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# A Defect in Lineage Fate Decision during Fetal Thymic Invariant NKT Cell Development May Regulate Susceptibility to Type 1 Diabetes[1]

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**A numerical and functional deficiency in invariant NKT (iNKT) cells detectable by 3 wk of age in the thymus and spleen mediates the pathogenesis of type 1 diabetes in NOD mice, but the stage of T cell development at which this deficiency first occurs is unknown. We report in this study that this deficiency develops after the $CD4^+CD8^+$ double-positive stage of thymic T cell development and is due to a lineage-specific depletion of $CD4^-CD8^-$ double-negative $\alpha\beta$ T cells and iNKT cells from the thymus between embryonic day 18 and day 1 after birth. Thus, an inheritable defect in a lineage fate decision that elicits a deficiency in fetal thymic iNKT cell development may predispose to susceptibility to type 1 diabetes.** ***The Journal of Immunology,*** **2005, 174: 6764–6771.**

The NOD mouse spontaneously develops a form of type 1 diabetes (T1D)[3] characterized by the progression from insulitis (infiltration of dendritic cells, macrophages, T cells, and B cells into pancreatic islets) at 3–4 wk of age to islet $\beta$ cell destruction and the onset of disease during 4–6 mo of age. Self-reactive T cells can be isolated from mice and humans free of autoimmune disease (1, 2). Because protection against an autoimmune disease in healthy mice and individuals may be mediated by different types of regulatory T cells, it follows that a deficiency in the number and function of a given subset(s) of regulatory T cells can mediate the onset of such a disease, including T1D (1–3).

Invariant NK T (iNKT) cells constitute a subset of regulatory T cells that express NK cell markers and a highly invariant TCR, V$\alpha$14J$\alpha$18, V$\beta$8.2/7/2 in mice that can recognize lipid Ags in a CD1-restricted manner (3). A numerical and functional deficiency in iNKT cells is detectable both in PBLs of diabetic patients (2) and in the thymus and spleen of prediabetic NOD mice as early as 3 wk of age (3–5). An increase in the number of iNKT cells that leads to a correction of this deficiency in NOD mice is sufficient to decrease insulitis and prevent T1D in several experimental systems, including intrathymic transfer, enhanced development through transgenic expression of the iNKT TCR$\alpha$ chain (V$\alpha$14J$\alpha$18), and proliferation induced by activation through the CD1d-restricted sphingoglycolipid Ag, $\alpha$-galactosylceramide ($\alpha$-GalCer) (6–8). Nonetheless, our understanding of the mechanism(s) of protection from T1D in NOD mice by iNKT cells requires further investigation.

Fully developed $CD4^+$ and $CD8^+$ T cells can first be detected in an embryonic murine thymus ex vivo on the day of birth (19 days postconception, embryonic day 19 (E19)) (9), and embryonic thymus-derived T cells from NOD mice are capable of infiltrating a syngeneic pancreas in vitro (10). It is conceivable, therefore, that T cells capable of infiltrating pancreatic islets might already be developed in these mice by day 1 after birth. Yet, despite this early generation of potentially autoreactive T cells, it is curious that a deficiency in iNKT cells in the thymus of NOD mice was reported to first manifest at the time of peri-insulitis, i.e., at ~3 wk of age (11). To resolve this apparent discrepancy and given that defective T cell activation occurs concomitantly with the onset of insulitis in NOD mice (12), we investigated whether a deficiency in iNKT cells arises earlier than 3 wk of age, and in particular during embryonic thymic T cell development. We reasoned that if this were the case, it might mediate the early pathogenesis of T1D and render NOD mice more susceptible to T1D at birth.

Similar to conventional T cells, iNKT cells pass through the $CD4^+CD8^+$ double-positive (DP) stage of T cell development and undergo positive and negative selection (13, 14). Moreover, several stages of embryonic T cell development are conserved with respect to specificity and order during adult T cell development. Embryonic T cells pass through the four $CD4^-CD8^-$ double-negative (DN) stages of development between E12 and E14, a DP stage that appears at E17-E18 and a mature T cell stage at E19-day 1 after birth (9, 15). $CD4^+$ or $CD8^+$ single-positive (SP) thymocytes that appear at E15-E17 are phenotypically ($CD4^+$ or $CD8^+$, TCR$\alpha\beta^-$) and functionally consistent with that of a precursor to DP thymocytes, initially described in the adult thymus as an immature SP (ISP) T cell (11, 16). Despite reports that common precursors mediate embryonic and adult T cell development and accumulating evidence for a conventional pathway of development for mainstream T cells and iNKT cells, an alternate T cell pathway

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[3] Abbreviations used in this paper: T1D, type 1 diabetes; $\alpha$-GalCer, $\alpha$-galactosylceramide; E, embryonic day; DN, double negative; DP, double positive; iNKT, invariant NK T cell; SP, single positive; ISP, immature SP.



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of development has been proposed for both types of T cells (17–21).

In this study, we further investigated the kinetics and pathway of development of iNKT cells in NOD mice by analyzing whether a deficiency in iNKT cells first occurs during embryonic intrathymic T cell development, and if so, at what stage of intrathymic T cell development. We found that an intrathymic iNKT cell deficiency in both embryonic and adult NOD mice is not present at the DP thymocyte stage of development, but rather manifests subsequent to positive selection of DP thymocytes from a lineage-specific depletion of DN $\alpha\beta$ T cells shortly before birth during E18-day 1. The latter DN $\alpha\beta$ T cells are enriched in DN iNKT cells. Our findings suggest that during embryonic thymic T cell development in NOD mice, conventional $\alpha\beta$ T cells and iNKT cells develop along a common pathway until the DP stage, after which iNKT cells and DN $\alpha\beta$ T cells are depleted and result in an iNKT cell deficiency. We also present data that suggest that the latter intrathymic iNKT cell deficiency may be corrected by stimulation of development with the Notch-1 ligand, Delta-1.

## Materials and Methods

### *Mice*

NOD/Del mice were bred in a specific pathogen-free environment at the Robarts Research Institute. C57BL/6 mice were obtained from Charles River Laboratories or The Jackson Laboratory and then bred at the Robarts Research Institute. The day of the vaginal plug was observed and counted as day 0 of gestation (E0).

### *Preparation of thymocytes*

Mice were euthanized by asphyxiation in a $CO_2$ chamber. Thymi were removed and kept on ice in Dulbecco's PBS (Invitrogen Life Technologies) supplemented with 10% FBS (Sigma-Aldrich) until further use. Embryonic and neonatal thymic lobes were isolated using a dissecting microscope (Niko type 102) and were kept on ice in PBS/FBS until used. Thymi were dissociated into single cells by passage through a 40-$\mu$m nylon mesh (BD Biosciences). Erythrocytes were removed from thymocyte preparations by lysis in potassium-ammonium chloride buffer (0.15 M $NH_4Cl$, 1.0 mM $KHCO_3$, and 0.1 mM $Na_2EDTA$, pH 7.3). Thymocyte viability was determined by trypan blue staining (Sigma-Aldrich). $CD4^-CD8^-$ (DN) thymocytes were isolated by using MiniMacs columns (Miltenyi Biotec), according to manufacturer's protocol, with the exception that PBS/FBS was used instead of PBS.

