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).

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 (Treg cells) in the thymic cortex. Both genetic inactivation models and anti-Dll4 antibody (Ab) treatment promote de novo natural Treg 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 Treg cells. Induction of these tolerogenic DN1-derived tDCs and the ensuing expansion of Treg cells are Fms-like tyrosine kinase 3 (Flt3) independent, occur in the context of transcriptional up-regulation of PU.1, Irf-4, Irf-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 Treg 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 Treg cell numbers in the pancreas-draining lymph nodes. These results identify Dll4–Notch as a novel Flt3-alternative pathway important for regulating tDC-mediated Treg cell homeostasis and autoimmunity.
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 thymi 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 (Radtké 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 (Radtké 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 (Treg cells; Watanabe et al., 2005). Treg 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 Treg 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 Treg 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 Treg 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 DCs originating from DN1 pro–T cells, independently of Flt3 and in the context of transcriptional up-regulation of PU.1, Ifn-γ, Ifn-β, and CSF-1, genes critical for DC differentiation. Furthermore, we showed that DN1-derived DCs promote the differentiation of naive CD4+ T cells into Treg cells in vitro and de novo CD25+FoxP3+ natural Treg cell (nTreg 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 Treg 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 Treg 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 Treg 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+) and immature DCs (iDCs; CD11c–B220−; P < 0.01), and immature DCs (imDCs; CD11c+MHCII+; 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 Treg 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 Treg cells compared with effector T cells (Teff cells; CD4+CD25–FoxP3−) in thymus, as demonstrated by a sixfold increase in the ratio of Treg cell versus Teff cell numbers (P < 0.001; Fig. 1 D, right). Thus, a majority of
and 16-, seven-, and fivefold increases of B cell (P < 0.001), DC (P < 0.001), and Treg 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 Treg 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 \( \gamma \)-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 Treg 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), CD4+ T cells within the thymus were FoxP3+CD25+, demonstrating that an enrichment of thymic Treg cells had occurred upon Notch signaling blockade. Treg 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 Treg cell homeostatic balance under steady-state conditions.

### Dll4–Notch signaling inhibition induces reversible tDC and Treg 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\(^{2+}\)/Dll4-COIN adult mice treated with tamoxifen (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 Treg 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 Treg 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 \( \gamma \)-secretase–Notch is needed to suppress the alternative DC enrichment in thymus.

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Figure 2. Dll4–Notch signaling inhibition induces a reversible tDC and Treg 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 Treg cell (FoxP3+CD25+ among CD3+CD4+; B) enrichment in thymus of vehicle (corn oil)- versus tamoxifen (TAM-induced Dll4 inactivation)-treated ROSA26-CreERt2+/Dll4-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-Dll4 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) Genetics of imDC (CD11c−MHCII−) enrichment in percentages (top) and absolute numbers (bottom) after treatment with anti-Dll4 or control Ab. Dot plots show day 7. (E) Dot plots (day 14, left) and graphs reflect Treg cell frequencies (right) in thymus upon anti-Dll4 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) Treg cell absolute numbers (left) in thymus upon anti-Dll4 versus control Ab treatment in C57BL/6 mice. Bar graphs (right) reflect the proportion of Treg cell numbers versus Teff 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-Dll4 Ab. mDC, Treg cell, and Teff 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 Treg cells (CD3+CD4+CD8−CD25−FoxP3+ and Treg 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 Treg cells among CD4+ T cells was observed within the thymus as demonstrated by a seven- to ninefold increase in the ratio of Treg cell/Teff cell numbers because of the severe drop in thymic cellularity.

Interestingly, we observed a significantly higher FoxP3 accumulation 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–prey-bred mice (Fontenot et al., 2005b; Lei et al., 2011). To determine whether anti-Dll4 treatment affects the anatomical distribution of DCs and Treg cells, FoxP33+ mice (Fontenot et al., 2005b) were treated with anti-Dll4 versus control Ab for 7 d. Thymic sections were stained with anti-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 Treg cell expansion in thymus 1 wk after treatment and an enrichment of these cell populations at later time points.

tDCs and CD25+FoxP3+ Treg 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 Treg cells, FoxP33+ 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+ Treg 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 Treg 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 Treg cells in the cortical area. To examine whether the homeostatic effect of anti-Dll4 Ab treatment on DCs and Treg 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 Treg cell numbers (P < 0.01). After cessation of treatment (4 wk, “recovery”), both tDC and Treg 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 Treg cell expansion.

