Cutting Edge: Discrete Functions of mTOR Signaling in Invariant NKT Cell Development and NKT17 Fate Decision

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Invariant NKT (iNKT) cells recently were classified into NKT1, NKT2, and NKT17 lineages with distinct transcription factor and cytokine profiles, but the mechanisms underlying such fate decisions remain elusive. In this article, we report crucial roles for mechanistic target of rapamycin (mTOR) signaling, especially mTORC2, in iNKT cell development and fate determination of NKT17 cells. Loss of Rictor, an obligatory component of mTORC2, decreased thymic and peripheral iNKT cells, which was associated with defective survival. Strikingly, Rictor deficiency selectively abolished the NKT17 lineage, as indicated by a marked reduction in RORγt and IL-17 expression. Moreover, deletion of phosphatase and tensin homolog (Pten) upregulated mTORC2 activity and enhanced NKT17 generation, but concomitant loss of Rictor reversed the NKT17 dysregulation. In contrast, mTORC1 regulators Raptor and Rheb are dispensable for NKT17 differentiation, despite their importance in iNKT cell thymic development. Our findings establish pivotal and unique roles for mTORC2 signaling, which is reciprocally regulated by Rictor and Pten, in NKT17 lineage determination. The Journal of Immunology, 2014, 193: 4297–4301.

Invariant NKT (iNKT) cells are a unique group of αβ T cells characterized by their expression of a semi-invariant TCRαβ (Vα14-Jα18) and a TCRβ-chain of limited repertoire (1, 2). iNKT cells play an important role in bridging innate and adaptive immunity. In response to self- and bacteria-derived lipid Ags presented by CD1d molecules, iNKT cells rapidly produce a broad range of cytokines, including IFN-γ, TNF-α, IL-4, and IL-17, and mediate key immune functions (1, 2). Traditionally, the development of iNKT cells has been divided into four distinct stages based on unique surface molecules CD24, CD44, and NK1.1: immature stage 0 (CD24+CD44+ NK1.1+), transitional stage 1 (CD24+CD44+CD122+ NK1.1+) and stage 2 (CD24+CD44+CD122+ NK1.1+), and mature stage 3 (CD24−CD44+ NK1.1+) cells (3). A number of transcription factors have been implicated in iNKT cell development, with the most notable example being promyelocytic leukemia zinc finger (PLZF), a lineage-specific factor crucial for iNKT cell early development and functional differentiation (4, 5). iNKT cells recently were classified into three effector lineages—NKT1, NKT2, and NKT17—based on their expression of the transcriptional factors T-bet, GATA3, and RORγt, respectively (6, 7). Despite the characterization of multiple regulators of iNKT cell development, the mechanisms underlying the diversity of iNKT effector lineages remain elusive. Moreover, little is understood about the relationship between developmental maturation and lineage diversification.

The mechanistic target of rapamycin (mTOR) signaling integrates immune signals and metabolic cues in orchestrating T cell responses (8, 9). mTOR signaling consists of two complexes, mTORC1 and mTORC2, which are defined by the respective signature components Raptor and Rictor. mTORC1 and mTORC2 exhibit distinct functions in peripheral T cell responses, especially T cell activation and differentiation (10–14), but their developmental roles are considerably less well understood (15). Of note, NKT thymocytes, unlike conventional T cells, undergo blasting and cell division during thymic development (1, 2). Consistent with this notion, loss of mTORC1 activity by deletion of Raptor was shown in very recent studies to block development of iNKT cells at early stages and impair their functionality (16, 17), while permitting the normal development of conventional T cells. However, the role of mTOR in lineage diversification of iNKT cells is less clear (18). In particular, there has been no evidence linking mTORC2 to iNKT cell development or effector differentiation.

