justice MJ. The homeobox gene Hex induces T-cell-derived lymphomas when overexpressed in hematopoietic precursor cells. *Oncogene* 2003 Oct 2; **22**(43)**:** 6764-6773.

23. Hunter MP, Wilson CM, Jiang X, Cong R, Vasavada H, Kaestner KH*, et al.* The homeobox gene Hhex is essential for proper hepatoblast differentiation and bile duct morphogenesis. *Dev Biol* 2007 Aug 15; **308**(2)**:** 355-367.

24. Hallaq H, Pinter E, Enciso J, McGrath J, Zeiss C, Brueckner M*, et al.* A null mutation of Hhex results in abnormal cardiac development, defective vasculogenesis and elevated Vegfa levels. *Development* 2004 Oct; **131**(20)**:** 5197-5209.

25. Nocka K, Tan JC, Chiu E, Chu TY, Ray P, Traktman P*, et al.* Molecular bases of dominant negative and loss of function mutations at the murine c-kit/white spotting locus: W37, Wv, W41 and W. *EMBO J* 1990 Jun; **9**(6)**:** 1805-1813.

26. Kuhn R, Schwenk F, Aguet M, Rajewsky K. Inducible gene targeting in mice. *Science* 1995 Sep 8; **269**(5229)**:** 1427-1429.

27. Lee PP, Fitzpatrick DR, Beard C, Jessup HK, Lehar S, Makar KW*, et al.* A critical role for Dnmt1 and DNA methylation in T cell development, function, and survival. *Immunity* 2001 Nov; **15**(5)**:** 763-774.

28. Srinivas S, Watanabe T, Lin CS, William CM, Tanabe Y, Jessell TM*, et al.* Cre reporter strains produced by targeted insertion of EYFP and ECFP into the ROSA26 locus. *BMC Dev Biol* 2001; **1:** 4.

29. Seibler J, Zevnik B, Kuter-Luks B, Andreas S, Kern H, Hennek T*, et al.* Rapid generation of inducible mouse mutants. *Nucleic Acids Res* 2003 Feb 15; **31**(4)**:** e12.

30. Barbier V, Nowlan B, Levesque JP, Winkler IG. Flow cytometry analysis of cell cycling and proliferation in mouse hematopoietic stem and progenitor cells. *Methods Mol Biol* 2012; **844:** 31-43.

31. Masuda K, Kakugawa K, Nakayama T, Minato N, Katsura Y, Kawamoto H. T cell lineage determination precedes the initiation of TCR beta gene rearrangement. *J Immunol* 2007 Sep 15; **179**(6)**:** 3699-3706.

32. Cleveland SM, Smith S, Tripathi R, Mathias EM, Goodings C, Elliott N*, et al.* Lmo2 induces hematopoietic stem cell-like features in T-cell progenitor cells prior to leukemia. *Stem Cells* 2013 May; **31**(5)**:** 882-894.

33. Tremblay M, Tremblay CS, Herblot S, Aplan PD, Hebert J, Perreault C*, et al.* Modeling T-cell acute lymphoblastic leukemia induced by the SCL and LMO1 oncogenes. *Genes Dev* 2010 Jun 1; **24**(11)**:** 1093-1105.

34. Tatarek J, Cullion K, Ashworth T, Gerstein R, Aster JC, Kelliher MA. Notch1 inhibition targets the leukemia-initiating cells in a Tal1/Lmo2 mouse model of T-ALL. *Blood* 2011 Aug 11; **118**(6)**:** 1579-1590.

35. Rodewald HR, Kretzschmar K, Swat W, Takeda S. Intrathymically expressed c-kit ligand (stem cell factor) is a major factor driving expansion of very immature thymocytes in vivo. *Immunity* 1995 Sep; **3**(3)**:** 313-319.

36. Kent D, Copley M, Benz C, Dykstra B, Bowie M, Eaves C. Regulation of hematopoietic stem cells by the steel factor/KIT signaling pathway. *Clin Cancer Res* 2008 Apr 1; **14**(7)**:** 1926-1930.

37. Mody R, Li S, Dover DC, Sallan S, Leisenring W, Oeffinger KC*, et al.* Twenty-five-year follow-up among survivors of childhood acute lymphoblastic leukemia: a report from the Childhood Cancer Survivor Study. *Blood* 2008 Jun 15; **111**(12)**:** 5515-5523.

38. Smith S, Tripathi R, Goodings C, Cleveland S, Mathias E, Hardaway JA*, et al.* LIM Domain Only-2 (LMO2) Induces T-Cell Leukemia by Two Distinct Pathways. *PLoS One* 2014 Jan 21; **9**(1)**:** e85883.

39. Martins VC, Busch K, Juraeva D, Blum C, Ludwig C, Rasche V*, et al.* Cell competition is a tumour suppressor mechanism in the thymus. *Nature* 2014 May 22; **509**(7501)**:** 465-470.

40. Treanor LM, Zhou S, Janke L, Churchman ML, Ma Z, Lu T*, et al.* Interleukin-7 receptor mutants initiate early T cell precursor leukemia in murine thymocyte progenitors with multipotent potential. *J Exp Med* 2014 Apr 7; **211**(4)**:** 701-713.

41. Treanor LM, Volanakis EJ, Zhou S, Lu T, Sherr CJ, Sorrentino BP. Functional interactions between Lmo2, the Arf tumor suppressor, and Notch1 in murine T-cell malignancies. *Blood* 2011 May 19; **117**(20)**:** 5453-5462.

42. Paietta E, Ferrando AA, Neuberg D, Bennett JM, Racevskis J, Lazarus H*, et al.* Activating FLT3 mutations in CD117/KIT(+) T-cell acute lymphoblastic leukemias. *Blood* 2004 Jul 15; **104**(2)**:** 558-560.

43. Lhermitte L, Ben Abdelali R, Villarese P, Bedjaoui N, Guillemot V, Trinquand A*, et al.* Receptor kinase profiles identify a rationale for multitarget kinase inhibition in immature T-ALL. *Leukemia* 2013 Feb; **27**(2)**:** 305-314.

575 **Figure Legends**

576 **Figure 1. Hhex is not required for Lmo2-induced T cell differentiation block**

577 **A**. Accumulation of immature $CD4^{-}CD8^{-}$ (double negative (DN)) thymocytes in 8 week old Lmo2;
578 $Hhex^{-/\Delta}$ mice. Representative FACS analysis of total (left panels) and DN thymocytes (right panels)
579 from individual mice of the indicated genotypes (ie: Tg; Lmo2-transgenic, $Tg^{+/\Delta}$; Lmo2; Lck-Cre;
580 $Hhex^{+/\Delta}$, $Tg^{-/\Delta}$; Lmo2; Lck-Cre; $Hhex^{-/\Delta}$). **B.** Thymic cellularity, **C.** analysis of the percentage DN
581 and **D.** analysis of the percentage of DN3 thymocytes (of DN thymocytes) in pre-leukemic mice.
582 Data are mean +/- SD of 5-8 mice per group and p-values were calculated using Student's T test (ie:
583 *=p<0.05, **=p<0.01, ***=p<0.001). **E.** Hhex is efficiently deleted in DN3 and DP thymocytes
584 from Lmo2;$Hhex^{+/\Delta}$ and Lmo2;$Hhex^{-/\Delta}$ mice. Genomic DNA was extracted from FACS sorted
585 DN2, DN3 and $CD4^{+}CD8^{+}$ (double positive (DP)) thymocytes and used in PCRs to reveal floxed
586 (fl), wild-type (WT) and null (Δ) Hhex alleles.

