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A Critical Role for Rictor in T Lymphopoiesis

Fei Tang,* Qi Wu,* Tsuneo Ikenoue,†,‡ Kun-Liang Guan,†,§ Yang Liu,*‡,‖ and Pan Zheng*§‖

Apart from a critical role for Notch and pre-TCR, the signaling pathway required for T lymphopoiesis is largely unknown. Given the potential link between Notch and mammalian target of rapamycin (mTOR) signaling in cancer cells, we used mice with conditional deletion of either Raptor or Rictor genes to determine potential contribution of the mTOR complex I and II in T lymphopoiesis. Our data demonstrated that targeted mutation of Rictor in the thymocytes drastically reduced the thymic cellularity, primarily by reducing proliferation of the immature thymocytes. Rictor deficiency caused a partial block of thymocyte development at the double-negative 3 stage. The effect of Rictor deficiency is selective for the T cell lineage, as the development of B cells, erythrocytes, and myeloid cells is largely unaffected. Analysis of bone marrow chimera generated from a mixture of wild-type and Rictor-deficient hematopoietic stem cells demonstrated that the function of Rictor is cell intrinsic. These data revealed a critical function of mTOR complex 2 in T lymphopoiesis.


With ~100 million thymocytes produced per day (1), the thymus is among the most generative organs in young adult mice. The mass production of new thymocytes provides ample choices for positive and negative selection to yield mature thymocytes with proper repertoire for adaptive immunity. Based on kinetic analysis of BrdU incorporation, it is now generally accepted that T lymphopoiesis is primarily fueled by massive proliferation of CD4+CD8+ (double-negative [DN]) thymocytes (1, 2), although more recent studies suggest that medullar thymocyte may further expand prior to emigration (3). Apart from pre-TCR complex and Notch activation (4), the molecular program for T lymphopoiesis is largely unknown.

Mammalian target of rapamycin (mTOR) has emerged as a central regulator for cellular proliferation and maturation (5). Recent studies from several groups, including us, have revealed a critical role for the two complexes in T cell differentiation and function 

Materials and Methods

Mice

Mice harboring conditional alleles of the Rictor gene (Rictor+/F) with floxed exon 11 were backcrossed for >10 generations onto the C57BL/6 background in the animal facility at the University of Michigan. Transgenic mice expressing Cre recombinase under the control of Mx1 gene (Mx1-Cre) were purchased from The Jackson Laboratory. The transgene was crossed into Rictor+/F mice to generate control (Ctrl) (Rictor+/F, Mx1-Cre or Rictor+/F; Mx1-Cre- or Rictor+/F, Mx1-Cre+ and conditional knockout (cKO) (Rictor+/F, Mx1-Cre-) mice. All the mice were kept in the Unit of Laboratory Animal Medicine at University of Michigan. All procedures involving experimental animals were approved by the University Committee on the Use and Care of Animals at the University of Michigan.

Induction of gene deletion by polyinosinic-polycytidylic acid

Polyinosinic-polycytidylic acid (polyP; Sigma-Aldrich) was dissolved in PBS at 2 mg/ml. Mice received 400 μg polyP once every day for seven times by i.p. injection, as described (6, 7). The deletion of Rictor exon 11 was assessed by the 7500 Real-Time PCR system (Applied Biosystems). The primers were as follows: Exon11-F, 5'-ATGAGGATCTTTGCA-TACC-3'; Exon11-R, 5'-TGTAGATGCCTAAGAGGACG-3'; Exon12-F, 5'-TCTGCTCTGAAGTGCTCTATG-3'; Exon12-R, 5'-TAGAACTGTTTGTCACAG-3'; GAPDH-F, 5'-GCCCACTTTAGGGCTGACG-3'; and GAPDH-R, 5'-CATGACGCCCCATCAATG-3'. Fold changes were calculated according to the △△CT method (22). Deletion frequency for DN subsets of thymocytes was determined by amplification of genomic DNA, followed by electrophoresis on a 2% agarose gel. PCR primers were as follows: F1, 5'-CAACATCATCGCCTCTC-3'; R1, 5'-TC-CCAGAATTTCAGGCT-3'; Del, 5'-CACACCTAACATGGCTTAG-3'. Primer combination F1/R1 gave PCR fragments of 389 bp (wild-type [WT] allele) and 520 bp (floxed allele), whereas F1/Del gave PCR fragments of ~1200 bp (undeleted floxed allele), 1030 bp (WT allele), and 700 bp (deleted floxed allele).

