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Mammalian Target of Rapamycin Complex 1 Orchestrates Invariant NKT Cell Differentiation and Effector Function

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Invariant NKT (iNKT) cells play critical roles in bridging innate and adaptive immunity. The Raptor containing mTOR complex 1 (mTORC1) has been well documented to control peripheral CD4 or CD8 T cell effector or memory differentiation. However, the role of mTORC1 in iNKT cell development and function remains largely unknown. By using mice with T cell–restricted deletion of Raptor, we show that mTORC1 is selectively required for iNKT but not for conventional T cell development. Indeed, Raptor-deficient iNKT cells are mostly blocked at thymic stage 1–2, resulting in a dramatic decrease of terminal differentiation into stage 3 and severe reduction of peripheral iNKT cells. Moreover, residual iNKT cells in Raptor knockout mice are impaired in their rapid cytokine production upon αGalCer challenge. Bone marrow chimera studies demonstrate that mTORC1 controls iNKT differentiation in a cell-intrinsic manner. Collectively, our data provide the genetic evidence that iNKT cell development and effector functions are under the control of mTORC1 signaling.

N
atural killer T cells have been well defined as a unique CD1d-restricted T cell lineage expressing NK cell markers (1–3). Invariant NKT (iNKT) cells are known as type I NKT cells that express a semi-invariant TCR consisting of α-chain (Vα14 in mouse) and Vβ2, Vβ7, or Vβ8.2. iNKT cells bridge the innate and adaptive arms of the immune system by producing a variety of cytokines very rapidly after stimulation (1, 2). In view of their transactivating properties, iNKT cells have been implicated in autoimmunity, viral infection, and tumor immunity (1, 4, 5). During thymic development, iNKT cells are branched off from conventional T cells at double-positive (DP) stage (6). Unlike conventional T cells, iNKT cells are selected by glycolipids presented on CD1d molecule expressed on DP thymocytes instead of thymic epithelial cells (6). In addition, the development of iNKT cells has been shown to involve distinct signaling requirements. For instance, DP thymocytes lacking expression of PDK1, c-myc, and Fyn fail to produce mature iNKT cells, despite normal development of conventional αβ T cells (7–11). Similarly, recent studies suggested that miR181a is a critical regulator for iNKT cell development (12, 13) by targeting phosphatase and tensin homologue expression, as the diminished iNKT development in miR181a knockout (KO) mice could be rescued by knocking out phosphatase and tensin homologue (12). It is thus evident that the PI3K-PDK1-Akt pathway plays crucial roles in regulating iNKT cell development, in particular during the crucial expansion phase characteristic of their early differentiation from DP thymocytes.

The mammalian target of rapamycin (mTOR) is a highly conserved serine/threonine kinase that integrates various environmental cues to regulate cell growth, proliferation, and metabolism (14, 15). mTOR can assemble into two structurally and functionally distinct complexes named, respectively, mTOR complex (mTORC)1 and mTORC2, by binding to different partner proteins. mTORC1 is composed of the DEP domain-containing mTOR-interacting protein (DEPTOR), the mammalian lethal SEC13 protein 8 (MLST8), the proline-rich AKT substrate (PRAS40), and the regulatory-associated protein of mTOR (Raptor). mTORC1 activity is directly induced by GTP-bound Rheb, which is inhibited by a heterotrimeric complex composed of TSC1 (hamartin), TSC2 (tuberin), and TBC1D7 (14, 16–18). As an essential component of mTORC1, Raptor has been well demonstrated to regulate both innate and adaptive immunity (19, 20). For example, mTORC1 is required for Th1, Th2, and Th17 differentiation in vitro and in vivo (20–22), and recent findings showed that Raptor/mTORC1 is essential for T regulatory cell–suppressive function (23). However, it remains largely undefined whether mTORC1 controls the thymic development of iNKT cells.

To this end, we have generated T cell–specific Raptor KO mice. Intriguingly, we found that mTORC1 was selectively required for thymic iNKT cell development. The developmental defects of iNKT cells in Raptor KO mice are due to a cell-autonomous effect, as shown by bone marrow (BM) chimera. Furthermore, the remaining iNKT cells in Raptor KO mice are functionally impaired. Therefore, our present study reveals that mTORC1 is a critical regulator of iNKT cell differentiation and effector function.

