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Mammalian Target of Rapamycin Complex 2 Modulates αβ TCR Processing and Surface Expression during Thymocyte Development

Po-Chien Chou,* Won Jun Oh,* Chang-Chih Wu,* Joseph Moloughney,* Markus A. Ruegg, ‡ Michael N. Hall, ‡ Estela Jacinto,* and Guy Werlen ‡

An efficient immune response relies on the presence of T cells expressing a functional TCR. Whereas the mechanisms generating TCR diversity for antigenic recognition are well defined, what controls its surface expression is less known. In this study, we found that deletion of the mammalian target of rapamycin complex (mTORC) 2 component rictor at early stages of T cell development led to aberrant maturation and increased proteasomal degradation of nascent TCRs. Although CD127 expression became elevated, the levels of TCRs as well as CD4, CD8, CD69, Notch, and CD147 were significantly attenuated on the surface of rictor-deficient thymocytes. Diminished expression of these receptors led to suboptimal signaling, partial CD4−CD8− double-negative 4 (CD25−CD44−/C211−) proliferation, and CD4−CD8− double-positive activation as well as developmental blocks at the CD4+CD8− double-negative 3 (CD25−CD44−) and CD8−immature CD8+ single-positive stages. Because CD147 glycosylation was also defective in SIN1-deficient fibroblasts, our findings suggest that mTORC2 is involved in the co/posttranslational processing of membrane receptors. Thus, mTORC2 impacts development via regulation of the quantity and quality of receptors important for cell differentiation. The Journal of Immunology, 2014, 193: 000–000.

The atypical protein kinase mammalian target of rapamycin (mTOR) associates with various proteins to form two distinct protein complexes. mTOR complex (mTORC) 1 is formed by mTOR, LST8, and raptor, whereas rictor, SIN1, LST8, and mTOR associate to form mTORC2. The mTORCs integrate environmental signals, including nutrients and growth factors, to promote cell growth and differentiation (1, 2). Whereas mTORC1 mediates growth via its function in translation initiation and metabolism, mTORC2 is also increasingly being shown to play a role in these processes, but the mechanisms remain to be elucidated (3, 4). mTORCs play a role in peripheral T cell differentiation (5). mTORC2 was also recently shown to be involved in the early stages of T cell ontogeny (6, 7), but the mechanisms as to how it controls these processes remain obscure.

Hematopoietic stem cells migrate from the bone marrow into the thymus where as immature thymocytes that do not yet express the coreceptors CD4 or CD8 (CD4−CD8− double-negative [DN]) they become committed to the T lymphocyte lineage that will express a polymorphic TCR as the hallmark (8–10) (Supplemental Fig. 1). The heterodimeric αβ TCR composed of an α- and a β-chain associates with the invariant CD3 complex for surface expression and signaling. The β-chain is rearranged first in CD25+CD44− DN3 cells and paired with an invariant pre−T α-chain to form the pre-TCR (8–10). Although ligands for the pre-TCR remain unknown, its signaling is required for thymocyte survival and maturation at the β-selection checkpoint (8, 9). Thymocytes that successfully undergo β-selection downregulate CD25 and differentiate into DN4 (CD25−CD44−) cells (8, 9). These highly proliferating cells start expressing the CD8 coreceptor and differentiate into the transitional CD8−immature CD8+ single-positive (ISP) subsets that rearrange TCR α-chains and express a functional αβ TCR (9, 11). The αβ TCR as well as the CD8 and CD4 coreceptors are concomitantly expressed on the surface of CD4+CD8+ double-positive (DP) thymocytes that differentiate from CD8-ISP cells (9, 10). Only DP thymocytes expressing TCRs with appropriate affinities for self-peptide ligands go through positive selection and differentiate into CD4+CD8+ or CD4−CD8+ SP thymocytes that egress the thymus and translocate to the peripheral lymphoid organs (9, 10). A number of studies have addressed the mechanisms generating TCR diversity for antigenic recognition as well as how downstream TCR signaling components affect thymocyte differentiation to the CD8 or CD4 SP lineage (9, 10, 12). Much less is known about the mechanisms controlling receptor expression at the surface of thymocytes or T cells.

