

Dll4–Notch signaling in Flt3-independent dendritic cell development and autoimmunity in mice

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Delta-like ligand 4 (Dll4)–Notch signaling is essential for T cell development and alternative thymic lineage decisions. How Dll4–Notch signaling affects pro-T cell fate and thymic dendritic cell (tDC) development is unknown. We found that Dll4 pharmacological blockade induces accumulation of tDCs and CD4⁺CD25⁺FoxP3⁺ regulatory T cells (T_{reg} cells) in the thymic cortex. Both genetic inactivation models and anti-Dll4 antibody (Ab) treatment promote de novo natural T_{reg} cell expansion by a DC-dependent mechanism that requires major histocompatibility complex II expression on DCs. Anti-Dll4 treatment converts CD4⁺CD8[−]c-kit⁺CD44⁺CD25[−] (DN1) T cell progenitors to immature DCs that induce ex vivo differentiation of naive CD4⁺ T cells into T_{reg} cells. Induction of these tolerogenic DN1-derived tDCs and the ensuing expansion of T_{reg} cells are Fms-like tyrosine kinase 3 (Flt3) independent, occur in the context of transcriptional up-regulation of *PU.1*, *lrf-4*, *lrf-8*, and *CSF-1*, genes critical for DC differentiation, and are abrogated in thymectomized mice. Anti-Dll4 treatment fully prevents type 1 diabetes (T1D) via a T_{reg} cell-mediated mechanism and inhibits CD8⁺ T cell pancreatic islet infiltration. Furthermore, a single injection of anti-Dll4 Ab reverses established T1D. Disease remission and recurrence are correlated with increased T_{reg} cell numbers in the pancreas-draining lymph nodes. These results identify Dll4–Notch as a novel Flt3–alternative pathway important for regulating tDC-mediated T_{reg} cell homeostasis and autoimmunity.

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Abbreviations used: Ab, antibody; cDC, conventional DC; CDP, common DC progenitor; DN, double negative; DP, double positive; DT, diphtheria toxin; imDC, immature DC; mDC, mature DC; MDP, macrophage DC progenitor; NOD, nonobese diabetic; nT_{reg} cell, natural T_{reg} cell; pDC, plasmacytoid DC; PLN, pancreas-draining LN; polyI:C, polyinosinic:polycytidylic acid; pStat5, phosphorylated Stat5; SP, single positive; T1D, type 1 diabetes; tDC, thymic DC; TEC, thymic epithelial cell.

Interaction between Notch receptors and their ligands represents an evolutionarily conserved signaling pathway important not only for cell fate commitment in the developing thymus (Radtke et al., 2010) but also for regulating

lineage decisions in hematopoiesis (Maillard et al., 2005). Genetic inactivation experiments showed that signaling mediated through the Notch 1 (N1) receptor plays an important role in T cell lineage commitment and maturation (Radtke et al., 1999, 2010; Wilson et al., 2001). It has been shown that Delta-like ligand 4 (Dll4)

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is the essential and nonredundant N1 receptor ligand (Koch et al., 2008), and its specific inactivation on thymic epithelial cells (TECs) leads to a block in T cell development accompanied by ectopic appearance of an alternative B cell lineage within the thymus (Koch et al., 2008). Recently, using pharmacological Dll4 blockade, we showed that ongoing Dll4–Notch signaling is required for T cell homeostasis in the thymus of both young and aged mice (Billiard et al., 2011). Thus, sustained Dll4–N1 signaling is necessary for maintaining the T cell lineage fate and inhibiting the potential of early T cell progenitors to generate alternative lineages (Hozumi et al., 2008).

The role of Notch signaling in the development of alternative lineages in thymus has been recently revisited, as controversial results about its specific impact on DC differentiation have been reported (Radtke et al., 2000; Feyerabend et al., 2009; Cheng et al., 2010; Di Santo, 2010). Although initial experiments using mice reconstituted with BM precursors deficient for N1 displayed normal DC frequencies in thymus and periphery (Radtke et al., 2000), a study showed that embryonic stem cells lacking N1 had a severely reduced capacity to generate DCs (Cheng et al., 2010). Moreover, a recent study demonstrated that deletion of N1 under the control of *Cpa3*, a gene encoding mast cell carboxypeptidase A, converted pro-T cells to mature DCs (mDCs) in thymus (Feyerabend et al., 2009). It has been established that thymic DCs (tDCs) originate from hematopoietic stem cells, yet their immediate precursors have been more difficult to identify (Wu et al., 1996; Merad and Manz, 2009). Current evidence suggests that a majority of DCs are generated from early intrathymic precursors (Merad and Manz, 2009; Zhou et al., 2009) versus a minority that derives extrathymically and continually migrates into the thymus (Wu and Shortman, 2005; Li et al., 2009). Thus, although there is increasing evidence implicating N1 in the development of intra-tDCs, the involvement of the Notch pathway and specifically the role of Dll4 in this process, as well as the molecular signals promoting Notch-mediated tDC expansion, remain unknown.

tDCs are thought to participate in central tolerance by negative selection of autoreactive T cells (Brocker et al., 1997) and/or induction of regulatory T cells (T_{reg} cells; Watanabe et al., 2005). T_{reg} cells ($CD4^+CD25^+FoxP3^+$ T cells) are specialized lymphocytes that play a major role in maintaining immune tolerance (Sakaguchi et al., 2008) and suppressing the development of organ-specific autoimmune diseases such as type 1 diabetes (T1D; Salomon et al., 2000; Tarbell et al., 2004; Darrasse-Jèze et al., 2009). Recently, a regulatory feedback loop between DCs and T_{reg} cells in the periphery has been described (Darrasse-Jèze et al., 2009; Swee et al., 2009). Moreover, it has been demonstrated that Fms-like tyrosine kinase 3 ligand (Flt3-L) is sufficient and essential for the development of blood and peripheral lymphoid organ DCs (Waskow et al., 2008) and that loss of T_{reg} cells increases DC division via an Flt3–dependent mechanism (Liu et al., 2009).

To explore the intriguing possibility that Dll4–Notch signaling functions as an Flt3–alternative pathway in regulating DC development and T_{reg} cell homeostasis, we used an anti-Dll4 antibody (Ab; Billiard et al., 2011), as well as genetic inactivation models to suppress this pathway. We found that Dll4–Notch signaling blockade induced expansion of immature tDCs originating from DN1 pro-T cells, independently of Flt3 and in the context of transcriptional up-regulation of *PU.1*, *Irf-4*, *Irf-8*, and *CSF-1*, genes critical for DC differentiation. Furthermore, we showed that DN1-derived DCs promote the differentiation of naive $CD4^+$ T cells into T_{reg} cells in vitro and de novo $CD25^+FoxP3^+$ natural T_{reg} cell (nT_{reg} cell) enrichment in vivo by a mechanism that required MHCII expression on the DC surface. Finally, we identified the physiological relevance of this treatment in a spontaneous autoimmune model (T1D). Thus, we reveal a novel function for Dll4–Notch as an Flt3–independent pathway essential for regulating tDC homeostasis and suppressing autoimmunity.

RESULTS

Nicastrin inactivation induces tDC and T_{reg} cell enrichment

Recent findings suggest that N1 is potentially implicated in alternative DC lineage development in thymus (Feyerabend et al., 2009; Cheng et al., 2010). In addition, the existence of a homeostatic feedback loop between DCs and T_{reg} cells (Darrasse-Jèze et al., 2009; Swee et al., 2009) has been revealed. To confirm and extend previous studies, we addressed the question of whether Notch receptor signaling inhibition could affect both tDC and T_{reg} cell homeostasis. We conditionally deleted Nicastrin ($Ncstn^{-/-}$), a γ -secretase signaling component located downstream of the Notch receptor (N1–4) signaling cascade (Klinakis et al., 2011). We found that Nicastrin deficiency induced an accumulation of pro-T cells at DN1 stage ($CD4^-CD8^-c-kit^+CD44^+CD25^-$) without affecting the final stage of T cell differentiation ($CD4^+$, $CD8^+$ T cells; not depicted). Within thymus, we observed an increased frequency (two- to fivefold) of plasmacytoid DCs (pDCs; $CD11c^+B220^+/PDCA-1^+$; $P < 0.01$), conventional DCs (cDCs; $CD11c^+B220^-$; $P < 0.01$), and immature DCs (imDCs; $CD11c^+MHCII^b$; $P < 0.05$) in $Ncstn^{-/-}$ mice (Fig. 1 A, left). The expansion of these cells was not reflected in absolute numbers because of a severe reduction in thymic cellularity upon Notch signaling blockade (Fig. 1, A [right] and B), as previously observed in several studies using genetic N1 inactivation models (Hozumi et al., 2008; Koch et al., 2008; Feyerabend et al., 2009). Thus, Nicastrin deletion mimics the effect of N1 deficiency on balancing T versus DC development (Feyerabend et al., 2009). Similarly, $Ncstn^{-/-}$ mice showed a fivefold increase in the frequency of T_{reg} cells ($P < 0.001$; Fig. 1 C), although this expansion was not reflected in absolute numbers (Fig. 1 D, left) because of the overall decrease of thymic cellularity. Surprisingly, we observed an enrichment of T_{reg} cells compared with effector T cells (T_{eff} cells; $CD4^+CD25^-FoxP3^-$) in thymus, as demonstrated by a sixfold increase in the ratio of T_{reg} cell versus T_{eff} cell numbers ($P < 0.001$; Fig. 1 D, right). Thus, a majority of

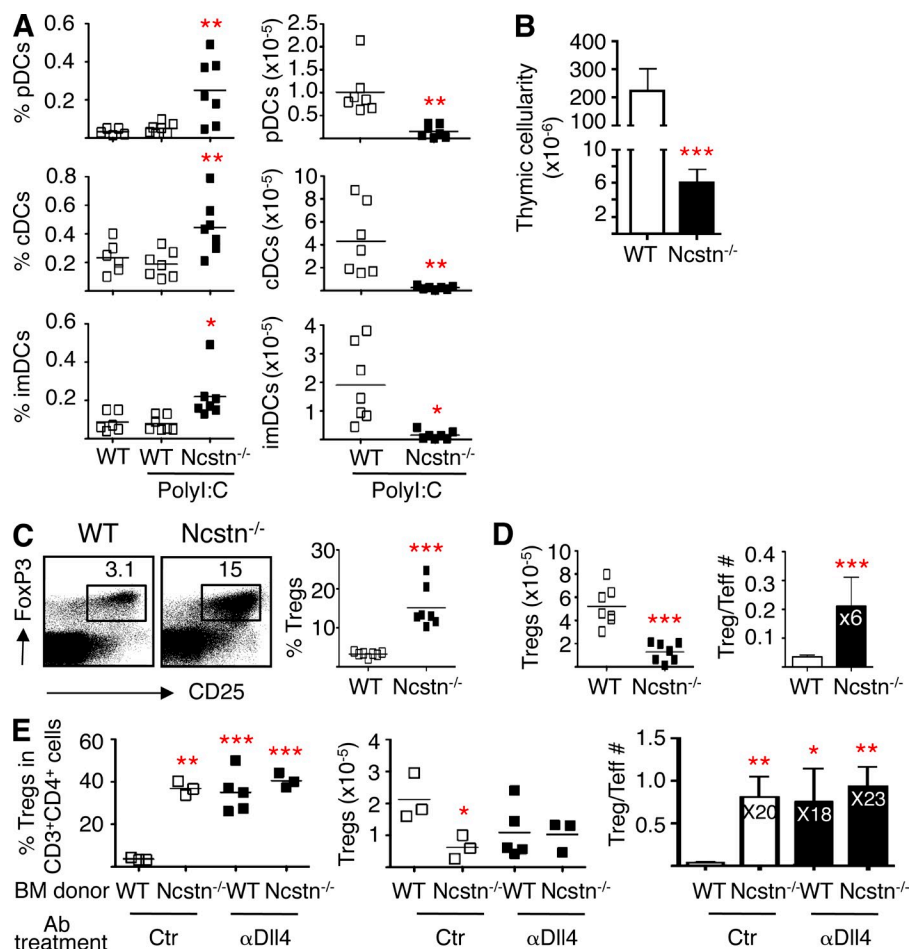


Figure 1. Nicastrin inactivation induces tDC and T_{reg} cell enrichment. (A) Percentages (left) and absolute numbers (right) of pDCs (CD11c⁺B220⁺/PDCA-1⁺), cDCs (CD11c⁺B220⁻), and imDCs (CD11c⁺MHCII^{lo}) in *Ncstn*^{fl/fl}Mx1-Cre⁺ mice (*Ncstn*^{-/-}) versus WT littermates after 3.5 wk of polyI:C treatment. Baseline levels in untreated WT mice are shown. (B) Thymus cellularity in *Ncstn*^{-/-} mice versus WT littermate controls. (C) Percentages of T_{reg} cells (FoxP3⁺CD25⁺) among CD3⁺CD4⁺ cells. (D) Absolute numbers of T_{reg} cells (left) and proportion of T_{reg} cell versus T_{eff} cell numbers in thymus (right). A–D show pooled data from three independent experiments with two to three mice per group. (E) T_{reg} cell enrichment in thymus of WT→ or *Ncstn*^{-/-}→WT BM chimeras upon anti-Dll4 or control Ab treatment for 2 wk. Percentages of T_{reg} cells (FoxP3⁺CD25⁺) among CD3⁺CD4⁺ cells (left), absolute numbers (middle), and proportion of T_{reg} cell versus T_{eff} cell numbers (right) are shown. Statistical significance was calculated for each group compared with the control (Ctr) group. Data are from one experiment with three to five mice per group. Error bars represent the mean ± SD. Horizontal bars indicate the mean. *, P < 0.05; **, P < 0.01; ***, P < 0.001.