Abs

The following Abs were purchased from eBioscience: FITC-conjugated anti-CD45.1 (A20), anti-CD3 (145-2C11), anti-CD4 (GK1.5), anti-CD8a (53-6.7), and anti-CD45.2 (104); PE-conjugated anti-CD3e (M1/69) and anti-CD4 (IM7); PE-Cy7–conjugated anti-CD4 (RM4-5); and allophycocyanin (al)fluor780–conjugated anti-CD4 (RM4-5); allophycocyanin-conjugated anti-CD45.1 (A20), anti-CD8a (53-6.7), and anti-CD25 (PC61.5). The following Abs were purchased from BD Biosciences: PerCP-Cy5.5–con-
jugated anti-CD25 (PC61.5); PE-Cy7–conjugated anti-CD45.2. The annexin V staining kit (BD Biosciences) was used to measure apoptosis in conjunction with 7-aminactinomycin D. Lineage markers included B220, CD3, Gr-1, Mac-1, and TER119. Anti-CD150 Ab was purchased from BioLegend, and all other Abs were obtained from eBioscience.

**Flow cytometry**

For surface staining, cells were stained with the indicated Abs in staining buffer (HBSS with 2% FBS) for 20 min at 4°C. For intracellular staining, cells were first stained with the indicated surface markers and then fixed with Fix buffer (BD Biosciences) for 2 h at 4°C, followed by incubation with BD cytoperm plus buffer for 10 min at room temperature and remixing for 10 min. Alexa Fluor 488–conjugated anti-p-AKT and isotype control Abs (Cell Signaling Technology) were diluted at 1:20 and incubated overnight at 4°C. All FACS analyses were performed on a LSR II flow cytometer (BD Biosciences), and data were analyzed with FlowJo software (Tree Star). BrdU (Sigma-Aldrich) was dissolved in PBS at a concentration of 10 mg/ml, and was injected i.p. into adult mice (100 mg/kg). The mice were sacrificed at 4 h after BrdU injection. BrdU incorporation was detected by flow cytometry with a FITC BrdU flow kit (BD Biosciences), according to the manufacturer’s instructions.

**Bone marrow transplantation**

Ctrl and cKO mice used in each experiment were sex-matched littermates. Recipient mice in bone marrow transplantation (BMT) were 6- to 8-wk-old C57BL/6J Ly5.2 (CD45.1) mice. The recipient mice were lethally irradiated with a 137Cs x-ray source delivering 170 rad per min for a total of 1100 rads. Ctrl and cKO mice were sacrificed 20 d after the final injection of plpC. Given numbers of bone marrow (BM) cells from Ctrl or cKO donor mice (CD45.2) were either injected alone or mixed with equal number of recipient-type (CD45.1) competitive BM cells. The BM cells were injected into recipients through tail vein within 24 h after irradiation. Reconstitution in the recipients’ thymus and peripheral blood were measured by flow cytometry.

**Statistics**

All the data are presented as mean ± SD. The two-tailed Student’s t tests were used for comparison between two experimental groups. Statistical significance was determined as *p < 0.05 (**p < 0.01, ***p < 0.001).