Materials and Methods

Mice

Raptor<lox/lox> mice have been described previously (24). T cell–specific Raptor KO mice were obtained by crossing Raptor<lox/lox> mice with transgenic mice that carried CD4 promoter-mediated Cre recombinase. All mouse strains have been backcrossed to C57BL/6J background and were maintained at the University of Lausanne’s specific pathogen-free facility. CD45.1 congenic C57BL/6 mice were purchased from Charles River. Age- and sex-
matched mice between 5 and 10 wk of age were used for all experiments. The present study was approved by the veterinary authority of the Canton Vaud, Switzerland, and all experiments were performed in accordance with Swiss ethical guidelines.

**Real-time PCR**

mRNA was extracted from total thymocytes or sorted CD4+CD8+ thymocytes (RNasy Plus Mini Kit; Qiagen), and cDNAs were generated using SuperScript III reverse transcriptase and oligo(dT) primers (Life Technologies). Quantitative PCR analysis was performed on the Applied Biosystems 7500 Fast Real-Time PCR System with SYBR Green as reporter (Fast SYBR Green Master Mix; Applied Biosystems). Measured cycle threshold values were normalized using the TATA box-binding protein gene. The primers were as follows: TBp forward, 5'-CCTCAC-CAATGACTCCTATGAC-3'; TBp reverse, 5'-CAGATTACAGCCCA-AGATTCAC-3'; Vx14 forward, 5'-TCCGTAGACGTCTGGTTGA-3'; Jx18 reverse, 5'-CAGGCTCTCAAGGCTGAA-3'.

**Flow cytometry**

Lymphocytes from peripheral lymph nodes (LN), thymus, and spleen were isolated and washed twice with ice-cold FACS buffer (PBS containing 2% FBS). LIVE/DEAD Aqua cell stain (Invitrogen) was used to exclude dead cells, and lymphocytes were gated on the basis of forward scatter and side scatter properties. Certain amounts of lymphocytes were first blocked by anti-mouse FcR mAb (2.4G2), followed by CD1d-tetramer staining at 4°C for 25 min. Cell suspensions were further incubated with the appropriate concentrations of Ab. Abs were obtained from BD Pharmingen (San Diego, CA) and eBioscience (San Diego, CA). For cell surface staining, the following anti-mouse Abs were used: anti-mouse CD3-Alexa 700 (145-2C11), anti-mouse CD4-FITC (RM4-5), anti-mouse CD8α-PE (53-6.7), anti-mouse CD8β-PE-Cy5 (A20), anti-mouse CD1d-PE-Cy5 (2G8), and anti-mouse CD44-PE-Cy7 (H129.19). Anti-mouse CD11c-PE (HL3), anti-mouse CD44-allophycocyanin-PerFP80 (IM7), anti-mouse CD122-Pacific Blue (TM-β1), and anti-mouse B220 FITC (RA3-6B2). Samples were acquired using a LSR-II or LSR-II SORP flow cytometer (BD Biosciences, San Jose, CA) and analyzed using FlowJo software (Tree Star, Ashland, OR). For liver mononuclear cell preparation, cells were carried out by Percoll gradient centrifugation, and the interface between 40 and 70% Percoll was collected and considered as lymphocytes.

**αGalCer challenge and cytokine quantification in serum**

Wild-type (WT) or Raptor KO mice received 0.5 μg αGalCer (Alexis Biochemicals) by i.v. injection. Two hours after injection, blood was collected and serum was prepared using Z-gel microtubes (Sarstedt). Multiple cytokine measurements were conducted by using BD Cytometric Bead Array kit Th1/Th2/Th17 (BD Biosciences), following the manufacturer’s instructions. Samples were acquired using a LSR-II and analyzed using FlowJo software (Tree Star).

