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Accessibility
Sex steroid blockade enhances thymopoiesis by modulating Notch signaling

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Abbreviations used: AR, androgen receptor; ARE, AR element; ChIP, chromatin immunoprecipitation; cTEC, cortical TEC; DHT, dihydrotestosterone; Dll4, Delta-like 4; EC, endothelial cell; GVHD, graft-versus-host disease; HSCT, hematopoietic stem cell transplantation; LCMV, lymphocytic choriomeningitis virus; LHRH, luteinizing hormone-releasing hormone; SL-TBI, sublethal total body irradiation; SSA, sex steroid ablation; TEC, thymic epithelial cell; TSC, thymic stromal cell.

Thymopoiesis is a complex process involving close interaction with the supporting nonhematopoietic stromal microenvironment, which is comprised of highly specialized thymic epithelial cells (TECs), endothelial cells (ECs), and fibroblasts (Takahama, 2006). It is these well-regulated cross talk interactions that guide sequential stages of T cell development by providing critical growth and differentiation factors as well as guiding the localization of thymocytes. In particular, expression of Notch ligands by TECs plays a crucial role and conditional deletion of Delta-like 4 (Dll4) within the thymic epithelium results in complete abrogation of thymopoiesis (Sambandam et al., 2005; Tan et al., 2005; Hozumi et al., 2008; Koch et al., 2008). Notably, TECs overexpressing Notch ligands are able to support T cell development ex vivo (Mohtashami et al., 2010). Although the role of Notch signaling in thymopoiesis has been extensively studied, the regulation of Notch ligands in TECs is poorly understood. Thus, identification and characterization of regulators...
that modulate Notch signaling will not only advance our understanding of T cell biology but also offer novel therapeutic targets for immune regenerative treatments.

Despite its importance for generating a diverse pool of naive T cells, thymic function progressively declines throughout life (Taub and Longo, 2005), which is particularly detrimental to the recovery of immune competence after cytoreductive chemotherapy, infection, and shock, as well as after radiation injury (Parkan and Weinberg, 1997; Edgar, 2008). Moreover, prolonged T cell deficiency is especially problematic in recipients of hematopoietic stem cell transplantation (HSCT), leading to increased morbidity and mortality from opportunistic infections and malignant relapses (Small et al., 1999; Storek et al., 2001). Therefore, development of strategies to improve thymic regeneration and reconstitution of the T cell pool represents a significant clinical challenge.

We and others have previously shown that sex steroid ablation (SSA) by surgical castration can boost BM and thymus lymphopoiesis, and promote recovery from autologous and allogeneic HSCT (allo-HSCT; Dudakov et al., 2009a,b; Goldberg et al., 2010). Importantly, reversible SSA can be achieved using agonists to the luteinizing hormone-releasing hormone (LHRH) receptor, which represents the gold standard clinical method for ablation of sex steroids in prostate cancer patients and is capable of mediating enhanced immune function (Sutherland et al., 2008; Goldberg et al., 2009). However, one major drawback of LHRH agonists (LHRH-Ag) is the initial spike in testosterone, which may actually result in increased morbidity and mortality (van Poppel and Nilsson, 2008).

Given the critical role of sex hormones in regulating thymic involution and its regeneration, we sought to investigate the molecular framework underlying these biological effects, and reveal Notch signaling as an important mediator in this pathway. Moreover, we identify LHRH antagonists (LHRH-Ant) as a rational alternate strategy to achieve SSA that circumvents the spike in sex steroids seen with LHRH agonists, thereby representing a safer approach for immune regeneration. Collectively, these findings will be crucial for developing a clinically appropriate regimen for improving immune function.