**Results**

**Targeted mutation of Rictor markedly reduced thymic cellularity**

Because the existing T lineage-specific Cre genes are expressed after the first proliferative phase of T lymphopoiesis (DN3) (23, 24), we crossed mice with floxed *Rictor* to the *Mx1-Cre* transgene (25). Gene deletion was induced by treatment with plpC. Efficient deletion was achieved in both BM and thymus, as revealed by quantitative PCR analysis of Rictor (Fig. 1A). This deletion causes a frameshift and thus creates a null allele. Among the thymic progenitor cells, efficient deletion was achieved at all stages (DN1–DN4), although a trace amount of undeleted band remained detectable in DN1 subset (Fig. 1B). Consistent with functional inactivation of TORC2 (20, 21), the intracellular level of pAKT473 (Akt phosphorylation at serine 473 by TORC2) was reduced in all subsets of DN thymocytes analyzed (Fig. 1C, 1D).

Remarkably, *Rictor* deletion markedly reduced thymic weight and cellularity (Fig. 2A). The reduction is more marked in thymocyte cellularity. Despite the major reduction of thymic cellularity, most of the thymocyte subsets are present in Rictor-deficient thymus (Fig. 2B). A subtle but statistically significant increase in DN percentage and decrease in double positive (DP) percentage were observed after *Rictor* deletion (Fig. 2C). The lack of gross defects in the frequency of mature T cells suggests that *Rictor* is not essential for thymocyte differentiation. Nevertheless, the number of mature T cells produced in the thymus is massively reduced (Fig. 2C). Subdivision of DN into DN1–DN4 revealed that, after *Rictor* deletion, percentage of DN3 was significantly increased, whereas percentages of DN1 and DN4 were significantly reduced (Fig. 2B, 2D). Due to the overall reduction of thymic cellularity, the cellularity in all DN subsets was reduced (Fig. 2D).

**Defective proliferation rather than increased apoptosis is responsible for reduced T lymphopoiesis**

Previous studies have established that thymic cellularity is increased through proliferation of DN thymocytes (1, 2). To understand the cellular basis for reduced thymic cellularity, we pulsed the thymocytes with BrdU and measured DN proliferation. As shown in Fig. 3A and Fig. 3B, *Rictor*-deficient DN showed much reduced proliferation. In contrast, staining with 7-aminactinomycin D and annexin V indicated that comparable rate of cell death was observed in both Rictor-sufficient and *Rictor*-deficient DN thymocytes (Fig. 3C, 3D). Therefore, reduced thymic cellularity is attributable to defective proliferation rather than increased apoptosis.

Consistent with defective DN proliferation, the effect of *Rictor* deletion on cell size was most profound on DN, although the size of DP was also reduced by the deletion (Fig. 4A, 4B). Among DN, significant reduction of cell size was observed in DN2, DN3, and DN4 (Fig. 4C, 4D). Therefore, Rictor regulates cellular metabolism during T lymphopoiesis in the thymus.

**Cell-intrinsic Rictor deficiency in hematopoietic cells is responsible for defective T lymphopoiesis**

T lymphopoiesis depends on interaction between thymocytes and stromal cells (26). To determine whether Rictor deficiency in BM-derived cells is sufficient to cause defective T lymphopoiesis, we generated BM chimera using donor-type (CD45.2) BM to reconstitute lethally irradiated recipient mice. Once the reconstitution was stabilized, the recipient mice were treated with plpC to delete *Rictor* in the *Rictor<sup>F/F</sup>;Mx1-Cre<sup>+</sup>* cells. Three weeks after completion of plpC treatment, the mice were sacrificed to analyze T lymphopoiesis (Fig. 5A). As shown in Fig. 5B, deletion of *Rictor* in BM-derived cells was sufficient to cause reduction in thymus weight and thymocyte cellularity. Moreover, the distribution of thymocyte subsets was also affected by selective *Rictor* deficiency in the BM cells (Fig. 5C–E). The phenotypes caused by BM cell-selective deletion and Rictor deletion that covers both hematopoietic and nonhematopoietic cells (Figs. 2, 5) are quite similar, suggesting that the function of *Rictor* in T lymphopoiesis is hematopoietic cell intrinsic.