587

588 **Figure 2. Hhex is essential for transplantation and radioresistance of Lmo2 transgenic**
589 **thymocytes**

590 **A**. Thymocytes were isolated from 8 week old Lmo2-transgenic mice of the indicated Hhex
591 genotypes and thymocytes equivalent to one quarter of a thymus were injected into sublethally
592 irradiated (6.5 Gy) Ly5.1 recipients. After 4 weeks, the proportion of donor thymocytes was
593 determined by flow cytometry. Points represent individual recipient mice, unique symbols denote
594 separate experiments and p-value was calculated using Student's T test (i.e. ***=p<0.001). **B.** Eight
595 week old Lmo2-transgenic mice of the indicated Hhex genotypes were sublethally irradiated (6.5
596 Gy) and injected with 10^7 Ly5.1 donor bone marrow cells, then 4 weeks later the donor chimerism
597 of the bone marrow and thymus was assessed by flow cytometry. In **C.** FACS plots of donor
598 chimerism analysis of recipient thymi from a representative wild-type (WT) and CD2-Lmo2
599 transgenic ($Tg^{+/+}$) mouse are shown. **D**. Cell cycle analysis of DN1-DN4 thymocytes. Thymocytes
600 from 6-8 week old wild-type and Lmo2-transgenic mice of the indicated Hhex genotypes were

stained with fluorescently labeled antibodies specific for markers of mature blood cells (CD4/CD8/Mac1/Gr1/B220/Ter119), CD44, CD25 and the marker for actively proliferating cells, Ki67 and DNA (DAPI). DN1-DN4 populations were gated and re-analysed for the quiescent (G_0; $Ki67^{lo}$/2N DNA content), G_1 ($Ki67^{hi}$/2N DNA content) and S,G_2,M populations ($Ki67^{hi}$/2N-4N DNA content). Data are mean + SD of 5-7 mice per group and p-values were calculated using Student's T test (i.e. **=p<0.01, ***=p<0.001).

Figure 3. Hhex is not required for leukemia development in CD2-Lmo2 mice

A. Kaplan-Meier survival curves of Lmo2-transgenic mice of the indicated Hhex genotypes (p=0.5 between $Hhex^{+/\Delta}$ and $Hhex^{-/\Delta}$ using Log-Rank (Mantel-Cox) test. **Inset.** Efficient Hhex deletion in thymomas of the indicated genotypes. **B**. Representative FACS plots of thymomas derived from Lmo2; $Hhex^{-/\Delta}$ mice. **C.** Representative FACS analysis of secondary thymoma arising in a Ly5.1 recipient mouse injected with tumor cells from Lmo2;$Hhex^{-/\Delta}$ #204.

Figure 4. Hhex regulates Kit expression, which is required for transplantation of Lmo2 transgenic thymocytes.

A. Analysis of Kit and CD93 expression on thymocytes from wild-type and CD2-Lmo2 transgenic mice of the indicated Hhex genotypes by flow cytometry. Relative Kit and CD93 expression was determined by dividing the Mean Fluorescence Intensity (MFI) attributed to binding of fluorescently labeled Kit or CD93 antibodies by the MFI attributed to the binding of fluorescently labeled Fluorescence Minus One (FMO) isotype control antibody. Data shown are mean+/- SD of 4-8 mice per group and p-values were calculated using the paired ratio Student's T test (ie: *=p<0.05,***=p<0.001). **B.** Aberrant Kit expression on DN thymocytes from aged Lmo2-transgenic mice. Representative FACS plots showing accumulation of DN3 thymocytes (left panels) in a 6 month old Lmo2-transgenic mouse and Kit expression (grey shaded) versus isotype control (dotted line) on DN and DP+SP thymocyte fractions (right panels). **C.** Sort populations used

to fractionate Kit^+ thymocytes from 2 month-old Lmo2-transgenic thymi. Cells equivalent to one quarter of a thymus were injected into sublethally irradiated (6.5 Gy) Ly5.1 recipients. **D.** Percent donor thymocytes in recipient mice receiving the indicated populations at 3 weeks post-transplant. Individual symbols indicate separate experiments. p-value was determined using paired ratio Student's T-test (ie: *=$p<0.05$). **E.** Retroviral overexpression of Hhex in thymocytes induces Kit expression. Flow cytometry analysis of DN T cells 2 weeks after transduction with MSCV-GFP (control; MIG), MSCV-GFP-Kit (Kit) and MSCV-GFP-Hhex (Hhex) retroviruses and propagated on OP9-DL1 feeder layers.

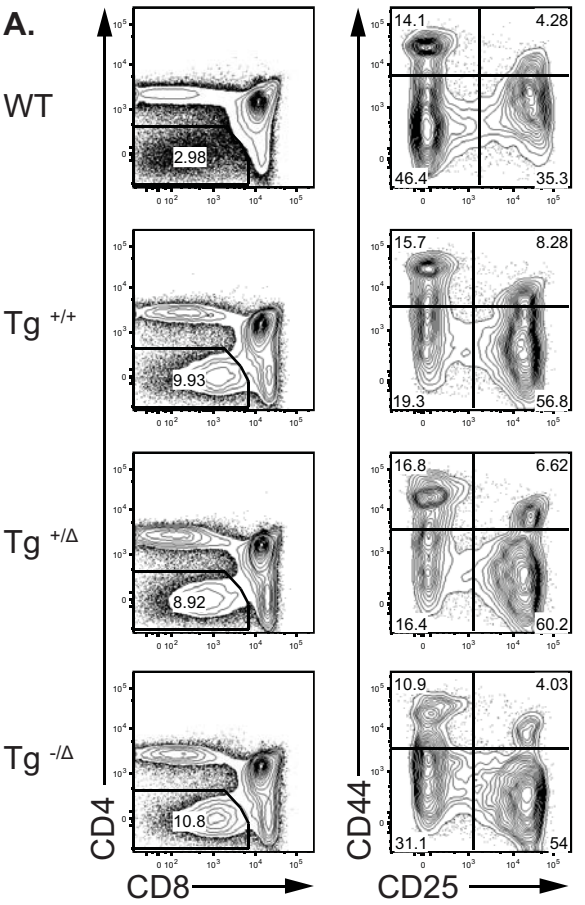
Figure 5. Functional Kit signalling is dispensable for T-cell differentiation block, but is required for transplantation of Lmo2 transgenic thymocytes.

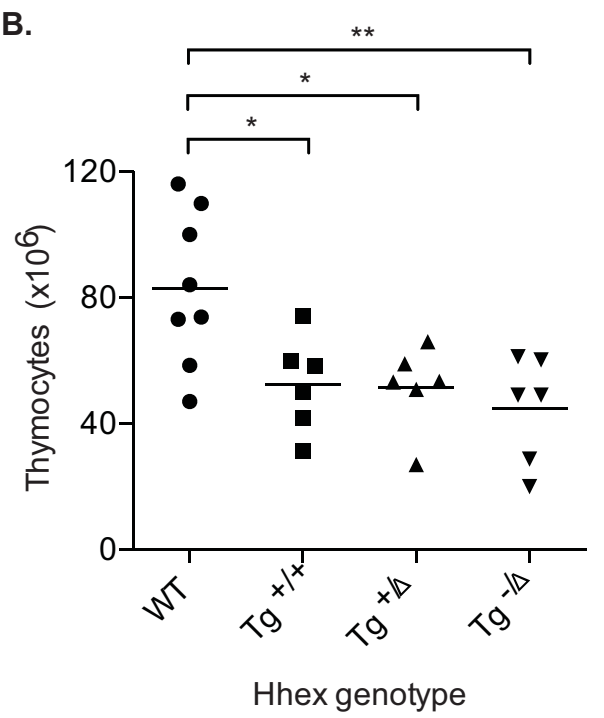
A.-B. Accumulation of DN thymocytes in 8-week old Lmo2; $Kit^{Wv/Wv}$ mice. **A.** Representative flow cytometry profiles of total (top panels) and DN thymocytes (middle and bottom panels). **B.** Summary of data showing the percentage of DN thymocytes from 8-12 week old mice of the indicated genotypes. **C.** Loss of functional Kit impairs transplantation capacity of Lmo2-transgenic thymocytes. Thymocytes were isolated from 8 week old Lmo2-transgenic mice of the indicated Kit genotypes. Thymocytes equivalent to one quarter of a thymus were injected into sublethally irradiated (6.5 Gy) Ly5.1 recipients. After 4 weeks, the proportion of donor thymocytes was determined by flow cytometry. Points represent individual recipient mice, unique symbols denote separate experiments and p-values were calculated using Student's T test (ie: ***=$p<0.001$).