CD4⁺ T cells within the thymus were FoxP3⁺CD25⁺, demonstrating that an enrichment of thymic T_{reg} cells had occurred upon Notch signaling blockade. T_{reg} cell enrichment was also observed in *Ncstn*^{-/-}→WT BM chimeras (P < 0.01), suggesting a cell-autonomous effect (Fig. 1 E). Dll4 blockade using anti-Dll4 Ab (Billiard et al., 2011) had no significant additive effect in these chimeras (Fig. 1 E). We conclude that Notch signaling is potentially important for maintaining both tDC and T_{reg} cell homeostatic balance under steady-state conditions.

Dll4–Notch signaling inhibition induces reversible tDC and T_{reg} cell expansion and accumulation in the thymic cortical region

To investigate the effect of Dll4–Notch signaling blockade on tDC homeostasis, we conditionally deleted Dll4 using ROSA26-CreER^{t2}/Dll4-COIN adult mice treated with tamoxifen (Economides, A., personal communication). We found that Dll4 deficiency led to a developmental delay between DN1 and double-positive (DP; CD4⁺CD8⁺) T cell stage in thymus without significantly affecting CD4⁺ or CD8⁺ T cell homeostasis (Billiard et al., 2011; unpublished data). Similar to Nicastrin deletion, genetic inactivation of Dll4 induced a strong reduction of thymic cellularity (not depicted)

and 16-, seven-, and fivefold increases of B cell (P < 0.001), DC (P < 0.001), and T_{reg} cell frequencies (P < 0.001), respectively, which were not reflected in absolute numbers, suggesting an enrichment but not an expansion of these populations in thymus (Fig. 2, A and B). These data support a role for Dll4 in B cell (Billiard et al., 2011) and potentially in DC and T_{reg} cell homeostasis. No significant changes were detected in splenic DC populations (not depicted). Overall, these results show that a sustained signal requiring both Dll4 and γ -secretase–Notch is needed to suppress the alternative DC enrichment in thymus.

We have recently shown that anti-Dll4 Ab induces accumulation of pro-T cells at DN1 stage and ectopic appearance of B cells within the thymus (Billiard et al., 2011). As genetic inactivation of Nicastrin and Dll4, respectively, induces an increase in the frequencies but a reduction in the absolute numbers of DCs and T_{reg} cells in thymus, we decided to investigate the effect of a blocking anti-Dll4 Ab treatment on DC homeostasis in adult mice before the severe reduction in thymic cellularity takes place. Indeed, pharmacological Dll4 blockade allowed us to study the kinetics of alternative DC development, which is impossible by conventional genetic inactivation models in which a severe reduction in thymic cellularity has already occurred at the time of sacrifice (Hozumi et al., 2008; Feyerabend et al., 2009). 7 d after anti-Dll4 Ab treatment, we found a significant increase in both frequency and absolute numbers of B cells (P < 0.001; Billiard et al., 2011),

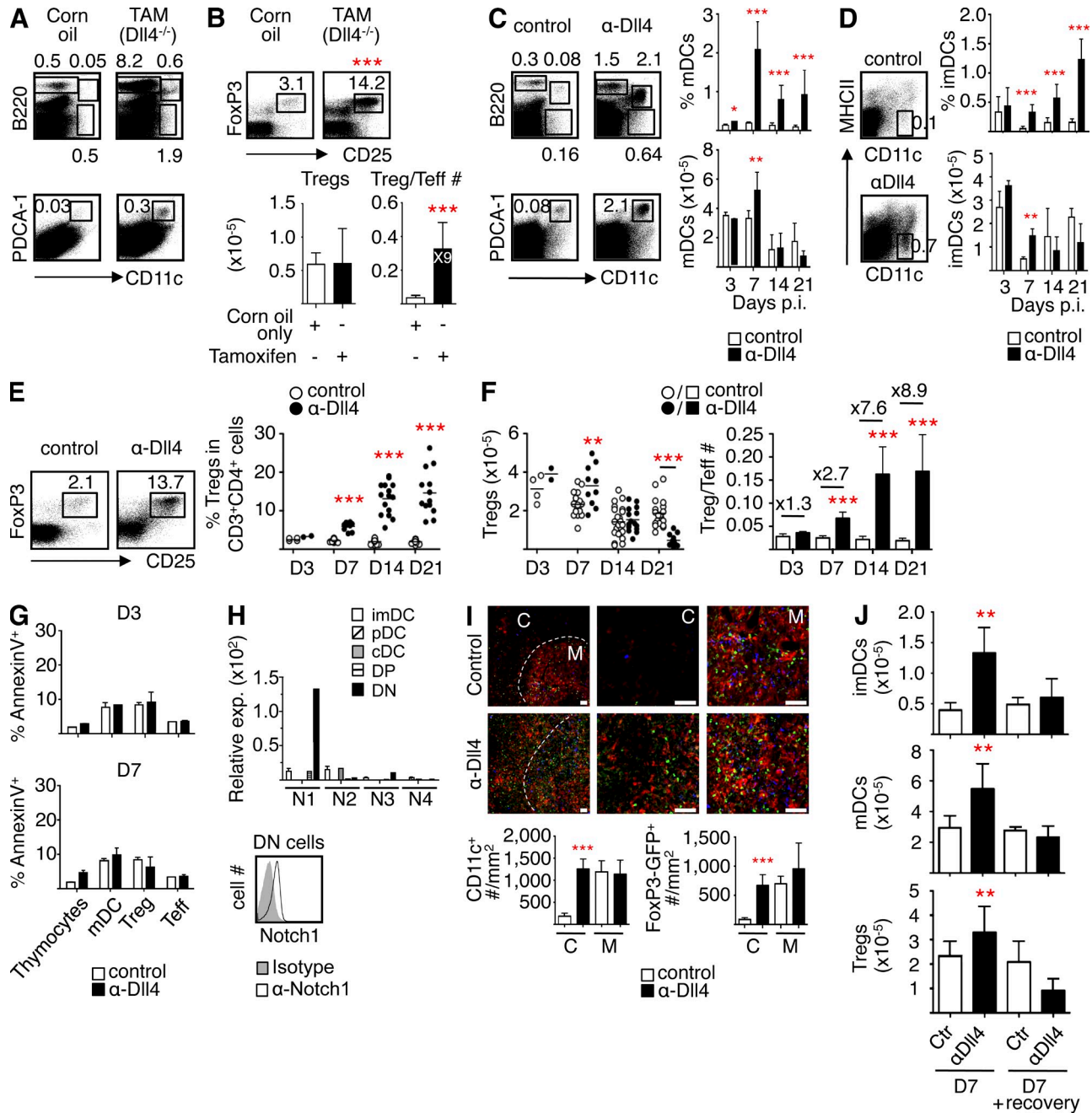


Figure 2. DII4–Notch signaling inhibition induces a reversible tDC and T_{reg} cell enrichment and accumulation in the cortical region. (A and B) pDC (CD11c⁺B220⁺/PDCA-1⁺), cDC (CD11c⁺B220⁻), and B cell (B220⁺CD11c⁻; A) and T_{reg} cell (FoxP3⁺CD25⁺ among CD3⁺CD4⁺; B) enrichment in thymus of vehicle (corn oil)- versus tamoxifen (TAM-induced DII4 inactivation)-treated ROSA26-CreERT2/DII4-COIN mice. Data are from one experiment with five mice per group. (C) C57BL/6 mice were treated every 3 d for 3, 7, 14, or 21 d with anti-DII4 or control Ab (p.i., postinjection). Dot plots show B cell, pDC, and cDC percentages among total thymocytes (day 7). Bar graphs show kinetics of enrichment of global mDCs (CD11c⁺MHCII⁺), which include pDCs and cDCs, in percentages (top) and absolute numbers (bottom). (D) Kinetics of imDC (CD11c⁺MHCII⁺) enrichment in percentages (top) and absolute numbers (bottom) after treatment with anti-DII4 or control Ab. Dot plots show day 7. (E) Dot plots (day 14, left) and graphs reflect T_{reg} cell frequencies (right) in thymus upon anti-DII4 versus control Ab treatment in C57BL/6 mice. C–E show pooled data from three independent experiments with four to five mice per group (day 3, one experiment). (F) T_{reg} cell absolute numbers (left) in thymus upon anti-DII4 versus control Ab treatment in C57BL/6 mice. Bar graphs (right) reflect the proportion of T_{reg} cell numbers versus T_{eff} cell numbers in thymus. Graphs show pooled data from two to four independent experiments with five mice per group (day 3, one experiment). (G) Apoptosis of cells in thymus (AnnexinV⁺), determined within viable cells (nonnecrotic), 3 or 7 d after treatment with anti-DII4 Ab. mDC, T_{reg} cell, and T_{eff} cell percentages were determined within total thymocytes. (H) Bar graphs (top) show relative messenger RNA expression of Notch receptors (N1–4) in sorted DC subsets (imDCs, pDCs, and cDCs) and pro-T cells (DP and DN). Histograms (bottom) show cell surface expression of N1 in DN T cells from nonmanipulated C57BL/6 mice. (I) Immunostaining of thymic sections (C, cortex; M, medulla)

mDCs (comprising cDCs and pDCs; not depicted; $P < 0.01$), and imDCs ($P < 0.01$) within the thymus (Fig. 2, C and D). Upon several injections with the anti-Dll4 Ab given over 14 or 21 d, the expansion of B cells and DCs was not reflected in absolute cell numbers (Fig. 2, C and D; and not depicted) as the result of a severe drop in thymic cellularity (Billiard et al., 2011), as expected and as observed upon genetic inactivation of Dll4 (Fig. 1 and Fig. 2, A and B). Furthermore, we found that Dll4 blockade induced an increase in both frequency and absolute numbers of T_{reg} cells ($CD3^+CD4^+CD8^-CD25^+FoxP3^+$) and T_{reg} cell precursors ($CD3^+CD8^+CD4^+CD25^+FoxP3^+$) in thymus at day 7 (Fig. 2, E and F [left]; and not depicted). As previously reported (Billiard et al., 2011), 2 wk after treatment, this was not reflected in absolute cell numbers because of the severe drop in thymic cellularity. However an enrichment of T_{reg} cells among $CD4^+$ T cells was observed within the thymus as demonstrated by a seven- to ninefold increase in the ratio of T_{reg} cell/ T_{eff} cell numbers ($P < 0.001$; Fig. 2 F, right). Furthermore, we found that anti-Dll4 Ab treatment did not promote apoptosis of thymocytes, mDCs, T_{reg} cells, or T_{eff} cells compared with control Ab treatment and that DCs and T_{reg} cells were not more resistant to apoptosis than T_{eff} cells or total thymocytes, as no difference in the apoptosis rates were detected 3 and 7 d after Ab treatment (Fig. 2 G; Billiard et al., 2011). In the periphery, no mDC enrichment was observed upon anti-Dll4 treatment (not depicted). We further examined the expression levels of Dll4 and Notch receptors on sorted pDCs, cDCs, imDCs, and early T cell progenitors. We found that Dll4 is not constitutively expressed on DCs and/or splenocytes (not depicted). Expression of N1–4 receptors remained undetectable on DCs, whereas N1 expression was high on double-negative (DN) pro-T cells (Fig. 2 H). We conclude that pharmacological Dll4 blockade induces DC and T_{reg} cell expansion in thymus 1 wk after treatment and an enrichment of these cell populations at later time points.

tDCs and $CD25^+FoxP3^+$ n T_{reg} cells are predominantly accumulated in the medullary region of the thymus and sparsely detectable in the cortex (Fontenot et al., 2005b; Lei et al., 2011). To determine whether anti-Dll4 treatment affects the anatomical distribution of DCs and T_{reg} cells, $FoxP3^{gfp}$ mice (Fontenot et al., 2005b) were treated with anti-Dll4 versus control Ab for 7 d. Thymic sections were stained for TECs and DCs. Measurement of tDC density in various thymic regions indicated that the number of tDCs and $FoxP3^{gfp+}$ (T_{reg}) cells per unit area in the deep cortical region was significantly ($P < 0.001$) increased in anti-Dll4-treated compared with control mice (Fig. 2 I). No detectable change in the absolute numbers of DCs and T_{reg} cells was observed in the medullary region (Fig. 2 I). We conclude that Dll4–Notch

signaling blockade induces ectopic appearance and accumulation of both DCs and T_{reg} cells in the cortical area. To examine whether the homeostatic effect of anti-Dll4 Ab treatment on DCs and T_{reg} cells was reversible, WT C57BL/6 mice were treated with control or anti-Dll4 Ab for 7 d. As previously described (Fig. 2, C, D, and F), we found that Dll4 inhibition induced a significant increase in imDC and mDC (tDC) and T_{reg} cell numbers ($P < 0.01$). After cessation of treatment (4 wk, “recovery”), both tDC and T_{reg} cell numbers returned to baseline levels (Fig. 2 J). This result is consistent with the previous finding showing that anti-Dll4 Ab washes out 2–3 wk after treatment arrest (Billiard et al., 2011). Thus, sustained Dll4–Notch signaling blockade is required for maintaining alternative tDC and T_{reg} cell expansion.