RESULTS AND DISCUSSION

Androgens regulate thymopoiesis by direct transcriptional control of Notch ligands

Previous studies have demonstrated that expression of androgen receptor (AR) in thymic stromal cells (TSCs) is indispensable for thymus rebound after surgical castration (Olsen et al., 2001; Lai et al., 2013). Given the primary role of the thymic stroma in thymopoiesis, we investigated the expression of key stromal-derived thymopoietic factors after testosterone treatment to identify candidate genes regulated by androgen signaling. Consistent with previous studies (Goldberg et al., 2007; Williams et al., 2008), we found significant down-regulation of thymic $IL7$ and $CCL25$ after androgen treatment; however, intriguingly we also found significantly lower levels of the Notch ligand $DLL4$ (Fig. 1 A). Consistent with previous reports (Koch et al., 2008), $DLL4$ was expressed by cortical TECs (cTECs) and ECs (Fig. 1 B); however, we observed that

**Figure 1.** AR negatively regulates $DLL4$ expression. (A) Molecular profiles of TSCs (CD45$^{-}$) 4 d after testosterone treatment ($n = 8$). (B) $DLL4$ expression in sorted TSC populations ($n = 12$). (C) $DLL4$ expression in sorted cTECs and ECs 4 d after testosterone treatment ($n = 12$). (A–C; 3.5-wk-old mice; mRNA expression relative to vehicle control and normalized to $β$-actin.) (D) Schematic representation of $DLL4$ promoter with ARE regions shaded in yellow and regions of high conservation in exons (dark blue), UTRs (light blue), or noncoding (pink). (E) ARE matrix logo as annotated in the JASPAR database is represented (top) with the ARE sequences identified in the $DLL4$ promoter. (F) $DLL4$ expression in C9 cells 24 h after treatment with DHT or MDV3100 (MDV). mRNA expression relative to untreated control ($n = 3$). (G) AR binding at region C of the $DLL4$ promoter 2 h after DHT and AR inhibitor MDV3100 treatment ($n = 4$). (H) HEK293T cells were transfected with a $DLL4$ promoter-driven reporter plasmid and luciferase activity was measured 48 h after transfection ($n = 4$). Data represent the mean + SEM of two independent experiments unless otherwise specified. *, $P ≤ 0.05$; **, $P ≤ 0.01$, unpaired Mann-Whitney U test.
testosterone treatment specifically down-regulatedDll4 expression in cTECs but not in ECs (Fig. 1 C).

One mechanism that AR uses to regulate its target genes is through its interaction with specific palindromic DNA binding consensus sequences containing two asymmetrical elements separated by a 3-bp spacer, 5′-GGA/TACANNTGTTCT-3′ (Roche et al., 1992). To determine if the observed transcriptional changes were the consequence of direct genomic regulation by the AR, we scrutinized the promoters of Il7, Ccl25, and Dll4 for putative AR elements (AREs). Although we could not detect any AREs in the promoters of Il7 or Ccl25 (unpublished data), suggesting an indirect mechanism of regulation, we identified eight AREs that were over-represented in the Dll4 promoter, six of which were equally distributed in two regions (Fig. 1, D and E).

To better evaluate the direct effect of sex steroids on Dll4 expression in cTECs, we treated the cortical cell line C9 with dihydrotestosterone (DHT). C9 cells treated with DHT exhibited a decrease in the expression of Dll4 24 h after treatment (Fig. 1 F), indicating the direct regulation of cTEC Dll4 expression by sex steroids. Importantly, the reduction in Dll4 expression after testosterone treatment was abrogated in the presence of the AR inhibitor MDV3100. To provide definitive evidence that AR directly regulates Dll4 transcription through physical interaction with its promoter, we performed chromatin immunoprecipitation (ChIP) using an antibody specific for AR in C9 cells. The Dll4 promoter was segmented into four regions according to the putative AREs (Fig. 1, D and E) and binding analyzed in each region with specific primers. We found fourth enrichment immunoprecipitated by AR antibody 2 h after DHT treatment in region C, in which three AREs clustered consecutively over a short sequence of 90 bp (Fig. 1 G). Once again, pretreatment with the AR inhibitor MDV3100 impeded this interaction. To provide functional evidence that region C was critical for AR-mediated inhibition of Dll4 expression, we generated mutant forms of the Dll4 promoter and analyzed their transactivation using a luciferase reporter assay. In the absence of region C, AR not only lost its inhibitory effects but also led to an increase in luciferase reporter activity. These results were also obtained when the CRE transgene was deleted under the control of CRE recombinase driven by the FoxN1 promoter. We observed an approximate 50% reduction of cTEC Dll4 expression (Fig. 2 C) and significantly decreased thymic cellularity in heterozygous Foxn1-cre::Dll4−/− mice (n = 7) and Foxn1-cre::Dll4−/− mice (n = 5). (E) Total thymic cellularity of K14-cre::Dll4−/− mice (n = 7) and K14-cre::Dll4−/− mice (n = 2). Data represents the mean + SEM of one experiment. *, P ≤ 0.05, unpaired Mann-Whitney U test.