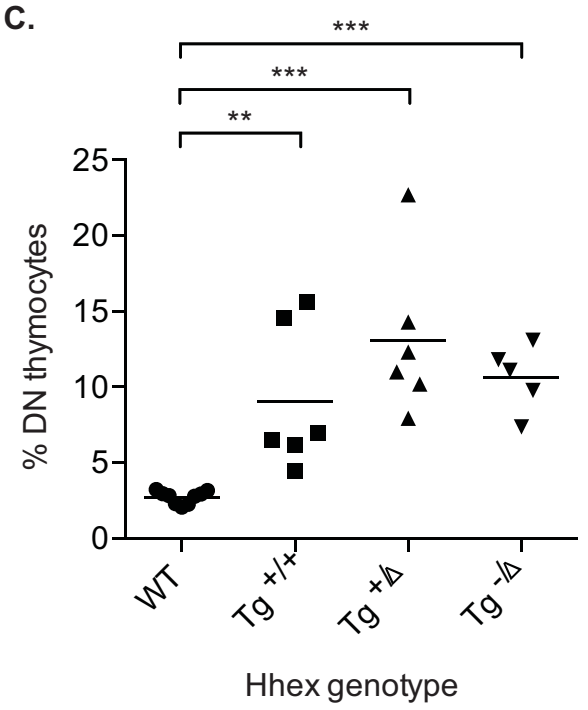
Figure 6. Self-renewal of Lmo2 transgenic thymocytes does not require functional Kit signaling. **A**. Two month old Mx-Cre;YFP mice of the indicated genotypes were treated with poly(I:C) to induce the expression of YFP in BM progenitors. Four weeks later, the extent of YFP expression in the $Lineage^-$ ($CD4^-/CD8^-/Mac1^-/Gr1^-/B220^-/Ter119^-$)/ Kit^+ BM fraction and thymus

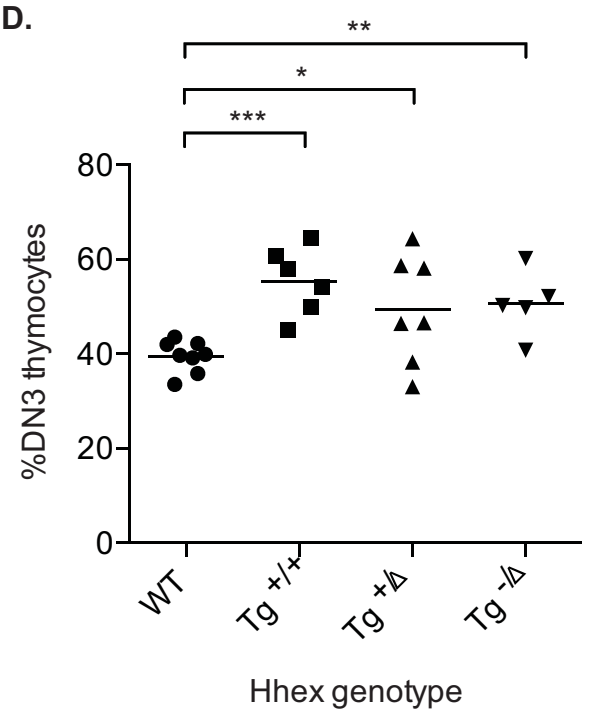
652 (THY) was assessed by flow cytometry. **B**. Representative FACS data showing YFP labeling of
653 $Lin^{-}Kit^{+}$ BM (top) and thymocytes (bottom).
654
655 **Figure 7. Functional Kit signalling is required for radioresistance of Lmo2 transgenic**
656 **thymocytes, but not for the development of Lmo2-induced leukemia. A.** Eight week old Lmo2-
657 transgenic mice of the indicated Kit^{Wv} genotypes were sublethally irradiated (6.5 Gy) and injected
658 with 10^7 Ly5.1 donor BM cells, then 4 weeks later the donor chimerism of the bone marrow and
659 thymus, was assessed by flow cytometry. **B.** Loss of functional Kit signaling promotes
660 leukemogenesis in Lmo2-transgenic mice. Kaplan-Meier survival curves of Lmo2-transgenic mice
661 of the indicated Kit^{Wv} genotypes ($p<0.001$ between $Kit^{+/+}$ and Kit^{WvWv} using Log-Rank (Mantel-
662 Cox) test).

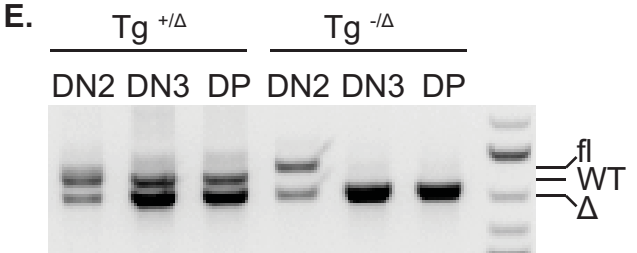
Figure 1: SHIELDS et al.











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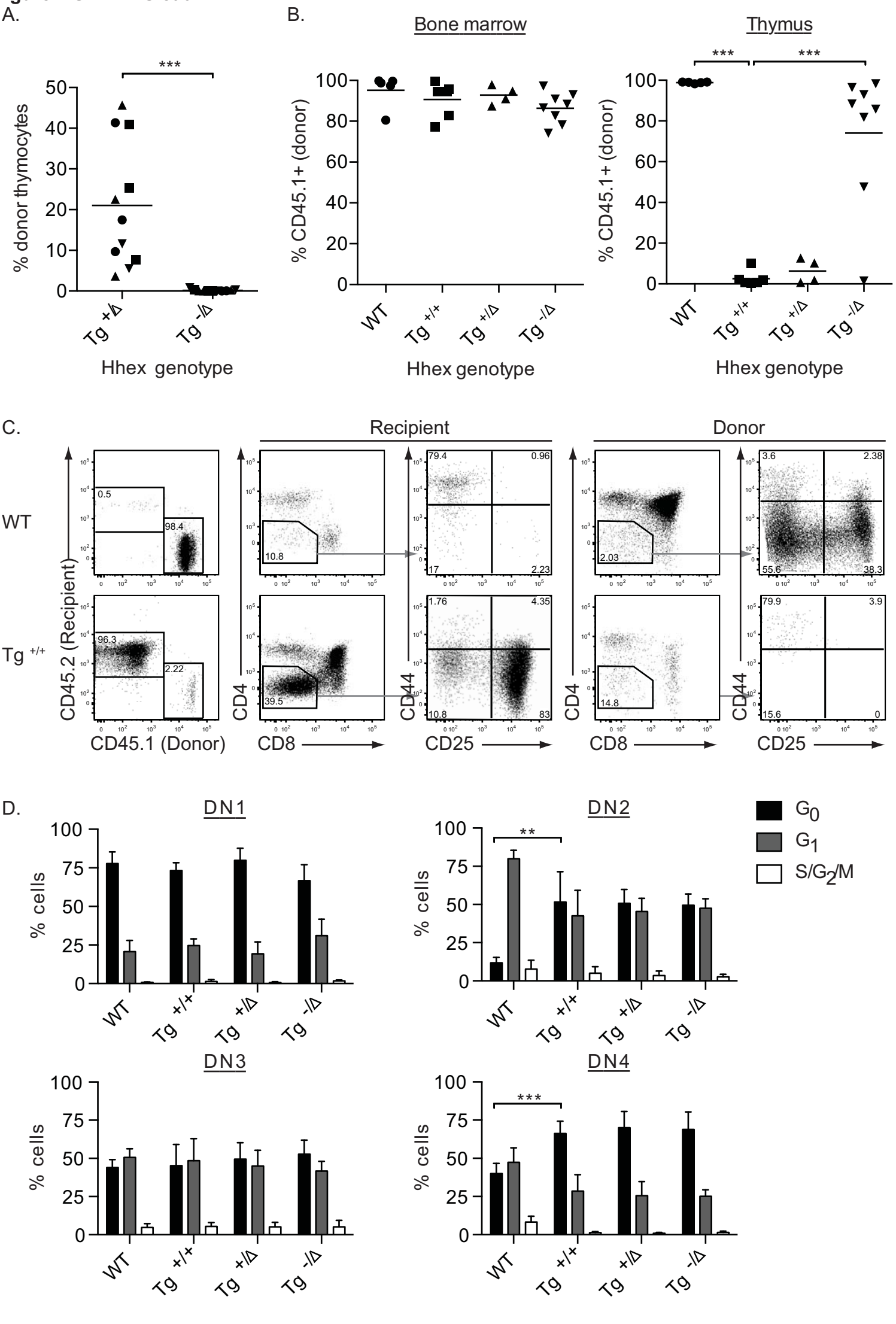