MHCII expression by DCs is required for in vivo

T_{reg} cell enrichment upon anti-Dll4 Ab treatment

It has been shown that tDCs contribute to T_{reg} cell induction (Watanabe et al., 2005). To examine whether DN1-derived DCs induce in vitro T_{reg} cell differentiation, $CD25^-FoxP3^-CD4^+$ single-positive (SP) or $CD25^-FoxP3^-DP$ T cells purified from the thymus of untreated mice (purity $>99\%$; not depicted) were incubated with tDCs sorted from anti-Dll4- or control-treated animals in the presence of IL-2, a cytokine required for T_{reg} cell differentiation and survival (Almeida et al., 2002). It is known that FoxP3 induction can occur at either the DP or CD4 SP stage in thymus or during the transition between these stages (Fontenot et al., 2005a). Interestingly, we observed a significantly higher FoxP3 acquisition in both DP and CD4 SP T cells (3.8- and 2.2-fold, respectively) upon culture with tDCs purified from mice previously treated with anti-Dll4 Ab- versus isotype control-treated animals (Fig. 3 A). This result suggests a potential tolerogenic effect of DN1-derived tDC populations. Furthermore, although T_{reg} cell proliferation in response to cultured DCs appears to be independent of MHCII (Swee et al., 2009), a separate study shows that homeostatic T_{reg} cell division requires self-antigen presentation by MHCII because it is impaired in MHCII KO mice (Shimoda et al., 2006). In addition, a recent work demonstrates that Flt3-dependent DC increase in the periphery leads to increased homeostatic T_{reg} cell division and accumulation by a mechanism requiring MHCII expression on DCs (Darrasse-Jèze et al., 2009). To determine whether Dll4-mediated thymic T_{reg} cell enrichment (Fig. 2 F) was DC dependent, $CD11c^{hi}$ DCs were ablated by administration of diphtheria toxin (DT) in $CD11c-DTR \rightarrow WT$ BM chimeras (Jung et al., 2002) throughout 3 wk of anti-Dll4 treatment. DC deficiency abrogated the effect of anti-Dll4 Ab treatment on T_{reg} cell frequency increase (Fig. 3 B), thus demonstrating the essential role of DCs

from WT $FoxP3^{gfp}$ C57BL/6 mice treated with anti-Dll4 versus control Ab for 7 d and stained for GFP (green), CD11c (red), and mTEC (blue). Bars, 50 μm . CD11c⁺ and GFP⁺ cell counts are shown (bottom). (J) imDC (top), mDC (middle), and T_{reg} cell (bottom) absolute numbers in thymus upon anti-Dll4 or control Ab treatment. Mice treated for 7 d were then allowed to recover without treatment for 4 wk (recovery). G–J show pooled data from two independent experiments with five mice per group. Error bars represent the mean \pm SD. Horizontal bars indicate the mean. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

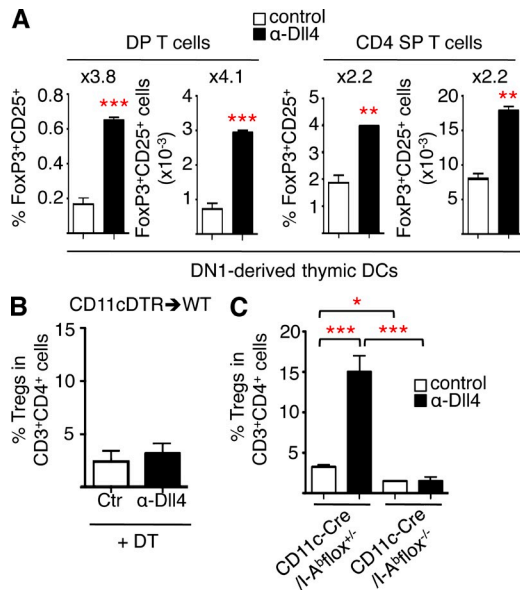


Figure 3. Anti-Dll4 Ab-mediated enrichment of T_{reg} cells is dependent on DC-expressing MHCII. (A) Thymic CD25⁺FoxP3⁺ DP (left) or CD4 SP (right) T cells from WT mice were cultured with CD11c⁺tDCs from anti-Dll4- or control Ab-treated mice and IL-2. The expression of FoxP3/CD25 was assessed after 3 d of culture. (B) CD11c-DTR→WT BM chimeras were injected with anti-Dll4 or control Ab twice a week for 14 d, along with DT to sustain DC deficiency. Percentages of T_{reg} cells were determined in thymus. (C) CD11c-Cre/I-A^bflox^{+/-} or CD11c-Cre/I-A^bflox^{-/-} mice were treated with anti-Dll4 or control Ab twice a week for 14 d, and percentages of T_{reg} cells were determined in thymus. Each panel shows pooled data from two independent experiments with four to five mice per group for each panel. Error bars represent the mean \pm SD. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

in Dll4-mediated T_{reg} cell enrichment. To investigate the role of MHCII expression by DCs in T_{reg} cell homeostasis in vivo, CD11c-Cre/I-A^bflox mice that lacked MHCII expression on CD11c^{hi} cells (not depicted) and had fewer T_{reg} cells than littermate controls (Darrasse-Jèze et al., 2009) were treated with control or anti-Dll4 Ab. We found that anti-Dll4-mediated T_{reg} cell enrichment was impaired in CD11c-Cre/I-A^bflox mice compared with control mice (Fig. 3 C). We conclude that Dll4–Notch signaling inhibition promotes thymic T_{reg} cell generation by a mechanism that requires MHCII expression on DCs.

Anti-Dll4 Ab treatment converts early T cell progenitors to imDCs

To determine whether tDC expansion at day 3 and/or 7 was caused by an extra-tDC migration via the blood stream (Wu and Shortman, 2005; Merad and Manz, 2009), DC homeostasis was evaluated in blood upon anti-Dll4 Ab treatment. We found that DC numbers were not affected in peripheral blood (not depicted), suggesting that anti-Dll4-mediated DC expansion could originate either intrathymically or via expansion of DC progenitors from the BM.

Early DC precursors (pre-DCs), imDCs, and mDC subsets (pDCs and cDCs) arise from the common precursors called macrophage DC progenitors (MDPs) and the common DC progenitors (CDPs) located in the BM (Waskow et al., 2008; Liu et al., 2009; Geissmann et al., 2010). To examine whether anti-Dll4 Ab treatment has a direct effect on BM-derived DC progenitors, Lin⁻Sca-1⁻c-kit⁺CSF-1-R⁺ (MDP) and Lin⁻Sca-1⁻c-kit^{lo}CSF-1-R⁺Flt3⁺ (CDP) populations (Liu et al., 2009) were assessed after 3, 7, 14, and 21 d of treatment. We found that MDPs and CDPs were undetectable in spleen and thymus but present in the BM. Surprisingly, no expansion of either MDPs or CDPs was observed in BM upon anti-Dll4 Ab treatment (Fig. 4 A). Furthermore, no effect was observed in imDC and mDC frequencies and absolute numbers (Fig. 4 B). Therefore, tDC enrichment is not caused by a homeostatic effect of anti-Dll4 Ab treatment on early or late BM-derived DC progenitors.

tDCs could either originate independently of the T cell pathway or branch off no later than the DN1 pro-T cell stage (Wu et al., 1996; Radtke et al., 2000). Deleting N1 in pro-T cells converts them to DCs, suggesting that intrathymically generated T cells and DCs could share a common early precursor (Feyerabend et al., 2009). In addition, N1 signaling has a role in the development of alternative lineages such as NK and B cells in thymus (Di Santo, 2010). To determine the origin of the early tDC expansion upon Dll4 blockade, we first examined the kinetics of appearance of imDCs within the DN1 population. We observed a threefold increase in the frequency and the absolute number of imDCs originating from the DN1 population as early as day 3 after anti-Dll4 Ab treatment, whereas an increase in mDC absolute number was detected only 7 d later in the same population (Fig. 4 C). We found that DCs did not show a higher proliferative potential (Fig. 4 C, top right) after anti-Dll4 Ab treatment, suggesting that this Ab treatment does not enhance proliferation of an existing tDC pool. No expansion in NK cells, granulocytes, macrophages, or B cells was detected after the same treatment within the DN1 population (Fig. 4 D). To further examine whether imDC expansion could originate from uncommitted early T cell precursors, an equal number of DN1 CD45.1⁺Lin⁻ sorted cells that did not express CD11c and/or MHCII (not depicted) was transferred by intrathymic injection into CD45.2⁺ host mice treated with anti-Dll4 or control Ab. As expected, anti-Dll4 treatment induced an endogenous DC expansion compared with control (Fig. 4 E, bottom; and Fig. 2, C and D). More importantly, we found that upon Dll4 blockade, CD45.1⁺ DN1 cells acquired an imDC phenotype and were significantly expanded in thymus (Fig. 4 E, top; $P < 0.01$). Interestingly, hardly any CD45.1⁺, CD4⁺, or CD8⁺ SP cells were detected in the control Ab-treated mice (not depicted), most likely because the vast majority of DN1-transferred cells were eliminated during T cell negative selection by deletion and neglect after their differentiation into TCR⁺ cells (Pénil et al., 1995; van Meerwijk et al., 1997). Indeed, based on the kinetics of thymocyte development and the fact that a

remarkably large fraction of thymocytes do not reach maturity as a result of overriding negative selection in the presence of physiological ligands expressed on hematopoietic cells, >90% of developing thymocytes die of neglect and 5–8% die as a result of deletion or another unknown mechanism (Huesmann et al., 1991; Surh and Sprent, 1994). Thus, we can speculate that because anti-Dll4 Ab treatment converts CD45.1⁺ DN1 cells into alternative cell types such as DCs, it rescues them from early T cell development checkpoints. We conclude that anti-Dll4 Ab treatment promotes the intrathymic development of imDCs originating from a common T/DC DN1 progenitor.