Figure 2. Notch ligand Dll4 regulates thymopoiesis in a dosage-sensitive manner. (A and B) Lymphoid differentiation of sorted LSK after 12 d of culture with IL-7, FLT3-ligand, SCF, and scalar concentrations of rDLL4. (A) Absolute numbers of DN2 cells. (B) DN cell proportions, gated on Lin−CD45+. A and B represent the mean + SEM of one of two representative experiments performed in triplicate. (C) Dll4 expression in FACSSorted cTEC from 6-wk-old Foxn1-cre::Dll4−/−/mice and Foxn1-cre::Dll4+/− mice. (D) Total thymic cellularity of 5–6-wk-old Foxn1-cre::Dll4−/− mice (n = 7) and Foxn1-cre::Dll4−/− mice (n = 5). (E) Total thymic cellularity of K14-cre::Dll4−/− mice (n = 7) and K14-cre::Dll4−/− mice (n = 2). Data represents the mean + SEM of one experiment. *, P ≤ 0.05, unpaired Mann-Whitney U test.

Concentration and availability of Notch ligands affects thymopoiesis

To support our hypothesis that modulation of Dll4 expression represents a feasible mechanism by which sex steroids control thymopoiesis, we sought to determine if the dose of Dll4 could impact on the efficiency and progression of T cell differentiation. We addressed this by seeding BM lineage− Sca−1+ c−Kit+ (LSK) cells in OP9 co-culture with scalar concentrations of recombinant DLL4 (rDLL4). Consistent with our hypothesis and previous in vitro studies (Wong et al., 2004; Dallas et al., 2005; Mohtashami et al., 2010), increased availability of rDLL4 led to enhanced thymocyte progression, suggesting a dosagesensitive effect of DLL4 (Fig. 2, A and B). To evaluate the role of Dll4 concentration in endogenous thymopoiesis, we generated heterozygous mice with one allele of Dll4 deleted under the control of CRE recombinase driven by the FoxN1 promoter. We observed an approximate 50% reduction of cTEC Dll4 expression (Fig. 2 C) and significantly decreased thymic cellularity in heterozygous Foxn1-cre::Dll4−/− mice compared with littermate controls (Fig. 2 D). Similar results were also obtained when the CRE transgene was under control of the K14 promoter (Fig. 2 E). These findings provide evidence of the importance of Dll4 availability for regulating thymopoiesis.

LHRH receptor antagonists promote thymopoiesis without the degenerative phase observed with LHRH agonists

Due to its mechanism of initial sensitization of the LHRH receptor, LHRH-Ag triggers an early surge in sex steroids before castrate levels are eventually reached (van Poppel and Nilsson, 2008). Given our findings demonstrating the direct effect of sex steroids on DLL4 expression, we sought to evaluate an alternate approach to SSA that would minimize this impact. Direct blockade of the LHRH receptor by LHRH-Ant...
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LHRH-Ag treatment caused a dramatic degenerative effect as expected, due to the initial increase in testosterone levels, through the rapid increase in sex steroid levels within 24–48 h, which causes immediate cessation of sex steroid production and castrate levels of circulating sex steroids within 24–48 h (Fig. 3 A). As expected, due to the initial increase in testosterone levels, LHRH-Ag treatment caused a dramatic degenerative effect on thymic cellularity at early time points (days 7 and 14) after treatment (Fig. 3 B). Conversely, LHRH-Ant mediated a rapid increase in thymic size compared with untreated control and LHRH-Ag–treated mice as early as 7 d after treatment. This rapid increase in thymic size is consistent with the kinetics of thymic cellularity increases within 7 d in surgically castrated mice (Heng et al., 2005; Sutherland et al., 2005). This effect on total thymic cellularity was reflected by an increase in all developing thymocyte subsets at days 7 and 14 (Fig. 3 C). Together, these data indicate that LHRH-Ant promote immediate thymic enhancement, without the characteristic LHRH-Ag–induced depletion of cellularity.