### *Fetal thymic organ culture*

Fetal thymic lobes were collected at E13 and cultured in complete RPMI 1640 medium supplemented with 10% FBS, 50 $\mu$M 2-ME, 100 IU/ml penicillin, 1 mM sodium pyruvate, and 15 mM HEPES buffer, pH 7.3. RPMI 1640 and all supplements were obtained from Life Technologies. Culture medium was also supplemented with soluble Delta-1-Fc or Jagged-1-Fc (a kind gift from M. Bhatia, Robarts Research Institute, London, Ontario, Canada), where indicated. Lobes (10–15 per well) were cultured in a six-well plate (Costar) at 37°C and 7% $CO_2$ for 3–21 days with a partial medium replacement every 3 days.

### *Flow cytometry*

Thymocytes ($10^5$-$10^6$) were stained at 4°C for 1.5 h and washed three times with PBS/FBS. Fetal thymic culture samples were preincubated in propidium iodide (Sigma-Aldrich) for 15 min before analysis. A FACSCalibur with CellQuest software (BD Biosciences) and FlowJo software (Treestar) was used for flow cytometry. Dead cells were excluded through gating based on forward and side scatter. For fetal thymic organ cultures, dead cells were further excluded by gating on cells negative for propidium iodide staining. Adult thymocytes were stained with $\alpha$-GalCer-loaded CD1d tetramers conjugated to PE, anti-TCR$\alpha\beta$ FITC, anti-CD8$\alpha$ CyChrome (PE Cy5), and anti-CD4 allophycocyanin. Fetal thymocytes were stained with $\alpha$-GalCer-loaded CD1d tetramers allophycocyanin, anti-TCR$\alpha\beta$ FITC, anti-heat-stable Ag FITC, anti-CD8$\alpha$ PE, or anti-CD4 allophycocyanin. To evaluate any nonspecific binding of the latter test Abs to NOD and B6 adult and embryonic thymocytes, the following isotype controls were used: hamster IgG2 FITC (for anti-TCR$\alpha\beta$ FITC), rat IgG2a PE Cy5 (for anti-CD8$\alpha$ PE Cy5), rat IgG2a allophycocyanin (for anti-CD4 allophycocyanin), rat IgG2b FITC (for anti-heat-stable Ag FITC), and rat IgG2a PE (for anti-CD8$\alpha$ PE). All mAbs used for staining were obtained from BD Biosciences. $\alpha$-GalCer-loaded PE or allophycocyanin-conjugated CD1d tetramers were prepared in our laboratory, as described (22).

### *RNA preparation and reverse transcription*

Thymocyte RNA was isolated using RNeasy spin columns (Qiagen), according to the manufacturer's instructions, with the addition of a poly(C) (Sigma-Aldrich) RNA carrier. RNA preparations were pretreated with DNase (Invitrogen Life Technologies), according to the manufacturer's instructions. Reverse transcription was performed with Superscript II (Invitrogen Life Technologies) and oligo(dT) (Invitrogen Life Technologies), according to manufacturer's instructions, and with the addition of an RNase inhibitor (Invitrogen Life Technologies). cDNA was then treated with RNase (Invitrogen Life Technologies), according to the manufacturer's instructions. Semiquantitative PCR analyses were performed by analysis of PCR samples at 15, 20, 25, 30, and 35 cycles. Real-time PCR analysis was performed by determining the fluorescence of a Syber I master mix (Sigma-Aldrich) measured by Stratagene MX 4000. The annealing temperature was 63°C for all PCR, and a brief 82°C step was added to the end of each cycle in real-time PCR cycles to melt primer dimers. The primers were designed using Primer3 software (⟨http://frodo.wi.mit.edu/cgi-bin/primer3/primer3.cgi⟩), as follows: V$\alpha$3J$\alpha$15, 5′-AAGGAGT GAATGGCTTCGAG-3′ and 5′-TGAGTCCCAGCTCCAAAATG-3′; V$\alpha$14J$\alpha$18, 5′-GACAAAACGTCAAATGGGAGA-3′ and 5′-CTGGG TCTGGATGTCAGGT-3′; Delta-1, 5′-TTAGCATCATTGGGGCTA CC-3′ and 5′-TAAGTGTTGGGGCGATCTTC-3′; and $\beta$-actin, 5′-TGT TACCAACTGGGACGACA and 5′-CTCTCAGCTGTGGTGGTGAA-3′.

## Results

### *iNKT cells are depleted after the DP stage in an adult NOD thymus*

The pathway of iNKT cell development is similar to that of conventional T cell development, and includes a precursor at the DP thymocyte stage of development (13). To determine whether iNKT cell development in a NOD thymus is blocked at or before the DP stage of thymocyte development, we examined female NOD and C57BL/6 (B6) thymi at 8 wk of age for their numbers of iNKT and conventional TCR$\alpha\beta$ T cells in the DP, mature $CD4^+$ or $CD8^+$ SP, and DN thymocyte subsets (Fig. 1). iNKT cells were detected by double staining with an anti-TCR$\alpha\beta$ mAb and $\alpha$-GalCer-loaded CD1d tetramers ($Tet^+$), as described (8). FACS analyses demonstrated that NOD mice are deficient in DN thymocytes at this age (Fig. 1*A*). This DN thymocyte deficiency consists of an iNKT cell ($Tet^+TCR\alpha\beta^+$) deficiency in a NOD thymus relative to a B6 thymus (Fig. 1*B*). Note that the number of iNKT cells is also reduced in $CD8^+$, $CD4^+$ SP, and DP thymocytes, but to a much lesser extent.

### *iNKT cell deficiency results from a lineage-specific depletion of DN $\alpha\beta$ T cells*

CD4 and CD8 coreceptors augment physical interactions between TCR and MHC in the periphery and define a Th cell or cytolytic T cell function. Due to their influence on T cell function, CD4 and CD8 lineage fate decisions in the thymus have been studied extensively, whereas DN T cell lineage decision has often been overlooked (23). To determine whether the depletion of DN iNKT cells may be due to a lineage decision event, we examined the numbers of conventional $\alpha\beta$ T cells ($Tet^-TCR\alpha\beta^+$) in the thymus of 8-wk-old NOD and B6 mice. We found that the number of conventional DN $\alpha\beta$ T cells was significantly decreased in a NOD thymus relative to a B6 thymus (Fig. 1*B*). In contrast, a significantly greater number of $CD4^+$ $\alpha\beta$ T cells and $CD8^+$ $\alpha\beta$ T cells were detected in a NOD thymus than a B6 thymus. These results indicate that a lineage-specific depletion of DN $\alpha\beta$ T cells after the DP stage of development may mediate an iNKT cell deficiency detectable in the thymus of an 8-wk-old NOD mouse.