Pharmacological Dll4 inhibition induces alternative tDC development via an Flt3-independent mechanism

The mechanism by which Notch signaling (N1 or Dll4) blockade induces alternative tDC development (Fig. 4; Feyerabend et al., 2009) remains unknown. Thus, we further investigated the molecular signals promoting alternative tDC enrichment. It has been shown that Flt3-L is able to drive differentiation of mouse BM progenitors into all DC subtypes (Brasel et al., 2000; Merad and Manz, 2009) and is sufficient and essential for the development of blood and peripheral lymphoid organ DCs (Waskow et al., 2008). Mice lacking Flt3-L show an eightfold reduction in the number of cDCs in the spleen

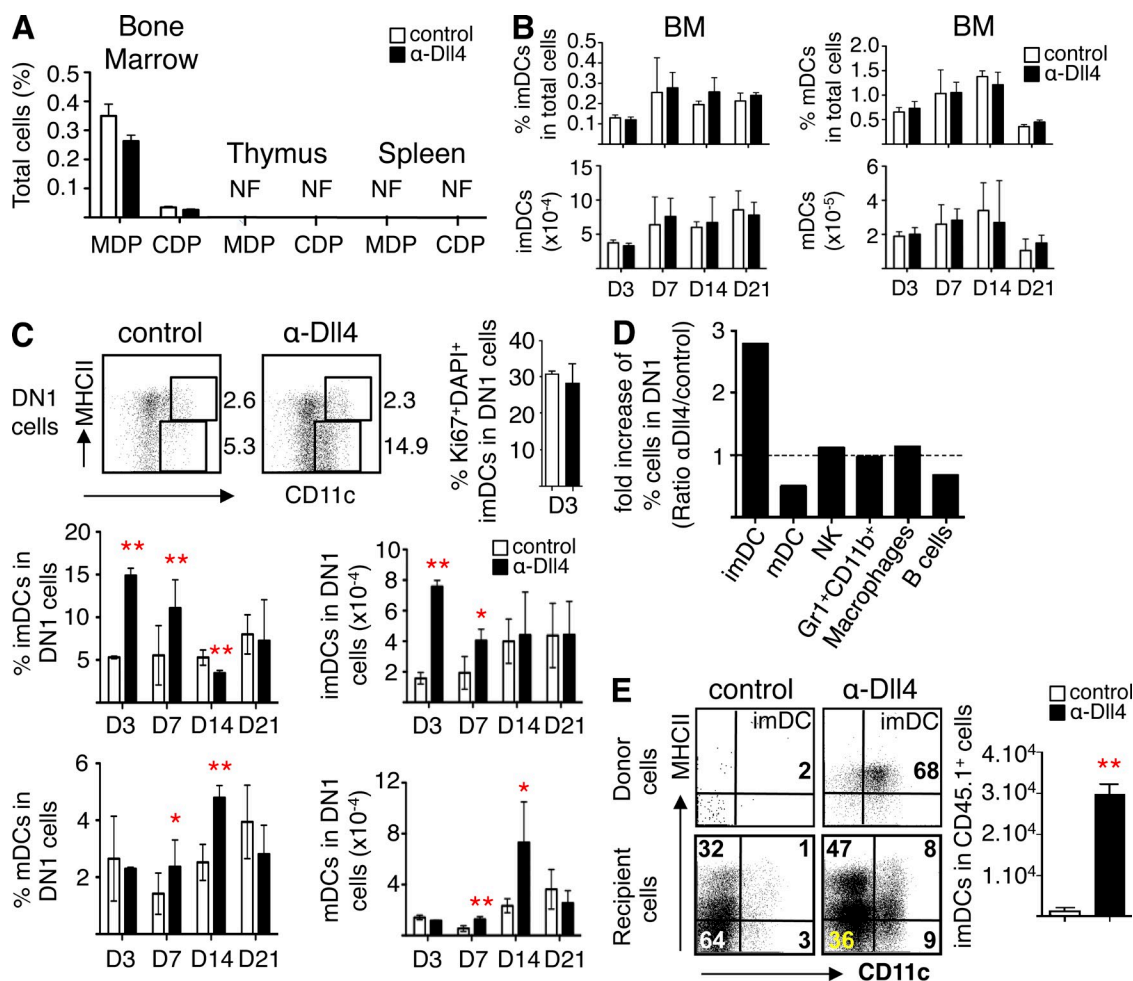


Figure 4. Anti-Dll4 Ab treatment converts pro-T cells to imDCs, independently of BM progenitors. (A) MDP (Lin⁻Sca-1⁻c-kit⁺CSF-1^{R+}) and CDP (Lin⁻Sca-1⁻c-kit⁺CSF-1^{R+}Flt3⁺) percentages in BM, thymus, and spleen of C57BL/6 mice 3 d after treatment with anti-Dll4 or control Ab. NF, not found. (B) CD11c⁺MHCII⁺ DC (imDC) and CD11c⁺MHCII⁺ mDC percentages (top) and absolute numbers (bottom) in BM upon anti-Dll4 versus control Ab treatment. A and B show pooled data from two independent experiments with five mice per group. (C) Dot plots (day 3) show percentages of imDCs and mDCs in DN1 pro-T cells (CD4⁻CD8⁻c-kit⁺CD44⁺CD25⁻) upon anti-Dll4 versus control Ab treatment. Percentages of proliferating (Ki67⁺DAPI⁺) imDCs found within DN1 are shown in anti-Dll4 Ab- or control Ab-treated mice (top right). Bar graphs show percentages (left) and absolute numbers (right) of imDCs (top) and mDCs (bottom) within DN1 cells. (D) Fold increase in percentages of imDCs, mDCs, NK cells (NK1.1⁺CD3⁻), Gr1⁺CD11b⁺ cells, macrophages (MAC-1⁺F4/80⁺), and B cells (B220⁺CD11c⁻) in DN1 pro-T cells in thymus upon treatment (day 3) with anti-Dll4 Ab. (E) Dot plots show imDCs among CD45.1⁺ DN1-transferred cells (top) or among CD45.2⁺ endogenous DN1 cells (bottom) in CD45.2⁺ C57BL/6 recipient mice treated every 3 d with anti-Dll4 or control Ab and sacrificed 9 d after first injection. Quadrants were set with isotype controls for CD11c and MHCII Abs. The bar graph shows absolute numbers of imDCs in CD45.1⁺ cells. C–E show data from three independent experiments with five mice per group. Error bars represent the mean \pm SD. *, $P < 0.05$; **, $P < 0.01$.

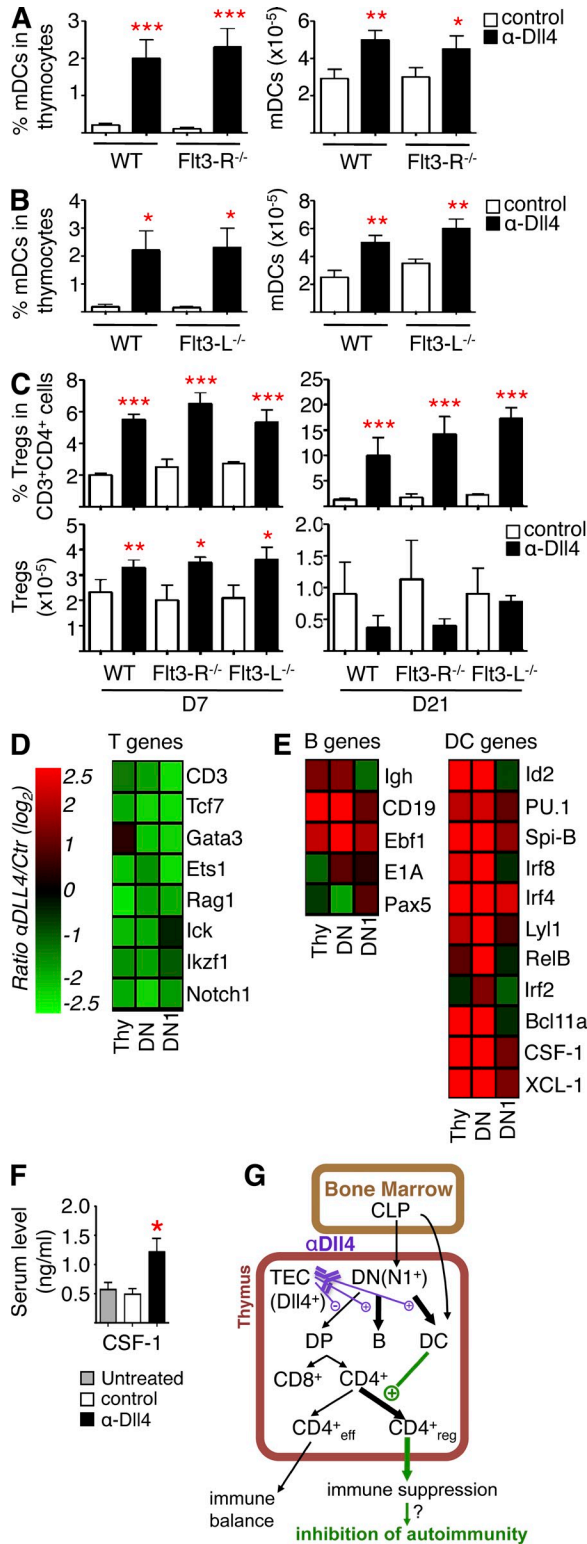


Figure 5. Pharmacological Dll4 inhibition induces alternative tDC development via an Flt3-independent mechanism. (A and B) CD11c⁺MHCII⁺ mDC percentages (left) and absolute numbers (right) in thymus of Flt3-R^{-/-} (A) and Flt3-L^{-/-} (B) mice 7 d after anti-Dll4 versus control Ab treatment. (C) T_{reg} cell percentages (top) and absolute numbers (bottom) in thymus of Flt3-R^{-/-} and Flt3-L^{-/-} mice 7 (left) or 21 d (right)

(McKenna et al., 2000; unpublished data), and additional studies show that the rate of cDC division in lymphoid organs is regulated in part by Flt3 receptor signaling (Steinman, 2007; Waskow et al., 2008). To determine whether the effect of anti-Dll4 Ab was mediated by Flt3-L, bioactive Flt3-L was measured in the serum of treated mice. No detectable modification of Flt3-L serum levels was observed (not depicted). To validate this result, mice lacking either Flt3-L (Flt3-L^{-/-}) or its receptor (Flt3-R^{-/-}) were treated with anti-Dll4 Ab for 7 or 21 d. Surprisingly, we found that a significant increase in both frequency and DC number indeed occurred in the thymus of both Flt3-R^{-/-} (P < 0.001; Fig. 5 A) and Flt3-L^{-/-} (P < 0.05; Fig. 5 B) mice at day 7. We further show that DC-dependent T_{reg} cell expansion observed 7 d after anti-Dll4 treatment took place in an Flt3-independent manner as demonstrated by an increase of T_{reg} cell frequency and absolute cell numbers in both Flt3-R^{-/-} (P < 0.05) and Flt3-L^{-/-} (P < 0.05) mice (Fig. 5 C, left). As expected, 21 d after anti-Dll4 treatment, this T_{reg} cell expansion was not reflected in absolute numbers (Fig. 5 C, right) because of a drop in thymic cellularity. Rather, the treatment induced an accumulation of T_{reg} cells among the CD4⁺ effector cell population in thymus, as demonstrated by an increase in the ratio of T_{reg} cells/T_{eff} cells (not depicted). We conclude that Dll4–Notch signaling blockade induces an Flt3-independent tDC and subsequent T_{reg} cell expansion. Therefore, we propose that Dll4–Notch functions as a novel alternative pathway regulating tDC homeostasis.

The ability of early T cell progenitors to rederive toward a non-T cell phenotype has been observed (Di Santo, 2010). Here, gene array analysis was performed in purified thymocytes and DN1 cells (defined and sorted as CD4⁻CD8⁻c-kit⁺CD44⁺CD25⁻; not depicted) to determine the effect of anti-Dll4 Ab treatment in genes implicated in T versus DC and B cell lineage specification. As previously described (Matsuzaki et al., 1993; Billiard et al., 2011), c-kit was used as a DN1 cell surface marker, as CD44 is also expressed in B cells and other myeloid cells. We recently showed that the Notch signaling pathway (Hes1, Ptcra, and Deltex-1) is inhibited upon anti-Dll4 treatment (Billiard et al., 2011). Here we found that *CD11c* gene present in fully differentiated DCs and DC precursors was intact (not depicted). In contrast, we observed a down-regulation of genes essential for T cell commitment,

after anti-Dll4 versus control Ab treatment. (D and E) Gene array analysis showing T, B, and DC gene signatures in total thymocytes and FACS-sorted Lin⁻CD11c⁻ DN (CD4⁻CD8⁻) and Lin⁻CD11c⁻ DN1 (CD4⁻CD8⁻c-kit⁺CD44⁺CD25⁻) from anti-Dll4- versus control Ab-treated C57BL/6 mice. Data reflect the ratio of expression (in log₂) between anti-Dll4/control (Ctr) treatment. (F) CSF-1 serum levels in C57BL/6 mice untreated or treated with anti-Dll4 or control Ab. (G) Proposed mechanism involving Dll4–Notch signaling in T cell versus DC/B fate decisions and expansion of T_{reg} cells. CLP, common lymphoid progenitor. A–F show pooled data from two independent experiments with five mice per group for each panel. Error bars represent the mean ± SD. *, P < 0.05; **, P < 0.01; ***, P < 0.001.

whereas genes (*Lyl1* and *PU.1*) that can each block T cell development (Di Santo, 2010) if sustained beyond this point were up-regulated (Fig. 5, D and E). Most interestingly, genes controlling DC (*PU.1* [Carotta et al., 2010] and *Spi-B*) and B cell development (Merad and Manz, 2009) were also up-regulated (Fig. 5 E). In addition, expression of *RelB* and *Id2*, as well as *IRF2* and *IRF4*, key transcription factors involved in DC subset development (Merad and Manz, 2009), were increased (Fig. 5 E). To validate this result, *PU.1*, *Spi-B*, and *IRF4* expression were also measured by quantitative PCR (not depicted). Finally, gene expression and serum levels of CSF-1 (M-CSF), a key cytokine involved in DC development (Fancke et al., 2008), were found to be up-regulated ($P < 0.05$; Fig. 5 F). Thus, Dll4–Notch signaling blockade down-regulated transcription factors specific for T cell lineage commitment, while up-regulating others crucial for DC development. Overall, we conclude that Dll4–Notch signaling is essential for sustaining proper T cell development and homeostasis, whereas its inhibition triggers an Flt3-independent pathway regulating tDC development and subsequent T_{reg} cell differentiation (Fig. 5 G). Thus, we sought to investigate the physiological relevance of this pathway and its potential consequences in immune suppression and autoimmunity.