Newly synthesized receptor polypeptides undergo posttranslational modification in the endoplasmic reticulum (ER), including...
the addition of N-linked glycans, proper folding, and, in some cases such as the TCR, assembly of protein complexes (13). The oligosaccharides are then further trimmed and modified in the Golgi to form functional receptors with complex N-linked glycan structures. Nascent membrane and secretory proteins that fail to attain their final folded structure and fully mature in the ER and/or in the Golgi are destroyed by proteasomal degradation. How signaling molecules interact with and regulate protein processing in response to cellular conditions remains poorly understood, particularly in higher eukaryotes. In the present study, by deleting rictor during early thymocyte development, we report a novel function by which mTORC2 controls the expression of receptors that are relevant for T cell ontogeny, such as the pre-TCR and TCR as well as Notch, CD127 (α-chain of the IL-7 receptor), CD147 (extracellular matrix metalloproteinase inducer), or the coreceptors CD4 and CD8.

Materials and Methods

Mice and cell lines

Homozygous C56BL/6 rictor−/− mice (14) were crossed with C56BL/6 Lck-Cre mice (Taconic Farms, Germantown, NY), which generates T cell–specific rictor knockout mice (rictor−/−) owing to the expression of Cre under the control of the proximal promoter of Lck. To specifically delete rictor in the OT-1 TCR background, we crossed C56BL/6/J OT-1 (β2m−/−/Rag-2−/−) mice (12) with homozygous rictor−/− to obtain OT-1/Rag-2−/−/ rictor−/− mice (referred to as OT-1/Trictor−/−). All mice were genotyped by PCR using the respective primers described in Supplemental Table I. Handling and experimentation protocols have been reviewed and used in accordance with Institutional Animal Care and Use Committee regulations of Rutgers University. Tissues were removed from 6-wk-old rictor−/− and rictor−/− littersmates, microsliced, and resuspended in RIPA buffer (50 mM Tris-HCl [pH 8.0], 100 mM NaCl, 5 mM EDTA, 1% Triton X-100, 0.2% SDS, and 0.5% sodium deoxycholate supplemented with protease inhibitors). Jurkat cells were electroporated and transfected with rictor short hairpin RNA (shRNA) or scrambled shRNA (Invitrogen, CA). Proteins were resolved as previously described (15).

Thymocyte proliferation and viability, immunostaining, and gene expression

Thymocytes were harvested in complete DMEM and counted by trypan blue exclusion. Cells were stained for receptor expression using Abs listed in Table I. Handling and experimentation protocols have been reviewed and used in accordance with Institutional Animal Care and Use Committee regulations of Rutgers University. Tissues were removed from 6-wk-old rictor−/− and rictor−/− littersmates, microsliced, and resuspended in RIPA buffer (50 mM Tris-HCl [pH 8.0], 100 mM NaCl, 5 mM EDTA, 1% Triton X-100, 0.2% SDS, and 0.5% sodium deoxycholate supplemented with protease inhibitors). Jurkat cells were electroporated and transfected with rictor short hairpin RNA (shRNA) or scrambled shRNA (Invitrogen, CA). Transfected cells were cultured in complete RPMI medium in the presence of 100 μM cycloheximide for the indicated times, followed by flow cytometric analysis to measure receptor turnover from the cell surface. Thymocytes were labeled with 2 μM of CFSE (Sigma-Aldrich, St. Louis, MO) prior to culturing in complete DMEM media at 37˚C for 24 h. Cells were stained and cell division was analyzed on the flow cytometer by monitoring the shift in CFSE fluorescence. To assess cell viability, ex vivo–cultured thymocytes were stained with annexin V (BD Pharmingen, San Diego, CA) and propidium iodide (PI) (Sigma-Aldrich) prior to flow cytometric analysis. RNA was extracted from 1 × 107 thymocytes/point using the RNeasy kit (Qiagen, Valencia, CA) and reverse transcribed by the iScript RT-PCR kit (Bio-Rad, Hercules, CA) before amplification of cDNA templates using the respective primers described in Supplemental Table I.