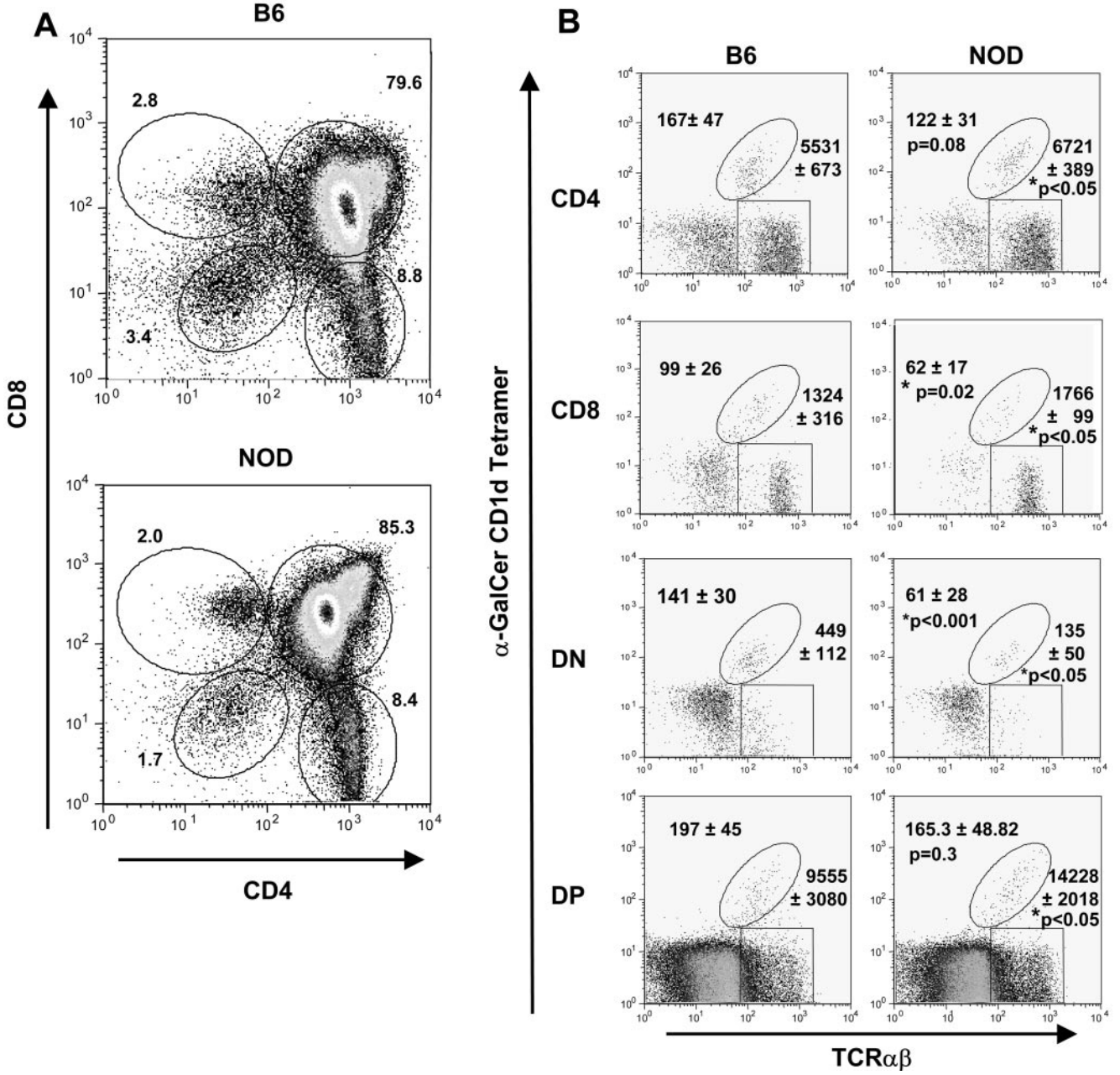


**FIGURE 1.** An iNKT cell deficiency arises after the DP thymocyte stage of development in a NOD thymus and is restricted mainly to the DN lineage of iNKT and conventional $\alpha\beta$ T cells. Mononuclear thymocytes isolated from 8-wk-old NOD ($n = 5$) and C57BL/6 (B6) ($n = 5$) mice were stained, as described in *Materials and Methods*, with either $\alpha$-GalCer CD1d PE tetramers (Tet), anti-TCR$\alpha\beta$ FITC, anti-CD4 allophycocyanin, anti-CD8$\alpha$ CyChrome, or IgG isotype controls. *A*, DP, DN, $CD4^+$ SP, or $CD8^+$ SP subsets were gated. *B*, iNKT cells ($Tet^+TCR\alpha\beta^+$) and conventional $\alpha\beta$ T cells ($Tet^-TCR\alpha\beta^+$) were detected within DP, DN, $CD4^+$ SP, or $CD8^+$ SP subsets. Mean values of absolute cell number ± SD of three flow cytometric samples are shown. *, $p < 0.05$.

### *iNKT cell deficiency arises during NOD fetal thymic development*

At E13 in a fetal mouse, the first wave of common lymphoid precursors enters into the thymic lobes. These precursors are predicted to give rise to T cells between days 1 and 14 after birth (24). To determine whether this first wave of thymic precursors in a NOD thymus results in an iNKT cell deficiency, NOD and B6 thymic lobes were removed from embryos at E13 and introduced into fetal thymic organ culture. This prevents further input of thymic precursors or other developmental stimuli. Interestingly, E13 NOD thymic precursors developed ~10-fold fewer iNKT cells than age-matched B6 thymic precursors (Fig. 2). This iNKT cell deficiency was apparent in an E13 NOD thymus despite the fact that ~4- to 5-fold more conventional $\alpha\beta$ T cells developed in a NOD thymus than in an age-matched B6 thymus (Fig. 2). Thus, an iNKT cell deficiency in NOD mice arises in an E13 thymus in the absence of in vivo developmental stimuli or further common lymphoid precursor input.

### *NOD and B6 fetal thymocytes develop at comparable rates*

Thymocyte development in a fetal B6 and BALB/c thymus parallels that of an adult thymus and progresses through three main precursor stages of mature T cells: DN $TCR\alpha\beta^-$ thymocytes, ISP

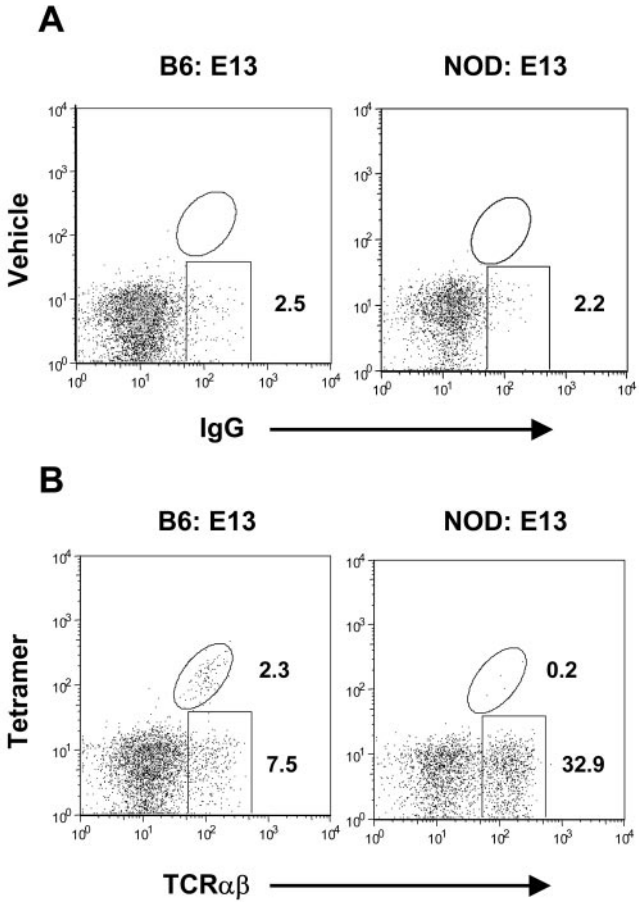


**FIGURE 2.** An iNKT cell deficiency is detectable in NOD fetal thymic organ cultures. Fetal thymic lobes from E13 NOD ($n = 46$) and C57BL/6 ($n = 49$) embryos were cultured for 21 days. Mononuclear thymocytes were then isolated, stained with vehicle (*A*) and α-GalCer CD1d allophycocyanin tetramers (*B*), and anti-TCRαβ FITC or IgG isotype control and iNKT cells ($Tet^+TCR\alpha\beta^+$), and conventional αβ T cells ($Tet^-TCR\alpha\beta^+$) were detected by flow cytometry. One of two reproducible flow cytometric analyses is shown.