Anti-Dll4 Ab treatment prevents T1D via a T_{reg} cell-mediated mechanism

We further examined whether anti-Dll4 Ab treatment could have an effect on peripheral T_{reg} cell homeostasis. It is known that imDCs have low MHCII and co-stimulatory molecule expression (Marguti et al., 2009), and we observed expansion of these cells in thymus upon anti-Dll4 Ab treatment under steady-state conditions. Our data (Fig. 3 A) suggest that DN1-derived imDCs could have a potential tolerogenic effect such as inducing T_{reg} cell expansion (Darrasse-Jèze et al., 2009; Swee et al., 2009). A nonsignificant increase in splenic T_{reg} cells was observed after 21 d of Dll4–Notch signaling blockade under steady-state conditions (Fig. 6 A), whereas in the presence of an inflammatory stimulus (CFA/OVA or polyinosinic:polycytidylic acid [polyI:C] in *Ncstn*^{-/-} mice), a significant increase in both T_{reg} cell frequency and absolute numbers was observed (Fig. 6, A and B; and not depicted). Importantly, no expansion of peripheral T_{reg} cells was observed in adult thymectomized mice compared with WT controls treated with anti-Dll4 Ab in the presence of CFA/OVA, suggesting that Dll4–Notch signaling blockade generated n T_{reg} cells in thymus (Fig. 6, A and B). To confirm that anti-Dll4 Ab treatment induced natural versus inducible T_{reg}

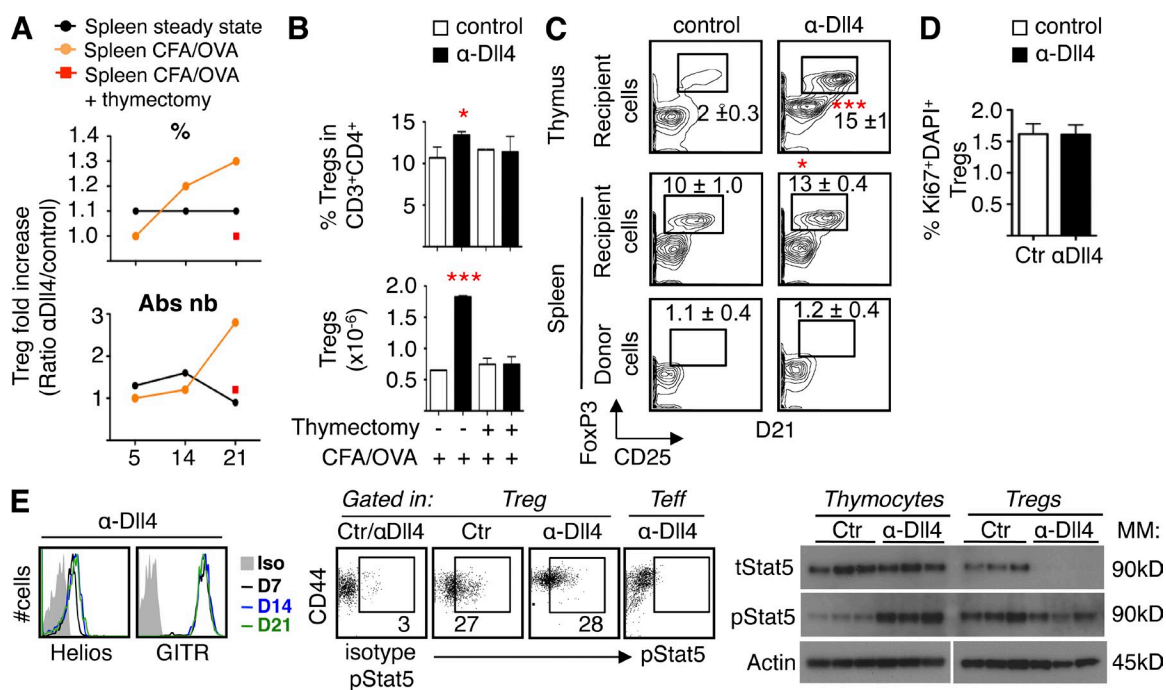


Figure 6. Anti-Dll4 Ab induces the expansion of thymus-derived n T_{reg} cells in the periphery upon immune stimulation. (A) T_{reg} cell expansion upon anti-Dll4 versus control Ab treatment in spleens of nonimmunized or CFA/OVA-immunized normal or thymectomized C57BL/6 mice. Fold increase in percentages (top) or absolute numbers (Abs nb; bottom) are shown. (B) Expansion of splenic T_{reg} cells in percentages (top) or absolute numbers (bottom) after treatment with anti-Dll4 or control Ab in WT or thymectomized C57BL/6 mice upon CFA/OVA immunization. (C) FoxP3/CD25 expression in CD4⁺ cells of CD45.2⁺ recipient or of CD45.1⁺CD4⁺FoxP3⁻CD25⁻ injected (donor) cells in C57BL/6 mice upon treatment with control or anti-Dll4 Ab for 21 d. (D) Percentages of proliferating (Ki67⁺DAPI⁺) T_{reg} cells in thymus of anti-Dll4- or control (Ctr) Ab-treated C57BL/6 mice at day 7 after start of treatment. (E) Expression of Helios and GITR (left) and pStat5 (day 14; middle) on thymic T_{reg} cells upon anti-Dll4 Ab treatment in C57BL/6 mice. T_{eff} cells are shown as a negative control for pStat5 expression. The gate of analysis was set with isotype control. (right) Detection of total Stat5 (tStat5) and pStat5 protein in total thymocytes and purified thymic T_{reg} cells upon 7 d of anti-Dll4 Ab treatment. mm, molecular mass. A–E show pooled data from two independent experiments with five mice per group. Error bars represent the mean \pm SD. *, $P < 0.05$; ***, $P < 0.001$.

cells, polyclonal CD45.1⁺CD4⁺FoxP3⁻CD25⁻ T cells (purity >99%; not depicted) were adoptively transferred into CD45.2⁺ recipient animals treated with control or anti-Dll4 Ab and immunized with CFA/OVA. We found that polyclonal CD4⁺ T cells remained negative for FoxP3 upon anti-Dll4 treatment, whereas expanded endogenous nT_{reg} cells were detected in thymus ($P < 0.001$) and spleen ($P < 0.05$; Fig. 6 C). We also assessed T_{reg} cell division by staining for the proliferation marker Ki67. A similar frequency of proliferating FoxP3⁺ T_{reg} cells was observed in thymus upon anti-Dll4 compared with isotype control treatment (Fig. 6 D). Finally, we further characterized the phenotype of anti-Dll4-mediated T_{reg} cells by showing expression of GITR, known to be associated with T_{reg} cell function (McHugh et al., 2002), and high expression of Helios and phosphorylated Stat5 (pStat5), both markers of nT_{reg} cell differentiation (Fig. 6 E; Thornton et al., 2010; Goldstein et al., 2011). Neither the percentage of pStat5⁺ T_{reg} cells nor the expression of the aforementioned markers was altered by anti-Dll4 treatment (Fig. 6 E). A more detailed quantitation of Stat5 and pStat5 levels showed a small but reproducible increase of pStat5 levels in thymocytes of mice treated with anti-Dll4 Ab. Remarkably, although treatment did not affect the levels of the phosphorylated form in T_{reg} cells, it resulted in a pronounced decrease of the non-phosphorylated form (tStat5, total Stat5). Therefore, the ratio between the phosphorylated/activatory and nonphosphorylated species of Stat5 was greatly increased upon Dll4 blockade (Fig. 6 E, right). Overall, these results suggest that Dll4 blockade does not induce expansion of a preexisting T_{reg} cell

population but rather induces a de novo generation of thymic nT_{reg} cells. We conclude that Dll4–Notch signaling regulates the homeostasis of nT_{reg} cells via a DC-mediated MHCII-dependent mechanism (Fig. 3 C), without promoting expansion of inducible T_{reg} cells.

We further investigated the physiological relevance of anti-Dll4 Ab treatment in blocking a spontaneous autoimmune disease (T1D) using nonobese diabetic (NOD) mice and examining whether T_{reg} cells mediated its effect. Compared with control Ab-treated animals that showed high diabetes incidence (>80%), we found that anti-Dll4 Ab treatment fully prevented disease development (Fig. 7 A). Disease protection was correlated with high T_{reg} cell frequency (Fig. 7 A, inset), high ratio of T_{reg} cell/T_{eff} cell numbers in pancreas-draining LNs (PLNs; not depicted), and lack of CD8⁺ cell infiltration in the pancreatic islets of anti-Dll4-treated animals (Fig. 7 B). In addition, as previously shown in WT C57BL/6 mice, a significant enrichment of T_{reg} cells (Fig. 2 E), DCs (Fig. 2 C), B cells, and DN1 pro-T cells (Billiard et al., 2011) was observed in thymus of anti-Dll4-treated NOD mice (not depicted). To determine whether anti-Dll4 Ab treatment could prevent T1D by DC-mediated T_{reg} cell expansion, mice were treated with anti-Dll4 Ab for 10 wk. After cessation of treatment, a cohort of mice was injected with anti-CD25 (PC61) T_{reg} cell-depleting Ab, whereas another cohort received PC61 isotype control. We found that anti-Dll4-mediated protection was significantly decreased in anti-CD25 Ab-treated animals. In contrast, mice treated with PC61 isotype control remained normoglycemic, suggesting that diabetes relapse was not caused by cessation of anti-Dll4 Ab treatment but by T_{reg} cell

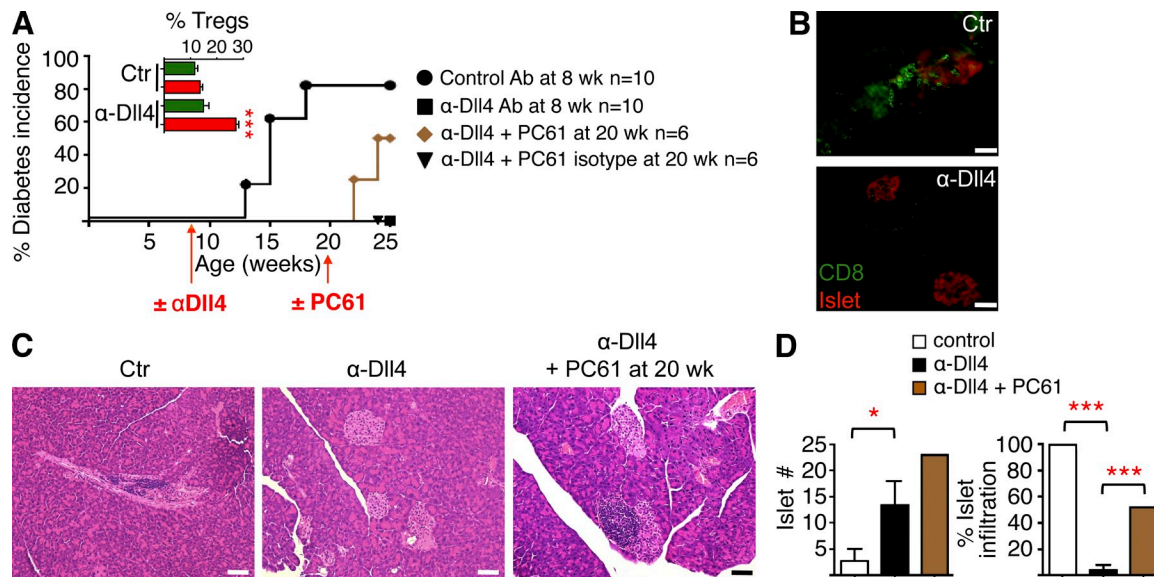


Figure 7. Anti-Dll4 Ab prevents T1D via a T_{reg} cell-mediated mechanism and inhibition of pancreatic islet infiltration. (A) Diabetes incidence of NOD mice treated with anti-Dll4 or control Ab starting at 8 wk of age. One injection of CD25-depleting Ab (PC61) or isotype Ab was administered to anti-Dll4-treated mice at 20 wk of age. (inset) Percentages of T_{reg} cells in nondraining LNs (green) or PLNs (red). The number of treated mice is indicated. (B) Immunostaining of pancreas sections from NOD mice treated with anti-Dll4 versus control (Ctr) Ab, stained for islets and CD8. (C) H&E staining of pancreatic sections from control (left)-, anti-Dll4 Ab (middle)-, or anti-Dll4/PC61 Ab-treated (right) NOD mice. Bars: (B) 50 μm; (C) 100 μm. (D) Islets were counted on whole pancreas section from the mice from C. Percentage of infiltration was determined (right). A–D show representative data from two independent experiments with five mice per group. Error bars represent the mean ± SD. *, $P < 0.05$; ***, $P < 0.001$.