**MHCI expression by DCs is required for in vivo Treg cell enrichment upon anti-Dll4 Ab treatment**

It has been shown that DCs contribute to Treg cell induction (Watanabe et al., 2005). To examine whether DN1-derived DCs induce in vitro Treg 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 DCs sorted from anti-Dll4– or control-treated animals in the presence of IL-2, a cytokine required for Treg 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 Treg cell proliferation in response to cultured DCs appears to be independent of MHCI (Swee et al., 2009), a separate study shows that homeostatic Treg cell division requires self-antigen presentation by MHCI because it is impaired in MHCI KO mice (Shimoda et al., 2006). In addition, a recent work demonstrates that Flt3-dependent DC increase in the periphery leads to increased homeostatic Treg cell division and accumulation by a mechanism requiring MHCI expression on DCs (Darrasse-Jèze et al., 2009). To determine whether Dll4-mediated thymic Treg cell enrichment (Fig. 2 F) was DC dependent, CD11c+ DCs were ablated by administration of diphtheria toxin (DT) in CD11c−DTR→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 Treg cell frequency increase (Fig. 3 B), thus demonstrating the essential role of DCs
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−CD45.1+CD11c+CD11b− (MDP) and Lin−Sca-1−c-kit−CD45.1+CD11b+ (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 DC expansion upon Dll4 blockade, we first examined the kinetics of appearance of imDCs within the DN1 population. We observed a threelfold 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 transplanted 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 (Pénit et al., 1995; van Meervijk et al., 1997). Indeed, based on the kinetics of thymocyte development and the fact that a...
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+) 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. 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+ donor 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 ± SD. *, P < 0.05; **, P < 0.01.

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.
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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 Treg cell expansion observed 7 d after anti-Dll4 treatment took place in an Flt3-independent manner as demonstrated by an increase of Treg 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 Treg 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 Treg cells among the CD4+ effector cell population in thymus, as demonstrated by an increase in the ratio of Treg cells/eff cells (not depicted). We conclude that Dll4–Notch signaling blockade induces an Flt3-independent tDC and subsequent Treg 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 FACSorted Lin−CD11c− DN (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,
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 Treg 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 Treg cell–mediated mechanism

We further examined whether anti-Dll4 Ab treatment could have an effect on peripheral Treg 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 Treg cell expansion (Darrasse-Jèze et al., 2009; Swee et al., 2009). A nonsignificant increase in splenic Treg 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 Treg cell frequency and absolute numbers was observed (Fig. 6, A and B; and not depicted). Importantly, no expansion of peripheral Treg 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 nTreg cells in thymus (Fig. 6, A and B). To confirm that anti-Dll4 Ab treatment induced natural versus inducible Treg...
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 nTreg cells were detected in thymus (P < 0.001) and spleen (P < 0.05; Fig. 6 C). We also assessed Treg cell division by staining for the proliferation marker Ki67. A similar frequency of proliferating FoxP3+ Treg 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 Treg cells by showing expression of GITR, known to be associated with Treg cell function (McHugh et al., 2002), and high expression of Helios and phosphorylated Stat5 (pStat5), both markers of nTreg cell differentiation (Fig. 6 E; Thornton et al., 2010; Goldstein et al., 2011). Neither the percentage of pStat5+ Treg 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 Treg cells, it resulted in a pronounced decrease of the nonphosphorylated form (sStat5, 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 Treg cell population but rather induces de novo generation of thymic nTreg cells. We conclude that Dll4–Notch signaling regulates the homeostasis of nTreg cells via a DC-mediated MHCII-dependent mechanism (Fig. 3 C), without promoting expansion of inducible Treg 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 Treg 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 Treg cell frequency (Fig. 7 A, inset), high ratio of Treg cell/Teff 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 Treg 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 Treg 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) Treg 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 Treg cell

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**Figure 7.** Anti-Dll4 Ab prevents T1D via a Treg 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 administrated to anti-Dll4–treated mice at 20 wk of age. (inset) Percentages of Treg 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 Treg 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 Treg 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 Treg cell–mediated mechanism. This hypothesis is strengthened by the fact that during T1D remission 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 Treg 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 Treg cell-mediated mechanism and inhibits pancreatic islet infiltration.