**BM chimera**

Recipient CD45.1+ congenic C57BL/6 mice were given 1000 rad γ-irradiation 16 h before BM transfer. A total of 5 × 10⁶ cells from either WT CD45.1 or WT/Raptor KO CD45.2 strains was mixed and injected i.v. into recipient mice. Eight weeks after reconstitution, lymphocytes from thymus, spleen, and liver were prepared and processed for FACs analysis.

**Intracellular cytokine staining**

Lymphocytes from spleen and liver were harvested and prepared for LIVE/DEAD Aqua and CD1d-tetramer staining, which was followed by other defined surface labeling, fixation, and permeabilization using the BD Fix/Perm kit. Intracellular cytokines were detected using fluorochrome-conjugated anti-IFN-γ and anti-TNF-α Abs. All reagents were purchased from BD Biosciences (San Diego, CA), and the fixation and staining were performed according to the manufacturer’s specifications.

**Statistical analysis**

All tests were performed using GraphPad Prism software (La Jolla, CA). All data are presented as the mean ± SD. Student’s unpaired t test for comparison of means was used to compare groups. A p value <0.05 was considered to be statistically significant (*p < 0.05, **p < 0.01, ***p < 0.001).

**Results**

**mTORC1 is essential for iNKT cell development in thymus**

To assess the role of mTORC1 in iNKT cell development, we generated CD4-Cre+/Raptor<sup>lox/lox</sup> mice with loss of mTORC1 activity specifically in T cells. In view of the CD4 promoter activity in the thymus, this model ensures the deletion of Raptor starting from late double-negative (DN3/DN4) stage, and thus, DP thymocytes lack mTORC1 activity. After several rounds of crossing, we obtained mice with homozygous Raptor deletion in T cells (CD4-Cre<sup>*</sup>/Raptor<sup>lox/lox</sup>; Raptor KO), and Raptor<sup>lox/lox</sup> littermates (WT) without Cre expression served as WT control. We first confirmed the deletion efficiency and found that mTORC1 activity was abolished in Raptor KO T cells upon activation, as shown by loss of pS6 expression (data not shown). Next, we determined the T cell compartment composition in thymus in the absence of Raptor. As shown in Fig. 1A, the frequencies and numbers of DN, DP, CD4 single-positive (SP), or CD8SP in Raptor KO thymi were identical to WT littermates. The proportion of thymic-derived CD4<sup>+</sup>CD25<sup>+</sup>Foxp<sup>+</sup> regulatory T cells was also indistinguishable between Raptor KO and WT mice (Fig. 1B), although their absolute numbers were slightly decreased (data not shown). In addition, we observed similar levels of γδ T cells in thymus between WT and Raptor KO mice (Fig. 1C). Intriguingly, we found dramatically decreased (>10-fold) frequency and number of iNKT cells in the absence of functional mTORC1 (Fig. 1D, 1E). The specificity of the residual α-galactosylceramide–CD1d tetramer<sup>+</sup> population in Raptor KO was confirmed by its absence when using unloaded CD1d tetramer (Fig. 1F). Therefore, Raptor deficiency leads to impaired development of thymic iNKT cells, whereas the development of conventional γδ/γδ T cells and CD4<sup>+</sup>CD25<sup>+</sup>Foxp<sup>+</sup> regulatory T cells is not affected, indicating a selective role of mTORC1 in iNKT cell development.

**FIGURE 1. mTORC1 is essential for iNKT cell development in thymus.** Thymocytes from 4- to 6-wk-old WT or Raptor KO mice were subjected to flow cytometric analysis. (A) Percentage and absolute number of distinct thymocyte subsets in Raptor KO mice and WT littermates. (B) Relative frequency of thymic T regulatory cells in WT or Raptor KO mice. (C) Percentage of thymic γδ T cells, gated on CD8<sup>+</sup> thymocytes. (D) WT or Raptor KO thymocytes were analyzed for iNKT cells by staining with CD3 and CD1d-tetramer either α-galactosylceramide loaded (left panel) or unloaded (right panel). (E) Relative frequency and absolute numbers of thymic iNKT cells were summarized. Data are shown as mean ± SD (five mice each group), and one representative of four independent experiments with identical results is illustrated. ***p < 0.001.
Defective iNKT cell maturation in thymus of Raptor KO mice