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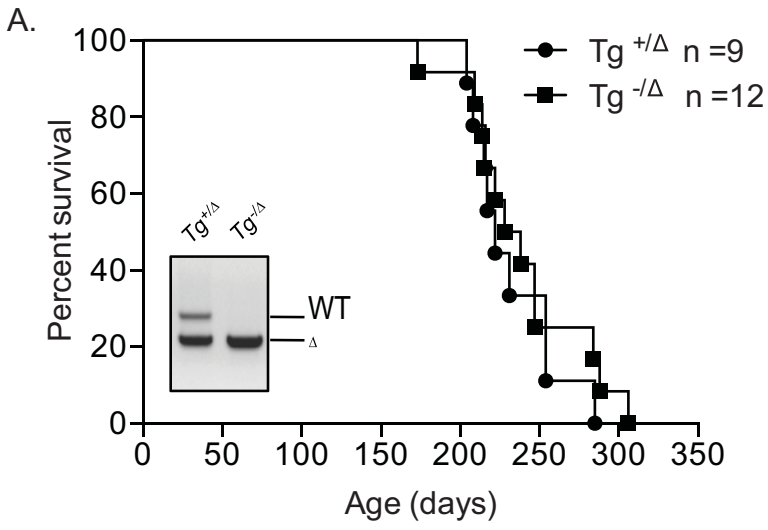

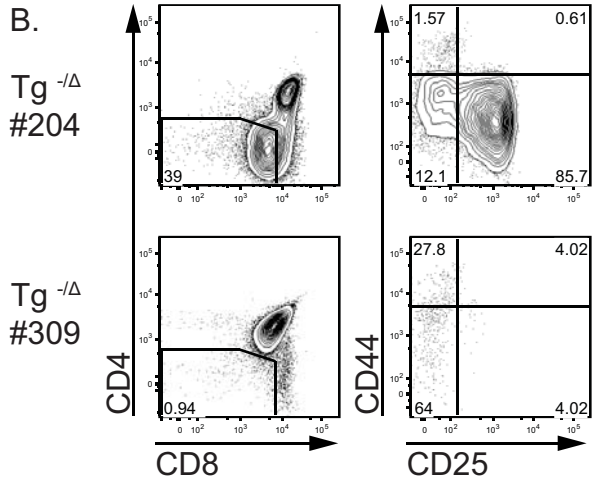

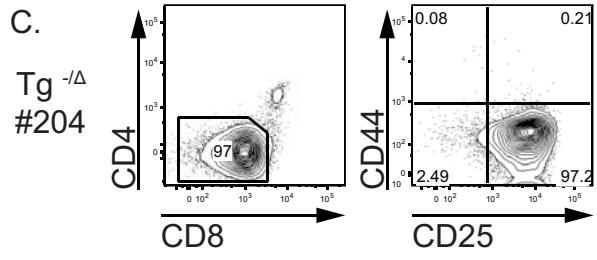

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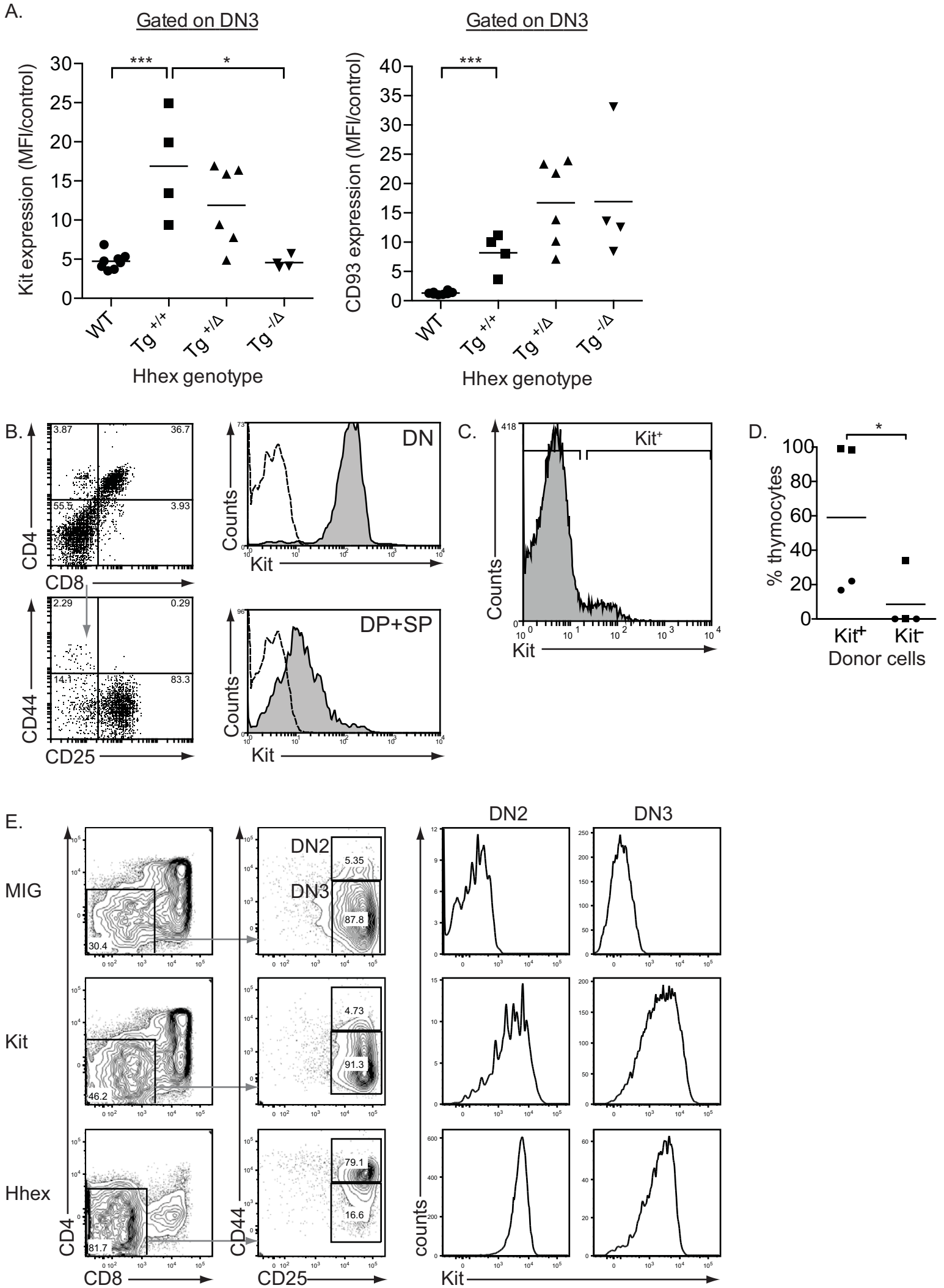