depletion (Fig. 7 A). Finally, anti-Dll4 Ab treatment significantly prevented pancreatic islet infiltration ($P < 0.001$) and subsequent destruction ($P < 0.05$; Fig. 7, C and D). We further investigated the effect of T_{reg} cell depletion (PC61 treatment) on pancreatic islet infiltration. Mice previously treated with anti-Dll4 Ab (disease free) that received a PC61 injection upon cessation of anti-Dll4 Ab treatment (T1D relapse) showed a severe increase in the percentage of islet infiltration ($P < 0.001$; Fig. 7 D). We conclude that anti-Dll4 Ab treatment fully prevents T1D development via a T_{reg} cell-mediated mechanism and inhibits pancreatic islet infiltration.

Anti-Dll4 Ab treatment reverses established T1D

Although several methods of preventing T1D in NOD mice have been described over the years (Atkinson and Leiter,

1999), few studies report disease treatment (Belghith et al., 2003; Tang et al., 2004). Recently, it has been shown that multiple injections of a low dose of IL-2 at diabetes onset can induce disease remission in 60% of mice (Grinberg-Bleyer et al., 2010). To determine whether anti-Dll4 Ab could treat T1D, NOD mice were injected with a single dose of anti-Dll4 Ab at the time of disease onset, with or without anti-CD25 Ab. We observed decreased blood glucose to normal levels (<200 mg/dl) in 100% of anti-Dll4-treated mice (Fig. 8 A and not depicted) for a sustained period (4 wk). When we simultaneously injected anti-Dll4 and anti-CD25 Abs, disease relapse took place 1 wk earlier versus injection with anti-Dll4 Ab alone, suggesting that anti-Dll4 Ab treatment has a therapeutic effect via a T_{reg} cell-mediated mechanism. This hypothesis is strengthened by the fact that during T1D remission

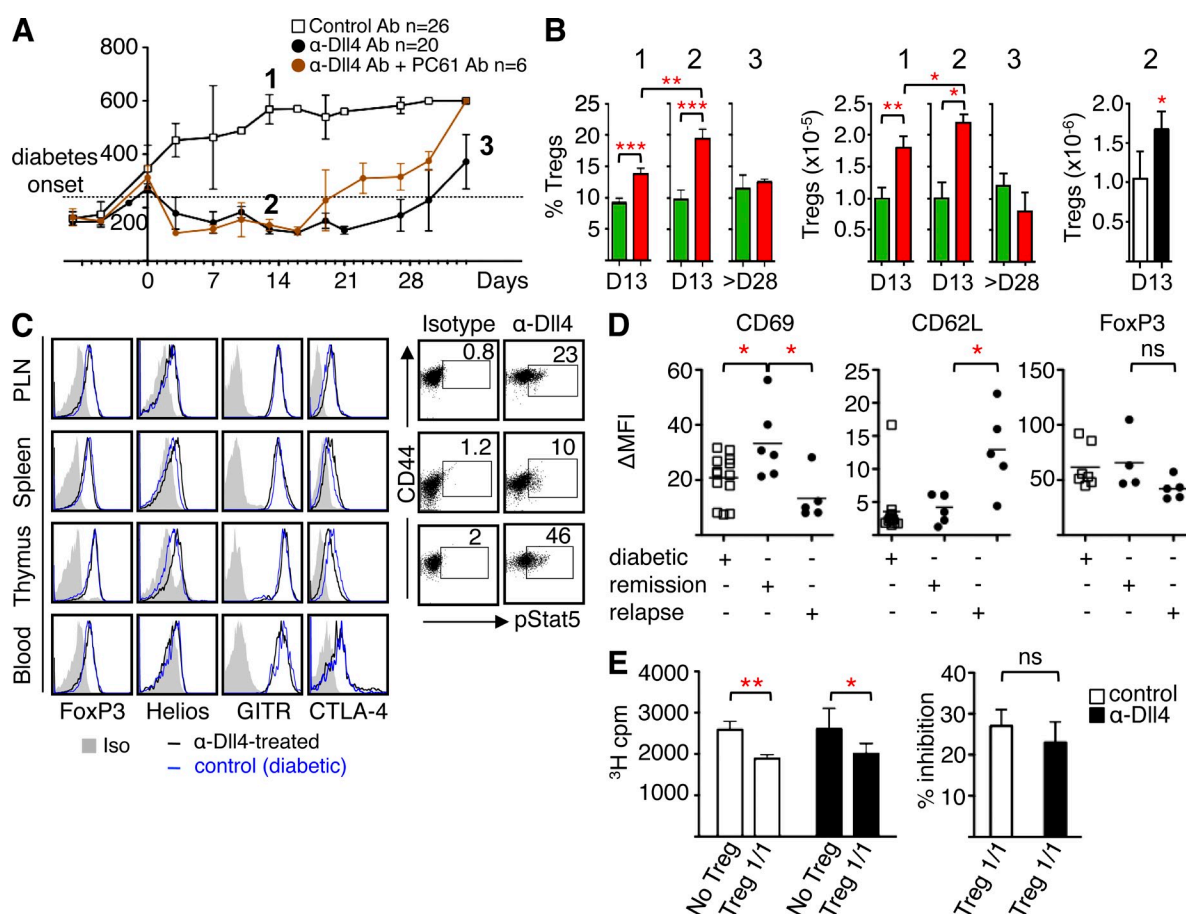


Figure 8. Anti-Dll4 Ab reverses established T1D. (A) Blood glucose levels in NOD mice treated at diabetes onset (250 < glucose level < 350 mg/dl) with control or anti-Dll4 Ab, alone or with 1 mg PC61 Ab the same day. The number of treated mice is indicated. Representative data are shown from three independent experiments. (B) Percentages (left) and absolute numbers (middle) of T_{reg} cells in nondraining LNs (green) or PLN (red) of NOD mice untreated (time point 1, diabetic) or treated with anti-Dll4 Ab (time points 2, remission; and 3, relapse). Absolute numbers of T_{reg} cells in spleen of NOD mice treated with control (open bar) or anti-Dll4 Ab (closed bar; time point 2) are shown (right). Data were pooled from three independent experiments with five mice per group. (C) Expression of FoxP3, Helios, GITR, CTLA-4, and pStat5 in organs of control (diabetic)- versus anti-Dll4 Ab-treated (diabetes remission) NOD mice. Graphs show representative data from two independent experiments with four mice per group. (D) Δ MFI (mean fluorescence intensity; as calculated by $MFI(Ab) - MFI(isotype)$) of CD69, CD62L, and FoxP3 on T_{reg} cells from PLN of NOD mice. Data were collected from mice at specific stages of disease/treatment across three independent experiments. (E) Suppressive activity of purified T_{reg} cells from spleen of diabetic versus anti-Dll4 Ab-treated mice, represented in cpm (left) or percent inhibition (right). Data were pooled from two independent experiments with three mice per group. Error bars represent the mean \pm SD. Horizontal bars indicate the mean. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

(2 wk after treatment), T_{reg} cell frequency ($P < 0.001$), absolute number ($P < 0.05$; Fig. 8 B), and T_{reg} cell/ T_{eff} cell number ratio ($P < 0.001$; not depicted) were significantly increased in the PLNs and spleen ($P < 0.05$; Fig. 8 B, right) of anti-Dll4- versus isotype control-treated mice. Confirming previous results shown in Fig. 6, we found that Dll4-mediated T_{reg} cells were nT_{reg} cells based on their sustained expression of FoxP3, Helios, GITR, CTLA-4, and pStat5 in thymus, spleen, and blood as well as in the PLNs of treated (disease free) animals. No differences in expression levels of these markers were detected compared with endogenous nT_{reg} cells found in diabetic animals (Fig. 8 C). However, we found that anti-Dll4 treatment induced up-regulation of CD69, an early activation marker (Sancho et al., 2005), in PLNs (Fig. 8 D) and spleen (not depicted). Upon T1D relapse, CD69 expression returned to low levels, whereas CD62L, a homing receptor, was up-regulated, suggesting a potential tissue relocalization of these cells (Cyster, 1999). FoxP3 expression was sustained upon T1D relapse (Fig. 8 D). Finally, we confirmed that anti-Dll4-mediated nT_{reg} cells were functional based on their ability to suppress T cell proliferation *ex vivo* (Fig. 8 E). Importantly, we found that after T1D recurrence (4 wk after anti-Dll4 treatment and Ab washout), the frequency and number of T_{reg} cells and the ratio of T_{reg} cells/ T_{eff} cells in PLNs returned to baseline levels (Fig. 8 B) as observed in diabetic mice, providing evidence for an existing link between anti-Dll4 Ab treatment, T_{reg} cell frequency, and disease incidence. Thus, we propose using Dll4 blockade to suppress established autoimmune diabetes.

DISCUSSION

In this study, we have described a novel function for Dll4–Notch signaling as an Flt3–alternative pathway essential for regulating tDC homeostasis with potential implications in the treatment of autoimmunity. Anti-Dll4 Ab treatment induced development of immature tDCs originating from early T cell precursors (DN1 stage, $CD4^{-}CD8^{-}c-kit^{+}CD44^{+}CD25^{-}$) in an Flt3-independent manner, implicating transcription factors critical for DC differentiation (*PU.1*, *Spi-B*, *If-4*, and *CSF-1*). DN1-derived tolerogenic DCs promoted *in vitro* differentiation of naive $CD4^{+}$ T cells into T_{reg} cells. Furthermore, Dll4–Notch signaling inhibition induced $CD25^{+}FoxP3^{+}$ nT_{reg} cell enrichment by a DC-dependent mechanism that required MHCII expression on DCs *in vivo*. Finally, pharmacological Dll4 blockade suppressed T1D via a T_{reg} cell-mediated mechanism and inhibited pancreatic islet infiltration. Importantly, a single injection of Dll4 Ab reversed established T1D, whereas disease remission and recurrence were correlated with increased and decreased T_{reg} cell numbers, respectively, in the PLNs.

