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Mammalian Target of Rapamycin Complex 2 Regulates Invariant NKT Cell Development and Function Independent of Promyelocytic Leukemia Zinc-Finger

Nicolas Prevot,* Kalyani Pyaram,* Evan Bischoff,* Jyoti Misra Sen,† Jonathan D. Powell,‡ and Cheong-Hee Chang*

The mammalian target of rapamycin (mTOR) senses and incorporates different environmental cues via the two signaling complexes mTOR complex 1 (mTORC1) and mTORC2. As a result, mTOR controls cell growth and survival, and also shapes different effector functions of the cells including immune cells such as T cells. We demonstrate in this article that invariant NKT (iNKT) cell development is controlled by mTORC2 in a cell-intrinsic manner. In mice deficient in mTORC2 signaling because of the conditional deletion of the Rictor gene, iNKT cell numbers were reduced in the thymus and periphery. This is caused by decreased proliferation of stage 1 INKT cells and poor development through subsequent stages. Functionally, iNKT cells devoid of mTORC2 signaling showed reduced number of IL-4-expressing cells, which correlated with a decrease in the transcription factor GATA-3–expressing cells. However, promyelocytic leukemia zinc-finger (PLZF), a critical transcription factor for iNKT cell development, is expressed at a similar level in mTORC2-deficient iNKT cells compared with that in the wild type iNKT cells. Furthermore, cellular localization of PLZF was not altered in the absence of mTOR2 signaling. Thus, our study reveals the PLZF-independent mechanisms of the development and function of iNKT cells regulated by mTORC2.


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The mammalian target of rapamycin (mTOR) is an evolutionarily conserved serine/threonine kinase that has a central role in the regulation of cell growth and metabolism (1, 2). mTOR, composed of two distinct complexes, mTOR complex 1 (mTORC1) and mTORC2, has been studied extensively in a variety of biological systems. mTOR integrates a range of different signals such as growth factors, amino acids, nutrients, cytokines, and stress factors from the microenvironment to ensure not only the delivery of most appropriate immune response during Ag recognition, but also in controlling various other cellular functions involved in cell growth and survival (3, 4). mTORC1 is involved in translation initiation, autophagy inhibition, and lipid biosynthesis, whereas mTORC2 promotes actin rearrangement and uptake of nutrients (5). For T cells, Ag recognition together with secondary signals by naive CD4 and CD8 T cells triggers mTOR activation, which, in turn, programs their differentiation into functionally distinct lineages (6). Studies have shown a central role of mTOR in determining the effector versus memory fate of CD8 T cells in infection and tumor immunity (7). mTORC1 and mTORC2 also regulate Th cell fate (8, 9). Th1 and Th17 cell differentiation require mTORC1, whereas mTORC2 is essential for Th2 cell generation. However, both mTOR complexes contribute to the inhibition of Foxp3 regulatory T cell differentiation. Although the role of mTOR in T effector cell functions has been studied, little is known about its role in regulating the thymocyte development. A study showed that mTORC2 is essential for proliferation and differentiation of thymic pre-T cells from double negative (DN) to double positive (DP) stage, which is driven by Notch signaling through Akt and NF-kB (10).