To exclude any direct effect of LHRH-Ant on hematopoietic cells and T cell differentiation, we cultured sorted LSK on OP9-DL1 stromal cells in the presence of the LHRH-Ant. Treatment with the LHRH-Ant not only provided no benefit in T cell differentiation compared with control groups but could actually lead to a reduction in T cell progression at high concentration (Fig. 3 D), potentially due to the direct effects of LHRH signaling on thymocyte proliferation (Marchetti et al., 1989).

**Sex steroid inhibition via LHRH antagonism increases the expression of Dll4 and downstream Notch targets**

Given the effect of SSA on the stromal microenvironment (Fig. 1 A), we examined the expression of key thymopoietic factors in TSCs 7 d after LHRH-Ant treatment. Although we did see a decrease in expression after testosterone treatment (Fig. 1 A), in contrast to previous studies using surgical castration (Williams et al., 2008), we did not observe any significant increase in the expression of Ptcra after LHRH-Ant treatment (Fig. 3 E), although this could be due to differences in the experimental approach. In agreement with our data using testosterone (Fig. 1 A), we found significant up-regulation in the expression of Dll4 (Fig. 3 F), which is required for SSA-mediated thymic regeneration (Goldberg et al., 2007) and Dll4, after LHRH-Ant treatment (Fig. 3 E). Similar to our findings after testosterone treatment, the effects of LHRH-Ant treatment on Dll4 expression were restricted to cTECs and not ECs (Fig. 3 F). Consistent with an association between sex steroids and Notch signaling, we found increased expression of the downstream Notch targets Hes1, Ptna, and Cd25 in developing T cells after treatment with LHRH-Ant (Fig. 3, G and H).

**LHRH-Ant administration enhances thymopoiesis after immune injury**

Given its potent effect on steady-state thymopoiesis, we tested if treatment with LHRH-Ant could accelerate thymic regeneration and peripheral immune reconstitution in mice after immune injury caused by sublethal total body irradiation (SL-TBI). As expected, thymic cellularity was strongly depleted 7 d after SL-TBI and returned to untreated levels by day 42 (Fig. 4 A). Administration of LHRH-Ant resulted in enhanced recovery of thymic cellularity starting at day 7 after SL-TBI and remained significantly enlarged at day 42 compared with vehicle-treated and untreated control mice (Fig. 4 A).
We next sought to determine if enhanced thymopoiesis after LHRH-Ant treatment translated into improved peripheral immune recovery after SL-TBI. Total splenic cellularity was increased by day 28 after SL-TBI (Fig. 4 B), comprised of both CD4+ and CD8+ T cells (Fig. 4 C), with naive (CD62L+CD44−) T cells the most affected by LHRH-Ant treatment. Functionally, although there were no significant differences in the production of IFN-γ and IL-2 (not depicted), proliferation of CD4+ T cells upon TCR stimulation was significantly increased in those derived from LHRH-Ant–treated mice (Fig. 4 D).

One of the major clinical challenges that immunocompromised patients encounter is their increased susceptibility to infection (Wils et al., 2011). To assess the function of T cells and their ability to clear an infection, mice treated with vehicle or LHRH-Ant were challenged with lymphocytic choriomeningitis virus (LCMV) 14 d after SL-TBI. Demonstrating functional superiority of the TCR repertoire, mice treated with LHRH-Ant exhibited a significantly lower viral burden compared with vehicle-treated mice at day 8 after infection (Fig. 4 E). These studies are in agreement with a recent report showing that surgical castration can improve T cell functionality and viral clearance in aged mice (Heng et al., 2012).