Thymocyte stimulation, lectin pull-down, immunoprecipitation, immunoblotting, and in vivo labeling

Aliquots of 5 × 106 DP thymocytes were stimulated for various times with either 10 μg/ml CD3ε mAb (145-2C11) or 16 nM phorbol ester (PMA). Where indicated, cells were incubated for 4 h at 37˚C in the presence of 50 μM MG132 (Tocris Bioscience, Ellisville, MO) or its vehicle. Cells were stained for receptor surface expression or lysed either in RIPA buffer or in 1% Triton X-100 buffer (15 mM Tris-HCl [pH 7.5], 150 mM NaCl, 2 mM EDTA, supplemented with protease inhibitors). Proteins were resolved by SDS-PAGE and analyzed by immunoblotting using the Abs listed in Supplemental Table II. Where indicated, MEF lysates were incubated for 1 h with 1500 U endoglycosidase H or peptide-N-glycosidase F (New England Biolabs, Ipswich, MA). For lectin binding assays, we incubated 300 μg thymocyte or MEF lysates overnight at 4˚C with 20 μl lectin-agaroose (Vector Laboratories, Burlingame, CA), followed by washing with buffer containing 0.25% Triton X-100. Lysates or pull-down precipitates were run on SDS-PAGE followed by immunoblot analysis. For immuno-precipitation of mTORC2, 5 × 107 wild-type or rictor-deficient thymocytes were harvested and lysed in 0.3% CHAPS buffer containing protease inhibitors (3) and proteins resolved as previously described (15). For 35S metabolic labeling experiments, 2 × 107 thymocytes were incubated for 90 min at 37˚C with methionine-free medium and then labeled for 30 min with 1 μCi/ml [35S]methionine (PerkinElmer, Waltham, MA). After labeling, cells were replaced with normal DMEM medium containing 5 mM methionine and incubated for the indicated "chase" times. Cells were lysed in RIPA buffer and TCR α-chains were immunoprecipitated overnight at 4˚C. SDS-PAGE–resolved proteins were transferred onto a polyvinylidene difluoride membrane and the incorporation of [35S] was assessed by autoradiography followed by immunoblotting for TCRα and ubiquitin. Densitometric analysis of protein expression or posttranslational phosphorylation was performed using the ImageJ software from the National Institutes of Health.

Results

Rictor deficiency in the thymus led to a marked decrease in thymocyte number and partial differentiation blocks at the DN3 and CD8-ISP stages

By gene ablation, we generated the rictor−/− mouse model in which rictor expression (Fig. 1A) and mTORC2 assembly (Fig. 1B) were exclusively disrupted in T cells starting at the DN2 stage of thymocyte development (Supplemental Fig. 1). Whereas T cell–specific ablation of rictor had no effect on size, viability, and reproduction of rictor−/− mice (data not shown), it dramatically affected the number of thymocytes in these animals (Fig. 1C). Because thymopoiesis fluctuates during the lifespan of an individual, thymocytes from different age groups ranging from day 15 embryos to 6-wk-old mice were analyzed (Fig. 1D). Whereas rictor ablation diminished the number of thymocytes by 25% in embryos, it led to a 50% reduction in 1-wk-old rictor−/− mice as compared with rictor+/+ littermates and a massive cell loss of up to 80% in 3- to 6-wk-old knockout animals (Fig. 1D). This age-associated thymocyte decline suggests that rictor plays an essential role in the generation or homeostasis of these cells. As previously reported (6, 7), we also found a stage-specific developmental block that could account for the severe thymocyte loss in rictor−/− mice (data not shown). A pronounced increase in the CD25+CD44− (DN3) population was accompanied by a striking attenuation of DN4 (CD25+CD44+) cells (Fig. 1E). Interestingly, the relative numbers of CD8-ISP cells were similar in wild-type and rictor-deficient 2-mo-old mice (data not shown). A pronounced increase in the CD25+CD44− (DN3) population was accompanied by a striking attenuation of DN4 (CD25+CD44+) cells (Fig. 1E), suggesting that rictor is required for DN3 to DN4 differentiation. Concomitantly, a modest elevation of the absolute number of rictor-deficient DN3 thymocytes was associated with a dramatic decrease in DN4 cells (Fig. 1F), implying that a defective α-chain of the IL-7 receptor (CD127) instead of CD147 (Fig. 1G). However, in rictor−/− mice the proportions were reversed, resulting in a substantial increase in the relative and absolute numbers of CD8-ISP cells (Fig. 1G). Interestingly, the relative numbers of CD8-ISP were similar in wild-type and rictor-deficient day 15 embryos, whereas the difference increased after birth to
reach an ∼4-fold greater proportion in 4-wk-old rictorT−/− mice as compared with the wild-type littermates (Fig. 1H). Thus, defective mTORC2 triggered a partial developmental block at the CD8-ISP stage that was exacerbated with age and could, together with delayed DN3 to DN4 differentiation, explain the pronounced reduction in thymocytes.