Invariant NKT (iNKT) cells express a semi-invariant b8 TCR in mice (conserved Vα14-Jα18 paired with a limited repertoire of Vβ-chains, mainly Vβ8.2, Vβ7, and Vβ2) and are restricted to or specific for lipids/glycolipids presented by nonpolymorphic MHC class I–like CD1d molecule (11). Characteristically, the iNKT cells express promyelocytic leukemia zinc-finger (PLZF), the signature transcription factor of the innate-like T cells, and the NK cell–associated marker NK1.1 (CD161) (12, 13). iNKT cell development and maturation occurs in the thymus, where CD1d-restricted double-positive (CD4+CD8+) thymocytes progress through four different stages to develop into mature iNKT cells: stage 0 (CD24+,CD44−,NK1.1−), stage 1 (CD24+,CD44−,NK1.1+), stage 2 (CD24+,CD44+,NK1.1−), stage 2 (CD24+,CD44+,NK1.1−), and stage 3 (CD24+,CD44+,NK1.1+) (14). iNKT cell development requires distinct signaling compared with conventional T cells. Regarding the upstream events of the mTOR signaling (1, 2), it is well established that inappropriate signaling from CD28 (15) and ICOS (16) results in a detrimental effect on NKT cell development. Similar observation has been reported with the potent mTORC1–inducer PI3K and its associated kinase and phosphatase phosphoinositide-dependent kinase 1 and phosphatase and tensin homolog, respectively (17, 18). In these studies, it is shown that adequate PI3K activity dictates the development and the homeostasis of the iNKT cells. Conversely, two different studies have shown that in...
mice deficient of TSC1, an mTORC1 suppressor, iNKT cell population is reduced in size. Although one study depicted a massive apoptosis during the iNKT cell lineage expansion (19) in the deficient mice, the other study reported defective terminal iNKT cell differentiation and predominance of NK1.1-positive lineage over NK1.1− (20). TSC1 also promotes iNKT cell anergy in response to Ag stimulation (21). More direct evidence was provided by two recent independent studies that used mice deficient in Raptor that is a component of mTORC1 complex (22, 23). It was clear from both the reports that Raptor-deficient mice showed drastically reduced iNKT cell numbers in the thymus and periphery because of defective proliferation of the early iNKT cell developmental stages. Further, an impaired cytokine production by iNKT cells was also observed (22, 23). These impairments were associated with a defect in the nuclear localization of PLZF. Given the fact that the two mTOR complexes work as a two-signal system and with studies demonstrating a critical role for mTORC1 in iNKT cell development, the question that arises is whether mTORC2 plays a similar role for iNKT cell maturation. It is especially of great interest because at the opposite of mTORC1, upstream events of mTORC2 are yet to be defined. This study reveals an important role of mTORC2 for iNKT cell development. We demonstrate in this study that, in the absence of Rictor that is a component of mTORC2, iNKT cell numbers were decreased in a cell-intrinsic manner. Unlike mTORC1 deficiency, mTORC2 is required for the proliferation of iNKT cells at the stage 1 to progress to the next stage. Moreover, IL-4–expressing iNKT cells were reduced in Rictor-deficient iNKT cells, which is likely caused by a reduction in the expression of transcriptional factor GATA-3. Surprisingly, neither the expression level nor the cellular localization of PLZF was compromised in Rictor-deficient iNKT cells. Therefore, we report that mTORC2 regulates iNKT cell development independent of PLZF.

Materials and Methods

Mice

C57BL/6Ncr were purchased from the National Cancer Institute (101C55), and mice deficient in Rictor in T cells (RictorCD4cre fl/fl) have been described earlier (9). B6.SJL-Ptpcra/BoyAiTac mice (CD45.1 congenic, 004007) were purchased from Taconic Farms. β-Catenin transgenic (CATtg) mice described previously (24) express β-catenin in thymocytes and T cells under the control of proximal Lck promoter. β-CATtg mice were bred with RictorCre mice to generate Rictor knockout (KO) β-CATtg mice. All the mice were bred and maintained under specific pathogen-free conditions at the University of Michigan animal facility and used at 8–12 wk of age. All animal experiments were performed under protocols approved by the University of Michigan Institutional Animal Care and Use Committee.

Bone marrow chimera mice

C57BL/6 recipient mice were lethally irradiated (950 rad) 24 h before receiving bone marrow (BM) transfers. Total BM cells were harvested from the femurs and tibias of donor mice (2–3 mo of age) and depleted of mature T cells, B cells, and MHC class II+ lymphocytes by using a mixture of Abs against CD4 (RL172), CD8 (TIB210), CD19 (1D3), and MHC class II (MS/114), followed by complement-mediated lysis. BM cells from RictorCD4cre fl/fl and B6.SJL-Ptpcra/BoyAiTac donor mice were then mixed at a ratio of 1:1, and each recipient mouse received 5 × 106 cells in 200 μl 1× PBS via tail vein. Recipient mice were housed in a barrier facility under pathogen-free conditions before and after BM transplantation. After BM transplantation, mice were provided autoclaved acidified water with anti-pathogen-free conditions before and after BM transplantation. After 14 d, the recipients were sacrificed, and the thymi and spleens were harvested.