In this study, we genetically defined the functions of various mTOR components in iNKT cell development and established a critical role for mTORC2 signaling in NKT17 lineage differentiation. Loss of Rictor decreases iNKT cells in the thymus and periphery, and this is associated with defective NKT cell survival but largely normal maturation. Strikingly, a deficiency in Rictor, but not Raptor or Rheb [a crucial upstream regulator of mTORC1 (8, 9)], abrogates the NKT17 lineage in the thymus and periphery. Moreover, NKT17 de-
development is markedly enhanced by the deletion of the PI3K inhibitory molecule phosphatase and tensin homolog (Pten), whereas concomitant loss of Rictor rescues this defect. Therefore, our studies identified crucial and unique roles for mTORC2 signaling, which is reciprocally regulated by Rictor and Pten, in establishing the NKT17 lineage.

Materials and Methods
Mice
C57BL/6J and Ptenfl mice were purchased from The Jackson Laboratory. Rptoifl, Rictorfl, Rhefl, and CD4-Cre mice were described previously (13, 14). All mice were backcrossed onto the C57BL/6J background and kept in specific pathogen−free conditions in the Animal Resource Center at St. Jude Children’s Research Hospital. Animal protocols were approved by the Institutional Animal Care and Use Committee of St. Jude Children’s Research Hospital.

Cellular assays
Cell isolation and flow cytometry of iNKT cells were as described (18, 19). For ex vivo stimulation, iNKT cells were enriched from total thymocytes by depleting CD8+ cells (20) and stimulated with PMA and ionomycin in the presence of monensin for 5 h. For in vivo −GalCer treatment, mice were injected i.p. with 200 μg brefeldin A and i.v. with 2 μg α-GalCer 30 min later; splenic iNKT cells were analyzed for cytokine production at 3 h after α-GalCer treatment. For α-GalCer stimulation in vitro, enriched thymocytes were stimulated with 125 ng/ml α-GalCer for 72 h.

Statistical analysis
One-way ANOVA with the Tukey test was performed to analyze statistical significance using GraphPad Prism 5.01.

Results and Discussion
Loss of Rictor impairs the development of iNKT cells
To investigate the roles of Rictor in iNKT cell development, we crossed mice with loxP-flanked Rictor with CD4-Cre mice to delete the Rictor conditional alleles specifically in T cells (Rictor−/− mice). Rictor−/− mice had significantly decreased frequencies and numbers of iNKT cells (CD1d-PBS57+TCRβ+) in the thymus, spleen, and liver, which were approximately one third of wild-type (WT) levels (Fig. 1A−C). To evaluate the role of Rictor in the developmental maturation of iNKT cells, we examined the expression of CD24, CD44, and NK1.1, which can be used to distinguish different stages of iNKT cells (3). Rictor−/− iNKT cells showed a modestly reduced frequency of stage 2 (CD24−CD44−NK1.1−) cells but a normal percentage of stage 3 (CD24−CD44+NK1.1+) cells (Fig. 1D, 1E). Therefore, Rictor contributes to normal development of iNKT cells but is largely dispensable for their terminal maturation.

To explore the underlying basis for the reduced cellularity of iNKT cells in Rictor−/− mice, we first examined the proliferation of thymic iNKT cells by measuring BrdU incorporation and Ki67 expression. WT and Rictor-deficient iNKT cells had comparable BrdU incorporation and Ki67 expression (Supplemental Fig. 1A, 1B), indicating a largely dispensable role for Rictor in the proliferation of iNKT cells. However, Rictor−/− iNKT cells exhibited elevated caspase activity, which is indicative of a survival defect (Supplemental Fig. 1C). Moreover, when stimulated with α-GalCer in vitro, Rictor−/− iNKT cells experienced more profound cell death compared with WT cells, as indicated by increased 7-aminoactinomycin D staining and caspase activity (Supplemental Fig. 1D, 1E). Collectively, these data indicate that Rictor deficiency impairs the survival of iNKT cells, which likely contributes to the defective development.