$CD4^+$ or $CD8^+$ $TCR\alpha\beta^-$ thymocytes, and DP $TCR\alpha\beta^{-/+}$ thymocytes (9, 15). To ensure that fetal NOD thymocytes develop at a rate comparable to that in B6 and BALB/c mice and with the same main precursor stages during positive and negative selection, we examined the main precursor stages and phenotype of mature T cells by FACS analyses beginning at E17, the initial time of appearance of DP thymocytes in fetal B6 mice. From E17 to day 14 after birth, NOD and B6 thymi consist of similar populations of DN, DP, and $CD4^+$ or $CD8^+$ SP T cells (Fig. 3*A*). Gating on DN, DP, and $CD4^+CD8^-$ or $CD4^-CD8^+$ populations, according to Fig. 3*A*, all NOD and B6 thymocytes were found to be $TCR\alpha\beta^-$ at E17 (Fig. 3*B*). These findings indicate that $CD4^+$ and $CD8^+$ SP thymocytes are ISP T cells at this stage, and that development has progressed in both strains to an early DP stage before TCRαβ-mediated positive or negative selection. At E18, DP thymocytes begin to express TCRαβ, allowing for positive and negative selection, and mature T cells expressing TCRαβ emerge among the $CD4^+$, $CD8^+$, and DN subsets from E18 to day 1 after birth. This finding suggests that positive and negative selection may occur for the first time at E18 in both strains. The distribution of thymocyte subsets from day 1 to 14 in NOD and B6 mice resembles that of adult mice as more DN, $CD4^+$, and $CD8^+$ SP T cells are evident. During this period of development, the latter subsets further mature, as indicated by the loss of CD24 expression (Fig. 3*B*). Thus, phenotypic analyses of fetal B6 and NOD thymocytes indicate that DP $TCR\alpha\beta^+$ thymocytes develop and allow for the positive and negative selection of mature T cells ($TCR\alpha\beta^+$, $CD24^{+/-}$) at E18.

### *Deficiency in DN αβ T cells and iNKT cells develops before birth*

An iNKT cell deficiency in NOD mice was initially demonstrated as a DN αβ T cell deficiency, which after correction by intrathymic transfer of syngeneic DN $TCR\alpha\beta^+$ thymocytes prevents insulitis and T1D (6). Our results indicate that reduced numbers of both DN iNKT cells and conventional T cells are present in an adult NOD thymus compared with a B6 thymus (Fig. 1). Furthermore, an iNKT cell deficiency is most prominent in DN $TCR\alpha\beta^+$ T cells after the DP stage of thymocyte development in an adult NOD thymus (Fig. 1*B*). To determine whether a similar depletion of DN $TCR\alpha\beta^+$ T cells begins with the emergence of $TCR\alpha\beta^+$ DP thymocytes at E18, we examined the distribution of thymocyte populations during late embryogenesis. At E18, a deficiency of mature DN αβ T cells ($TCR\alpha\beta^+CD24^-$) was found to develop in a NOD thymus relative to a B6 thymus (Fig. 3*B*). This deficiency arises concomitantly with the emergence of DP $TCR\alpha\beta^+$ thymocytes, which time it is presumably followed by TCR-mediated positive and negative selection. A role for TCR-mediated positive and negative selection in DN αβ T cell depletion is strengthened by the finding of consistently lower numbers of $CD24^+$ DN αβ T cells in a NOD thymus. The $CD24^+$ DN αβ T cell is a precursor to mature DN αβ T cells ($TCR\alpha\beta^+CD24^-$), and it arises immediately after the DP thymocyte stage. This $CD24^+$ DN αβ T cell subset was consistently low in number in a NOD thymus beginning at E18, and was detectable in statistically significant numbers only by day 1 after birth. At the latter time, significantly more mature $CD4^+$ and $CD8^+$ SP T cells develop in a NOD thymus than B6 thymus (Fig. 3*B*). These results are similar to that found in an adult NOD thymus, indicating that in both embryonic and adult NOD there is a lineage-specific deficiency of DN T cells.

Previously, the earliest detection of iNKT cells by flow cytometry was reported to be 0.01% of thymocytes on day 5 after birth in a B6 thymus (25). iNKT cells comprise only ~0.3–0.4% of adult NOD thymocytes, and are therefore difficult to detect by flow cytometry until adequate numbers of mature T cells have accumulated. To circumvent this technical difficulty and estimate the relative number of iNKT cells during thymic development, we used real-time PCR to quantify the expression of invariant TCR Vα14Jα18 chain mRNA specific for iNKT cells relative to that of β-actin mRNA. This approach was used to determine whether selection of NOD $TCR\alpha\beta^+$ DP thymocytes at E18 depletes iNKT cells and results in an iNKT cell deficiency at birth. Fig. 3 shows that both NOD and B6 thymocytes progress to an early DP stage at E17 without detectable TCRαβ surface expression. As TCRα rearrangement is reported to occur at E17 (26), we were unable to detect Vα14Jα18 mRNA expression at E13 or E15 (our unpublished observations). As TCRα mRNA is readily detected at E17, it is reasonable to assume that the majority of TCRα mRNA detected at this stage is expressed by the large group of $TCR\alpha\beta^-$ DP thymocytes, which first develop at this time with a rather low level of TCRα mRNA expression in DN4 and ISP thymocytes. Thus, Vα14Jα18 mRNA levels at E17 should be indicative of the relative number of iNKT cells at an early DP or less mature precursor stage.

At E17, the ratio of Vα14Jα18 mRNA:β-actin mRNA was found to be greater in NOD thymocytes than in B6 thymocytes (Fig. 4). This finding suggests that a larger number of iNKT cell precursors and/or a higher level of TCRαβ mRNA expression is

