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Temporal regulation of Wnt/ β -Catenin signaling is important for Invariant NKT Cell development and Terminal Maturation

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Abstract

The Wnt/ β -catenin signaling pathway plays important roles during various cellular functions including survival and proliferation of immune cells. The critical role of this pathway in conventional T cell development is established but little is known about its contributions to innate T cell development. In this study, we found that β -catenin level, an indication of the strength of Wnt/ β -catenin signaling, is regulated during invariant NKT (iNKT) cell development. β -catenin levels were greatly increased during iNKT cell selection from double positive thymocytes to Stage 0 of iNKT cell development and during subsequent development to Stage 1. Thereafter, β -catenin levels decrease from Stage 2, which is essential for the terminal maturation of iNKT cells. Failure to dampen Wnt/ β -catenin signaling as in mice expressing a stabilized active form of β -catenin (CATtg) resulted in increased Stage 2 and decreased Stage 3 iNKT cells. Inefficient transition from Stage 2 to 3 in CATtg iNKT cells seems to be contributed by poor expression of IL-15R (CD122) and transcription factor T-bet, both of which are necessary for terminal maturation of iNKT cells in the thymus. Consequently, IFN- γ + iNKT cells were greatly reduced in CATtg mice. Together, our findings reveal that proper regulation of β -catenin and in turn Wnt signaling plays an important role in the terminal maturation and function of iNKT cells.

1. INTRODUCTION

Invariant natural killer T (iNKT) cells are a subset of T cells that recognize glycolipid antigens presented by the MHC class I-like CD1d molecule and perform both innate and adaptive immune functions¹. They express a semi-invariant $\alpha\beta$ TCR (V α 14-J α 18 which pairs with V β 8.2, V β 7 and V β 2 in mice). In addition to TCR, iNKT cells express natural killer (NK) cell-associated marker NK1.1 (CD161) and the master transcription factor of the

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Conflict of Interest

The authors declare no conflict of interest.

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innate-like T cells, promyelocytic leukemia zinc-finger (PLZF). iNKT cells respond rapidly to stimulation to secrete cytokines, an innate feature that they acquire during their maturation in the thymus ^{1, 2}. iNKT cells play an important role in anti-tumor immunity, microbial clearance, and autoimmune disease conditions ³.

In the thymus, iNKT cells branch out from the conventional $\alpha\beta$ T cell development pathway at the CD4⁺ CD8⁺ DP (double positive) stage of thymocytes. Post-selection, immature Stage 0 (CD24⁺, CD44⁻, NK1.1⁻) iNKT cells lose the expression of CD24 as they undergo a burst of proliferation and progress to Stage 1 (CD24⁻, CD44⁻, NK1.1⁻) and then Stage 2 (CD24⁻, CD44⁺, NK1.1⁻) by acquiring CD44 and the ability to express cytokines. Final maturation to Stage 3 (CD24⁻, CD44⁺, NK1.1⁺) is marked by the expression of NK1.1 and the full capacity to produce cytokines ^{1, 4}. While this developmental pattern is widely accepted to define iNKT cell maturation and function, more recent belief is that the different stages in the thymus are comprised of terminally differentiated iNKT cells with different effector functions overlapping with Th1, Th2 and Th17 cells, named iNKT1, iNKT2 and iNKT17 respectively ⁵. Irrespective of the two views, all the factors and mechanisms controlling the development of these stages or effector types are yet to be identified.

Molecular mechanisms that control the iNKT cell development and effector function acquisition and signaling pathways regulating iNKT cell maturation are distinct from conventional T cells. Recent emerging data indicate that many different signaling pathways orchestrate the maturation of iNKT cells. The SLAM/SAP pathway, which is recruited during the TCR signaling in precursor iNKT cells, controls the thymic expansion and differentiation of these cells eventually ⁶. IL-15 signaling mediated by the up-regulated IL-15R (CD122) expression in the late stages is important for terminal maturation of Stage 2 cells to Stage 3 ⁷. Both the complexes of mammalian target of rapamycin (mTOR), complex 1 (mTORC1) and complex 2 (mTORC2) regulate early iNKT cell development and effector function by different mechanisms. While mTORC1 controls the nuclear localization of PLZF, mTORC2 works in PLZF independent manner ⁸⁻¹⁰. Different branches of TGF- β signaling have been shown to work together to control early, intermediate and late differentiation of iNKT cells ¹¹. Other factors that have been reported to play a role in iNKT cell maturation are RelA ¹², Itk ¹³, Calcineurin ¹⁴, Id2 ¹⁵ and CYLD ¹⁶ including transcriptional regulators PLZF ^{17, 18}, T-bet ^{19, 20}, c-Myc ^{21, 22}, EGR-2 ¹⁴, IRF-1 ²³ and β -Catenin ²⁴.