Selective roles of mTOR signaling components in iNKT cell development
We next compared the effects of Rictor deficiency with the disruption of mTORC1 signaling on iNKT cell development. To this end, we used the CD4-Cre system to delete Raptor and Rheb (Rptoifl and Rhefl), key molecules associated with mTORC1 activity (8, 9). In contrast to the modest reduction in iNKT cells in Rictor−/− mice, Raptor deficiency caused a more profound impairment of iNKT cellularity in the thymus, spleen, and liver (Fig. 1A−C). These defects were largely recapitulated in mice lacking both Rictor and Raptor in T cells (Rictorfl/Riptofl/CD4-Cre; Rictor−/−/Riptofl−/−) (Fig. 1A−1C). Additionally, thymic development of Riptofl−/− iNKT cells was blocked at an early stage, as indicated by the accumulation of stage 0 and stage 1 cells and a marked loss of stage 3 cells (Fig. 1D, 1E). Severe defects also were observed in Rictor−/−/Riptofl−/− iNKT cells (Fig. 1D, 1E). These results indicate an essential role for Raptor in iNKT cell development.
In contrast, a deficiency in Rheb resulted in a small reduction in iNKT cells in the thymus (Supplemental Fig. 2A–C), with marginal effects on their distribution at different developmental stages (Supplemental Fig. 2D). These results indicate that Rheb-independent mTORC1 signaling plays a dominant role in promoting terminal maturation of iNKT cells and highlight the discrete requirement of mTOR signaling in iNKT cell development.

Rictor, but not Raptor or Rheb, promotes NKT17 lineage differentiation

iNKT cells recently were classified into three functional lineages according to the expression of the signature transcription factors (6). Therefore, we used intracellular staining of PLZF, RORγt, T-bet, and GATA3 in iNKT cells from the thymus. NKT1, NKT2, and NKT17 cells were labeled as described (6). NKT1: WT versus Rictor−/−, p < 0.05; WT versus Raptor−/−, p < 0.001. NKT2: WT versus Rictor−/−, p > 0.05; WT versus Raptor−/−, p < 0.001. NKT17: WT versus Rictor−/−, p < 0.001; WT versus Raptor−/−, p > 0.05 (n = 4–7 mice/group). (B) Intracellular staining of PLZF and RORγt in iNKT cells from the spleen (Spl), peripheral lymph nodes (PLN), and liver. Spl, p < 0.001; PLN, p < 0.001; liver, p < 0.001 (n = 4 mice/group). Cytokine production by iNKT cells (gated on CD1d−PBS57+TCRγ−) cells from the thymus (C) and spleen, peripheral lymph nodes, and liver (D) after stimulation with PMA and ionomycin in the presence of monensin for 5 h (n = 4–6 mice/group). (E) Cytokine production of splenic iNKT cells in response to in vivo α-GalCer challenge (n = 3–5 mice/group). (F) IL-17 production by thymic iNKT cells stimulated with α-GalCer in vitro. Thymocytes were cultured with 125 ng/ml α-GalCer for 72 h and treated with PMA, ionomycin, and monensin for the last 5 h (n = 4–5 mice/group). Results are representative of two to four independent experiments. Data shown are mean ± SEM.

Consistent with the reduction in NKT17 cells in the thymus, Rictor−/− mice also exhibited diminished NKT17 cells in peripheral lymphoid organs, including spleen, peripheral lymph nodes, and liver (Fig. 2B). NKT1, NKT2 and NKT17 cells are enriched at different developmental stages: NKT1 cells in stage 3, NKT2 cells in stages 1 and 2, and NKT17 cells in stage 2 (6). Rictor−/− iNKT cells showed a marked reduction in NKT17 cells at all stages examined (data not shown), indicating an intrinsic effect of mTORC2 on the promotion of NKT17 differentiation. We next examined cytokine production of NKT cells in vitro and in vivo. Consistent with the transcription factor analysis, thymic Rictor−/− iNKT cells had substantially reduced IL-17 production but normal IFN-γ and TNF-α production (Fig. 2C). Reduced IL-17 production also was manifested in Rictor−/− iNKT cells in spleen, peripheral lymph nodes, and liver (Fig. 2D). Moreover, in response to in vivo challenge or in vitro stimulation with α-GalCer, Rictor−/− iNKT cells exhibited a substantial decrease in IL-17 production (Fig. 2E, 2F). Collectively, these results indicate that Rictor is selectively required for RORγt expression, IL-17 production, and NKT17 cell generation.