To more vigorously demonstrate the cell-intrinsic function of *Rictor*, we carried out a competitive BMT using a 1:1 mixture of either cKO plus recipient type or Ctrl plus recipient type BM. The contribution of donor-type cells to thymocytes was analyzed by their CD45 markers at 12 wk after BMT (Fig. 6A). As shown in Fig. 6B, the cKO BM-derived thymocytes accounted for only ~10% of the thymocytes, whereas the Ctrl BM-derived thymocytes constituted ~50% of the total thymocytes. Again, Rictor deficiency did not prevent differentiation of thymocytes, as all major thymocyte subsets were produced among CD45.2<sup>+</sup> cKO progenitors (Fig. 6C). However, a significant increase of DN percentages and an appreciable decrease of DP percentages were observed in the cKO populations (Fig. 6D). Among the DN subsets, an increase of DN3 percentages, and reductions in DN1 and DN4 percentages were observed among the CD45.2<sup>+</sup> cKO thymocytes (Fig. 7A, 7B). However, due to reduced cKO thymocyte numbers, no increase was found in the numbers of any DN subsets (Fig. 7C). Interestingly, the selective reduction of proliferation in DN3 was also recapitulated in the chimera mice (Fig. 7D). Since the donor- and recipient-type cells were present in the same hosts, these data demonstrated that the requirement for *Rictor* is cell intrinsic.
Rictor is not essential for hematopoiesis

The Mx1-Cre transgene is known to cause efficient deletion of Rictor in BM (23) and the hematopoietic stem cells (HSC) (6–8, 21). As shown in Fig. 1A, the efficacy of gene deletion is similar in BM cells and in thymocytes. In contrast to thymic cellularity, we observed no effect of Rictor deletion on BM cellularity (Fig. 8A). We further evaluated whether Rictor is required for hematopoiesis based on the frequencies of HSC, progenitors as B cell

**FIGURE 1.** Efficient deletion of Rictor in thymocytes. (A) Relative copy number of the floxed Rictor exon 11. BM cells and thymocytes were prepared from Ctrl mice and cKO mice on day 20 after the final injection of pIpC. The deletion of exon 11 in genomic DNA was assayed by real-time PCR, and the nondeleted exon 12 served as an internal control. n = 5 for Ctrl mice; n = 5 for cKO mice. (B) Deletion of Rictor exon 11 in subsets of DN thymocytes. Genomic DNA isolated from sorted DN subsets was amplified by PCR. (C) Representative FACS profile for intracellular staining of pAkt 473 in CD3-DN thymocytes. (D) pAkt levels in DN thymocyte subsets as measured by phosphor-flow. Data shown are the relative mean fluorescence intensity (MFI) values from cKO mice compared with that from Ctrl mice. n = 5 for Ctrl mice; n = 3 for cKO mice. Similar data were obtained from another independent experiment. **p < 0.01, ***p < 0.001.

**FIGURE 2.** Defective T lymphopoiesis induced by targeted mutation of Rictor. (A) Significant reduction of thymic cellularity upon Rictor deletion. The weight (left) and absolute numbers (right) per thymus are shown. (B) Representative FACS profiles showing thymocyte subsets based on surface markers CD4, CD8, CD3, CD25, and CD44. (C) Percentages (left) and absolute numbers (right) of indicated thymocyte subpopulations in Ctrl and cKO mice. (D) Percentages (left) and absolute numbers (right) of DN1–DN4 subsets from Ctrl mice and cKO mice. n = 8 for Ctrl mice; n = 5 for cKO mice for data in (A), (C), and (D). These data have been reproduced in three independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001.
myeloid and erythroid lineages. For flow cytometry of hematopoiesis, Lin<sup>−</sup>Sca-1<sup>−</sup>c-Kit<sup>−</sup>CD150<sup>−</sup>CD48<sup>−</sup> was used for long-term HSC; Lin<sup>−</sup>Sca-1<sup>−</sup>c-Kit<sup>−</sup>CD150<sup>−</sup>CD48<sup>−</sup> was used for short-term HSC; myeloid progenitors were defined as Lin<sup>−</sup>Sca-1<sup>−</sup>c-Kit<sup>−</sup>; LSK population was marked as Lin<sup>−</sup>Sca-1<sup>−</sup>c-Kit<sup>−</sup>; and multipotent progenitors were defined as Lin<sup>−</sup>Sca-1<sup>−</sup>c-Kit<sup>−</sup>CD150<sup>−</sup>CD48<sup>−</sup>. As shown in Fig. 8B, the frequency of long-term HSC, short-term HSC, multipotent progenitors, LSK, and myeloid