**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 Treg cells in nondraining LNs (green) or PLNs (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 Treg 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) MFI (mean fluorescence intensity; as calculated by MFI(Ab) – MFI(isotype)) of CD69, CD62L, and FoxP3 on Treg cells from PLNs of NOD mice. Data were collected from mice at specific stages of disease/treatment across three independent experiments. (E) Suppressive activity of purified Treg 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 ± 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 $T_{reg}$ 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 $T_{reg}$ 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 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 n$T_{reg}$ 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, Ifi-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+ n$T_{reg}$ cell enrichment by a DC–dependent mechanism that required MHCI 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 n$T_{reg}$ cell generation rather than endogenous $T_{reg}$ cell proliferation or conversion of n$T_{reg}$ 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 n$T_{reg}$ 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\textsubscript{reg} cell expansion followed by an enrichment of T\textsubscript{reg} cells in thymus, and induce T\textsubscript{reg} cell expansion in the periphery upon immune stimulation.

Anti-Dll4 Ab treatment did not induce accumulation of tDCs and T\textsubscript{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\textsubscript{reg} cell numbers in thymic medulla, suggesting a potential role for this protein in the medullary accumulation of tDCs and T\textsubscript{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\textsubscript{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\textsubscript{β}1–J\textsubscript{β}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 required 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\textsuperscript{+} mDCs. However, its selective requirement in CD8\textsuperscript{+} 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 DC 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\textsuperscript{−/−}) or its receptor (Flk3-R\textsuperscript{−/−}) 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, Ifi4, and Ifi8) 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, Ifi4, and Ifi8 (Carotta et al., 2010). Furthermore, anti-Dll4 Ab treatment induced enrichment of CD25\textsuperscript{+}Foxp3\textsuperscript{+} nT\textsubscript{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\textsubscript{reg} cell regulatory feedback loop (Darrasse-Jèze et al., 2011), our results revealed the existence of two signaling pathways (Flt3 and Dll4–Notch) that both can promote a reversible DC-dependent nT\textsubscript{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\textsubscript{reg} cells in the periphery (Darrasse-Jèze et al., 2009), whereas Dll4–Notch promotes a de novo nT\textsubscript{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\textsubscript{reg} cells. Overall, this finding reveals a novel role for Dll4 in regulating tDC and the subsequent T\textsubscript{reg} cell homeostasis.

Using the NOD spontaneous autoimmune T1D mouse model, we showed that anti-Dll4 Ab treatment prevented diabetes development by inhibiting CD8\textsuperscript{+} cell pancreatic islet infiltration and promoted enrichment of functional nT\textsubscript{reg} cells able to suppress T cell proliferation ex vivo. In addition, simultaneous injection of anti-Dll4 and anti-CD25 (PC61) T\textsubscript{reg} cell–depleting Ab caused T1D relapse, suggesting an important role for anti-Dll4–mediated T\textsubscript{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 Treg cell–dependent mechanism. The correlation between disease remission and increased Treg cell numbers in both PLNs and spleen, Ab washout after treatment cessation (Billiard et al., 2011), and the return of Treg 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 Treg 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 Treg cell–mediated mechanism, the fast remission we observed is unlikely to be solely the result of Treg 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 Treg cell expansion necessary for disease remission. Finally, we showed that nTreg cells generated upon anti-Dll4 Ab treatment were not qualitatively different from the endogenous nTreg cells found in the diabetic mice, but rather were outnumbered. Our data are in agreement with a previous study showing that Treg cells from diabetic and diabetes-free NOD mice (20–25 wk old at this stage) similarly suppress the proliferation of Teff 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 Treg cells in T1D patients compared with healthy controls could participate in the development of the disease (Kukreja et al., 2002) and that polyclonal Treg cell enrichment is sufficient and essential to suppress autoimmunity (Darrasse-Jéze 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 Treg cell amplification cascade originating in thymus and leading to peripheral Treg 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 DC development and subsequent MHCII–dependent Treg 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 Treg cell homeostasis that could be used as therapeutic treatment in autoimmunity.