Cell surface expression of receptors relevant for thymocyte development was defective in the absence of rictor

We noticed that whereas rictorT−/− newborns expressed slightly less CD147 on the surface of CD8-ISP cells as compared with wild-type cells, ∼40% less of this membrane glycoprotein was found on CD8-ISP thymocytes of 4-wk-old rictorT−/− mice (Fig. 2A). Similarly, in the absence of rictor, CD8 expression was significantly reduced on the surface of mature CD8-SP cells (Fig. 2B), whereas CD127 was upregulated on these cells (Fig. 2C). In accordance with a role of mTORC2 in Notch-driven thymocyte differentiation (7), the surface expression of Notch was also consistently decreased on rictor-ablated thymocytes (Fig. 2D). Furthermore, significantly diminished expression of pre-TCRs or TCRs was observed in the absence of rictor at each of the respective stages wherein they affect thymocyte development (Fig. 2E). This attenuation became particularly pronounced at later (DP and SP) stages and seemed to persist beyond T cell maturation in the thymus, as we also observed it on rictor-deficient peripheral T cells (Supplemental Fig. 2A). CD8 and CD4 expression was also significantly diminished on the surface of rictor-deficient DP and SP cells (Fig. 2B, 2F) as well as peripheral T cells (Supplemental Fig. 2A). Overall, our results suggest that mTORC2 could play a role in controlling the expression of receptors relevant for T cell ontogeny.

Decreased pre-TCR or TCR expression resulted in suboptimal signaling and cell responses in the absence of rictor

Among developing thymocytes, DN4 cells are the main population undergoing proliferation, and although ligands for the pre-TCR remain unknown, its surface expression is required for DN4 clonal expansion (8, 9, 16). Thus, we asked whether decreased pre-TCR expression would affect the division of rictor-deficient DN4 cells and consequently partially account for their reduced cell number. We labeled wild-type and rictor-deficient thymocytes with the fluorescent dye CFSE and assessed proliferation by flow
cytometry after 24 h of cell culture. As expected, neither DP nor SP cells spontaneously divided (data not shown); however, 37% of wild-type DN4 thymocytes but only 17% of rictor-deficient counterparts accomplished at least one division in 24 h (Fig. 3A). Both the surface expression of CD147 and the relative number of CD4 and CD8 subsets among CD8α+CD4− and CD8α−CD4+ thymocytes were similarly found to be increased in the absence of rictor as compared with its presence (Fig. 3B), supporting a defect in the cell cycling machinery in those cells. Whereas the addition of CD3ε mAb in the culture media further increased the percentage of dividing wild-type DN4 thymocytes, it had no effect on rictor-deficient counterparts (Fig. 3A), suggesting that the reduced pre-TCR surface expression accounted in part for defective DN4 clonal expansion in rictor−/− mice. To further assess the consequence of lower receptor expression, we measured the TCR-dependent expression of CD69, a marker for T cell activation (17). CD3ε mAb was markedly less potent in triggering CD69 expression on rictor-deficient thymocytes as compared with wild-type cells. Indeed, both the relative number of CD69+ cells (median 0.7%) as well as the surface amount of this receptor were significantly reduced in the absence of rictor (Fig. 3C, 3D), corroborating that lower TCR levels affected the functionality of rictor−/− thymocytes by probably delaying the transduction of required intracellular signals.