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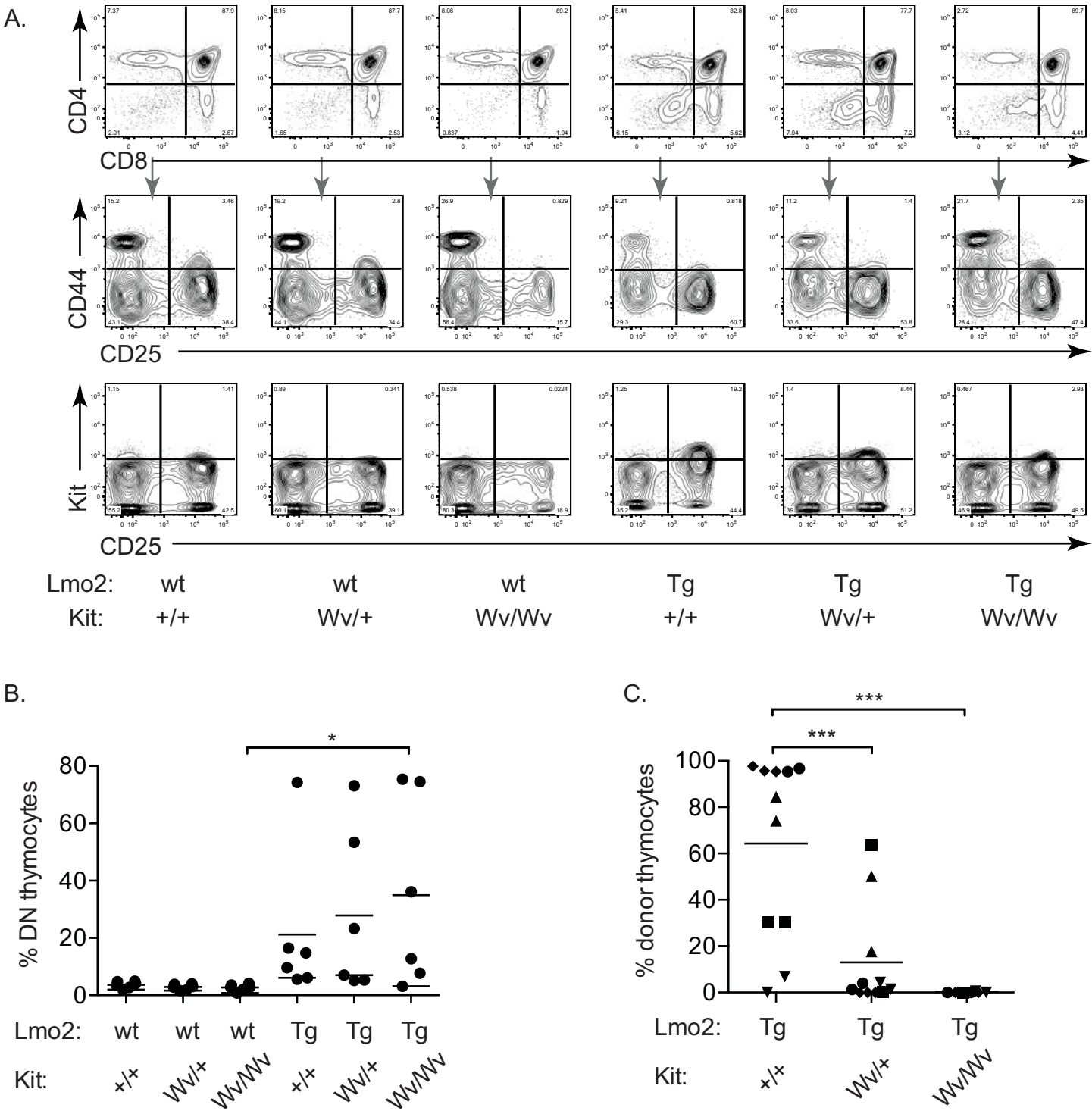

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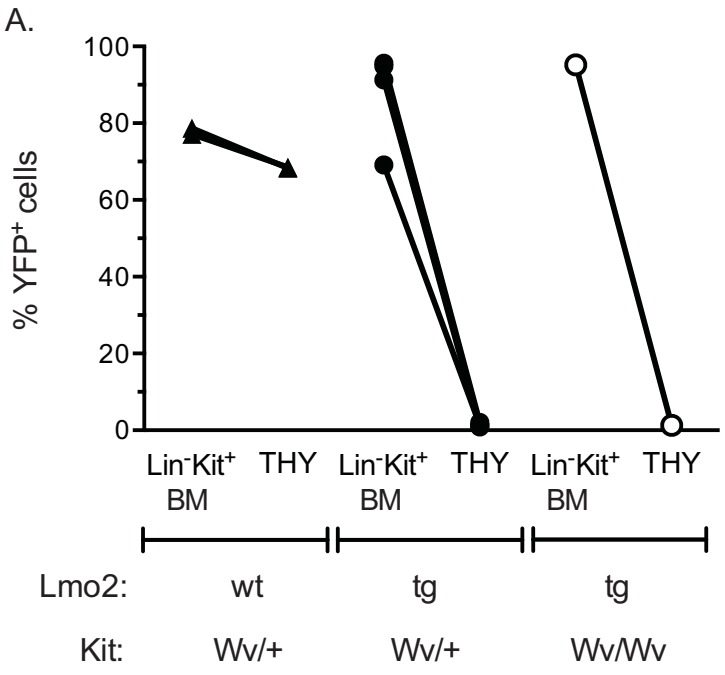