**FIGURE 3.** Rictor deletion reduced DN thymocyte proliferation. (A) Representative profiles of BrdU incorporation in DN thymocytes at 4 h after BrdU pulse. (B) Percentages of BrdU<sup>+</sup> cells among DN1–DN4 subsets. *n* = 3 for Ctrl mice; *n* = 3 for cKO mice. (C) Representative staining profiles for cell death in DN thymocytes. (D) Percentages of annexin V<sup>+</sup> DN thymocytes. *n* = 5 for Ctrl mice; *n* = 5 for cKO mice. Data shown have been reproduced in two independent experiments. *p* < 0.05, **p** < 0.01.

**FIGURE 4.** Rictor deficiency reduces cell sizes of immature thymocytes. (A) and (C) show representative histograms of forward scatters of area (FSC-A) of thymocytes. Thymocytes are divided into DN, DP, and single-positive subsets (A) or DN1–DN4 subsets (C). (B) and (D) show means and SD of relative cell sizes, involving seven control and five cKO mice. The mean FSC-A of control mice is artificially defined as 1.0. These data have been reproduced in five independent experiments. **p** < 0.01, ***p** < 0.001.
progenitors was unaffected by the Rictor deletion. Based on the distribution of B220, CD43, IgM, and IgD, Rictor deficiency caused no defects in B cell differentiation (Fig. 8C). Likewise, based on distribution of CD71 and Ter119, as well as that of CD11b and Gr-1, development of erythrocytes (Fig. 8D) and myeloid cells (Fig. 8E) was largely unaffected by Rictor defi-

FIGURE 5. Abnormal T lymphopoiesis induced by Rictor deletion was BM cell intrinsic. (A) Diagram of experimental design. BM cells from either Ctrl or cKO donors were transplanted into lethally irradiated CD45.1 recipients (10^6 cells/mouse). pIpC treatment commenced at 6 wk after transplantation and lasted for 2 wk. At 3 wk after pIpC treatment, thymus was analyzed by flow cytometry. (B) Reduction of thymic cellularity in the recipients of Rictor cKO BM cells. The weight (left) and absolute cell numbers (right) per thymus are shown. (C) Representative FACS profiles and (D) summary data for T cell subsets in Ctrl and cKO recipients. (E) Percentage and absolute number of DN subsets. n = 4 for recipient mice of Ctrl donor; n = 4 for recipient mice of cKO donor. *p < 0.05, **p < 0.01, ***p < 0.001.

FIGURE 6. Cell-intrinsic requirement of Rictor for T lymphopoiesis. (A) Diagram of experimental design. BM cells were isolated from Ctrl or cKO mice at 20 d after pIpC treatment. They were mixed with equal number of recipient-type BM cells and injected into lethally irradiated host (CD45.1^+). (B) Representative profiles of donor-type (CD45.2^+) and recipient-type (CD45.1^+) cells in the thymus of recipient mice at 12 wk after BMT are shown on the left, whereas summary of percentages for donor-derived cells in recipient thymus is presented on the right. (C) Donor-derived thymocyte subpopulations based on expression of CD4 and CD8 markers. Data shown are representative profiles. (D) Percentages (left) and absolute numbers (right) of thymocyte subpopulations were shown. Data shown are representative of two independent similar BM chimera studies. *p < 0.05, **p < 0.01, ***p < 0.001.
ciency. These data further suggest that T cell development is more dependent on Rictor.