Wnt/ β -Catenin signaling pathway, also known as canonical Wnt pathway is evolutionarily conserved and is the best characterized of the three known Wnt pathways. β -Catenin is the central player of this pathway, which is regulated at the protein-level by glycogen synthase kinase 3 β (GSK3 β) by targeting it for ubiquitylation and proteosomal degradation in the absence of Wnt ligand binding ²⁵. Upon binding of a Wnt ligand, the stable β -Catenin translocates to the nucleus to form a complex with T cell factor (TCF)/lymphoid enhancer factor (LEF1) leading to activation of Wnt target genes identified as those associated with proliferation, anti-apoptosis, cell adhesion and other cellular functions ^{25, 26}. In developing T cells, TCR-signaling is known to augment β -Catenin expression and its role in the conventional T cell development is well established ²⁶⁻²⁹. In the thymus, Wnt signaling provides proliferative signals to immature T cells, aids in DN to DP transition of

thymocytes, and regulates the positive selection of thymocytes^{28, 30–32}. However, the role of Wnt/ β -Catenin signaling in the development of innate T cells is not clearly understood. Recently, β -Catenin was demonstrated to be important for controlling iNKT cell numbers and balancing the iNKT effector subsets²⁴. However, what is not clear so far is if β -Catenin expression changes during iNKT cell development and if that in turn contributes to the acquisition of effector functions.

In the current study using mice with transgenic expression of stabilized, active β -Catenin (CATtg), we demonstrate that the level of β -Catenin is temporally controlled during iNKT cell development. β -Catenin level is induced during DP to Stage 0 transition, reaches the maximum level at Stage 1, but decreases during terminal maturation of Stage 2 and 3. Accordingly, in CATtg mice, reduction of terminally mature Stage 3 cells is observed. The down regulation of β -Catenin at Stage 2 is critical for the expression of CD122 (IL-15R subunit) and Tbet, supporting IL-15 signaling which is required for terminal maturation of iNKT cells. In summary, proper regulation of β -Catenin mediated signaling is essential for the terminal maturation of iNKT cells in the thymus.

2. MATERIALS AND METHODS

2.1. Mice

β -Catenin transgenic (CATtg) mice described previously³² express an active form of β -catenin in thymocytes and T cells under the control of proximal *Lck* promoter. β -Catenin knock out (CAT-KO) mice were generated by breeding previously described β -CAT^{flox/flox} mice³³ with mice expressing Cre recombinase under the control of *Cd4* promoter (CD4-Cre mice). ICAT (inhibitor of β -catenin-TCF interactions) transgenic (ICATtg) mice expressing ICAT under the control of proximal *Lck* promoter have been described previously³⁴. PLZF-deficient mice (a kind gift of Dr. Derek Sant'Angelo) were described previously³⁵. CATtg mice were bred with PLZF-deficient mice to generate CATtg-PLZF^{+/-} mice. Age matched littermate controls (8 to 16 weeks of age) or C57BL/6 mice were used in all experiments. All the mice were bred and maintained under specific pathogen-free conditions at the University of Michigan animal facility. All animal experiments were performed under protocols approved by the University of Michigan Institutional Animal Care and Use Committee.

2.2. Cell preparation and flow cytometry

Primary cell suspensions were prepared from thymi and spleens as per standard protocol. Up to 3×10^6 cells per sample were resuspended in 100 μ l of FACS buffer (2% FBS, 1X PBS) in FACS tubes and stained for surface molecules expression with FITC-, PE-, PerCP-Cy5.5-, PE-Texas Red-, PeCy-7, APC-, APC-Cy7- or Pacific Blue-conjugated antibodies in the presence of anti-Fc γ R mAb 2.4G2 to block nonspecific binding of the antibodies. For intracellular staining of cytokines and β -Catenin, the cells stained for surface molecules were fixed, permeabilized and stained using Cytofix/Cytoperm kit (BD Bioscience). For intranuclear staining of transcription factors, PLZF, Tbet, GATA3 and ROR γ -T, surface-stained cells were fixed, permeabilized and stained using the Foxp3/Transcription Factor Staining Buffer Kit (eBioscience) as per manufacturer's recommendation. Cell fluorescence

was assessed using FACSCanto II (Becton Dickinson) and data were analysed using FlowJo software (Tree Star).