In agreement with previous studies demonstrating that genetic inactivation of N1 or Dll4 severely reduces thymic cellularity (Hozumi et al., 2008; Koch et al., 2008; Feyerabend et al., 2009; Billiard et al., 2011), we observed that both Nicastrin and Dll4 deletion induced a significant increase in tDC and T_{reg} cell frequency that was not reflected in cell

numbers because of the decreased cellularity in thymus the day of sacrifice. Anti-Dll4 Ab gave us the ability to examine the kinetics of tDC expansion before the reduction of thymic cellularity occurs, a task impossible to perform by conventional genetic inactivation studies (Hozumi et al., 2008; Koch et al., 2008; Feyerabend et al., 2009). Importantly, we observed a significant increase in imDC absolute number within DN1 pro-T cells as early as 3 d after anti-Dll4 treatment, followed by an expansion of mDC and T_{reg} cell absolute numbers 7 d later when thymic cellularity was not yet severely reduced. The same treatment did not promote expansion of NK cells, granulocytes, or macrophages within the DN1 population. These findings suggest that although the majority of the enriched populations detected within the DN1 cells and the cortical thymic area are DCs and T_{reg} cells, the effect of Notch signaling blockade in DC-dependent T_{reg} cell expansion is reflected in absolute numbers only 1 wk after treatment, as at later time points (days 14 and 21) this increase is not sufficient to compensate for the robust decrease in the overall thymic cellularity. Furthermore, anti-Dll4 treatment did not promote apoptosis of thymocytes, DCs, T_{reg} cells, or T_{eff} cells, and no difference in the apoptosis rate between DCs and T_{reg} cells versus T_{eff} cells and thymocytes was detected upon treatment. Notably, we have previously shown that our anti-Dll4 Ab specifically blocks Dll4 function and does not promote death of Dll4-expressing cells (TECs) or T helper cells; therefore, it does not alter the thymic environment and the final stages of T cell development (Billiard et al., 2011). Moreover, we demonstrated that anti-Dll4 Ab converts early canonical T cell progenitors to DCs, which subsequently promote *de novo* nT_{reg} cell generation rather than endogenous T_{reg} cell proliferation or conversion of nT_{reg} cells to inducible T_{reg} cells. Overall, we conclude that as DCs do not show a higher proliferative potential and have a similar apoptosis rate to thymocytes and T_{eff} cells, it seems that anti-Dll4 Ab treatment does not enhance proliferation of an existing tDC pool but rather converts DN1 progenitors to DCs. This Dll4-mediated conversion represents a source of continuous tDC generation that could account for the increase in absolute tDC number. To further support the functional relevance of the anti-Dll4-mediated T_{reg} cell expansion, we demonstrated that in an inflammatory setting (CFA/OVA administration and T1D), anti-Dll4 treatment of NOD mice at diabetes onset induces a significant increase in T_{reg} cell number in the periphery, as opposed to what we observed under steady-state conditions. This T_{reg} cell expansion directly correlates with T1D remission. Indeed, T_{reg} cell-depleting PC61 treatment together with anti-Dll4 administration significantly accelerated T1D relapse compared with the treatment with anti-Dll4 Ab alone. Thus, we demonstrated that anti-Dll4 treatment initiates an expansion of T_{reg} cells necessary for T1D remission. These findings suggest a role for Dll4–Notch signaling in T_{reg} cell homeostasis and provide evidence for the functional relevance of Dll4-mediated T_{reg} cells in the treatment of T1D. Finally, we found that the effect of the anti-Dll4 Ab in tDC and nT_{reg} cell expansion is

reversible. Overall, these results demonstrate that a sustained Dll4–Notch signaling blockade is required to maintain alternative DC development in the thymus, promote early DC and T_{reg} cell expansion followed by an enrichment of T_{reg} cells in thymus, and induce T_{reg} cell expansion in the periphery upon immune stimulation.

Anti-Dll4 Ab treatment did not induce accumulation of tDCs and T_{reg} cells in the medullary area, but rather it favored accumulation of these cells in the cortex. In a recent study, Lei et al. (2011), using *Xcl1*-deficient mice, showed a reduction of tDC and T_{reg} cell numbers in thymic medulla, suggesting a potential role for this protein in the medullary accumulation of tDCs and T_{reg} cell generation. Here, gene array data showed an up-regulation of *XCL1*, along with chemokine receptors (*CCR2*, *CCR5*, *CCR7*, and *CXCR4*) known for their role in cell migration (Sallusto et al., 1998). Further investigation is required to address whether overexpression of *XCL1* and chemokine receptors could lead to ectopic appearance and accumulation of DCs and T_{reg} cells in the thymic cortex.

Current evidence suggests that a majority of DCs are generated from early intrathymic T cell precursors (Wu and Shortman, 2005; Li et al., 2009; Merad and Manz, 2009; Zhou et al., 2009). However, a recent study proposed a DC potential of intrathymic precursors found within the DN1 population that originate from BM DC progenitors and do not share a common origin with pro-T cells (Luche et al., 2011). In contrast, another study demonstrated that genetic inactivation of N1 converted early T cell progenitors to DCs that contain TCR $D\beta 1$ – $J\beta 1$ rearrangements, thus providing evidence for the existence of a common T/DC precursor (Feyerabend et al., 2009). In agreement with this work, we found that alternative tDC differentiation originating from early T cell progenitors is mediated by Dll4–Notch signaling blockade and that a sustained signal requiring the Dll4–Notch signaling pathway is needed to suppress early tDC expansion. Indeed, a gene array study on purified DN1 cells showed that anti-Dll4 Ab treatment does not modify the expression of genes (*CD11c*) found in DC precursors and in fully differentiated DCs, but it rather down-regulates genes involved in T cell differentiation while up-regulating others (*PU.1* and *SpiB*) crucial in DC development. Moreover, no major modification was observed in peripheral DC homeostasis upon pharmacological blockade or Dll4 deficiency, suggesting that peripheral DCs are not directly affected by this treatment. Our findings are in agreement with a previous study showing that N1 deficiency has no effect on splenic DC subsets (Feyerabend et al., 2009). In contrast, a separate study shows that recombinant binding protein (RBP-Jk), a molecule implicated in Notch receptor signaling cascade, seems to play an intrinsic role in the maintenance and survival of peripheral $CD8^-$ mDCs. However, its selective requirement in $CD8^-$ DCs is correlated with the specific and RBP-Jk-dependent expression of Notch target *Deltex1* by this population (Caton et al., 2007). In addition, it is known that Dll4 is not constitutively expressed on DCs and/or splenocytes, but rather its expression is inducible (Skokos and Nussenzweig, 2007;

Takeichi et al., 2010). N1–4 expression remains undetectable on the DC surface (Merad and Manz, 2009), whereas N1 is highly expressed on DN thymocytes as expected (Fiorini et al., 2009). Thus, we hypothesized that anti-Dll4 Ab does not have a direct effect on DC precursors or differentiated splenic DCs but must preferentially inhibit the interaction between TECs expressing Dll4 and DN thymocytes expressing N1 to convert these DN1 thymocytes to a DC phenotype.

The underlying mechanism by which Notch signaling blockade induces and sustains alternative tDC development has not been explored to date. Here, we demonstrate that anti-Dll4 Ab treatment is sufficient to promote an immature tDC differentiation, surprisingly via an Flt3-independent mechanism, as mice lacking either Flt3-L (*Flt3-L^{-/-}*) or its receptor (*Flt3-R^{-/-}*) showed a significant increase in DC numbers upon Dll4–Notch signaling blockade. A gene array experiment using purified thymic pro-T cells showed that suppression of Dll4–Notch signaling up-regulated transcription factors (e.g., *PU.1*, *RelB*, *Id2*, *Irf2*, *Irf4*, and *Irf8*) and CSF-1 cytokine, which are crucial for DC subset development and differentiation. Consistent with our findings, a recent study showed that conditional ablation of *PU.1* leads to a severe reduction in both cDC and pDC numbers in thymus mediated by Flt3-L or other key targets of *PU.1* such as *GM-CSF*, *GM-CSFR*, *CD11b*, *CSF-1*, *Irf4*, and *Irf8* (Carotta et al., 2010). Furthermore, anti-Dll4 Ab treatment induced enrichment of $CD25^+FoxP3^+$ nT_{reg} cells via a DC-mediated MHCII-dependent mechanism. Although Flt3 is considered to be essential for DC development in the BM and in healthy mouse brain (Liu and Nussenzweig, 2010; Anandasabapathy et al., 2011) and an important component in controlling the DC– T_{reg} cell regulatory feedback loop (Darrasse-Jéze et al., 2009), our results revealed the existence of two signaling pathways (Flt3 and Dll4–Notch) that both can promote a reversible DC-dependent nT_{reg} cell expansion but function independently in two anatomically distinct locations, periphery and thymus, respectively. Indeed, the Flt3 pathway favors a sustained proliferation of nT_{reg} cells in the periphery (Darrasse-Jéze et al., 2009), whereas Dll4–Notch promotes a de novo nT_{reg} cell differentiation in thymus. In addition, our study provided in vitro and in vivo evidence for the tolerogenic potential of DN1-derived DCs in generating T_{reg} cells. Overall, this finding reveals a novel role for Dll4 in regulating tDC and the subsequent T_{reg} cell homeostasis.

Using the NOD spontaneous autoimmune T1D mouse model, we showed that anti-Dll4 Ab treatment prevented diabetes development by inhibiting $CD8^+$ cell pancreatic islet infiltration and promoted enrichment of functional nT_{reg} cells able to suppress T cell proliferation ex vivo. In addition, simultaneous injection of anti-Dll4 and anti-CD25 (PC61) T_{reg} cell-depleting Ab caused T1D relapse, suggesting an important role for anti-Dll4-mediated T_{reg} cells in disease prevention. An additional potential mode of action for anti-Dll4 Ab in T1D prevention could implicate the inhibition of T cell differentiation toward a Th1 phenotype important in T1D development (Esensten et al., 2009; Takeichi et al., 2010).

Strikingly, a single injection of anti-Dll4 Ab reversed established T1D via a T_{reg} cell-dependent mechanism. The correlation between disease remission and increased T_{reg} cell numbers in both PLNs and spleen, Ab washout after treatment cessation (Billiard et al., 2011), and the return of T_{reg} cell numbers to baseline upon disease recurrence suggests an important role for the Dll4–Notch signaling pathway in the treatment of T1D. Few studies have reported the reversal of an early diabetes onset with sufficient efficiency: only successive daily injections of low doses of IL-2 (Grinberg-Bleyer et al., 2010) or anti-CD3 Ab (Belghith et al., 2003) or high quantities of in vitro-expanded islet-specific T_{reg} cells (Tang et al., 2004) had this effect. Our treatment is highly efficient in that a single injection reverses hyperglycemia in 100% of mice within a few days, although a certain therapeutic window of 250–350 mg/dl of blood glucose level must apply. Although anti-CD25 Ab treatment shortens the diabetes remission time, thus strongly suggesting a T_{reg} cell-mediated mechanism, the fast remission we observed is unlikely to be solely the result of T_{reg} cell proliferation, as it would take several days for these cells to expand enough. Because Dll4 and Notch signaling have been shown to play multiple roles in diverse biological pathways, such as in cell development and differentiation (i.e., of endothelial cells [Hellström et al., 2007] and intestinal stem cells [Pellegrinet et al., 2011]) and in angiogenesis (Gale et al., 2004; Noguera-Troise et al., 2006), it is possible to speculate that multiple modes of action could be occurring here. In fact, it has recently been shown that pharmacological blockade of Notch signaling with γ -secretase inhibitors raises insulin sensitivity in both lean and obese insulin-resistant mice (Pajvani et al., 2011). It is conceivable that the fast reduction of blood glucose levels observed in our system may be insulin mediated. Although further investigation is needed to better understand this complex mechanism, our data suggest that Dll4 blockade promotes a DC-dependent T_{reg} cell expansion necessary for disease remission. Finally, we showed that nT_{reg} cells generated upon anti-Dll4 Ab treatment were not qualitatively different from the endogenous nT_{reg} cells found in the diabetic mice, but rather were outnumbered. Our data are in agreement with a previous study showing that T_{reg} cells from diabetic and diabetes-free NOD mice (20–25 wk old at this stage) similarly suppress the proliferation of T_{eff} cells from prediabetic NOD mice, as in our system (Gregori et al., 2003). The percentage of inhibition was low (30%), as previously described in NOD mice of this age (You et al., 2005). In addition, it has been suggested that lower frequencies of T_{reg} cells in T1D patients compared with healthy controls could participate in the development of the disease (Kukreja et al., 2002) and that polyclonal T_{reg} cell enrichment is sufficient and essential to suppress autoimmunity (Darrasse-Jèze et al., 2009). Therefore, the overall outcome of the anti-Dll4 Ab treatment in an autoimmune disease setting could be caused by a DC-dependent T_{reg} cell amplification cascade originating in thymus and leading to peripheral T_{reg} cell expansion that subsequently down-regulates the autoimmune process.

In summary, our results support the existence of a novel Flt3-alternative pathway involved in tolerogenic tDC development and subsequent MHCII-dependent nT_{reg} cell enrichment. Under physiological conditions, this pathway is suppressed in the presence of ongoing Dll4–Notch signaling. These features of Dll4–Notch signaling extend our knowledge by linking Notch and DC biology and revealing a potential pathway for controlling T_{reg} cell homeostasis that could be used as therapeutic treatment in autoimmunity.