Cell preparation and flow cytometry

Primary cell suspensions were prepared from thymus, spleens, and livers. Livers were perfused with 1× PBS solution via the portal vein until most of the RBCs were washed out to prepare liver mononuclear cells. Tissues were then mechanically disrupted between two frosted microscope slides, and resultant homogenates were filtered through a 70-μm filter. Splenic and thymic erythrocytes were lysed in 1.66% NH4Cl solution (at room temperature for 6 min). Liver cells were resuspended in a 40% isotonic Percoll solution and a 70% Percoll solution was carefully underlay. After centrifugation at 900 × g at room temperature with no brakes for 30 min, liver mononuclear cells were then washed at the interface of the two layers of Percoll, washed, and resuspended.

Up to 10 × 106 cells per sample were resuspended in 200 μl FACS buffer (2% FBS, 1× PBS) in FACS tubes and preincubated with anti-FcγR mAb 2.4G2 to block nonspecific Ab binding. Cells were then washed and stained for surface molecule expression with FITC–PE–, PerCP-Cy5.5–, PE–Texas Red–, PeCy7–, allophycocyanin–, allophycocyanin-Cy7–, Pacific Blue–, or biotin-conjugated Abs. The following Abs were purchased from eBioscience: CD4 (AK1.5), CD5 (53-7.3), CD8α (53-6.7), CD44 (IM7), CD45.1 (A20), NK1.1 (PK136), IFN-γ (XM2G12), IL-4 (BV6D10), IL-13 (10F1), IL-6 (67-20Raj1), and TCR-β (H57-597). Abs against CD24 (M1/69), CD45.2 (104), and 17-17 (TC11-18H10) were purchased by BD Pharmingen. Conjugated mAbs against PLZF (Mags.21F7) were generously provided by Prof. Derek Sant’Angelo (Rutgers, The State University of New Jersey, New Brunswick, NJ). Allophycocyanin– or Pacific Blue–conjugated murine CD4tetramers loaded with PBS-57 were provided by the National Institutes of Health Tetramer Facility.

For intracelular staining, postsurface staining, the cells were fixed in 4% paraformaldehyde solution for 10 min at room temperature, washed, and then permeabilized with 0.2% saponin. Cytokine staining was performed for 30 min at 4°C directly with appropriate Abs. For intranuclear staining, surface-stained cells were fixed and permeabilized with the Foxp3/Transcription Factor Staining Buffer Kit from eBioscience, according to manufacturer’s recommendation, and then stained with corresponding Abs for 1 h at 4°C. Cell fluorescence was assessed using FACS Canto II (BD Biosciences), and data were analyzed with FlowJo software (version 9; Tree Star, Ashland, OR). For analysis, forward and side scatter parameters were used for exclusion of doublets.

In vitro stimulation assay

Freshly isolated cells (10 × 106 cells) at a concentration of 5 × 106 cells/ml were stimulated with 50 ng/ml PMA (Sigma-Aldrich) and 500 ng/ml ionomycin (Sigma-Aldrich) and complete RPMI (1640), 10% FBS, antibiotics, 2-ME). Stimulation was performed for 5 h in the CO2 incubator in FACS tubes with loosened caps. Monensin (Sigma-Aldrich) at a final concentration of 3 μM was added during the last 3 h of stimulation. Cells were then washed twice in FACS buffer (2% FBS, 1× PBS) and stained for flow cytometry analysis as described earlier.

In vivo BrdU incorporation

Mice were injected i.p. with 0.3 ml of 1 mg/ml BrdU solution (Sigma-Aldrich) every 4 h for three times. Eighteen hours after the last injection, mice were euthanized and thymi were processed to obtain primary cell suspension as described earlier. After surface staining, cells were stained for BrdU incorporation using BrdU flow kit (BD Biosciences), as per manufacturer’s instructions.

Apoptosis assay

Apoptosis rate of freshly isolated thymic cells was studied by flow cytometry using a PE-conjugated Annexin V Ab and Apoptosis Detection Kit (eBioscience), following the manufacturer’s recommendation.