All the antibodies were purchased from eBioscience or BD Biosciences. The following antibodies conjugated to FITC-, PE-, PerCP-Cy5.5-, PE-Texas Red-, PeCy-7, APC-, APC-Cy7- or Pacific Blue- were used - CD4 (GK1.5), CD8a (53-6.7), TCR- β (H57-597), CD44 (IM7), NK1.1 (PK136), CD24 (M1/69), CD122 (5H4), IFN- γ (XMG1.2), IL-4 (BVD6-24G2), IL-17 (TC11-18H10), β -Catenin (15B8), PLZF (Mags.21F7), T-bet (eBio4B10), GATA3 (TWAJ) and ROR γ T (Q31-378). APC- or Pacific Blue-conjugated murine CD1d tetramers loaded with PBS-57 were kindly provided by the National Institutes of Health Tetramer Facility.

2.3. *In vitro* cytokine stimulation assay

Freshly isolated total thymocytes (5×10^6 cells in 2ml) were stimulated with 50 ng/ml of phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich) and 1.5 μ M of ionomycin (Sigma-Aldrich), in complete RPMI media (RPMI 1640, 10% FBS, antibiotics Penicillin and Streptomycin, and β -mercaptoethanol) for 5 hours in the CO₂ incubator. Monensin (Sigma-Aldrich) at a final concentration of 3 μ M was added during the last 3 hours of stimulation. Cells were washed in FACS buffer before staining for flow cytometry analysis as described above.

2.4. Quantitative real-time PCR

CD1d-PBS57 tetramer-positive iNKT cells from the thymus were sorted by flow cytometry after staining the cells as described above. Total RNA was isolated from the sorted populations using RNeasy kit (QIAGEN) and 50ng of RNA was reverse transcribed to cDNA using poly(dT) and MuLV RT Reverse Transcriptase (Applied Biosystems). RT-PCR was performed in the presence of cDNA, SYBR green mix and specific primers for the target gene on an Applied Biosystems 7400 PCR machine. The primers (synthesized from IDT) used were as follows: PLZF (*zbtb16*), 5'-AACGGTTCCTGGACAGTTTG-3' and 5'-CCCACACAGCAGACAGAAGA-3'; CD122 5'-GAAGTGCTCGACGGAGATTC-3' and 5'-GAAGTAGCCCTGGTTGGTGA-3'. Gene expression of the target genes was normalized to the GAPDH expression levels. The data analysis was performed using the 2^{-CT} method. Relative expression was calculated as fold-difference by considering WT iNKT cell as one.

2.5. Statistical analysis

All data were obtained from at least three independent repeated experiments as mentioned in each figure legend. Data are represented as Mean $\pm$ SEM and were analysed with Prism software (GraphPad). Unpaired Student's *t*-test was used for comparison of two experimental groups and one-way analysis of variances (ANOVA) was used for multiple comparisons. *p* values < 0.05 were considered statistically significant, represented as < 0.05 (*), < 0.01 (**), < 0.001 (***) and < 0.0001 (****).

3. RESULTS

3.1. Aberrant terminal maturation of iNKT cells in mice expressing constitutively active β -catenin

To study the role of Wnt/ β -Catenin signaling in iNKT cell development, we used two mouse models; transgenic mice expressing a dephosphorylated active form of β -Catenin (β -Catenin transgenic; CATtg) under the *Lck* promoter and β -Catenin knock out mice (CAT-KO) with a conditional deletion of β -Catenin under the CD4 promoter³². We first examined the iNKT cell compartment in the thymus as well as in the spleen of CATtg and CAT-KO mice compared to the wild type (WT) littermate controls (Fig. 1A and 1B). Specific detection of iNKT cells with PBS-57-loaded CD1d tetramers was confirmed by including unloaded tetramers as controls (Fig S1). Consistent with the previous report²⁴, we found increased and decreased percentages of CD1d-tetramer positive iNKT cells in CATtg and CAT-KO thymus, respectively and increased numbers of iNKT cells in the CATtg thymus. In the periphery however, there was no significant difference in the iNKT cell percentages or numbers of CATtg spleen, while those of CAT-KO spleen were slightly lower (Fig. 1A and 1B).