MATERIALS AND METHODS

Mice. 6-wk-old C57BL/6 (CD45.2⁺ and CD45.1⁺) and female NOD mice (The Jackson Laboratory) were housed in our animal facility. COIN (conditionals by inversion) Dll4 transgenic mice were obtained by crossing ROSA26-CreERT⁺ with Dll4-COIN mice, resulting in F1 mice that expressed the Dll4-COIN gene in all tissues. Inversion of the reading sequence and thus inactivation of Dll4 was induced by the injection of tamoxifen (2 mg/animal/d) emulsified in corn oil for 5 d. Mice were sacrificed 2.5 wk later. Control mice were injected with corn oil only. *Ncstn*^{fl/Mx1-Cre^{+/+} mice (Klinakis et al., 2011; *Ncstn*^{-/-}) were injected starting at 3 wk of age with polyI:C for 2 wk to induce inactivation of Notch receptor signal transduction. The mice were sacrificed 3.5 wk later. *Ncstn*^{-/-}→WT BM chimeras were obtained by BM transplantation from *Ncstn*^{-/-} mice (or from WT C57BL/6 for controls) into lethally irradiated C57BL/6 recipient mice. 8 wk later, mice were injected twice a week for 3 wk with control or anti-Dll4 Ab. CD11c-Cre/I-A^bflox mice (Darrasse-Jèze et al., 2009) and *FoxP3*^{fl} mice (Fontenot et al., 2005b) were used at 6 wk of age. CD11c-DTR→WT chimeras were obtained by BM transplantation from CD11c-DTR mice into lethally irradiated C57BL/6 recipient mice. 8 wk later, mice were injected twice a week for up to 13 d with DT along with control or anti-Dll4 Ab. *Flt3-R*^{-/-} and *Flt3-L*^{-/-} mice (Darrasse-Jèze et al., 2009) were injected at 12 wk of age with control or anti-Dll4 Ab. Animal housing and procedures were reviewed and approved by Regeneron Pharmaceuticals, Inc.'s internal animal care and use committee.}

Anti-Dll4 Abs. By immunizing VelocImmune mice, which express the full repertoire of human Ab variable domains in the endogenous locus of the mouse immunoglobulin gene, we discovered an array of Dll4 Abs that bind to various domains of Dll4 and efficiently block the interaction of Dll4 with Notch receptors. Further characterization was performed on 40 of these Abs, which represent a significant diversity of repertoire and bind Dll4 with an affinity of <1 nM, in cell-based and biochemical assays. A subset of these Abs was further tested using in vivo assays, and one (anti-Dll4 Ab) that recognizes both human and mouse Dll4 was selected for use in these experiments, which allowed investigation of thymic changes in WT mice (i.e., non-Dll4 humanized). These Abs were both previously tested (Billiard et al., 2011). Human Fc (IgG1; control Ab) was used as a control.

Ab treatments. C57BL/6, WT→ or *Ncstn*^{-/-}→WT BM chimeras, *Flt3-R*^{-/-}, *Flt3-L*^{-/-}, CD11c-Cre/I-A^bflox mice, and CD11c-DTR→WT chimeras were injected subcutaneously with 25 mg/kg anti-Dll4 or control Ab twice a week for 1, 2, or 3 wk depending on the experiment. For prophylactic treatment, NOD mice were injected at 8 wk of age, once a week for 10 wk. For therapeutic treatment, NOD mice were injected once at diabetes onset, with or without 1 mg anti-CD25 PC61 Ab. For T_{reg} cell depletion in prophylactic treatment, NOD mice were left 2 wk without anti-Dll4 Ab treatment and then were injected intraperitoneally with 1 mg anti-CD25 PC61 Ab or isotype control (rat IgG1).

Cell preparations. Thymi and spleens were collected and gently dissociated between frosted slides. BM was collected by flushing femurs. All cells were strained after dissociation or flushing and washed in PBS 1× 3% fetal bovine serum for staining.

Abs for flow cytometric analysis. The following Abs were used: for DCs, Sirp- α (P84), B220 (RA3-6B2), PDCA-1 (eBio927), CD8 (53-6.7), CD11b/MAC-1 (M1/70), MHCII (M5/114.15.2), CD11c (N418), and CD135 (A2F10); for T cells, CD45.1 (A20), CD4 (GK1.5 or L3T4), CD3 (145-2C11), CD25 (PC61 or 7D4), FoxP3 (FJK16s), N1 (mN1A), Ki67 (20Raj1), Helios (22F6), GITR (DTA-1), CTLA-4 (WKH203), pStat5 (47), CD69 (H1.2F3), and CD62L (MEL-14); for progenitors, Sca-1 (D7), c-kit (2B8), CD115 (CSF-1-R; AFS98), and CD135 (Flt3-R; A2F10); and Lin gate was NK1.1 (PK136), Ter119 (TER-119), Gr1 (RB6-8C5), CD19 (eBio1D3); all from eBioscience except Sirp- α , CD45.1, and pStat5 from BD and Helios from BioLegend). DAPI was used at 10 μ g/ml for proliferation assay (Invitrogen). Necrotic cells were excluded from analysis using Live/Dead Fixable Dead Cell Stain kit (Invitrogen). FoxP3 staining buffer set (eBioscience) was used to stain T_{reg} cells. Cells were acquired on an LSRII (BD), and data were analyzed using FlowJo software (Tree Star).

Diabetes and glucose monitoring. NOD mice were checked for glucose levels once a week (or twice at the time of the onset) using a OneTouch mini glucometer (LifeScan). The mice were considered diabetic after two consecutive readings >250 mg/dl. Diabetes occurred between 15 and 20 wk of age in our animal facility.

Histology. Pancreases were harvested in 10% buffered formalin (Thermo Fisher Scientific). After 24 h, the organs were transferred in ethanol 70%. Sections and hematoxylin and eosin (H&E) staining were performed by Histoserv, Inc.

Immunofluorescence analysis. Tissues were fixed in 4% PFA and perfused with 30% sucrose before freezing in optimal cutting temperature compound (Sakura). Frozen tissue was sliced into 10- μ m-thick sections, postfixed with acetone, and stained with the following Abs: for thymus, mTEC-specific Ab MTS-10 (BD), Alexa Fluor 647-conjugated anti-IgM (Invitrogen), DC-specific CD11c-biotin (clone N418; BD), and Alexa Fluor 555-conjugated streptavidin (Invitrogen); FoxP3 promoter-driven GFP was detected by Alexa Fluor 488-conjugated anti-GFP (Invitrogen); for pancreas, islets were stained with guinea pig anti-insulin (Dako) and DyLight-649-anti-guinea pig (Jackson), human antilugucagon (Regeneron Pharmaceuticals, Inc.) and DyLight-649-anti-human (The Jackson Laboratory), CD8-biotin (BD), and Alexa Fluor 488-streptavidin (Invitrogen). Images were acquired on an inverted laser-scanning confocal microscope (SP2 [Leica] or LSM 510 [Carl Zeiss]) using a 40 \times or 25 \times objective. Images were analyzed using ImageJ software (National Institutes of Health).

Quantitative PCR. Total RNA was isolated using the RNeasy Plus Mini kit (QIAGEN), and cDNA was synthesized using the SuperScript First-Strand kit (Invitrogen). Quantitative PCR was performed using SYBR green iMaster and a LightCycler 480 (Roche). The following primer sequences were used (melting temperature = 60°C used for all primers): mN1 forward, 5'-TGCCTGTGCACACCATTCTGC-3'; and reverse, 5'-CAATCAGAGATGTTGGAATGC-3'; mNotch2 forward, 5'-ATGCACCATGACATCGTTCC-3'; and reverse, 5'-GATAGACTCACTGAGCTCTCG-3'; mNotch3 forward, 5'-TTGGTCTGCTCAATCCTGTAGC-3'; and reverse, 5'-TGGCATTGGTAGCAGTTGCTG-3'; and mNotch4 forward, 5'-AAGCGACACGTACGAGTCTGG-3'; and reverse, 5'-ATAGTTGC-CAGCTACTTGTGG-3'.

Whole mouse genome oligonucleotide microarray. RNA from each sample was amplified by TargetAmp 2-Round Aminoallyl-aRNA Amplification kit (Epicentre). Amplified cRNAs were labeled with Cyanine 3 and hybridized to custom arrays comprising of 43,538 60-mer oligonucleotides covering the whole mouse transcriptome (Agilent Technologies). Hybridized microarrays were scanned on a Microarray scanner (Agilent Technologies). Raw data were extracted from scanned array images using Feature Extraction 9.5.1.1 (Agilent Technologies). Global normalization was performed by normalizing each chip to the 75th percentile of the measurements

taken from that chip. The transcripts that were differentially abundant between anti-Dll4 Ab and control Ab treatments were identified. Comparisons between two groups for continuous variables were made with a Student's *t* tests. Results were considered statistically significant if *P* < 0.05. Microarray data were deposited in GEO DataSets under accession number GSE24296.

ELISA. For the detection of Flt3-L and CSF-1, sera from mice were collected, and manufacturer's instructions were followed (R&D Systems).

Cell injections and immunization. For intrathymic injections, DN1 pro-T cells from thymus of CD45.1⁺ C57BL/6 mice were magnetically enriched using anti-CD4/CD8 beads (Miltenyi Biotech) and then sorted on a MoFlo (Beckman Coulter) based on their Lin⁻CD3⁻CD4⁻CD8⁻c-kit⁺CD25⁻CD11c⁻ expression. Recipient CD45.2⁺ mice were anesthetized with ketamine and xylazine, the thoracic cage was carefully opened to expose the thymus, and 10⁵ DN1 cells were injected using a 10- μ l Hamilton syringe. Recipient mice were injected with 25 mg/kg anti-Dll4 or control Ab 24 h before the surgery and right after recovery. The injections were pursued every 3 d until day 9 after surgery, the time of sacrifice. For T_{eff} cell transfers, OVA-specific (OTII) cells from C57BL/6 mice were magnetically enriched using a Regulatory T Cell Isolation kit (Miltenyi Biotech). The CD4⁺CD25⁻ fraction was kept and assessed for FoxP3 expression before injection. Mice were immunized subcutaneously with a 100- μ g emulsion of CFA and OVA.

In vitro culture of tDCs and T cells. tDCs were enriched from anti-Dll4- or control-treated mice using anti-CD11c magnetic beads (Miltenyi Biotech) with two consecutive positive selections. Purity was >90%. Thymic CD4⁺CD25⁻FoxP3⁻ T cells were enriched from WT C57BL/6 mice using a Regulatory T Cell Isolation kit with two consecutive negative selections for CD25. T cells were cultured at 10⁶/ml with DCs (ratio T/DC, 5:1) and 0.5 μ g/ml hIL-2 (R&D Systems) for 3 d.

Western blot of pStat5/Stat5. Enriched thymic CD4⁺CD25⁻FoxP3⁻ T cells (with a Regulatory T Cell Isolation kit) were lysed by boiling in SDS sample buffer (Invitrogen). Proteins were analyzed by PAGE and transferred to polyvinylidene fluoride membranes. After blocking, the indicated proteins were probed using Abs specific for Stat5 or pStat5 (Cell Signaling Technology) or actin (Abcam). Membranes were then incubated with an anti-rabbit or anti-mouse Ab conjugated to horseradish peroxidase. Proteins were visualized by addition of SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific) and exposure to x-ray film.

Proliferation assay. Enriched splenic T_{reg} cells (CD4⁺CD25⁺) and T_{eff} cells (CD4⁺CD25⁻) from NOD mice were obtained with a Regulatory T Cell Isolation kit. The purity of CD4⁺CD25⁺ cells exceeded 98% (cells were >98% FoxP3⁺) after three consecutive positive selections, and the contamination of T_{eff} cells by CD25⁺FoxP3⁺ cells was <3%. 2 \times 10⁴ T_{eff} cells and irradiated syngeneic T cell-depleted splenocytes from 4-wk-old prediabetic NOD mouse were cultured with or without 2 \times 10⁴ T_{reg} cells for 3 d in U-bottomed 96-well plates, with 3 μ g/ml anti-CD3 ϵ mAb (145-2C11; BD) and pulsed with [Methyl-3H]thymidine (1 μ Ci/well) for the last 6–8 h of culture. Cells were collected on a Filtermate Harvester, and thymidine disintegration (cpm) was counted on a TopCount (PerkinElmer). The percentages of inhibition were calculated as follows: percent inhibition = 100 - (cpm with T_{reg} cells/cpm T_{eff} cells alone) \times 100.

Statistical analysis. Statistical significances were calculated using the two-tailed unpaired Student's *t* test with 95% confidence intervals (*, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.001).

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