Statistical analysis

Data for all experiments were analyzed with Prism software (GraphPad, San Diego, CA). Unpaired Student t test was used for comparison of experimental groups. The following p values were considered statistically significant: *p < 0.05, **p < 0.01, and ***p < 0.001.

Results

Rictor controls T cell development

To study the role of mTORC2 in iNKT cell development, we used Rictor conditional KO mice (RictorCD4cre fl/fl, designated T-Rictor−/−) that were previously reported (9). CD4 promoter-driven Cre was used to study the effect of Rictor deficiency during T cell development. We first measured the total numbers in the thymus and the spleen from T-Rictor−/− mice and compared them with the wild type (WT) mice. We observed decreased cell numbers in the thymus, but not in the spleen of T-Rictor−/− mice compared with the WT mice (Fig. 1A). We then assessed and compared the...
subsets of thymocytes between the T-Rictor$^{-/-}$ and WT mice by analyzing the percentages and cell numbers of DN, DP, SPCD4, and SPCD8 cells. Interestingly, although the percentages of thymic DP, SPCD4, and SPCD8 were comparable between the two groups (Fig. 1B, 1C), careful examinations of these subpopulations revealed that the total cell numbers of thymic DP and SPCD4 T cells were significantly lower in the T-Rictor$^{-/-}$ mice (Fig. 1C). Next, we calculated and compared the selection efficiency of SPCD4 and SPCD8 cells using the ratio of the percentages of cells of interest to the percentages of DP (Fig. 1D). A ratio of 1 would indicate the equivalent selection efficiency between T-Rictor$^{-/-}$ and WT cells. The data demonstrated that Rictor plays a modest role for SPCD4 T cell development, whereas SPCD8 T cell development was not affected in T-Rictor$^{-/-}$ mice.

Rictor is important for iNKT cell development and homeostasis

Next, we assessed iNKT cell development in Rictor-deficient mice. We compared the iNKT cell compartment in the thymus, spleen, and liver between the T-Rictor$^{-/-}$ and WT mice. The percentages as well as the cell numbers of CD1d-tetramer$^+$ iNKT cells were greatly reduced in the T-Rictor$^{-/-}$ thymus (Fig. 2A, 2B). In contrast with the conventional T cells for which the developmental defect is limited to the thymus, both the percentages and the cell numbers of iNKT cells were significantly decreased in the spleen as well as in the liver of the T-Rictor$^{-/-}$ mice (Fig. 2A, 2B).

iNKT cell development occurs as the CD1d-restricted immature progenitor DP cells progress through four different stages defined by the expression of surface molecules CD24, CD44, and NK1.1 (Fig. 2C). When we examined the iNKT maturation in the thymus, we observed that the percentages of CD24$^+$ CD44$^+$NK1.1$^-$ Stage 2 iNKT cells were greatly reduced, whereas CD24$^+$ CD44$^+$ NK1.1$^+$ Stage 1 iNKT cells were increased (Fig. 2D, upper panels). CD24$^+$ stage 0 and CD24$^+$CD44$^+$NK1.1$^+$ stage 3 iNKT cells did not show significant differences. However, the cell numbers in all stages except stage 1 were reduced in the T-Rictor$^{-/-}$ thymus, which resulted in the lower number of total thymic iNKT cells in T-Rictor$^{-/-}$ mice as compared with the WT (Fig. 2B, 2D). Thus, Rictor participates in iNKT cell development in the thymus.

To further investigate a possible role of Rictor during iNKT cell development, we compared the generation of stage 0 cells from DP because this step would be equivalent to positive selection of iNKT cells, and subsequent stages reflect proliferation and maturation of selected iNKT cells. The results showed that although stage 0 cell numbers were reduced in T-Rictor$^{-/-}$ mice, the selection efficiency was comparable between WT and T-Rictor$^{-/-}$ mice likely because of the reduced DP in T-Rictor$^{-/-}$ mice (Fig. 2E). Next, we compared the transition 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. 2F). As expected, WT iNKT cells showed an increase in cell numbers at each transition period during maturation. In contrast, stage 1 to 2 transition of T-Rictor$^{-/-}$ iNKT cells was impaired, whereas stage 0 to 1 and 2 to 3 were similar to the WT. Together, mTOR is indispensable for stage 0 iNKT cells to develop into stage 1 iNKT cells.