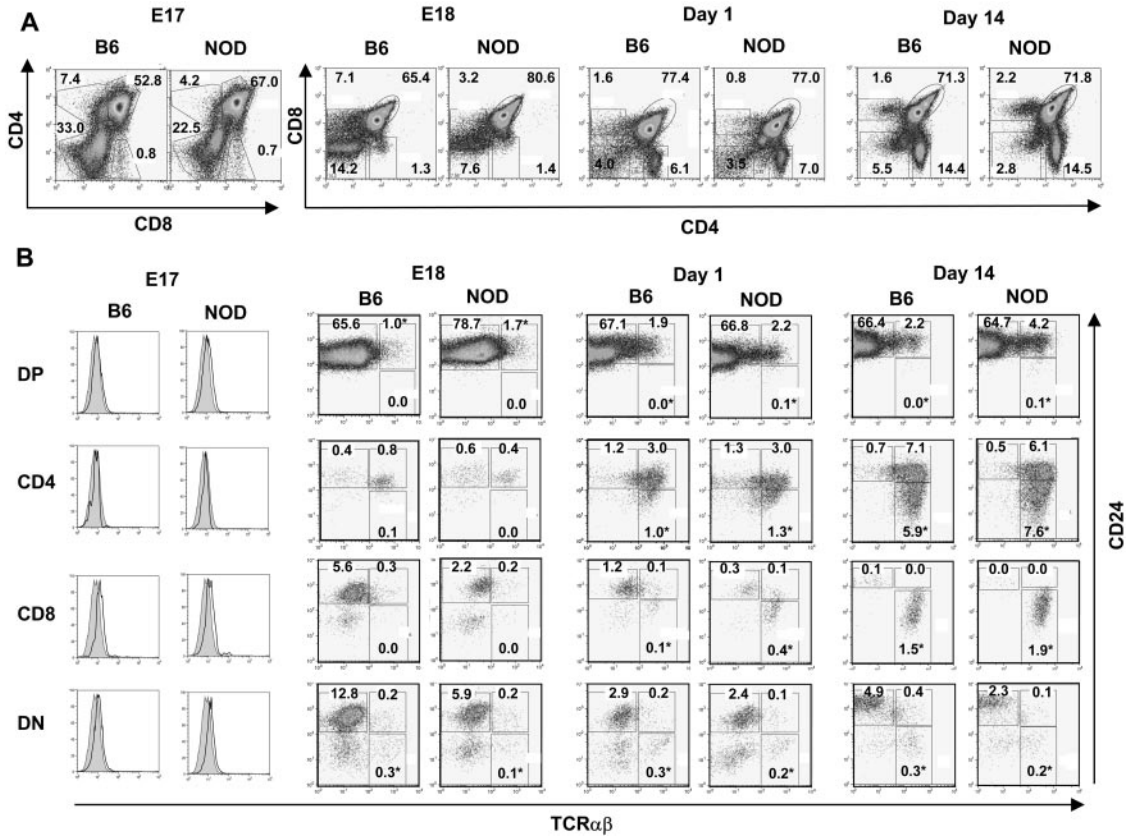


**FIGURE 3.** A deficiency in DN $\alpha\beta$ T cells develops in NOD mice before the day of birth (E19). Mononuclear thymocytes were isolated from NOD and B6 embryos at E17 (B6, $n = 15$; NOD, $n = 15$), E18 (B6, $n = 20$; NOD $n = 20$), day 1 (B6, $n = 18$; NOD, $n = 20$), and day 14 (B6, $n = 4$; NOD, $n = 4$) after birth. *A*, The percentages of DP, DN, $CD4^+$ SP, and $CD8^+$ SP thymocytes present at E17, E18, day 1, and day 14 were analyzed by flow cytometry as in Fig. 2. *B*, The $CD4^+$ SP $\alpha\beta$ thymocytes ($CD4^+CD8^-$), $CD8^+$ SP $\alpha\beta$ thymocytes ($CD4^-CD8^+$), DP $\alpha\beta$ thymocytes ($CD4^+CD8^+$), and DN $\alpha\beta$ thymocytes ($CD4^-CD8^-$) detected in *A* were gated and then analyzed for their respective levels of CD24 and TCR$\alpha\beta$ surface expression by flow cytometry as in Fig. 2. The percentages of cells with varying levels of CD24 and TCR$\alpha\beta$ expression for each of the gated SP, DP, and DN subsets are presented as the mean values ± SD of three flow cytometry samples. $CD4^+$ SP $\alpha\beta$ thymocytes ($CD4^+CD8^-TCR\alpha\beta^+CD24^-$), $CD8^+$ SP $\alpha\beta$ thymocytes ($CD4^+CD8^-TCR\alpha\beta^+CD24^-$), DP $\alpha\beta$ thymocytes ($CD4^+CD8^+TCR\alpha\beta^+CD24^-$), and DN $\alpha\beta$ thymocytes ($CD4^-CD8^-TCR\alpha\beta^+CD24^-$) were detected. SDs ranged from 5 to 15% of the mean values and were generally <10%. *, $p < 0.05$.

present in a NOD than B6 thymus at and before the $TCR\alpha\beta^-$ DP stage of thymocyte development. At E18, DP thymocytes express TCR$\alpha\beta$ on their surface, which enables TCR-mediated positive and negative selection. This results in the emergence of mature DN T cells and a decrease in the level of V$\alpha$14J$\alpha$18 mRNA in NOD thymocytes, which most likely accounts for the similar ratio of V$\alpha$14J$\alpha$18 mRNA:$\beta$-actin mRNA observed in NOD and B6 thymocytes at E18 (Fig. 4). At days 1 and 7 after birth, the estimated iNKT cell deficiency becomes more pronounced in a NOD thymus relative to a B6 thymus. Similar to the findings in adult NOD thymocytes, this kinetic analysis of iNKT cell depletion in fetal NOD thymocytes suggests that depletion occurs during the positive and negative selection of DP thymocytes between E18 and day 1 after birth.

### *Delta-1 stimulates the development of thymic iNKT and conventional $\alpha\beta$ T cells*

The Notch family of molecules is linked to many aspects of T cell development, including the CD4/CD8 lineage decision (27, 28). The Notch ligand, Delta-1, was recently shown to promote conventional $\alpha\beta$ T cell development during the first three stages of DN thymocyte development (29, 30). Delta-1, but not Jagged-1, enhances the development of cells with a phenotype of conventional $\alpha\beta$ T cell and NKT precursors in cultures of human cord blood cells (31). To determine the effect of Delta-1 and Jagged-1 on iNKT cell development, soluble Fc-conjugated forms of these ligands were added to cultures of E13 B6 DN thymocytes, and the development of iNKT cells was estimated by semiquantitative RT-PCR analyses of V$\alpha$14J$\alpha$18 mRNA. Delta-1-Fc, but not Jagged-1-Fc, stimulated V$\alpha$14J$\alpha$18 mRNA expression in a dose-dependent manner (Fig. 5*A*). To determine whether Delta-1 enhances iNKT cell development by a pathway independent of conventional $\alpha\beta$ T cells, as previously suggested (17–19), fetal thymic organ cultures established in the presence or absence of Delta-1-Fc and Jagged-1-Fc were examined for V$\alpha$14J$\alpha$18 and control V$\alpha$3J$\alpha$15 expression by RT-PCR. TCR V$\alpha$3J$\alpha$15 is a gene that was characterized in the B6 T cell repertoire, and primers were designed to study the rearrangement of this gene based on analyses of its expression in a conventional $\alpha\beta CD4^+$ T cell reactive to proinsulin-derived C peptide (32) (National Center for Biotechnology Information accession: Z86026). Delta-1-Fc and Jagged-1-Fc did not yield detectable changes in the number of thymocytes in these cultures (our unpublished observations). However, the levels of TCR V$\alpha$14J$\alpha$18 (iNKT cell-specific) and TCR V$\alpha$3J$\alpha$15 (conventional $\alpha\beta$ T cell-specific) mRNA were significantly increased in thymocytes cultured in the presence of Delta-1-Fc, but not Jagged-1-Fc (Fig. 5, *B* and *C*). These observations suggest that Delta-1 can stimulate the development of iNKT cells in addition to conventional $\alpha\beta$ T cells.