To examine if the changes observed in the iNKT cell percentages in the transgenic and KO mice was due to a stage-specific defect during iNKT cell development, we performed a stage-wise analysis of the iNKT cells (Fig 1C and D). In CATtg mice, CD24⁺ Stage 0 iNKT cells were not different from the WT littermates but the percentages of Stage 1 and the Stage 2 iNKT cells were increased compared to WT cells, albeit a modest increase at Stage 1 (Fig 1C and 1D). Stage 3 iNKT cells were significantly reduced in CATtg mice consistent with an increased percentage of Stage 2 cells. This difference was also reflected in the cell numbers with Stage 2 and 3 iNKT cell numbers being higher and lower, respectively, compared to the WT (Fig 1D, lower panels). We further compared the efficiency of transition of the CATtg iNKT cells through different stages compared to the WT controls. For this, we calculated the transition efficiency from Stage 0 to 1 (S1/S0), Stage 1 to 2 (S2/S1), and Stage 2 to 3 (S3/S2) using the cell numbers at each Stage (Fig. 1F). The data showed that Stage 1 to 2 transition was enhanced, whereas Stage 2 to 3 transition was severely impaired in CATtg iNKT cells. Stage 0 to Stage 1 transition was similar to the WT. We also looked if the defective maturation of iNKT cells is also reflected in the periphery and found that in both spleen and liver, NK1.1- stage 2 iNKT cells were most frequent in the CATtg mice compared to the wild type, the difference being more prominent in spleen (Fig S2A). Together these results indicate that expression of constitutively active Wnt/ β -Catenin in iNKT cells delays the terminal maturation of iNKT cells.

A previous study of CAT-KO mice showed reduced thymic iNKT cells but developmental stages were not examined²⁴. When we examined the stage-wise development of CAT-KO iNKT cells in the thymus, an increase in the percentage but not the cell number of Stage 0 cells was observed, but the rest of stages were comparable to WT (Fig 1E). The efficiency of transition was not significantly different from the WT (Fig 1F). To further confirm the results from CAT-KO mice, we employed another genetically modified mouse model that expresses an inhibitor of β -Catenin (ICAT) in thymocytes³⁴. ICAT is a naturally occurring

inhibitor of β -Catenin -TCF1 interactions and thus blocks the downstream activation of TCF1 specific genes³⁶. Similar to CAT-KO mice, we observed a significant reduction in the thymic iNKT percentages and a slight reduction in splenic iNKT percentages in ICATtg mice compared to the WT littermates (Supplemental Fig 3A). Again, no significant differences were noticed in cell numbers. Altogether, these data demonstrate that the proper regulation of β -Catenin is important for the later stage of iNKT cell development.

3.2. Signaling of Wnt/ β -Catenin is regulated during iNKT cell maturation

β -Catenin protein levels, which reflect the strength of Wnt signaling, are regulated during the development of T cells in the thymus³⁷ but its expression in iNKT cells has not yet been studied. To address this, we measured the relative change in the β -Catenin level from double positive thymocytes (DP) stage to iNKT, CD4 and CD8 T cells. All the three subsets up-regulated the expression of β -catenin (Fig 2A and 2B). iNKT cells depicted a higher increase than CD4 T cells but comparable to CD8 T cells. As expected, we could detect high levels of β -Catenin in CATtg iNKT cells compared to WT iNKT cells (Fig 2C). When we looked into the stage-wise regulation of Wnt/ β -Catenin signaling, we observed a prominent increase in β -Catenin levels during the iNKT cell commitment stage (from DP to Stage 0) as well as through progression to Stage 1 but the levels were down-regulated in subsequent stages (Fig 2D and E). Thus, β -catenin expression is regulated during iNKT cell development in the thymus and its down-regulation at later stages seems to be important for their terminal maturation. Indeed, our data showed that β -Catenin expression in Stage 2 and Stage 3 (high CD44-expressing late stages) iNKT cells from CATtg thymus was significantly higher than those from WT thymus while the expression in stages 0 and 1 (low CD44-expressing early stages) was similar in both mice (Fig 2F).

3.3 β -Catenin up-regulates the expression of PLZF in iNKT cells

Previous reports and our data showed that the frequency of PLZF-expressing iNKT cells were augmented in CATtg mice^{24, 38}. Thus, it is possible that the defect in iNKT cell maturation in CATtg mice is contributed by the higher expression of PLZF or by Wnt signalling independent of PLZF. To distinguish these possibilities, we looked into the expression of PLZF at each stage. CATtg iNKT cells expressed higher levels of PLZF protein and mRNA compared to the WT iNKT cells (Fig 3A and 3B). In addition, unlike WT iNKT cells that show proper up- and down-regulation of PLZF during development, CATtg iNKT cells expressed consistently high levels of PLZF throughout development (Fig 3C). We also investigated PLZF levels in CAT-KO, and I-CATtg mice to see if Wnt/ β -Catenin signaling is important for PLZF expression. PLZF expression did not require Wnt/ β -Catenin signaling evidenced by normal levels of PLZF in CAT-KO (Fig 3D) or I-CATtg iNKT cells (Supplemental Fig 3B).