mTORC1-deficient mice displayed an accumulation of CD24\(^+\) stage 0 iNKT cells as compared with WT counterparts (Fig. 2A), suggesting an early blockade of iNKT cells. Similarly, we found an accumulation of CD4\(^+\)CD8\(^+\) iNKT cells in Raptor KO mice (Fig. 2A). Next, we examined whether loss of mTORC1 altered the recombination and expression of the V\(\alpha\)14-Jo18 TCR\(\alpha\)-chain, which occurs at early DP stage. A marked decrease of V\(\alpha\)14-Jo18 rearrangement was evidenced in total thymocytes of Raptor KO mice, possibly reflecting the very low number of residual iNKT cells in these mice (Fig. 2B). However, when analyzing sorted WT or Raptor KO DP thymocytes, similar V\(\alpha\)14-Jo18 TCR\(\alpha\) rearrangement was obtained (Fig. 2B), indicating that the V\(\alpha\)14-Jo18 recombination in Raptor KO iNKT precursor cells remained intact. Upon positive selection, iNKT cells undergo a series of further differentiation steps to acquire functional maturation (1, 6). This process is characterized by a distinct expression pattern of the activation markers CD44 and NK1.1. Stage 1 duration (1, 6). This process is characterized by a distinct expression pattern of the activation markers CD44 and NK1.1. Stage 1 duration (1, 6). This process is characterized by a distinct expression pattern of the activation markers CD44 and NK1.1. Stage 1 duration (1, 6). This process is characterized by a distinct expression pattern of the activation markers CD44 and NK1.1. Stage 1 duration (1, 6). This process is characterized by a distinct expression pattern of the activation markers CD44 and NK1.1. Stage 1 duration (1, 6). This process is characterized by a distinct expression pattern of the activation markers CD44 and NK1.1. Stage 1 duration (1, 6). This process is characterized by a distinct expression pattern of the activation markers CD44 and NK1.1. Stage 1 duration (1, 6). This process is characterized by a distinct expression pattern of the activation markers CD44 and NK1.1. Stage 1 duration (1, 6). This process is characterized by a distinct expression pattern of the activation markers CD44 and NK1.1. Stage 1 duration (1, 6). This process is characterized by a distinct expression pattern of the activation markers CD44 and NK1.1. Stage 1 duration (1, 6). This process is characterized by a distinct expression pattern of the activation markers CD44 and NK1.1. Stage 1 duration (1, 6). This process is characterized by a distinct expression pattern of the activation markers CD44 and NK1.1. Stage 1 duration (1, 6). This process is characterized by a distinct expression pattern of the activation markers CD44 and NK1.1. Stage 1 duration (1, 6). This process is characterized by a distinct expression pattern of the activation markers CD44 and NK1.1. Stage 1 duration (1, 6). This process is characterized by a distinct expression pattern of the activation markers CD44 and NK1.1. Stage 1 duration (1, 6).

FIGURE 2. Thymic iNKT cells are blocked at early stage in Raptor KO mice. (A) WT or Raptor KO thymocytes were stained with α-galactosylceramide/CD1d-Tetra and CD24 (upper) or CD8 and CD4 (lower); results are shown as representative dot plots (left) and as graphs of the means (right). (B) Quantification by real-time quantitative PCR of the iNKT-specific V\(\alpha\)14-Jo18 TCR\(\alpha\) rearrangement in total (upper) and sorted DP thymocytes (lower); results are expressed as relative expression normalized to the TATAA box-binding protein. (C) Live CD3\(^+\)CD1d-Tetra\(^+\) iNKT cells were stained for CD44 and NK1.1. (D) Relative frequencies and absolute number of stage 1, 2, or 3 iNKT cells are summarized. Data are shown as mean ± SD (five mice each group). One representative of three separate experiments with identical results is illustrated. **p < 0.01, ***p < 0.001.