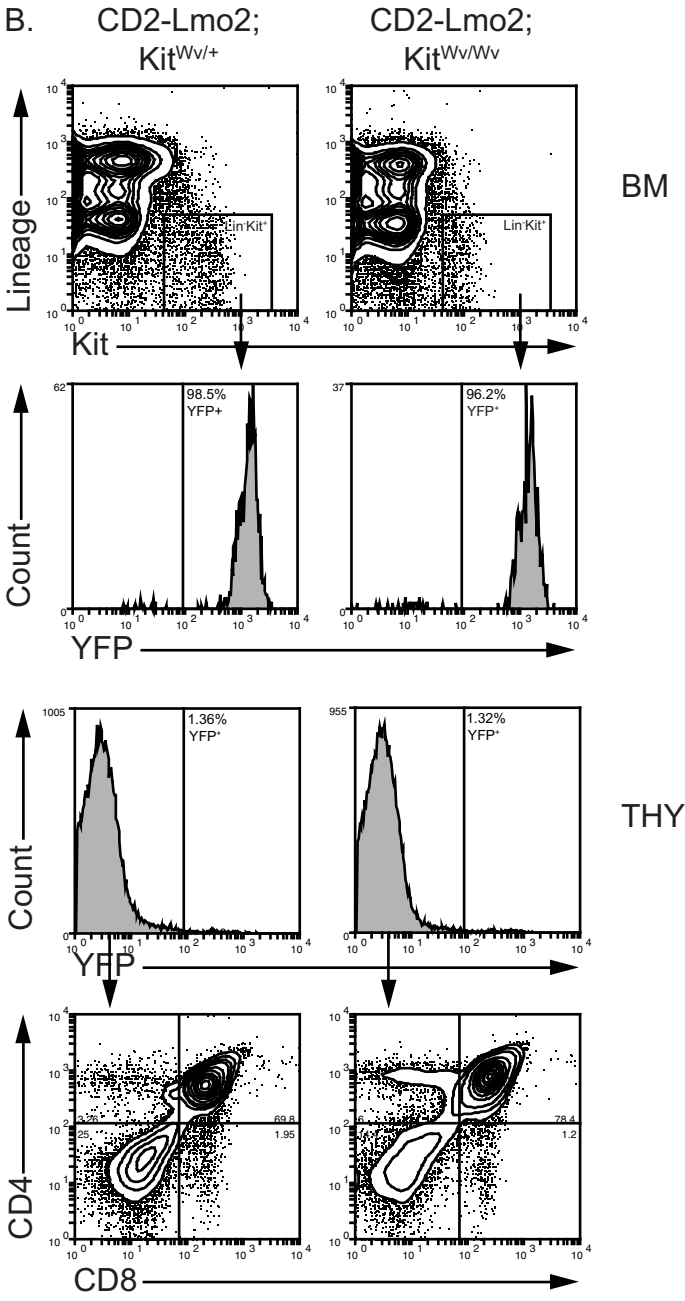

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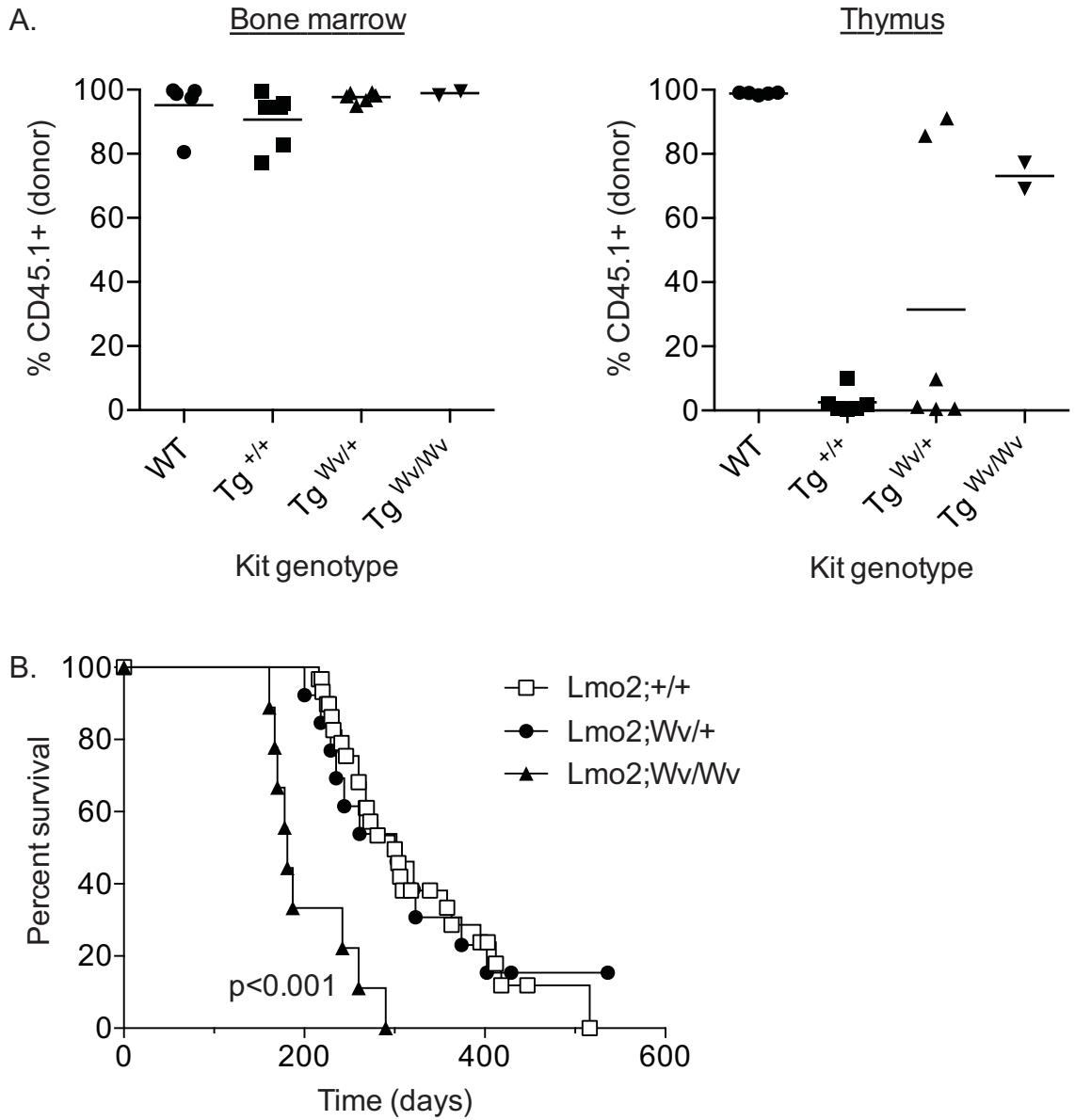


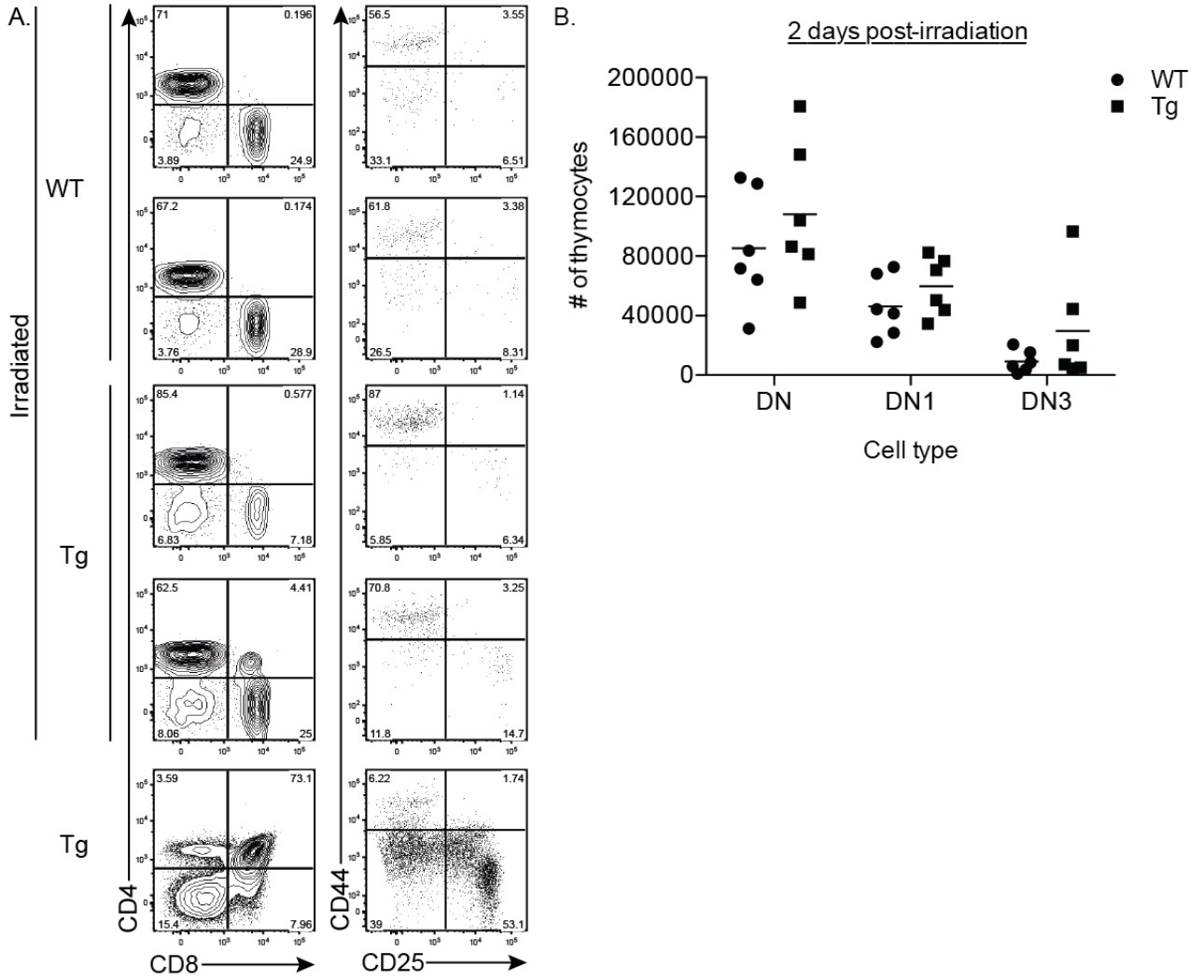
Table 1: Phenotyping of Lmo2;Hhex$^{+/\Delta}$ and Lmo2;Hhex$^{-/\Delta}$ thymomas

Comparison of T cell cellularity and transplantation capacity

	DN1(%)	DN2	DN3	DN4	DP	Transplant	Mean latency
$Tg^{+/\Delta}$							
#125	3.63	27.3	8.69	1.37	57.6	0 of 3	ND
#154	4.49	70.4	5.47	1.12	16.4	0 of 3	ND
#293	3.61	6.86	37.2	48.9	2.99	4 of 4	48
#326	2.59	30.4	12.9	47.6	4.05	4 of 4	24
#327	31.3	0.90	0.06	30.7	29.5	0 of 4	ND
#637	0.13	17.5	1.35	0.00	82	0 of 4	ND
$Tg^{-/\Delta}$							
#204	0.83	0.57	33.3	4.47	41.8	4 of 4	25
#291	4.05	0.43	1.76	53.1	39.3	3 of 3	45
#308	0.23	11.1	0.24	0.04	86.2	1 of 4	32
#309	0.24	0.02	0.03	0.51	97.1	0 of 4	ND
#595	0.15	0.60	0.10	0.22	97.9	0 of 4	ND