Discussion
We have presented several lines of evidence to substantiate a critical role for Rictor in T lymphopoiesis. The function of Rictor is cell intrinsic as it is recapitulated in BM chimera consisting of both WT and Rictor-deficient HSC.

The fact that the phenotype is transferrable to new host that received no poly(I:C) treatment ruled out a possibility that cytokine storm associated with this treatment plays a role in the reduction in thymocyte cellularity, as has been observed in the presence of strong Ag-induced activation of T cells in the periphery (27).

Based on normal cellularity and composition of BM from the same mice, the Rictor appears to function selectively in T lymphopoiesis. However, it must be cautioned that Rictor’s function is clearly not restricted to lymphopoiesis as the deletion of the Rictor gene is embryonic lethal (28). Although Rictor does not affect general hematopoiesis under noncompetitive setting, we have observed that in competitive transplantation setting, deletion of Rictor in donor cells makes them less competitive than WT cells (F. Tang, Y. Liu, and P. Zheng, unpublished observation). Therefore, it remains likely that Rictor may play other function in hematopoiesis.

Our data also revealed that the function of Rictor in T lymphopoiesis is at least in part through promoting proliferation of DN3. The molecular mechanism remains to be elucidated. Because TORC2 phosphorylates and activates AKT (20, 21), a key regulator for proliferation, we propose that Rictor promotes T lymphopoiesis at least in part by phosphorylating Akt. Consistent with this interpretation, targeted mutations of both Akt1 and Akt2 in the thymus recapitulate the phenotypes of Rictor deletion reported in this work, namely reduced T lymphopoiesis, defective DN proliferation, decreased metabolism, and developmental block of DN3–DN4 transition (29, 30).

While this manuscript was in revision, Lee et al. (31) reported largely similar impacts of Rictor deletion on T cell development using Cre that were activated either prior to T cell commitment (Vav-Cre) or during DN3 (Lck-Cre). Moreover, using an in vitro model, they observed a significant function of Rictor in Notch-driven thymocyte proliferation and differentiation. Because the Rictor defects were partially rescued by a combination of Foxo silencing and constitutive NF-κB activation, they suggest Foxo and NF-κB as likely downstream targets. Nevertheless, because the effects were partial and were observed also in WT cells, the effector mechanism downstream of Akt remains to be fully elucidated.

T lymphopoiesis provides large number of T cells for both positive and negative selection. Because chronic infections cause deletion of Ag-specific T cell in the periphery, continuous production of naive T cells is essential for maintaining immunity to persistent infections (32). Moreover, it has been demonstrated that thymic cellularity is dynamically regulated by inflammatory response in the periphery (33, 34). In case of HIV infection, it has been suggested that part of T cell depletion may be attributed to depletion of thymocytes (35, 36). Given the critical role for Rictor in T lymphopoiesis presented in this work, it is of great interest to determine whether defective Rictor function may limit T lymphopoiesis during infection. Likewise, because defective thymopoiesis is associated with aging (33), it is interesting to consider whether defective Rictor function may be involved in thymic involution.
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Disclosures
The authors have no financial conflicts of interest.

References

FIGURE 8. Rictor is not required for adult hematopoiesis. Targeted mutation of Rictor did not affect BM cellularity (A), stem/progenitor cells (B), and development of B cells (C), erythrocytes (D), and myeloid cells (E) in BM. The data have been reproduced in three independent experiments, n=7 for Ctrl, and n=6 for cKO. LSK, Lin"Sca-1"c-Kit+; LT-HSC, Lin"Sca-1"c-Kit+ CD150+CD48−, long-term hematopoietic stem cells; MP, Lin"Sca-1"c-Kit+, myeloid progenitor cells; MPP, Lin"Sca-1"c-Kit+ CD150−CD48+, multipotent progenitors; ST-HSC, Lin"Sca-1"c-Kit+ CD150−CD48+, short-term hematopoietic stem cells.