Raptor KO mice, about two-thirds of the iNKT cells stayed blocked in the early immature stage 1 and less than one-third proceeded to stage 2 (Fig. 2C, 2D), but very rare iNKT cells reached stage 3, as seen by the selective loss of mature CD44\(^+\) NK1.1\(^+\) iNKT cells. We further confirmed the iNKT cell specificity by gating on the few unloaded CD1d-tetramer\(^+\) cells, which were mostly CD4\(^+\)CD8\(^-\) and CD44\(^-\)NK1.1\(^-\) regardless of WT or Raptor KO background (Supplemental Fig. 1). Given the importance of mTORC1 in controlling T cell proliferation, we hypothesize that iNKT cells may require mTORC1 to meet the metabolic demands for high proliferative expansion during transition from stage 1 to stage 2. In this context, we determined the proliferative capacity of iNKT cells with Ki67 staining. A dramatic decrease of Ki67-expressing stage 1–2 iNKT cells was observed in Raptor KO mice (Supplemental Fig. 2A), which suggests that mTORC1 is necessary for iNKT early stage proliferation and differentiation. The promyelocytic leukemia zinc finger protein (PLZF) is the key transcription factor governing iNKT cell programming and acquisition of rapid effector functions (6, 25), and PLZF expression is gradually decreased from stage 1 to stage 3. In this study, we found that Raptor KO iNKT cells showed an elevated PLZF expression comparable to stage 1 iNKT cells in WT mice (Supplemental Fig. 2B), which is supporting an early block of iNKT cell differentiation between stages 1 and 2. Functional maturation of iNKT cells is also characterized by acquisition of Ly49 family molecules. In line with the developmental blockade in stage 1 or 2, we found that Raptor KO iNKT cells also lacked the Ly49-A, Ly49C/I, and Ly49G NK cell markers (Fig. 3A). Finally, T-bet and IL-15 signaling components are required for iNKT cell terminal differentiation (26, 27), and, consistent with the loss of stage 3 iNKT cells, we found a significant decrease of T-bet and its target CD122 expression in Raptor KO thymic iNKT cells (Supplemental Fig. 2C). Recently, the maturation process of iNKT cells has been challenged by Lee et al. (28) claiming that differential expression of T-bet, PLZF, and ROR\(\gamma\)t by thymic iNKT cells defines diverse lineages of, respectively, NKT1, NKT2, and NKT17 rather than developmental stages. Consistent with NKT1 being predominantly stage 3 (28), the drastic loss of T-bet expression in Raptor KO iNKT cells may correlate with a decreased NKT1 subset (Fig. 3B, Supplemental Fig. 2C), associated with a concomitant enrichment of the NKT2 subset characterized by increased PLZF expression (Fig. 3B).

The murine iNKT V\(\alpha\)14Jo18 chain preferentially pairs with a restricted number of V\(\beta\)-chains, in particular V\(\beta\)7 and V\(\beta\)8.2 (29). We found that in Raptor KO mice, the few residual thymic iNKT cells showed a significant decreased V\(\beta\)7 and V\(\beta\)8.2 usage as compared with WT littermates (Fig. 3C, 3D), resulting in a 2-fold increased frequency of iNKT cells negative for these two V\(\beta\)s, which suggested a bias for other V\(\beta\)-chains shown to be used by iNKT cells (30, 31). Collectively, our data suggest that mTORC1 deficiency leads to a severe developmental blockade of iNKT cells between thymic stages 1 and 2.

Remarkably reduced iNKT cells in liver, spleen, and LN of Raptor KO mice

Consistent with their reduced levels in thymus, iNKT cells were significantly decreased in spleen and LNs of Raptor KO mice (Fig. 4). We also found an even more dramatic loss of iNKT cells in the liver that is known as the major reservoir for iNKT cells in periphery (Fig. 4). Moreover, there was a significant decrease of the CD4\(^+\) or the NK1.1\(^+\) iNKT cell subset in Raptor KO mice (Fig. 5). In this regard, our observations suggest that mTORC1 may play important roles in regulating peripheral homeostasis of distinct subsets of iNKT cells.
mTORC1 is required for thymic iNKT cell maturation. (A) FACS plots analysis of thymic iNKT cells from WT or Raptor KO mice for CD44 or CD1d-tetramer, together with Ly49C/I, Ly49A, and Ly49G staining. (B) Live CD3+CD1d-Tetra+ thymic iNKT cells from WT and Raptor KO were stained intracellularly for T-bet and PLZF. (C) iNKT cells from thymus of WT or Raptor KO mice were stained for Vβ8.2 or Vβ7. (D) Percentage of Vβ8.2- or Vβ7-positive thymic iNKT cells in WT or Raptor KO mice was summarized. One representative of three separate experiments with identical results is illustrated. *p < 0.05, **p < 0.01.