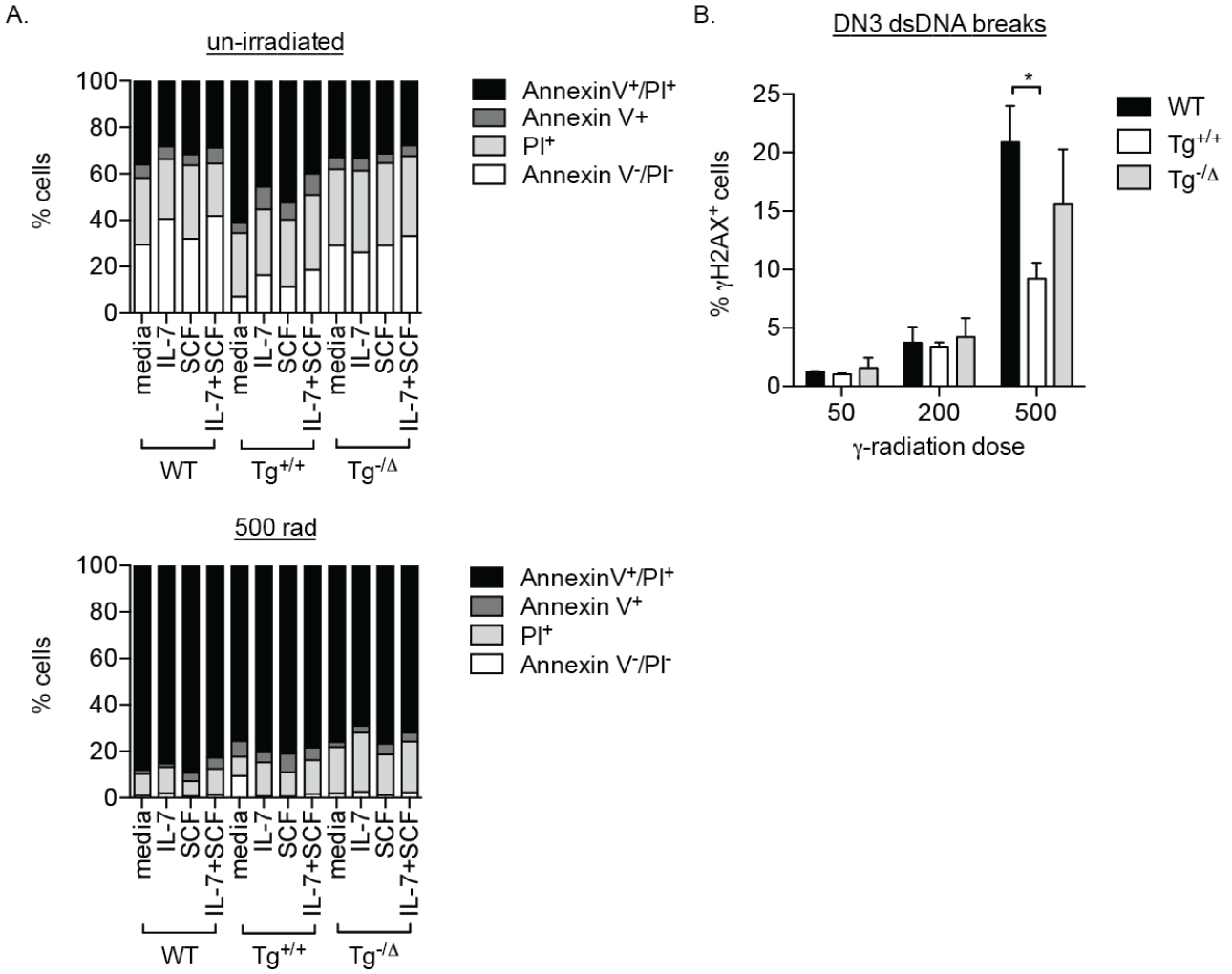
DN1(%) ($CD44^+CD25^-$); DN2 ($CD44^+CD25^+$); DN3 ($CD44^-CD25^+$); DN4 ($CD44^-CD25^-$); DP ($CD4^+CD8^+$). Transplant= number of mice which develop secondary leukemia within 130 days. Mean latency= Mean number of days of survival post transplant; N/D = No Disease up to 130 days after transplant.

Supplementary Figure 1. SHIELDS et al.



Supplementary Figure 1. Lmo2-transgenic thymocytes are not intrinsically radioresistant
A. Representative flow cytometry analysis of thymi from WT and CD2-Lmo2 (Tg) mice, 2 days after γ-irradiation (650 Rad). As a control for antibody staining, analysis of the thymus of an un-irradiated Tg mouse is also shown. Left panels show T-Cell cellularity in the thymus and right panels the proportion of DN1-DN4 thymocytes. In **B.** the number of total DN and DN1 and DN3 cells in the thymi of 6 WT and 6 Tg mice 2 days after γ-irradiation (650 Rad) was determined. Bars represent the mean for each population.

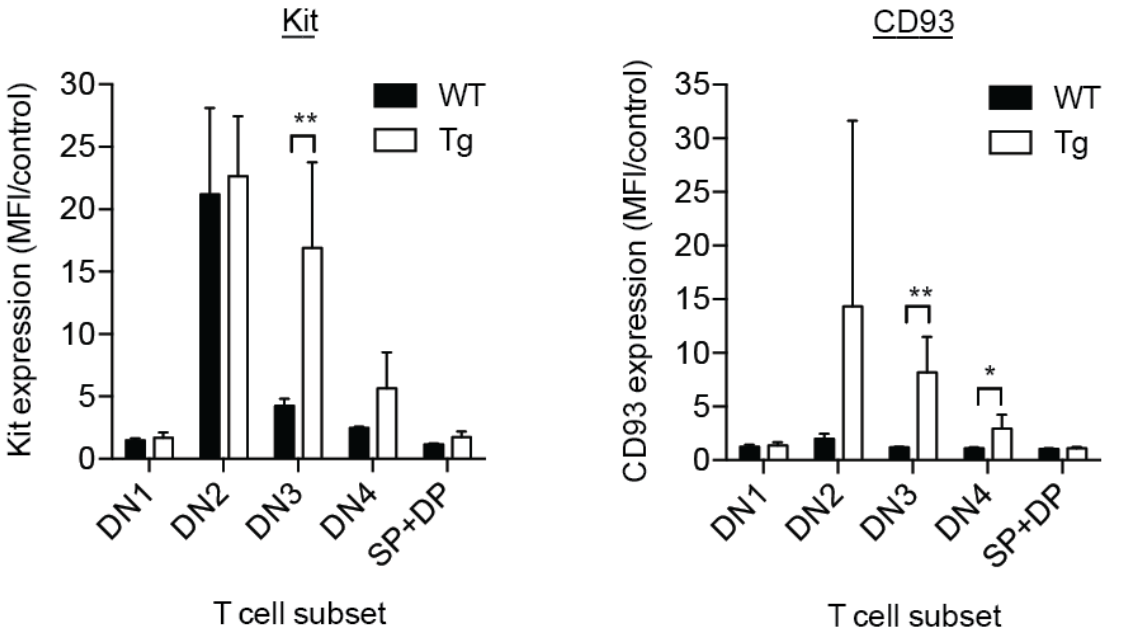
Supplementary Figure 2. SHIELDS et al.



Supplementary Figure 2. Lmo2-transgenic thymocytes exhibit reduced DNA damage post-irradiation

A-B. DN3 thymocytes of the indicated genotypes were FACS sorted then exposed to the indicated doses of γ-irradiation. In **A.** representative data is shown where cells were plated in IMDM+10% FCS with the indicated cytokines, then harvested 24hr later, stained with PI and Annexin V-FITC, then analysed by flow cytometry. In **B.** cells were fixed and stained with γH2AX-A647 antibodies and the percentage of γH2AX positive cells in γ-irradiated samples determined by flow cytometry. Data are the mean +/SD of 3-4 mice and p-values were calculated using Student's T test (*= $p<0.05$).