MATERIALS AND METHODS

**Mice.** C57BL/6 (CD45.2+ and CD45.1+) and female NOD mice (The Jackson Laboratory) were housed in our animal facility. COIN (conditioned media by inversion) Dll4 transgenic mice were obtained by crossing ROSA26-CreERT2 with Dll4-COIN mice, resulting in Fl1 mice that expressed the Dll4–COIN gene in all tissues. Inversion of the reading sequence and inactivation of Dll4 was induced by the injection of tamoxifen (2 mg/animal/d) emulsified in corn oil for 3 d. Mice were sacrificed 2.5 wk later. Control mice were injected with corn oil only. Nestn/+/Mx1-Cre+ mice (Klinakis et al., 2011; Nestn−/−) 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. Nestn−/−→WT BM chimeras were obtained by BM transplantation from Nestn−/− 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/-/Alox mice (Darrasse-Jéze et al., 2009) and FoxP3gfp mice (Fontenot et al., 2005b) were used at 6 wk of age. CD11c–DTR→WT chimeras were obtained by BM transplantation from CD11c–DTR into lethally irradiated C57BL/6 recipient mice. 8 wk later, mice were injected twice a week for up to 13 d with WT along with control or anti-Dll4 Ab. Flt3–R–/– and Flt3−/− mice (Darrasse-Jéze 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 IgG1 (control Ab) was used as a control.

**Ab treatments.** C57BL/6, WT→WT or Nestn−/−→WT BM chimeras, Flt3−/−, Flt3−/−, CD11c–Cre/-/Alox 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 Abs. For Treg 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 into single-cell suspensions. The 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, Surp-α (P84), BI220 (RA3-6B2), POCA-1 (eBio297), CD3 (53-67), CD11b/MAC-1 (M1/70), MICHI (M5/114.15.2), CD11c (N418), and CD135 (A2F10); for T cells, CD45.1 (A20), CD4 (GK1.5 or L3T4), CD3 (145-2C11), CD25 (PC6I or 7D4), FoxP3 (FJK161), N1 (mN1A), K67 (20Raj1), Helios (22F6), GITR (DTA-1), CTLA-4 (WKH203), pStat5 (47), CD69 (HI.2F3), and CD62L (MEL-14); for progenitors, Sca-1 (D7), c-kit (2B8), CD115 (CSF-1–R; AFS98), and CD115 (Flt3-R; A2F10); and Lin gate was NK1.1 (PK136), Ter119 (TER-119), Gr1 (RB6-8C5), CD19 (eBio1D3; all from eBioscience except Surp-α, CD45.1, and pStat5 from BD and Helios from BioLegend). DAPI was used at 10 µg/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 Treg cells. Cells were acquired on an LSRII assay (Invitrogen). Necrotic cells were excluded from analysis using Live/Dead Fixable Dead Cell Stain kit (Invitrogen).

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%. Pancreases were harvested in 10% buffered formalin (Thermo Fisher Scientific). 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-µm-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 anti-glucagon (Regeneron Pharmaceuticals, Inc.) and Dylight-649–anti–human (The Jackson Laboratory), CD135 (A2F10); for T cells, CD45.1 (A20), CD4 (GK1.5 or L3T4), CD3 (174-5G12), CD11b/MAC-1 (M1/70), MHCII (M5/114.15.2), CD11c (N418), and Helios from BioLegend). DAPI was used at 10 µg/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 Treg cells. Cells were acquired on an LSRII assay (BD) and data were analyzed using FlowJo software (Tree Star).

In vitro culture of tDCs and T cells. tDCs were enriched from 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 by 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-µg emulsion of CFA and OVA.

In vitro culture of tDCs and T cells. tDCs were enriched from anti-CD14– 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 by a Regulatory T Cell Isolation Kit with two consecutive negative selections for CD25. T cells were cultured at 10^6/ml with DCs (ratio T/DC, 5:1) and 0.5 µg/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 with 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 Treg cells (CD4+CD25+) and Teff 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 Treg cells by CD25+FoxP3+ cells was <3%. 2 × 10^6 Treg cells and irradiated syngeneic T cell–depleted splenocytes from 4-wk-old prediabetic NOD mouse were cultured with or without 2 × 10^4 Treg cells for 3 d in U-bottomed 96-well plates, with 3 µg/ml anti-CD3e mAb (145-2C11; BD) and pulsed with [Methyl-3H]thymidine (1 µCi/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 percentage of inhibition was calculated as follows: percent inhibition = 100 – (cpm with Treg cells/cpm Treg cells alone) × 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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