Cell-intrinsic defect of Rictor-deficient iNKT cells

To study whether the developmental defect of Rictor KO iNKT cells is cell intrinsic, we performed mixed BM chimera experiments. Equal proportions of Rictor KO (CD45.2) BM cells were transferred together with WT (CD45.1/CD45.2) BM cells to irradiated hosts (CD45.1) and analyzed for T cell development. Rictor KO cells were poorly reconstituted in the thymus as shown by the percentages and the recovery of total cells from each donor (Fig. 3A). We then compared the iNKT compartment in the thymus, spleen, and liver. Very few iNKT cells from Rictor KO donor were detected in the thymus, as well as in the spleen or liver of the chimeric mice (Fig. 3B). Moreover, similar to our observations from Rictor KO mice, the thymic selection efficiency was lowest for iNKT cells followed by CD4 T cells, whereas the efficiency of Rictor KO CD8 T cells was slightly reduced (Fig. 3C). Together, our data demonstrate that Rictor controls iNKT development in a cell-intrinsic manner.

Rictor is required for optimum proliferation of stage 1 iNKT cells

A reduced thymic iNKT cell output in the absence of Rictor could be explained by at least two independent and nonexclusive possibilities. First, Rictor-deficient cells might undergo a higher rate of spontaneous apoptosis. Alternatively, iNKT lineage expansion is reduced in the absence of Rictor. To test these two possibilities, we first assessed apoptotic cell death by staining cells for Annexin V and 7-aminoactinomycin D (7-AAD). In WT mice, the most immature stage 0 iNKT cells showed greater cell proportion that underwent apoptosis (Annexin V$^+$ 7-AAD$^+$) than stages 1–3 (Fig. 4A). Although the percentages of apoptotic immature iNKT cells, namely, stage 0 cells, were slightly lower in the T-Rictor$^{-/-}$ mice, the difference was not statistically significant (Fig. 4A). T-Rictor$^{-/-}$ iNKT cells in stages 1–3 had comparable fractions of apoptotic cells to the WT iNKT cells. Furthermore,
both WT and T-Rictor−/− iNKT cells showed similar proportions of apoptotic cells after stimulation with anti-CD3, suggesting that cell death was not altered in T-Rictor−/− mice (data not shown). Overall, our data suggest that an increased rate of spontaneous apoptosis in absence of functional mTORC2 is unlikely to explain the reduced iNKT population observed in T-Rictor−/− mice.

We next measured the proliferation capacity of T-Rictor−/− cells by using two approaches. First, we measured the expression of Ki-67 in each stage of cells to assess the cell-cycling activity reflecting proliferation. It is reported that stage 1 iNKT cells undergo high proliferation, whereas the fully mature Stage 3 cells are quiescent in the absence of stimuli (11, 25). Consistent with the reported studies, our data demonstrated that most of the stage 0 iNKT cells from WT mice were Ki-67+, indicating that they were in an active phase of their cell cycle (Fig. 4B). As iNKT cells matured, a significant fraction of cells became Ki-67− and stage 3 cells were mostly Ki-67−. T-Rictor−/− iNKT cells showed a similar pattern of Ki-67+ cells except that stage 1 cells had a significant reduction of Ki-67+ cells compared with WT (Fig. 4B, 4C), indicating that the stage 1 iNKT cells in T-Rictor−/− mice might undergo decreased rates of proliferation. To further confirm this difference in cell-cycling activity, we performed in vivo BrdU incorporation experiments. Consistent with the Ki-67 data, stage 1 iNKT cells from the T-Rictor−/− thymus showed a significantly lower percentage of BrdU+ cells than those from the WT thymus (Fig. 4D). Thus, these results indicate that Rictor is important for...
stage 1 iNKT cells to proliferate and progress to stage 2 during iNKT cell maturation.