LHRH-Ant treatment rapidly restores thymopoiesis after allo-HSCT and boosts peripheral immune reconstitution up to 3 mo. Delayed T cell reconstitution is a major clinical hurdle to allo-HSCT and we, and others, have previously shown that SSA using LHRH-Ag promotes recovery after autologous and allo-HSCT (Goldberg et al., 2007; Sutherland et al., 2008). We therefore investigated the effects of LHRH-Ant pretreatment on thymic and peripheral reconstitution of allo-HSCT recipients after SL-TBI.

Figure 4. LHRH-Ant treatment restores thymopoiesis and accelerates peripheral reconstitution in immunocompromised recipients after SL-TBI. (A–E) Mice were pretreated 5 d before SL-TBI with vehicle or LHRH-Ant. (A) Total thymic cellularity. (B) Total number of splenocytes. (C) Absolute number of total and naive (N, CD62Lhi CD44lo) T cells. (D) CD5+ enriched splenocytes obtained from vehicle and LHRH-Ant treated mice 42 d after SL-TBI were assessed in vitro for proliferation. (E) Viral titer of LCMV in the spleen 8 d after infection (where mice were infected 14 d after SL-TBI). (F–H) Allo-HSCT was performed by lethally irradiating B6 mice and transplanting with 5 × 10^6 B10.BR TCD BM cells (mice were pretreated with vehicle or LHRH-Ant). (F) Total thymic cellularity. (G) Absolute number of total, effector memory, central memory, and naive T cells in the spleen 3 mo after transplant. (H) Allo-HSCT recipients were spiked with 2 × 10^6 B10. BR T cells to induce GVHD and median survival time measured. Survival data were analyzed with the Mantel-Cox log-rank test. (I) Total thymic cellularity of 9-mo-old male mice 28 d after treatment. (J) Total thymic cellularity of 8–12-wk-old or 9-mo-old female mice 28 d after treatment. (K) Total thymic cellularity at day 7 after SL-TBI of young C57BL/6 female mice pretreated with vehicle or LHRH-Ant 5 d before SL-TBI. Results are expressed as combined mean ± SEM of 8–15 mice for each group representing at least two independent experiments. */^, P ≤ 0.05; **/^^, P ≤ 0.01; ***/^^^, P ≤ 0.001, compared with untreated (*) and vehicle-treated (^) mice. Statistical analysis between two groups was performed with an unpaired Mann-Whitney U test. ANOVA was used for comparisons between more than two groups.
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The analysis of developing thymocytes revealed a significant increase in all subsets for at least 3 mo after transplant, suggesting that the effects of LHRH-Ant were long-lasting (unpublished data). Characterization of peripheral T cell reconstitution 3 mo after transplant revealed a significant increase in the number of CD4+ and CD8+ T cell subsets (Fig. 4 G). Of note, the most abundant populations among these peripheral T cell subsets were naïve T cells, indicating a robust thymopoiesis in LHRH-Ant–treated mice compared with controls. Importantly, we did not observe significant differences in graft-versus-host disease (GVHD) mortality between LHRH-Ant–treated and control mice (Fig. 4 H). LHRH-Ant treatment therefore enhances thymic output and peripheral T cell function without exacerbating post-transplant complications.

LHRH antagonists enhance thymopoiesis in aged and female mice

We next investigated the capacity of LHRH-Ant to reverse the physiological decrease in thymic cellularity observed in aging mice. 9-mo-old male mice, which already have considerable age-related thymic involution (Heng et al., 2005), responded to the regenerative effects of LHRH-Ant with increased levels of total thymic cellularity and all thymic subsets compared with control mice (Fig. 4 I and not depicted), although the durability of this effect is not yet clear given recent reports (Griffith et al., 2012). In addition to the well-known effects of androgens on thymopoiesis, estrogen has also been shown to negatively impact thymic function and can contribute to its involution (Zoller and Kersh, 2006). Given the direct influence of LHRH on both androgens and estrogens, and the profound effect of LHRH-Ant on the regeneration of thymopoiesis in young and aged male mice, we tested the efficacy of LHRH-Ant on the thymic function in female mice. Consistent with our findings in male mice, and valuable for its wider clinical application, we found that LHRH-Ant treatment caused a significant increase in thymic cellularity 28 d after treatment in both young and aged female mice (Fig. 4 J). Importantly, the regenerative effect of LHRH-Ant treatment after SL-TBI in young male mice was also evident in female mice starting from 7 d after injury (Fig. 4 K).