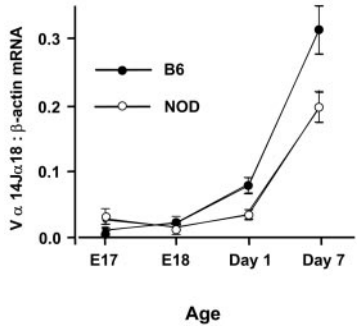


**FIGURE 4.** A deficiency in iNKT cells develops in a NOD thymus on the day of birth. Mononuclear thymocytes from NOD and B6 embryos were isolated at E17 (B6, $n = 16$; NOD, $n = 13$), E18 (B6, $n = 20$; NOD, $n = 20$), day 1 (B6, $n = 8$; NOD, $n = 7$), and day 7 (B6, $n = 5$; NOD, $n = 5$) after birth. The number of iNKT cells was estimated by a standard curve method of analysis of real-time PCR quantitation of Vα14Jα18 mRNA expression normalized to β-actin mRNA expression. Mean values ± SD of three quantitative PCR are shown. ∗, $p < 0.05$.

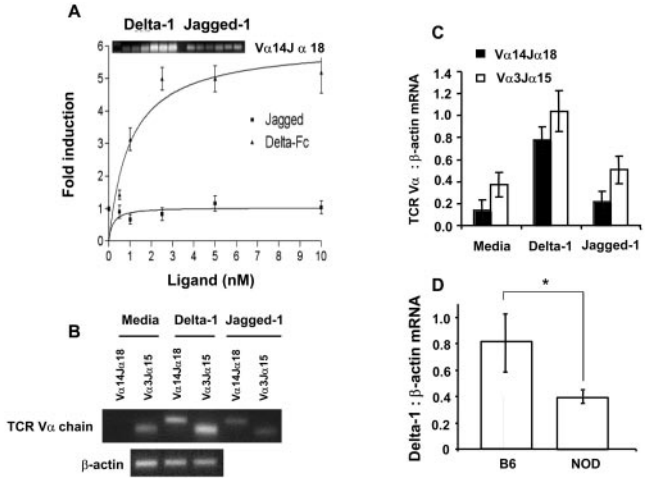


**FIGURE 5.** Delta-1 enhances the development of iNKT and conventional T cells, and its expression is reduced in a NOD fetal thymus. *A*, Notch-mediated TCR Vα14Jα18 mRNA induction is Notch ligand specific and dose dependent. DN thymocytes from 25 E13 B6 embryos were cultured in triplicate in the presence of various concentrations of Delta-1 or Jagged-1 for 24 h. Semiquantitative RT-PCR was performed for each replicate to obtain relative levels of TCR Vα14Jα18 mRNA expression. Mean values of TCR Vα14Jα18 mRNA levels are shown. *B* and *C*, Delta-1 induces TCR Vα mRNA expression characteristic of iNKT cells and DN TCRαβ cells. Thymic lobes from E13 B6 embryos ($n = 28$) were cultured in complete RPMI 1640 supplemented with 5 nM soluble Delta-1. Typical bands and mean values ± SD from three separate and reproducible semiquantitative PCR are shown. *D*, Delta-1 mRNA expression is reduced in E13 NOD ($n = 22$) thymocytes compared with age-matched B6 ($n = 20$) thymocytes. Data shown represent mean values ± SD of three separate semiquantitative PCR experiments. ∗, $p < 0.05$.

Next, we determined whether the relative level of Delta-1 expression is decreased in a NOD fetal thymus, and if so, whether this decreased expression mediates the development of an iNKT cell deficiency in NOD mice. Comparison of the ratios of Delta-1:β-actin mRNA levels expressed in NOD and B6 E13 thymocytes by real-time PCR revealed that the relative level of Delta-1 mRNA expression is reduced ~2-fold in NOD vs B6 thymocytes (Fig. 5*D*). These findings suggest that a decrease in Delta-1 expression during T cell development may mediate the generation of the intrathymic iNKT cell deficiency noted in NOD embryos.

## Discussion

Our results demonstrate for the first time that an iNKT cell deficiency, which is known to be associated with susceptibility to T1D, develops in NOD mice before birth. Previous studies of this deficiency failed to detect iNKT cells either in a NOD fetal thymus or in a newborn NOD thymus before 3 wk of age (26). An earlier attempt to detect Vα14Jα18 mRNA in a B6 fetal thymus by PCR analysis was unsuccessful possibly due to the fact that E9 embryos were used and DP thymocytes with Vα14Jα18 gene rearrangement appear in the thymic primordium 8–9 days later. Indeed, we were also unable to detect Vα14Jα18 mRNA in E13 or E15 thymic lobes (our unpublished observations). Moreover, the inability to detect iNKT cells in a NOD thymus by flow cytometry before 3 wk of age is not surprising, because this population comprises only 0.3% of thymocytes in a B6 thymus that contains a higher percentage of iNKT cells at 3 wk after birth (25). iNKT cells comprise a relatively small subset in the thymus, and require several rounds of T cell development to accumulate and become detectable by tetramer staining and flow cytometry.

Application of a real-time PCR approach enabled us to show that an iNKT cell deficiency begins with a depletion of these cells at E18, and that this deficiency is evident in NOD mice on day 1 after birth. Although adult and embryonic T cell development are often assumed to differ (21), we contend that many of these differences may be minor and ascribable to the different stages of development that occur in the thymus at a given day during embryonic life. Our results indicate that in NOD mice the development of an autoimmune T cell repertoire develops before birth and is then conserved during adult T cell development. This raises the issue that further investigation of the similarities between embryo and adult T cell development is required. Moreover, the fact that a day 7 neonatal mouse is considered to be the immunological equivalent of a newborn human (33) coupled with the finding that an iNKT cell deficiency is established by day 1 after birth in NOD mice further emphasizes the need for additional research to be directed toward the early detection and treatment of T1D in humans.

A defect in central tolerance (negative selection) has been reported to be causal to susceptibility to T1D in NOD mice (34, 35). In addition, NOD thymic epithelium, which is enriched in self Ag for positive and negative selection, was shown to induce insulitis upon transfer into diabetes-resistant B6 mice (36). Furthermore, our demonstration in this study that an intrathymic deficiency in iNKT cells appears after the DP thymocyte stage in embryonic NOD mice points toward a defect in positive selection of iNKT cells in these mice. Collectively, these findings suggest that central tolerance, including defects in the negative selection of autoreactive T cells and positive selection of regulatory T cells, such as iNKT cells, is an important factor that determines susceptibility to T1D in NOD mice.

We have obtained evidence that the depletion of iNKT cells from the thymus that occurs after the DP stage of development may be lineage specific. This conclusion is strengthened by our detection of a numerical deficiency in DN conventional αβ T cells and iNKT cells both in a NOD adult thymus at 8 wk of age and in a NOD fetal thymus at E18. Recent reports indicate that iNKT cells and conventional αβ T cells follow a similar developmental pathway, including progression through the DP stage of T cell development and positive and negative selection (13, 14). Based on our results in this study, we propose that DN conventional αβ T cells and DN iNKT cells make a common lineage decision, and that this lineage decision is defective in a developing NOD thymus according to a model shown in Fig. 6. Pre-iNKT DP thymocytes