Raptor KO iNKT cells show impaired response to αGalcer challenge

To determine the role of mTORC1 in controlling iNKT effector function, we administered α-galactosylceramide i.v. into WT or Raptor KO mice. We found significantly decreased production of serum IFN-γ, IL-2, IL-4, IL-6, and TNF-α 2 h after α-galactosylceramide injection (Fig. 6A), which could be largely explained by the dramatically reduced iNKT cells in Raptor KO mice. IL-10 and IL-17 were hardly detectable in both WT and Raptor KO mice. Next, we aimed to further explore the role of mTORC1 in controlling iNKT cell cytokine production on a single cell level. Consistent with reduced cytokine levels in serum, the few residual mTORC1-deficient iNKT cells in both liver and spleen were severely impaired in their capacity to produce IFN-γ or TNF-α (Fig. 6B, 6C). In addition, CD69 expression was significantly decreased on Raptor KO iNKT cells (Fig. 6B, 6C). Altogether, these observations suggest that, in addition to a thymic development defect, iNKT cell function is also severely impaired in the absence of mTORC1 signaling.

Considering T-bet as a regulator of IFN-γ production, we thus compared intracellular T-bet expression in iNKT cells between WT and Raptor KO mice. While in line with the impaired cytokine production, we observed remarkably reduced frequency of T-bet–expressing iNKT cells in the absence of Raptor (Fig. 6D). Moreover, we observed that CD122, a known target of T-bet, was significantly reduced in Raptor KO iNKT cells, as shown by the decreased proportion of CD44+CD122+ iNKT cells in both spleen and liver (Fig. 6E). In contrast, we did not observe any change in the frequency of virtual memory CD8+ T cells (CD62L+CD44+CD122+) in mTORC1-deficient mice, although the level of CD122 expression is also decreased on this subset (data not shown).

Defective iNKT cell maturation in mTORC1-deficient mice is T cell intrinsic

Unlike conventional T cells, iNKT precursor cells are selected by CD1d-expressing DP thymocytes. Similar CD1d expression on DP thymocytes in WT and Raptor KO mice rules out the possibility that the block in iNKT cell development was due to a defect in CD1d expression (Fig. 7A, 7B). Homotypic interactions mediated by Slamf1 (SLAM) and Slamf6 (Ly108) are essential for differentiation of the iNKT cell lineage (32). Raptor deficiency seems not to affect the expression pattern of Slamf1 and Slamf6, as we observed comparable Slamf1 and Slamf6 expression between WT and Raptor KO DP thymocytes (Fig. 7C, 7D). To investigate whether the developmental defects of iNKT cells in Raptor-deficient mice were cell intrinsic, we established BM chimeras by reconstituting lethally irradiated CD45.1 recipients with 1:1 mixture of WT CD45.1 and Raptor KO CD45.2 BM cells (Fig. 8A). Eight weeks after BM reconstitution, we found only 2–3% of iNKT cells in thymus, spleen, or liver that were derived from Raptor KO BM cells (Fig. 8B, 8C). As a control, we found equal contributions to the iNKT pool in the WT CD45.1 and WT CD45.2 littermate chimera group (Supplemental Fig. 3). In contrast, we observed almost similar levels of CD4SP and CD8SP cells in the recipient thymus derived from either WT (60%) or Raptor KO origins (40%; Fig. 8B), confirming that Raptor deficiency does not affect hematopoietic stem cell engraftment or common lymphoid progenitor commitment. Consequently, these data also excluded the possibility that the defect in Raptor KO mice was due to impaired endogenous glycolipid presentation on CD1d, as WT DP thymocytes in the BM chimeric mice did not rescue iNKT cell maturation. Altogether, our observations suggest that mTORC1 selectively controls iNKT cell development in a cell-intrinsic manner.