Transgenic expression of PLZF does not affect iNKT cell development^{18, 39}, however, it is possible that constitutively high levels of PLZF together with high amounts of active β -Catenin in CATtg could be detrimental for iNKT cell development in these mice. To test this, we introduced haplodeficiency of PLZF into CATtg mice by crossing CATtg mice with mice deficient in PLZF (PLZF^{-/-}) to generate CATtgPLZF^{+/-} mice. We then analysed the iNKT cell numbers and the developmental progression in these mice (Fig 4). The iNKT cell

frequency in CATtgPLZF^{+/-} mice was as poor as in PLZF^{+/-} mice (Fig 4A). However, PLZF levels in CATtgPLZF^{+/-} iNKT cells were lower than CATtg iNKT cells, yet greater than WT or PLZF^{+/-} iNKT cells (Fig 4B). When we analysed each stage of iNKT cells, CATtgPLZF^{+/-} iNKT cells followed a developmental pattern similar to CATtg mice and not to that of PLZF^{+/-} mice with significantly increased Stage 2 cells and decreased Stage 3 cells compared to WT mice (Fig 4C). Therefore, the defect in the transition from Stage 2 to Stage 3 in CATtg iNKT cells is not due to high levels of PLZF. Rather, the high level of active β -Catenin and thus constitutively delivered Wnt signaling at Stage 2 in both CATtg and CATtgPLZF^{+/-} mice likely contributes to the alteration of iNKT cell maturation.

3.4. Signaling of Wnt/ β -Catenin controls the expression of CD122 and Tbet

Next, we investigated how β -catenin regulates terminal maturation of iNKT cells. Among several factors that control iNKT cell maturation, IL-15 signaling has been shown to be critical, particularly for the Stage 2 to Stage 3 transition⁷. In the absence of IL-15 signaling, Stage 2 to Stage 3 transition of iNKT cells in the thymus is very poor⁷ and further, IL-15R expression is upregulated during iNKT cell development⁴⁰. CATtg iNKT cells show a similar developmental defect as in mice deficient of IL-15 signaling^{7, 40-42}, which prompted us to examine IL-15 signaling in these mice. To do this, we first measured the levels of CD122, a subunit of the IL-15 receptor. We observed that both surface expression and mRNA levels of CD122 were significantly lower in CATtg iNKT cells compared to those from WT mice (Fig 5A and B). In contrast, CAT-KO iNKT cells did not show a measurable difference in CD122 expression (Fig 5A). Because CD122 is expressed at Stage 2 of WT iNKT cells⁴⁰, we examined CD122 expression during iNKT cell development. CD122 expression was increased from Stage 0 to 1 and then greatly elevated at Stage 2 and 3 of iNKT cells in WT mice consistent with the published studies (Fig 5C). On the contrary, CATtg iNKT cells showed a small increase of CD122 expression at Stage 1 but failed to elevate the expression at Stages 2 and 3. It has been reported that IL-15 signaling mediated by increased CD122 is important for induction of transcription factor Tbet²⁰. This raised a question if expression of both CD122 and Tbet is compromised in CATtg iNKT cells. Indeed, iNKT cells expressing both CD122 and Tbet were negligible in the CATtg thymus (Fig 5D). In the CAT-KO iNKT cells however, CD122 expression level was comparable to the WT cells (Supplemental Fig 2B). Altogether, down regulation of β -Catenin at the Stage 2 seems to be necessary to induce the expression of CD122 and Tbet, both of which are critical requirements for terminal maturation of iNKT cells.

3.5. Signaling of Wnt/ β -Catenin regulates the cytokines and transcription factors in iNKT cells

It was reported that CD122 expression during iNKT cell maturation is important for the up-regulation of Tbet expression in these cells²⁰. Based on this and our data that CD122⁺ Tbet⁺ iNKT cell population has been changed, we asked if there is any change in iNKT cell effector functions in CATtg mice. To investigate this, we compared the expression levels of three transcription factors, Tbet, GATA-3 and ROR- γ t that define the iNKT functions. As expected, CATtg iNKT cells expressed significantly lower levels of Tbet than those from WT mice (Fig 6A). However, GATA3 was upregulated but ROR- γ -T levels were reduced in CATtg iNKT cells. The differences in expression of transcription factors Tbet and GATA-3