It is known that Wnt signaling pathway functions similarly to mTOR and overexpression of active dephosphorylated form of β-catenin leads to a significant increase in iNKT cells (26). This raises a question whether Wnt and mTOR signaling may regulate each other so that constitutively active Wnt signaling may restore the defect of T-Rictor <sup>−/−</sup> iNKT cell development. To test this, we crossed T-Rictor <sup>−/−</sup> mice with the transgenic mice expressing active dephosphorylated form of CATtg, resulting in CATtg<sup>3</sup> KO. We found that iNKT cell development in CATtg<sup>3</sup> KO mice was not rescued (Supplemental Fig. 1), suggesting that mTORC2 and Wnt signaling pathway seem to work independent of each other.

Rictor regulates the generation of iNKT cells expressing IL-4, but not IFN-γ or IL-17

Having observed that the iNKT cell maturation is controlled by mTORC2, we next asked whether the Rictor deficiency also affects the effector function of iNKT cells. iNKT cells are defined by their ability to secrete various cytokines like IL-4, IFN-γ, and IL-17. In addition, previous studies have demonstrated that cytokine production varies with thymic iNKT cell development, and these cells produce different cytokines at maximum levels at different developmental stages (27–29). Rictor/mTORC2 has been shown to be important for Th2 cell differentiation, but not for Th1 or Th17 phenotypes in conventional CD4 T cells. We therefore asked whether Rictor also regulates the generation of IL-4–expressing iNKT cells. To test this, we stimulated thymocytes including the thymic iNKT cells with PMA and ionomycin; then the expression of IL-4, IFN-γ, and IL-17 was measured by intracellular cytokine staining. Assessment of the cytokine expression showed that IL-4– but not IFN-γ– or IL-17–expressing cells were decreased in iNKT cells from T-Rictor <sup>−/−</sup> mice compared with that in WT iNKT cells (Fig. 5).

Considering cytokine expression has been reported to be maturation dependent, we further assessed the cytokine production at each stage of iNKT cells. IL-4–expressing cells were reduced in all the stages of iNKT development in the T-Rictor <sup>−/−</sup> mice compared with the WT mice, whereas a modest decrease in IFN-γ-expressing
Rictor deficiency results in decreased GATA-3–expressing cells

It is known that PLZF is the most critical transcription factor for the development as well as IL-4 expression of iNKT cells as illustrated in PLZF−/− mice (12). In addition, PLZF overexpression was shown to induce IL-4 production in CD4 T cells (30). Therefore, we asked whether Rictor controls the expression of PLZF, which, in turn, affects iNKT cell development and IL-4 expression. Expression of PLZF in total iNKT cells as well as at each stage of iNKT cell development showed that the absence of Rictor did not interfere with the expression of PLZF (Fig. 6A). However, it is possible that PLZF function may be compromised in the absence of Rictor. In fact, PLZF localization, but not the expression level, was found to be affected in mTORC1-deficient iNKT cells (22). We found that the cellular distribution of PLZF was similar between the WT and T-Rictor−/− iNKT cells, which was mostly confined to the nucleus (Fig. 6B). Thus, unlike mTORC1 deficiency, absence of Rictor does not affect either the PLZF expression or its nuclear localization.

It is known that the transcription factors T-bet, GATA-3, and RAR-related orphan receptor γt (RORγt) control the differentiation of Th1, Th2, and Th17 cells, respectively. Furthermore, the same transcription factors are expressed in iNKT cells generating functionally distinct iNKT cells (31). Therefore, we compared the expression of T-bet, GATA-3, and RORγt. There was no difference in RORγt- or T-bet-expressing cell populations between the two groups (Fig. 6C). However, the frequencies of GATA-3–expressing iNKT cells were significantly lower among the T-Rictor−/− iNKT cells than the WT cells (Fig. 6C, 6D). Therefore, the reduction in GATA-3–expressing iNKT cells in T-Rictor−/− mice correlated with less IL-4+ iNKT cells observed. Together, Rictor controls the generation of GATA3-expressing iNKT cells, which, in turn, is responsible for differentiation of IL-4-producing cells.