Pten deficiency potentiates NKT17 differentiation in a Rictor-dependent manner

To determine whether NKT17 development is controlled by regulators of mTOR activity, we analyzed mice deficient in Pten, a negative regulator of mTOR signaling (8), using the CD4-Cre system (Ptenfl/flCD4-Cre; Pten−/−). As expected,
Pten−/− mice had increased AKT S473 phosphorylation, a signature of mTORC2 activity in mature thymocytes (Fig. 3A). iNKT cells were increased in the thymus of Pten−/− mice, but this was largely rescued after the concomitant loss of Rictor in Pten−/−Rictor−/−CD4-Cre mice (Pten−/− Rictor−/−) (Fig. 3B). The development of Pten−/− iNKT cells was blocked at the transition from stage 2 to stage 3, as shown by the marked accumulation of stage 2 cells and the loss of stage 3 cells (Fig. 3C), similar to the phenotype observed following Lck-Cre–mediated deletion of Pten (21). Notably, this phenotype persisted in Pten−/− Rictor−/− mice (Fig. 3C), indicating the involvement of an mTORC2-independent activity. Strikingly, Pten−/− iNKT cells contained markedly increased RORγt+ NKT17 cells and a corresponding reduction in NKT1 cells, and these defects were completely reversed upon the additional loss of Rictor (Fig. 3D). Consistent with these observations, IL-17 production from iNKT cells was greatly upregulated in Pten−/− mice, but not in Pten−/− Rictor−/− mice, whereas IFN-γ production from iNKT cells was reduced in Pten−/− mice but not in Pten−/− Rictor−/− mice (Fig. 3E). Therefore, Rictor-mediated signaling drives the expansion of NKT17 cells in Pten−/− mice but does not contribute to the defective terminal maturation in these mice.

Much progress has been made in identifying the mechanisms of peripheral CD4 T cell differentiation, as well as the control of thymic iNKT cell development. Emerging evidence also suggests that iNKT cells diverge into distinct effector lineages, similar to Th cell differentiation (6, 7), but how iNKT lineage determination is orchestrated remains obscure. We compared and contrasted the roles of mTORC1 and mTORC2 signaling in the coordination of iNKT cell development and lineage determination. Raptor-mediated mTORC1 signaling plays a dominant role in iNKT cell development, terminal maturation, and NKT1 differentiation, and these effects are largely independent of Rheb functions. Rictor-mediated mTORC2 signaling also contributes to the development and peripheral maintenance of iNKT cells, at least in part, by facilitating their survival. Moreover, mTORC2 is selectively required for NKT17 lineage determination, because loss of Rictor and Pten reciprocally affects NKT17 cell generation, RORγt expression, and IL-17 production. Interestingly, although deletion of Rictor reversed NKT17 dysregulation in Pten-deficient cells, it did not affect the blocked maturation of stage 2 iNKT cells to stage 3 iNKT cells. These results provide crucial genetic evidence that developmental maturation and lineage differentiation of iNKT cells can be uncoupled, lending additional support for the recently proposed model of their lineage diversification (6, 7). Furthermore, despite the analogy and shared transcription factors between effector differentiation of iNKT cells in the thymus and Ag-stimulated CD4 Th cells in the periphery (6, 7), these processes use distinct mTOR signaling. For instance, although Raptor and Rheb, but not Rictor, have been implicated in Th17 cell generation (10–12), we show that Rictor, but not Raptor or Rheb, is crucial for NKT17 cell differentiation. Altogether, the new insights into mTOR-dependent programming of iNKT cell differentiation contribute to our understanding of fundamental mechanisms of lineage commitment and fate determination in adaptive immunity.

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**Disclosures**

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