Although it is well known that castration can reverse age-related thymic involution, increase thymic function, and boost T cell output in the periphery in mouse and human, the mechanisms underlying these effects are still poorly understood. This study offers one important mechanism by which sex steroids, and by extension SSA, can mediate its effect on thymic function: the direct regulation of Notch signaling. Importantly, we also demonstrated that this sensitivity to sex steroids of Notch ligand availability can have profound implications on thymopoiesis, with in vivo depletion (but not abrogation) of DLL4 expression leading to significantly reduced thymopoiesis. Given the prevalence in use of LHRH-Ag to achieve castrate levels of sex steroids, and our findings that the surge in testosterone can reduce expression of DLL4 and consequently lead to significant depletion of thymus cellularity, alternate clinical approaches to achieve reversible SSA are desirable. Here, we also present data to demonstrate that use of an LHRH-Ant can forego any surge in sex steroids and lead to rapid promotion of thymopoiesis. Collectively, these findings reveal not only a novel mechanism by which sex steroids regulate thymopoiesis but also provide evidence for a novel therapeutic alternative for regeneration of the thymus and immune function.

MATERIALS AND METHODS

Mice and BM transplantation. C57BL/6 (H-2b) male mice (The Jackson Laboratory) between 8 and 12 wk of age were used unless otherwise stated. Specifically, 3.5-wk-old male mice in Fig. 1, 6-wk-old male mice in Fig. 2 (C and D), and 9-mo-old male and female mice in Fig. 4 (I–J) were used. DLL4 flox/flox mice were provided by D. Shima (UCL, London, England) and generated as previously described (Hozumi et al., 2008). B6(Cg-Foxn1<tm1Wtsi>)/N and B6N-Cg-Tg(KRT14-cre)1Amc/J were obtained from The Jackson Laboratory. To model thymic damage and lymphoid depletion, C57BL/6 received SL-TBI with no hematopoietic rescue. All SL-TBI experiments were performed with a Cs-137 γ-radiation source. The HSCT procedure was performed as previously described (Goldberg et al., 2009), with 1,100 cGy split-dosed lethal irradiation of C57BL/6 hosts receiving 5 × 10^6 T cell–depleted MHC-mismatched BM cells from B10.BR (H-2k) donor mice (The Jackson Laboratory). BM cells were T cell depleted by incubation with anti–Thy-1.2 for 40 min at 4°C and incubation with Low-TOX-M rabbit complement (Cedarlane Laboratories) for 40 min at 37°C. Cells were transplanted by tail vein infusion (0.2 ml total volume) into lethally irradiated recipients (C57BL/6) on day 0. To model GVHD, donor splenic T cells (5 × 10^6 B10.BR) were enriched using MACS CD5 purification (routine purity > 90% purity: Miltenyi Biotec). Recipient mice were monitored weekly for survival and clinical GVHD symptoms as previously described (Goldberg et al., 2009). All animal protocols were approved by the Memorial Sloan-Kettering Cancer Center (MSKCC) Institutional Animal Care and Use Committee (IACUC).