were consistent with the decrease and increase in IFN- γ ⁺ and IL-4⁺ iNKT cells, respectively (Fig 6B and C). Next, we compared the frequency of cytokine expressing cells within iNKT cell developmental stages (Fig 6D). In CATtg iNKT cells, the frequency of IFN- γ ⁺ cells in all stages was reduced, whereas IL-4⁺ cells showed a trend of increase without reaching to a statistical significance compared to WT iNKT cells (Fig 6D). Similar frequency of IL-17⁺ iNKT cells were observed in WT and CATtg mice (Fig 6B and C). iNKT cells from CAT-KO mice did not show any measureable difference in T-bet expression or cytokine expression (Fig 6C and Supplemental Fig 2B and C). Together, these results suggest that down-regulation of Wnt/ β -Catenin is important for iNKT cell maturation marked by the expression of cytokines and transcription factors especially for IFN- γ and T-bet.

4. DISCUSSION

We had previously shown that β -catenin expression promotes the development and function of iNKT cell effector subsets. The underlying mechanisms however were not known. In the current study we report that regulation of β -catenin at later developmental stages in the thymus is crucial for terminal maturation and function.

Here, we demonstrated for the first time that the protein levels of β -Catenin are regulated during iNKT cell development. We observed that the levels of endogenous β -Catenin increase during early stages (Stage 0 and Stage 1) and decrease at the later stages (Stage 2 and Stage 3) of iNKT cell development in the thymus. It is widely accepted that cytoplasmic β -Catenin levels directly correlate with the activity of canonical Wnt signaling²⁵, which suggests that the Wnt signaling activity is temporally regulated as the iNKT cells progress from one stage to another. While Stage 0 cells were slightly increased in CAT-KO thymus, the frequency and cell numbers of the rest of the stages were comparable to the WT littermate along with no major changes in the effector program. Furthermore, after the β -Catenin deficient iNKT cells pass the Stage 0, the absence of β -Catenin does not affect the next steps of iNKT development or the acquisition of effector function. Therefore, Wnt signaling is not a requirement to initiate the commitment and early development of iNKT cells. On the other hand, down regulation of β -Catenin at Stage 2 plays an important role for the terminal maturation of these cells to Stage 3. During the late or terminal stages (Stages 2 and 3), iNKT cells undergo acquisition of the distinct effector programs after the proliferation. A defect in terminal maturation is often accompanied by alterations in the expression of cytokines and transcription factors in addition to poor acquisition of NK marker as seen in CATtg iNKT cells. In fact, the low number of stage 3 CATtg iNKT cells exhibited a defective effector program (IFN- γ and T-bet) and could be labelled as 'pre-stage3' cells. At this checkpoint, Wnt signaling controls the terminal maturation by regulating the expression of T-bet and CD122, a subunit of IL15R. IL-15 signaling mediated by up-regulated CD122 at Stage 2 is important for iNKT cell maturation and homeostasis^{7, 40, 42}. In mice deficient of IL-15 signaling, T-bet expression is significantly reduced in iNKT cells and in iNKT cells from T-bet-deficient mice, CD122 mRNA levels were reduced indicating that these two pathways work in a feedback loop mechanism^{7, 42}. Importantly, both IL15^{-/-} and Tbet^{-/-} mice show a developmental and cytokine defect in Stage 2 and Stage 3 iNKT cells, a phenotype similar to CATtg mice that we observed. It is

possible that β -Catenin controls T-bet expression independent of development by cross-talking with NF- κ B and/or mTORC1 signaling pathways, both of which have been shown to control T-bet^{43, 44}. In CD8 T cells, mTORC1-mediated inactivation of Foxo1 has been shown to be critical for T-bet expression⁴⁴. β -catenin has been reported to bind to and enhance FOXO transcription factors and enhance their transcriptional activity in mammalian cells^{45, 46}. Regardless of the possibilities, Wnt/ β -Catenin signaling checks the terminal maturation of iNKT cells by regulating the expression CD122 and T-bet. On the other hand, increase of GATA-3 expression in CATtg iNKT cells could be explained by at least three possibilities. Firstly, the increased GATA-3 in CATtg iNKT cells could simply be the result of low T-bet expression in these cells as it is well established that T-bet and GATA-3 are expressed in an opposing manner⁴⁷. Secondly, this could be an effect of high PLZF expression in CATtg iNKT cells, as PLZF is known to direct the IL-4 effector program in innate T cells^{48, 49}. Lastly, β -Catenin is known to interact with LEF1 to influence its transcriptional activity and recent study demonstrated transcriptional activation of *Gata3* gene by direct binding of LEF1⁵⁰. Upon examination of iNKT effector cytokines, we did not find any difference in the frequency of IL-17+ iNKT cells in the CATtg thymus compared to its WT littermate, which was consistent with the previous study where no significant difference in IL-17 expression was reported in CATtg thymic iNKT cells²⁴.