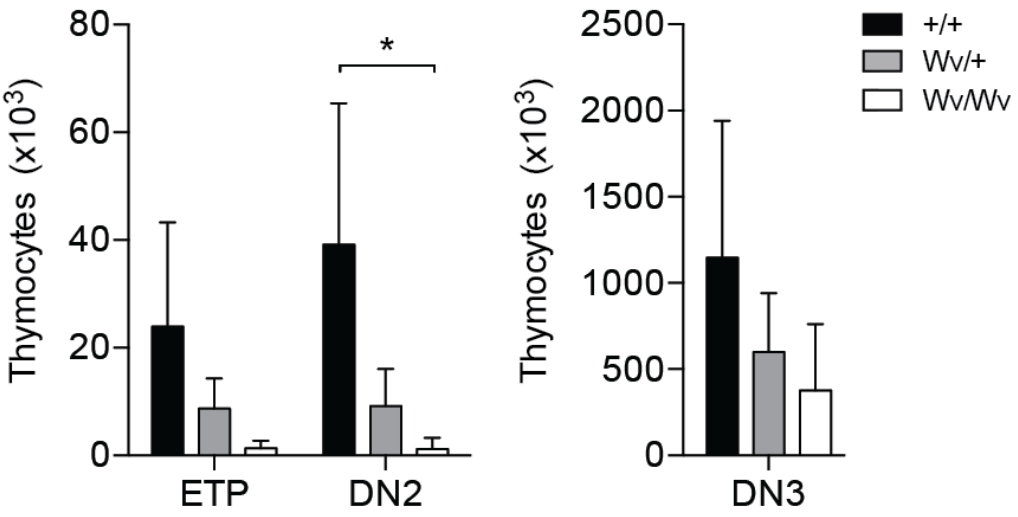
Supplementary Figure 3. SHIELDS et al.



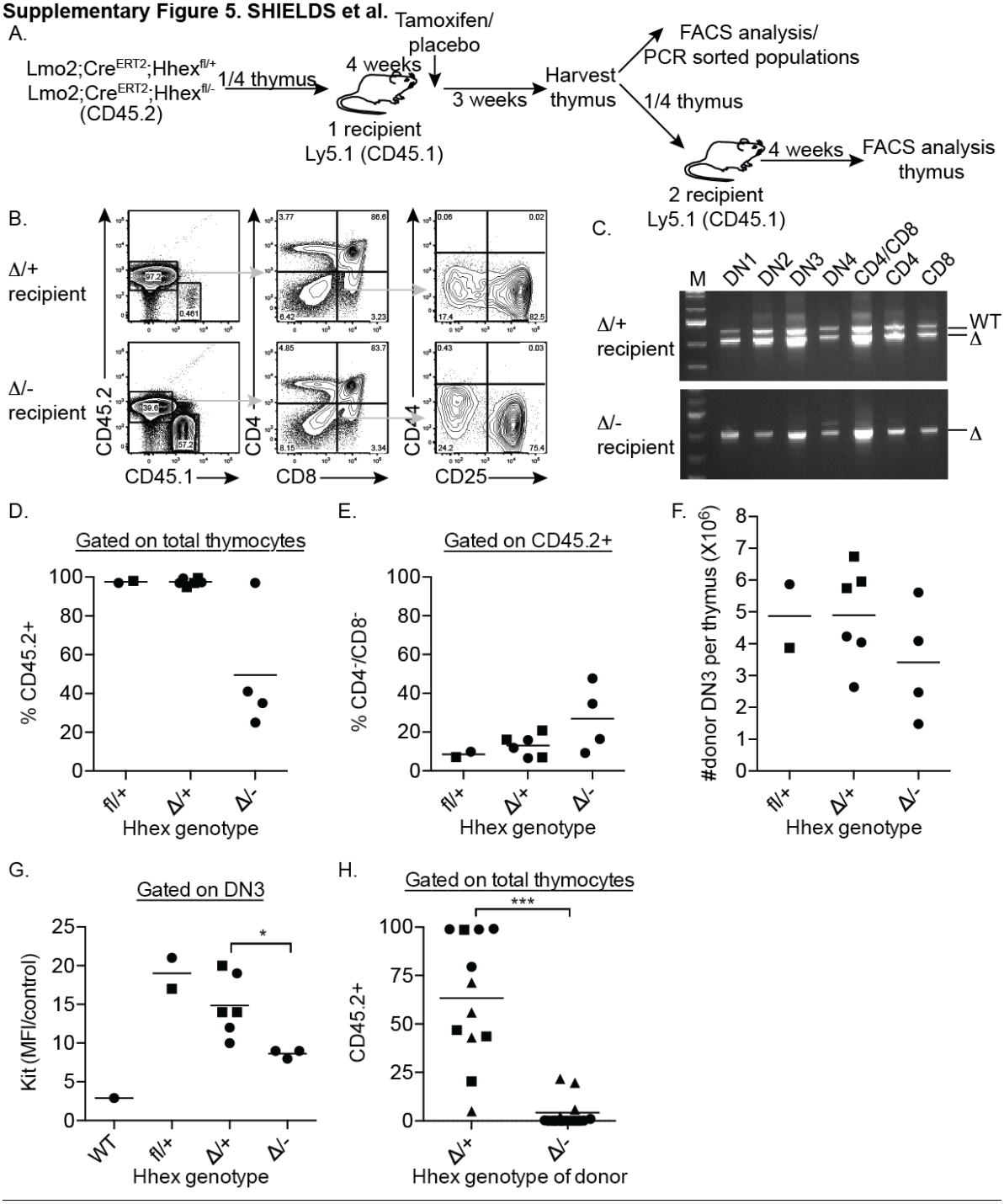
Supplementary Figure 3. Kit and CD93 expression on wild-type and Lmo2-trangsenic thymocytes

Relative Kit and CD93 expression was determined by dividing the Mean Fluorescence Intensity (MFI) attributed to binding of fluorescently labeled Kit or CD93 antibodies by the MFI attributed to the binding of fluorescently labeled isotype control antibody (Fluorescence Minus One (FMO)). Data shown are +SD of 4 mice per group and p-values were calculated using the Student's T test (*=$p<0.05$, **=$p<0.01$).

Supplementary Figure 4. SHIELDS et al.



Supplementary Figure 4. Immature thymocyte subsets are absent in Kit$^{Wv/Wv}$ mice
The percentage of ETP and DN2 populations were determined by flow cytometry. The number of ETPs and DN2s per thymus was calculated by multiplying the frequency of each population by the %DN cells and the total number of thymocytes. Data shown are from 4 mice of each genotype. p-values were calculated using the Student's T test (*=$p<0.05$).



Supplementary Figure 5. Hhex maintains transplantation capacity of Lmo2-induced DN3 thymocytes.

A. Schematic flow diagram of experimental design using Lmo2;Cre^{ERT2};Hhex$^{fl/-}$ and Lmo2;Cre^{ERT2};Hhex$^{fl/+}$ mice. **B.** Representative flow cytometry analysis of donor (CD45.2)/ recipient (CD45.1) chimerism in recipient thymus 7 weeks after transplant and 4 weeks after tamoxifen administration. In **C.** the Hhex deletion status of sorted DN1-DN4 fractions, CD4$^+$/CD8$^+$, CD4$^+$ and CD8$^+$ cells was determined by PCR. **D.** Analysis of % donor, **E.** analysis of % donor DN and **F**. analysis of total number of donor derived DN3s in recipient mice**.** In **G.** the relative levels of Kit expression on DN3 thymocytes (from **F.**) is shown. As a control, Kit expression on DN3 thymocytes from a Ly5.1 mouse (WT) is shown. In **H.** % donor thymocytes in secondary Ly5.1 recipient mice injected with thymocytes of the indicated Hhex genotypes (from **E.-G.**) is shown. Different symbols denote individual donors. p-values were calculated using Student's T test (*= $p<0.05$, ***= $p<0.0001$).