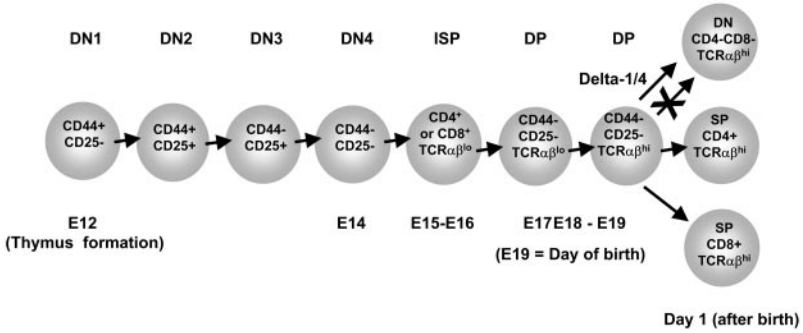


**FIGURE 6.** Proposed role for Delta-1 in NOD T cell development. Delta-1 may play a role in the selection of DP thymocytes by enhancing the positive selection of DN $\alpha\beta$ T cells, including iNKT cells. In contrast, a deficient amount of Delta-1 in a NOD thymus may decrease the number of DN $\alpha\beta$ T cells positively selected in a lineage-specific manner, as shown (X). Thus, relative to that in a B6 thymus, a Delta-1 deficiency in a NOD thymus may be responsible for the selection of a decreased number of DN $\alpha\beta$ T cells, including iNKT cells, and an increased number of conventional $CD4^+$ and $CD8^+$ $\alpha\beta$ T cells.

and earlier precursors are not deficient in a fetal NOD thymus; in fact, these pre-iNKT thymocytes appear to be more numerous in a NOD than B6 thymus at E17. TCR protein expression in DP thymocytes at E18 allows for a selection event that preferentially depletes DN $\alpha\beta$ T cells. This lineage-specific depletion of DN $\alpha\beta$ T cells results in an iNKT cell deficiency at day 1 after birth, and this deficiency is conserved after birth in the adult NOD thymus. Hence, our studies not only highlight the importance of analysis of the relationship of DN $\alpha\beta$ T cell fates and lineage decisions as they relate to the pathogenesis of autoimmune disease, but also provide the first indication that lineage decision in early intrathymic T cell development may govern susceptibility to autoimmune T1D.

In addition to thymic epithelial stimulation, other factors may also affect the positive or negative selection of iNKT cells in a NOD thymus. Intrathymic cotransfer of thymic precursors (1:1) from diabetes-susceptible NOD and diabetes-resistant ACK mice can normalize the frequency of $TCR\alpha\beta^+DX5^+$ iNKT cells in a NOD thymus (37). The Notch ligand, Delta-1, may be the source of such a survival signal during the positive and negative selection of DP thymocytes. Previously, Delta-1 was shown to provide survival signals to all of three subsets of DN $TCR\alpha\beta^-$ T cell precursors (29, 30), and to enhance T and possibly NKT cell development from human cord blood cells (31). Nonetheless, loss of function experiments in Delta-1 knockout mice demonstrated that T cell development was not altered in these mice possibly due to a compensation of function by Delta-4 in these mice (38). Despite functional overlap between Delta-1 and Delta-4, our finding that Delta-1, but not Jagged-1, can enhance conventional $\alpha\beta$ T and iNKT cell development supports the notion that Notch ligands differentially promote T cell development. Furthermore, our detection of a dose-dependent effect of Delta-1 on iNKT cell development demonstrates that despite overlap in Delta-1/Delta-4 function, the local concentration of Delta-1 in the thymus may affect T cell development. Thus, our observation that the amount of Delta-1 mRNA is significantly decreased in E13 NOD thymocytes compared with an age-matched B6 thymus supports the notion that a reduced level of Delta-1 protein in a NOD thymus may elicit in part the deficiency in DN $\alpha\beta$ T cells and iNKT cells in NOD mice. Future experimentation is required to investigate the effects of Delta-1 on the development of DN $\alpha\beta$ T cells, iNKT cells, and other T cell populations in NOD mice and the effects of altered T cell development on T1D development in NOD mice. Such analyses may also reveal whether a reduced intrathymic level of Delta-1 gives rise to a general thymic lymphopenia in NOD mice, which is purported to be a susceptibility factor for T1D in these mice (8, 29, 30, 39).

In conclusion, this study further underscores the important role that central tolerance may play in controlling the susceptibility to autoimmune disease, and T1D in particular. Our observations demonstrate that lineage fate decisions in positive selection during the intrathymic development of conventional DN $\alpha\beta$ and iNKT cells before birth may be an important feature of central tolerance that, if defective, can predispose to the outcome of autoimmune disease.

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## Disclosures

The authors have no financial conflict of interest.

## References

1. Delovitch, T. L., and B. Singh. 1997. The nonobese diabetic mouse as a model of autoimmune diabetes: immune dysregulation gets the NOD. *Immunity* 7: 727–738.
2. Sakaguchi, S., N. Sakaguchi, J. Shimizu, S. Yamazaki, T. Sakihama, M. Itoh, Y. Kuniyasu, T. Nomura, M. Toda, and T. Takahashi. 2001. Immunologic tolerance maintained by $CD25^+$ $CD4^+$ regulatory T cells: their common role in controlling autoimmunity, tumor immunity, and transplantation tolerance. *Immunol. Rev.* 182: 18–32.
3. Wilson, S. B., and T. L. Delovitch. 2003. Janus-like role of regulatory iNKT cells in autoimmune disease and tumor immunity. *Nat. Rev. Immunol.* 3: 211–222.
4. Wilson, S. B., S. C. Kent, H. F. Horton, A. A. Hill, P. L. Bollyky, D. A. Hafler, J. L. Strominger, and M. C. Byrne. 2000. Multiple differences in gene expression in regulatory V$\alpha$24J$\alpha$Q T cells from identical twins discordant for type I diabetes. *Proc. Natl. Acad. Sci. USA* 97: 7411–7416.
5. Hammond, K. J., D. G. Pellicci, L. D. Poulton, O. V. Naidenko, A. A. Scalzo, A. G. Baxter, and D. I. Godfrey. 2001. CD1d-restricted NKT cells: an interstrain comparison. *J. Immunol.* 167: 1164–1173.
6. Baxter, A. G., S. J. Kinder, K. J. Hammond, R. Scollay, and D. I. Godfrey. 1997. Association between $\alpha\beta TCR^+CD4^-CD8^-$ T-cell deficiency and IDDM in NOD/Lt mice. *Diabetes* 46: 572–582.
7. Laloux, V., L. Beaudoin, D. Jeske, C. Carnaud, and A. Lehuen. 2001. NK T cell-induced protection against diabetes in V$\alpha$14-J$\alpha$281 transgenic nonobese diabetic mice is associated with a Th2 shift circumscribed regionally to the islets and functionally to islet autoantigen. *J. Immunol.* 166: 3749–3756.
8. Sharif, S., G. A. Arreaza, P. Zucker, Q. S. Mi, J. Sondhi, O. V. Naidenko, M. Kronenberg, Y. Koezuka, T. L. Delovitch, J. M. Gombert, et al. 2001. Activation of natural killer T cells by $\alpha$-galactosylceramide treatment prevents the onset and recurrence of autoimmune type 1 diabetes. *Nat. Med.* 7: 1057–1062.
9. Xiao, S. Y., Y. Li, and W. F. Chen. 2003. Kinetics of thymocyte developmental process in fetal and neonatal mice. *Cell. Res.* 13: 265–273.
10. Wilson, S. S., and D. DeLuca. 1997. NOD fetal thymus organ culture: an in vitro model for the development of T cells involved in IDDM. *J. Autoimmun.* 10: 461–472.
11. Gombert, J. M., A. Herbelin, E. Tancrede-Bohin, M. Dy, C. Carnaud, and J. F. Bach. 1996. Early quantitative and functional deficiency of $NK1^+$-like thymocytes in the NOD mouse. *Eur. J. Immunol.* 26: 2989–2998.
12. Zipris, D., A. H. Lazarus, A. R. Crow, M. Hadzija, and T. L. Delovitch. 1991. Defective thymic T cell activation by concanavalin A and anti-CD3 in autoimmune nonobese diabetic mice: evidence for thymic T cell anergy that correlates with the onset of insulitis. *J. Immunol.* 146: 3763–3771.
13. Gapin, L., J. L. Matsuda, C. D. Surh, and M. Kronenberg. 2001. NKT cells derive from double-positive thymocytes that are positively selected by CD1d. *Nat. Immunol.* 2: 971–978.
14. Chun, T., M. J. Page, L. Gapin, J. L. Matsuda, H. Xu, H. Nguyen, H. S. Kang, A. K. Stanic, S. Joyce, W. A. Koltun, et al. 2003. CD1d-expressing dendritic cells but not thymic epithelial cells can mediate negative selection of NKT cells. *J. Exp. Med.* 197: 907–918.