PLZF is crucial for iNKT cell development evidenced by the lack of iNKT cells in PLZF deficient mice¹⁸. Here, we show that the expression of PLZF does not require β -catenin but the continuous delivery of Wnt signaling results in increased PLZF levels throughout iNKT cell development. According to the previous study, β -Catenin levels are high in double negative thymocytes but reduced in DP thymocytes followed by upregulation in single-positive CD4 and CD8 T cells³⁷. The same study showed that CATtg thymocytes express the transgenic form of β -Catenin from the DN stage of thymocytes. Thus, it is possible that carrying high levels of β -Catenin during the DP stage of thymocytes in CATtg mice triggers upregulation of PLZF. Mechanisms behind initiation of PLZF gene expression in iNKT cells are not well understood except that strong TCR signaling induces the expression of PLZF mediated by EGR2⁵¹. Previously, *egr* genes were shown to be up-regulated in CATtg thymocytes⁵² and thus β -Catenin may directly bind and regulate the PLZF gene expression. Regardless of the molecular mechanisms upregulating PLZF expression in CATtg iNKT cells, the high PLZF level is not likely a contributor of the developmental defect in these cells. Our premise is further supported by an earlier study showing that overexpression of PLZF transgene in thymocytes under *lck* promoter (like CATtg mice) did not have an adverse effect on the iNKT cell numbers or development despite elevated PLZF in iNKT cells³⁹. Similar results were observed in another study wherein mice expressed PLZF under the *cd4* promoter¹⁸. Lastly, it should be noted that in CD4 T cells from CATtg mice, the expression of PLZF was induced but their development was not altered indicating that iNKT cells are more sensitive than CD4 T cells to Wnt/ β -Catenin signaling³⁸.

In summary, our findings revealed that the generation and development of iNKT effector cells requires an optimum amount of Wnt/ β -Catenin signaling in a stage specific manner. iNKT cell selection from DP stage of thymocytes as well as early stages of iNKT cell development requires high Wnt signaling activity, but the strength of Wnt signaling must be

weakened at later stages to complete the terminal maturation and become functionally competent iNKT cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- β -catenin protein level is upregulated at early stages and is reduced at later stages of iNKT cell development.
- Stage 2 to Stage 3 transition is defective in β -catenin transgenic iNKT cells
- Expression of T-bet is reduced in β -catenin transgenic iNKT cells.
- Down-regulation of β -catenin at Stage 2 is important for the expression of CD122 (IL-15R subunit) and terminal maturation of iNKT cells.

Figure 1. Defective iNKT cell development in CATtg mice

(A) Thymic and splenic iNKT cells identified as TCR β ⁺ and CD1d tetramer⁺ cells in CATtg and CAT-KO mice were compared with the respective WT littermate control mice. Numbers in the dot plots indicate the percentages of iNKT cells. (B) Summary of iNKT cell percentages (top) and numbers (bottom) in indicated tissues from WT, CATtg (left group) and CAT-KO (right group) mice. (C) Representative CD1d tetramers and CD24 profiles of total thymocytes derived from WT and CATtg thymus showing CD24⁺ Stage 0 cells and stages 1–3 cells (top panels). CD24[−] cells were further analyzed using CD44 and NK1.1 for Stages 1, 2 and 3 (bottom panels). (D and E) The percentages (upper) and cell numbers

(lower) of iNKT cells at each stage in the thymus of CATtg (D) and CAT-KO (E) compared to respective WT littermates are shown (F) Progression efficiency at each stage in CATtg (left group) and CAT-KO (right group) iNKT cells was calculated by dividing the cell numbers of each stage by the numbers of the previous stage. S0, S1, S2, and S3 represent Stage 0, Stage 1, Stage 2, and Stage 3, respectively. All data are shown as Mean $\pm$ SEM from four to six independent experiments. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