Reagents. Degarelix (as acetate), a third generation LHRH-Ant (Firmagon), was resuspended in sterile water for injection and administered s.c. to mice at a dose of 40 μg/g. Lupron (11.25-mg 3-mo depot), an LHRH-Ag, was prepared according to the manufacturer’s instructions and administered intramuscularly to mice at a dose of 20 μg/g. Degarelix and Lupron were purchased from the MSKCC Pharmacy. Testosterone propionate (Sigma-Aldrich) was resuspended in peanut oil and injected daily s.c. (1 mg/mouse) in 100 μl. Surface antibodies against CD44 (IM7), EpCAM (G8.8), PDGFRα (AP5), PECAM-1 (390), CD45 (30-F11), and H-2Kk (AF3-12.1.3) were purchased from eBioscience; anti-Ly-51 (BP-1), CD34 (RAM34), CD62L (MEL-14), H-2Kb (AF6-88.5), IFN-γ (XM1G2.1), IL-2 (JES6-5H4), c-Kit (2B8), CD3e (145-2C11), CD25 (PC61), TER-119 (TER-110), and CD8α (53–6.7) were purchased from BD; anti-CD4 (RM4-5) and B220 (RA3-6B2) were purchased from Invitrogen; anti-CD44 (IM7), CD90.2 (30-H12), and H-2Kk (AF3-12.1.3) were purchased from BioLegend; and Ulex europaeus agglutinin 1 (UEA-1) was purchased from Vector Laboratories. For in vitro cultures of T cell differentiation (Fig. 2, A and B), cells were stained with antibodies to lineage (Lin)-specific markers: CD8, CD11c, NK1.1, CD3, CD4, B220, CD11b, and GR-1.

Flow cytometry and cell isolation. Cells were incubated for 15 min at 4°C with antibodies and washed twice with FACS buffer. Flow cytometric analysis was performed on an LSRII (BD) using FACSDiva (BD) or FlowJo (Tree Star). To isolate LSK cells, murine BM cells were first lineaged depleted using a MACS-based cell depletion kit (Miltenyi Biotec), stained with a lineage panel (CD3, NK1.1, Gr-1, CD11b, CD19, CD4, CD8), Sca-1, and c-kit antibodies, and then Lineage–Sca-1–c-Kit– cells were selected using a
FACS Aria II cell sorter (BD). Individual or pooled single cell suspensions of freshly dissected thymi were obtained by either mechanical dissociation or enzymatic digestion, as previously described (Gray et al., 2008). CD45^-cells for quantitative PCR experiments were enriched by magnetic bead separation using an Auto MACS (Miltenyi Biotech) or MACS separation LD columns (Miltenyi Biotech). Thymic cells were sorted on a FACS Aria II (BD) as follows: cTECs, CD45^-MHCII^+EpCAM^-UEA-1^+Ly-51^hi; and ECs, CD45^-EpCAM^-MHCII^-CD31^+.

Cell culture. cTEC cell line C9 cells were maintained in culture in DME supplemented with 10% FCS, 100 U/ml penicillin, and 100 µg/ml streptomycin. For experiments with DHT (Sigma-Aldrich) and MVD3100 (Selleck), cells were maintained in DME supplemented with 10% charcoal/dextran-stripped FCS (Gemini Bioproducts). MVD3100 was reconstituted in DMSO and used in culture at the final concentration of 10 µM. For experiments with DHT and MVD3100, cells were pretreated with MVD3100 30 min before DHT treatment. Splenocytes for in vitro studies were cultured in RPMI supplemented with 10% FCS, 2 mM l-glutamine, 1 mM sodium pyruvate, 50 µM 2-mercaptoethanol, 100 U/ml penicillin, and 100 µg/ml streptomycin.

OP9-DLL4 cultures. OP9-DLL1, a mouse BM stromal cell line of (C57BL/6 x C3H)F1 progeny, was obtained under an MTA from J.-C. Zúñiga-Pflücker (University of Toronto, Toronto, Canada). Cell culture medium consisted of αMEM (Invitrogen) supplemented with 20% heat-inactivated FBS, 100 U/ml penicillin (Invitrogen), and 100 µg/ml streptomycin (Invitrogen). T cell precursors were generated in vitro as described previously with modifications (Min et al., 2002). In brief, LSK cells were sorted as described above and added to tissue culture-treated polysyrene 24-well tissue culture plates that were seeded with 4,000 OP9-DLL1 cells per cm² the day before. The tissue culture media was supplemented with 10 ng/ml IL-7 (Miltenyi Biotech) and 10 ng/ml FLT3-ligand (Miltenyi Biotech) and different concentrations of Dregarelix or vehicle (man-nilotid). Cultures were passaged every 4 d.