15. Douagi, I., I. Andre, J. C. Ferraz, and A. Cumano. 2000. Characterization of T cell precursor activity in the murine fetal thymus: evidence for an input of T cell precursors between days 12 and 14 of gestation. *Eur. J. Immunol.* 30: 2201–2210.
16. Shortman, K., A. Wilson, M. Egerton, M. Pearse, and R. Scollay. 1988. Immature $CD4^-$ $CD8^+$ murine thymocytes. *Cell. Immunol.* 113: 462–479.
17. Ohteki, T., S. Ho, H. Suzuki, T. W. Mak, and P. S. Ohashi. 1997. Role for IL-15/IL-15 receptor $\beta$-chain in natural killer $1.1^+$ T cell receptor-$\alpha\beta^+$ cell development. *J. Immunol.* 159: 5931–5935.
18. Eberl, G., B. Lowin-Kropf, and H. R. MacDonald. 1999. NKT cell development is selectively impaired in Fyn-deficient mice. *J. Immunol.* 163: 4091–4094.
19. Voyle, R. B., F. Beermann, R. K. Lees, J. Schumann, J. Zimmer, W. Held, and H. R. MacDonald. 2003. Ligand-dependent inhibition of CD1d-restricted NKT cell development in mice transgenic for the activating receptor Ly49D. *J. Exp. Med.* 197: 919–925.
20. Elewaut, D., R. B. Shaikh, K. J. Hammond, H. De Winter, A. J. Leishman, S. Sidobre, O. Turovskaya, T. I. Prigozy, L. Ma, T. A. Banks, et al. 2003. NIK-dependent RelB activation defines a unique signaling pathway for the development of V$\alpha$14i NKT cells. *J. Exp. Med.* 197: 1623–1633.
21. Kincade, P. W., J. J. Owen, H. Igarashi, T. Kouro, T. Yokota, and M. I. Rossi. 2002. Nature or nurture? Steady-state lymphocyte formation in adults does not recapitulate ontogeny. *Immunol. Rev.* 187: 116–125.
22. Matsuda, J. L., O. V. Naidenko, L. Gapin, T. Nakayama, M. Taniguchi, C. R. Wang, Y. Koezuka, and M. Kronenberg. 2000. Tracking the response of natural killer T cells to a glycolipid antigen using CD1d tetramers. *J. Exp. Med.* 192: 741–754.
23. Germain, R. N. 2002. T-cell development and the CD4-CD8 lineage decision. *Nat. Rev. Immunol.* 2: 309–322.
24. Jotereau, F., F. Heuze, V. Salomon-Vie, and H. Gascan. 1987. Cell kinetics in the fetal mouse thymus: precursor cell input, proliferation, and emigration. *J. Immunol.* 138: 1026–1030.
25. Pellicci, D. G., K. J. Hammond, A. P. Uldrich, A. G. Baxter, M. J. Smyth, and D. I. Godfrey. 2002. A natural killer T (NKT) cell developmental pathway involving a thymus-dependent $NK1.1^-CD4^+$ CD1d-dependent precursor stage. *J. Exp. Med.* 195: 835–844.
26. Makino, Y., R. Kanno, H. Koseki, and M. Taniguchi. 1996. Development of V$\alpha4^+$ NK T cells in the early stages of embryogenesis. *Proc. Natl. Acad. Sci. USA* 93: 6516–6520.
27. Jimenez, E., A. Vicente, R. Sacedon, J. J. Munoz, G. Weinmaster, A. G. Zapata, and A. Varas. 2001. Distinct mechanisms contribute to generate and change the CD4:CD8 cell ratio during thymus development: a role for the Notch ligand, Jagged1. *J. Immunol.* 166: 5898–5908.
28. Radtke, F., A. Wilson, S. J. Mancini, and H. R. MacDonald. 2004. Notch regulation of lymphocyte development and function. *Nat. Immunol.* 5: 247–253.
29. Schmitt, T. M., and J. C. Zuniga-Pflucker. 2002. Induction of T cell development from hematopoietic progenitor cells by Delta-like-1 in vitro. *Immunity* 17: 749–756.
30. Schmitt, T. M., M. Ciofani, H. T. Petrie, and J. C. Zuniga-Pflucker. 2004. Maintenance of T cell specification and differentiation requires recurrent notch receptor-ligand interactions. *J. Exp. Med.* 200: 469–479.
31. Jaleco, A. C., H. Neves, E. Hooijberg, P. Gameiro, N. Clode, M. Haury, D. Henrique, and L. Parreira. 2001. Differential effects of Notch ligands Delta-1 and Jagged-1 in human lymphoid differentiation. *J. Exp. Med.* 194: 991–1002.
32. Doffinger, R., T. C. Klein, M. B. Pepys, J. L. Casanova, and B. A. Kyewski. 1997. The MHC class II-restricted T cell response of C57BL/6 mice to human C-reactive protein: homology to self and the selection of T cell epitopes and T cell receptors. *Mol. Immunol.* 34: 115–124.
33. Adkins, B., C. Leclerc, and S. Marshall-Clarke. 2004. Neonatal adaptive immunity comes of age. *Nat. Rev. Immunol.* 4: 553–564.
34. Kishimoto, H., and J. Sprent. 2001. A defect in central tolerance in NOD mice. *Nat. Immunol.* 2: 1025–1031.
35. Mathis, D., and C. Benoist. 2004. Back to central tolerance. *Immunity* 20: 509–516.
36. Thomas-Vaslin, V., D. Damotte, M. Coltey, N. M. Le Douarin, A. Coutinho, and J. Salaun. 1997. Abnormal T cell selection on NOD thymic epithelium is sufficient to induce autoimmune manifestations in C57BL/6 athymic nude mice. *Proc. Natl. Acad. Sci. USA* 94: 4598–4603.
37. Yang, Y., M. Bao, and J. W. Yoon. 2001. Intrinsic defects in the T-cell lineage results in natural killer T-cell deficiency and the development of diabetes in the nonobese diabetic mouse. *Diabetes* 50: 2691–2699.
38. Hozumi, K., N. Negishi, D. Suzuki, N. Abe, Y. Sotomaru, N. Tamaoki, C. Mailhos, D. Ish-Horowicz, S. Habu, and M. J. Owen. 2004. Delta-like 1 is necessary for the generation of marginal zone B cells but not T cells in vivo. *Nat. Immunol.* 5: 638–644.
39. King, C., A. Ilic, K. Koelsch, and N. Sarvetnick. 2004. Homeostatic expansion of T cells during immune insufficiency generates autoimmunity. *Cell* 117: 265–277.