Discussion

iNKT cells play crucial roles in bridging innate and adaptive immunity. However, the signaling pathway that regulates iNKT cell development remains poorly understood. Using the T cell–specific Raptor KO mice, we have demonstrated that mTORC1 is selectively required for iNKT but not for conventional T cell development, as also shown in a very recent parallel study (33).
Unlike conventional T lymphocytes, iNKT cells exhibit high proliferative capacity during early stage development in thymus. In this context, mTORC1 and c-Myc represent two core regulators of T cell proliferation and metabolism, and, interestingly, the block in iNKT cell development in mTORC1-deficient mice is reminiscent of similar observations in c-Myc KO mice (8, 11). The wave of c-Myc–mediated intrathymic proliferation occurring immediately after agonist selection was shown to be necessary for mature iNKT cell generation. Similarly, our results demonstrate that, in mTORC1-deficient mice, the developmental blockade of iNKT cells is also associated with decreased proliferation. Ongoing experiments are dedicated to understand the connection between mTORC1 and c-Myc in controlling iNKT cell development.

In the absence of mTORC1, iNKT cells are blocked between stages 1 and 2 of thymic development. This developmental blockade is independent of the key transcription factor PLZF that controls iNKT cell lineage differentiation, as its expression is similar in Raptor KO and WT mice. However, it was recently
Interestingly, mTORC1 seems to influence the Vβ repertoire used by iNKT cells. In Raptor KO mice, we indeed found that the few residual thymic iNKT cells showed remarked decreased Vβ7 and Vβ8.2 usage compared with WT littermates. Thus, it will be worthwhile to test whether mTORC1 deficiency modifies the positive selection of iNKT cells with a bias for Vα14.1a18 invariant chain pairing with additional Vβs.

Importantly, mTORC1-deficient stage 2 iNKT cells could not further differentiate into NK1.1-expressing stage 3 cells that are characterized by their rapid effector function, such as IFN-γ production. T-bet and IL-15 were shown to be critical for iNKT cell terminal differentiation (27), and our data show that the severe loss of stage 3 iNKT cells in Raptor KO mice was associated with decreased expression of T-bet and of its target CD122/IL-15R. However, it is unlikely that the decreased levels of T-bet and CD122 are explaining the early developmental blockade in Raptor KO mice, because, in these studies, iNKT cells are blocked in stage 3 and not in stages 1–2. Moreover, CD4 and CD8 T cell activation are also dependent on mTORC1-mediated T-bet activity (34, 35), which excludes this signaling pathway to be responsible for the iNKT-restricted defect in Raptor KO mice.

One of the unique features of iNKT cells is to rapidly produce effector cytokines upon α-galactosylceramide stimulation. In the current study, we found that the residual iNKT cells in Raptor KO mice failed to upregulate CD69 and produce either IFN-γ or TNF-α, suggesting that iNKT cells could not be properly activated without mTORC1. Similarly, we have observed that mTORC1 is required for priming and cytokine production by Ag-specific CD8+ T cells in viral or bacterial infection settings (unpublished observations), suggesting that T lymphocytes in general, including iNKT cells, may rely on mTORC1 for activation and cytokine production (19, 20). Recently, one elegant study showed that aerobic glycolysis is specifically required for effector cytokine production but not for fueling proliferation or T cell survival (36). Further experiments are needed to explore whether mTORC1 is required for iNKT cell cytokine production by engaging glycolysis.

mTORC1 signaling is required for distinct CD4 effector lineage specification, whereas T regulatory cell differentiation depends on decreased mTORC1 activity (22, 23). It has also been suggested that higher mTORC1 activity during CD8 T cell priming promotes terminal effector differentiation. In contrast, lower mTORC1 activation drives CD8 T cell differentiation into long-lived memory lineage (37). Apparently, mTORC1 signaling needs to be finely tuned to fulfill proper iNKT cell development and maturation. Indeed, it has been shown that hyperactivation of mTORC1 in T cell–specific TSC1 KO mice also results in defective iNKT development and survival, which could be rescued by Bcl-2 overexpression (20, 38).