DLL4 cultures. LSK cells were cultured in non-tissue culture-treated polystyrene 24-well plates that were precoated with 5 µg/ml CH-296 fibronectin (R&D Systems). Cell culture medium consisted of αMEM (Invitrogen) supplemented with 20% heat-inactivated FBS, 100 U/ml penicillin (Invitrogen), and 100 µg/ml streptomycin (Invitrogen). The media was supplemented with 5 ng/ml IL-7 (Miltenyi Biotech), 100 ng/ml FLT3-ligand (Miltenyi Biotech), and 100 ng/ml stem cell factor (SCF; Miltenyi Biotech) and changed every 4 d.

T cell in vitro assay. To evaluate T cell proliferation and cytokine production, spleens were harvested 42 d after SL-TBI and T cells were purified by CD5^-MACS selection. Half of the cells were stimulated with 50 ng/ml PMA, 1 µg/ml Ionomycin, and 1 µl/ml Golgi Plug (BD) and cytokines evaluated by intracellular flow cytometric analysis. The remaining cells were CFSE (Invitrogen)-labeled and plated on aCD3/aCD28 (5 and 1 µg, respectively)-precocated plates. Proliferation was assessed by measuring the number of cell divisions 2 d after stimulation by flow cytometric analysis.

Real-time PCR. Reverse transcription PCR was performed with Quantitect reverse transcription kit (QIAGEN). For real-time PCR, the following specific primer and probe sets were obtained (Applied Biosystems): β-actin (Mm01265674_g1), CD25 (Mm00436443_m1), CD12 (Mm00445553_m1), Dll4 (Mm01279269_m1), Dll4 (Mm00446199_m1), Foxn1 (Mm00439460_m1), Ho1 (Mm01342805_m1), B15 (Mm00432100_m1), B18 (Mm04342255_m1), Il7rB (Mm00434228_m1), Il7r (Mm01298903_m1), Kgf (Mm04339211_m1), Pten (Mm01281478_m1), and Sip1 (Mm04492792_m1). PCR was done on an ABI 7500 (Applied Biosystems) or Step-One Plus (Applied Biosystems) with TaqMan Universal PCR Master Mix (Applied Biosystems). Relative amounts of mRNA transcripts were calculated by the comparative ΔCt method.

Identification of transcription factor–binding sites. Whole genome rVISTA (Zambon et al., 2005) at a stringency of P < 0.005 was used to predict potential AR binding sites 5000 bp upstream of the transcription starting site. Putative transcription factor–binding sites were then further characterized using JASPAR database (Bryne et al., 2008).

Luciferase assay. Dll4 promoter containing the putative ARE A, B, and C regions was amplified from the BMC clone RPCI-23 46Δ4 using the following primers: forward, 5’-AGAGCTACCCGTGAAAGG-3’; and reverse, 5’-TACGTTGACCGAGGAGCGAAACCC-3’. PCR product was cloned using the pCR2.1 TOPO TA cloning kit (Invitrogen) and then subcloned into pGL4.23 vector (Promega) using the restriction sites Kpn1 and Xho1. The truncated form of Dll4 promoter containing only putative ARE A and B regions, −1644 bp, was cloned using the following primers: forward, 5’-GGTACCTCACCTGGGAGCGAAACCC-3’; and reverse, 5’-TACGGTGCAGGAGGAAACCC-3’. The PCR products were similarly subcloned into pGL4.23 vector. The Dll4 promoter-pGL4.23 vectors were then co-transfected into HEK293T cells using lipid-based 293T TransIT Reagent (Mirus Bio) with either pUC19 empty vector or pWZL-AR vector (Berger et al., 2004), as well as pHRL-TK vector as an internal control. After 48 h, cells were collected and the luciferase activity was evaluated using Dual-Luciferase Reporter Assay System (Promega) as per the manufacturer’s instructions.

Statistics. All experiments were performed at least twice. All statistics were calculated and graphs generated using Prism 6 (GraphPad Software).

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