Figure 2. β -Catenin expression is regulated during iNKT cell maturation

(A) Representative histograms of intracellular β -Catenin expression in double positive (DP) thymocytes and T cell subsets in the thymus. The values indicate the mean fluorescence intensities (MFI) of each peak. (B) Fold induction in β -Catenin expression from DP stage in indicated T cell subsets was calculated by dividing the MFI from each subset by the MFI from DP thymocytes. Data are shown as Mean $\pm$ SEM from three independent experiments. (C) Representative histograms comparing the intracellular expression of β -Catenin in CATtg and wildtype (WT) littermate iNKT cells from thymus. (D) Intracellular β -Catenin expression in each stage of thymic iNKT cells was compared. The values indicate the MFI of each peak. (E) The summary of MFI of β -Catenin in DP thymocytes and at each iNKT stage are shown as Mean $\pm$ SEM from four independent experiments. (F) Representative histograms comparing the intracellular expression of β -Catenin in low CD44 expressing early stages (CD44lo) and high CD44-expressing late (CD44hi) stages of thymic iNKT cells from CATtg and wildtype (WT) littermate mice. Bar graph is the summary of MFI of β -

Catenin from CD44^{lo} and CD44^{hi} iNKT cells shown as Mean $\pm$ SEM from four independent experiments. *p<0.05; **p <0.01; ****p<0.0001.

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Figure 3. Dysregulation of PLZF expression in CATtg iNKT cells

(A) Representative histogram overlays comparing PLZF expression in total thymic iNKT cells from CATtg (open) and WT littermate (filled) mice. The summary of PLZF protein expression as mean fluorescence intensities (MFI) in total thymic iNKT cells is shown as Mean $\pm$ SEM from four independent experiments. *** $p < 0.001$. (B) PLZF mRNA expression was assessed by real-time qPCR and the relative quantity of mRNA in the sorted thymic iNKT cells is shown as Mean $\pm$ SEM from three independent experiments. *** $p < 0.001$. (C) Representative histograms comparing PLZF expression at each developmental stage of thymic iNKT cells between CATtg and WT littermate from four independent experiments. (D) Representative overlays and summary of PLZF expression in total thymic iNKT cells from CAT-KO and WT littermate mice. Data are represented as Mean $\pm$ SEM from four independent experiments.

Figure 4. Developmental defect in CATtg iNKT cells is not due to the high level of PLZF
 (A) Representative dot plots of iNKT cell populations in the thymus of the indicated mice.
 (B) Mean fluorescence intensity (MFI) of PLZF expression in total thymic iNKT cells was compared among the CATtg, PLZF^{+/-}, CATtgPLZF^{+/-} and their WT littermate mice. (C) The percentage of iNKT cells at each stage of the four indicated mice were compared. Statistical analysis was performed using one-way ANOVA between multiple groups. WT vs. CATtg - Stg2 p=0.0001, Stg3 p=0.0006; WT vs. CATtgPLZF^{+/-} - Stg2 p=0.0004, Stg3 p=0.001; All data are shown as Mean ± SEM from three independent experiments.

Figure 5. β -Catenin regulates expression of CD122

(A) Representative histogram overlays comparing surface expression of CD122 in total thymic iNKT cells from CATtg and CAT-KO mice with respective WT littermate control mice from four independent experiments (B) Relative quantity of CD122 mRNA levels in the total thymic iNKT cells in WT and CATtg mice were assessed by real-time qPCR. (C) Surface expression of CD122 at each stage of thymic iNKT development were compared between the CATtg and WT littermate mice. (D) Representative T-bet and CD122 profiles of total thymic iNKT cells from WT and CATtg mice. Numbers in the dot plots indicate the percentages of T-bet⁺CD122⁺ cells in the corresponding regions. The bar graph shows the summary of percentage of T-bet⁺CD122⁺ iNKT cells. Data are shown as Mean $\pm$ SEM from three independent experiments unless otherwise mentioned. **p < 0.01; ***p < 0.001; ****p < 0.0001.

Figure 6. Effector program is altered in CATtg iNKT cells

(A) Expression of T-bet, GATA-3 and ROR γ -T in thymic iNKT cells from WT littermate and CATtg mice was analyzed. Bar graphs show the summary of expression of T-bet and GATA-3 and percentage of ROR γ -T⁺ cells from four independent experiments. (B) Total thymocytes including thymic iNKT cells were stimulated for 5 hours with PMA and Ionomycin as described in the Experimental Procedures. IL-4 and IFN- γ (left group) and IL-17 and IFN- γ (right group) expression from total iNKT cells are shown. Numbers in the dot plots indicate the percentages of cytokine expressing iNKT cells. (C) Summary of percentages of iNKT cells expressing indicated cytokines from the WT and CATtg mice from six independent experiments (D) Summary of IFN- γ + cells (upper panel) and IL-4+ cells (lower panel) within the Stage 1, Stage 2 and Stage 3 iNKT cells from WT littermate and CATtg mice from four independent experiments. Data are represented as Mean $\pm$ SEM from the indicated sets of experiments. * p <0.05; ** p <0.01; *** p <0.001; **** p <0.0001.