Discussion

In this study, we reveal a critical role of mTORC2 in the iNKT development and maturation. We observed that, in the absence of functional mTORC2 due to Rictor deficiency in the T cells, early iNKT cell development is impaired in the thymus as well as in the peripheral organs including the spleen and the liver. Although
mTORC1 deficiency also results in reduced iNKT cell numbers, mTORC1 and mTORC2 appear to play a different role during iNKT cell development. In mTORC1-deficient mice, the percentage of stage 0 and 1 iNKT cells are higher, whereas the percentage of stage 3 iNKT cells were greatly reduced (22, 23). In contrast, we observed that iNKT cells from T-Rictor+/− mice display a stage 1-specific defect in proliferation. T-Rictor+/− iNKT cells showed the higher percentage of stage 1 cells, but significantly lower level of stage 2 cells compared with the WT. Stage 3 cells were comparable between the two groups of mice.

This indicates that the initial stages of iNKT development require both mTORC1 and mTORC2, and as cells mature, mTORC2 is especially critical for the transition from stage 1 to stage 2. Interestingly, more mature long-term resident thymic stage 3 iNKT subset seems to depend solely on mTORC1 for their survival. This differential dependence on the two mTOR complexes by the different developmental stages of iNKT cells may be dictated by their different metabolic needs as they proceed through the maturation process, thus changing their responsiveness to either mTORC1 or mTORC2. A more striking difference was revealed with PLZF. Unlike mTORC1-deficient NKT cells in which PLZF nuclear localization was impaired, we observed no difference either in the expression level or the cellular localization of PLZF in iNKT cells deficient of Rictor. Thus, mTORC1 and mTORC2 control the iNKT cell development and maturation by mechanisms dependent and independent of PLZF, respectively.

Nevertheless, there appear to be similarities between mTORC1- and mTORC2-mediated iNKT cell development. Both mTORC1 and mTORC2 indeed incorporate upstream proliferation signals, and thus promote the cell-cycling activity and cell proliferation (Fig. 4). Deficiency in either mTORC1 or mTORC2 would lead to poor proliferation. Interestingly, we report in this article that lack of functional mTORC2 induces a greater developmental defect in iNKT cells than in conventional CD4 and CD8 T cells. Given the fact that iNKT lineage cells undergo two consecutive rounds of expansion in the thymus (DN-to-DP transition and then stage 0 to stage 2 for iNKT commitment) to become fully mature and functional cells, it is not surprising that iNKT cells rely more on mTORC1 and mTORC2 signaling. Conventional CD4 T cells, in contrast, undergo the second round of expansion during differentiation into the Th cell subsets in the periphery. During this differentiation and expansion stage, the two mTOR complexes have been shown to be requisite regulators for different Th cell lineage numbers and function.

It is well established that mTORC1 and mTORC2 signals promote Th1 and Th2 responses, respectively, in conventional CD4 T cells (9). Unlike conventional CD4, Th cell differentiation programming that is initiated upon Ag exposure in the periphery, iNKT cells express cytokines during their differentiation and maturation in the thymus (27). It is known that stage 1 cells express IL-4; then both IL-4 and IFN-γ are produced by more mature stage 2 and 3 cells. Therefore, decreased stage 1 cell proliferation would lead to the reduction of IL-4+/ but not IFN-γ+/ cells, which we have shown in this study. In line with this assumption, absence of functional mTORC2 in iNKT cells greatly decreases IL-4+/cells, particularly in stages 2 and 3 (Fig. 5 and Supplemental Fig. 1). A recent study proposed simultaneous generations of three effector types of NKT cells, which are designated NKT1, NKT2, and NKT17 (31). According to this new model, mTORC2 signaling is important for the differentiation of NKT2, but not NKT1 and NKT17, which mirrors the requirement of mTORC2 signaling for Th2 but not Th1 or Th17 cells. The main difference is that development and effector iNKT cell generation is tightly linked and occurs in the thymus.

In conclusion, mTORC2, together with mTORC1, is an indispensable modulator of iNKT cell development and function by promoting the active proliferation during iNKT cell maturation in the thymus. Further studies to identify signaling molecules and pathways that regulate the mTOR complexes should shed light on the molecular mechanisms to control iNKT cell development.

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Disclosures

The authors have no financial conflicts of interest.

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