According to the recent definition of iNKT cell subsets based on the expression of the transcription factors T-bet, PLZF, and RORγt (28, 39, 40), the drastic loss of T-bet+ thymic iNKT cells in Raptor KO mice may suggest that the iNKT1 subset did not develop, whereas iNKT2 characterized by PLZF expression seemed less affected. In view of their natural scarcity, iNKT17 cells were hardly identified in Raptor KO mice. However, additional functional studies should be performed to confirm the differential development of iNKT sublineages in Raptor KO mice.

Intriguingly, one recent study has shown that T cell–specific deletion of TSC1 induces a developmental block of iNKT cells at stage 2, resulting from an increased mTORC1 activity, as shown by a partially rescued phenotype upon rapamycin treatment (41). Taken together, all these observations suggest that iNKT cell development and acquisition of their effector functions are under the

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**FIGURE 7.** Expression of CD1d, Slamf1, and Slamf6 on DP thymocytes of WT and Raptor KO mice. (A) Histogram to show the expression of CD1d on DP thymocytes of WT or Raptor KO mice. (B) Quantification analysis of CD1d mean fluorescence intensity on DP thymocytes. (C) Histogram to show the expression of Slamf1 and Slamf6 on DP thymocytes of WT or Raptor KO mice. (D) Mean fluorescence intensity of Slamf1 and Slamf6 on DP thymocytes of WT or Raptor KO mice was summarized. One representative of two independent experiments with identical results is illustrated. **p < 0.001.

**FIGURE 8.** T cell–autonomous defect of iNKT differentiation in mTORC1-deficient mice. (A) Schematic representation of the BM chimera generation and analysis. (B) Representative FACS plots showing CD45.1 and CD45.2 expression by gating on thymic CD4SP, CD8SP, and iNKT cells of BM chimeric mice. (C) Frequency of iNKT cells in thymus, spleen, and liver of recipients reconstituted with 1:1 mixture of WT and Raptor KO BM cells. Data are shown as mean ± SD (three to four mice each group). One representative of two independent experiments with identical results is illustrated. **p < 0.001.
control of a complex regulation of mTORC1 signaling. Last, but not least, there is a considerable interaction between mTORC1 and mTORC2 signaling, and much less is known about the role of mTORC2 in T cell biology to date. Is there any role of mTORC2 in regulating iNKT cell development and function? All of these questions are currently under investigation.

Together with one recent study (33), we have provided convincing genetic evidence that mTORC1 is essential for iNKT cell development and maturation.

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Disclosures

The authors have no financial conflicts of interest.

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12. T-cell exit from quiescence and metabolic reprogramming.
Unspecific staining pattern by unloaded CD1d-Tetramer. Representative FACS plots to show the staining for CD4, CD8 or CD44, NK1.1 by gating on unloaded/CD1d-Tetramer positive thymocytes in WT (upper panel) and Raptor KO mice (bottom panel).
Impaired iNKT development in Raptor KO mice was due to decreased proliferation but independent of PLZF expression. (A) Thymic iNKT cells were stained with Ki67 and percentage of Ki67 positive cells (stage 1-2 iNKT cells) was summarized. (B) Representative FACS plot analysis showing PLZF expression in thymic iNKT cells. MFI of PLZF in WT and Raptor KO iNKT cells was summarized. (C) Representative overlay histograms showing the expression of T-bet and CD122 in thymic iNKT cells of WT (shaded grey) and Raptor KO mice (solid black line).
T cell autonomous defect of iNKT differentiation in Raptor KO mice. Representative FACS plots showing CD45.1 and CD45.2 expression by gating on live iNKT cells in liver of BM chimeric mice. Both WT CD45.1+ WT LC CD45.2 group and WT CD45.1+ Raptor KO CD45.2 chimera group were shown. Data are shown as Mean±SD (3-4 mice each group). One representative of two independent experiments with identical results is illustrated.