To test the latter idea, we examined the phosphorylation of ERK, a kinase essential for CD69 expression (17) and thymocyte differentiation (12) that is activated by TCR engagement. Compared to wild-type cells, ERK phosphorylation was markedly reduced following CD3ε mAb stimulation of rictor-deficient thymocytes (Fig. 4A). In contrast, the addition of PMA led to maximal phosphorylation of ERK after 10 min of stimulation (Fig. 4B), as well as to the expression of CD69 in most thymocytes following 6 h of treatment (Fig. 3C), independently of the presence or absence of a functional mTORC2 complex. These results indicated that the abrogation of rictor only affected the activation of ERK and consequently CD69 expression in response to TCR engagement, but not to stimuli that bypass the receptor. In contrast, both anti-CD3ε mAb and PMA treatment induced significantly less CD69 on the surface of rictor−/− thymocytes as compared with rictor+/+ cells (Fig. 3D), supporting a defect in the molecular mechanisms controlling CD69 expression in the absence of rictor. The activation of Akt was also affected in rictor-deficient cells following TCR engagement (Fig. 4A). Whereas the loss of rictor completely abolished the phosphorylation of Akt at both mTORC2 target sites, Ser240 (hydrophobic motif [HM]) and Thr450 (turn motif) (Fig. 4B, 4C), anticD3ε mAb only marginally rescued this phosphorylation (Fig. 4A) and PMA treatment had no effect whatsoever (Fig. 4B). Concomitantly, the Akt substrate Foxo1 was hypophosphorylated in rictor−/− and PMA treatment had no effect whatsoever (Fig. 4B). Similarly, the loss of mTORC2 accounted for diminished HM site phosphorylation of another AGC kinase, PKCs, that could also be rescued by PMA stimulation of rictor+/+ thymocytes (Fig. 3B, 3C), suggesting that rictor ablation broadly affected sig-
We then examined how mTORC2 can affect TCR surface expression in the absence of rictor. Defective processing of newly synthesized polypeptides could lead to defective receptor expression even in absence of ligand stimulation, as reported recently for the TCR (18). However, in the presence of cycloheximide, turnover of the TCR or the CD4 and CD8 coreceptors from the surface of rictor-deficient thymocytes was unchanged or slightly slower as compared with wild-type cells (Fig. 5B and data not shown), indicating that increased endocytosis was unlikely responsible for the reduced receptor expression in the absence of rictor. Thus, we considered whether rictor deficiency might alter de novo synthesis of the TCR complex. To facilitate biochemical analysis of the TCR α- or β-chains that, in contrast to CD3ε, were present in meager amounts in whole-cell extracts of rictor-deficient thymocytes (Fig. 5C, lane 1 versus 2 and data not shown), we crossed transgenic mice expressing exclusively the Vα2/TCRα and Vβ5/TCRβ chains (OT-1 TCR) (12) onto the rictor-deficient background (OT-1/rictor<sup>+/−</sup> mice). Compared to wild-type OT-1 cells, significantly less OT-1 TCR was also found on the surface of OT-1/rictor<sup>+/−</sup> thymocytes, as measured by Vα2 expression (Fig. 5D). Nevertheless, sufficient amounts of Vα2 could now be obtained by immunoprecipitation from rictor-deficient cells to allow examining the synthesis of this TCR α-chain (Fig. 5E, middle panel). After [35S]methionine labeling, the level of the radioactive Vα2 band (molecular mass of ~38 kDa) gradually decreased over time in wild-type thymocytes (Fig. 5E, x symbol, lanes 1–4, upper panel), whereas it was barely discernible in rictor-deficient cells after 1 h of chase (Fig. 5E, lanes 6–8, upper panel). Instead, a faster migrating band of ~29 kDa that likely corresponded to the native TCRα polypeptide lacking N-linked oligosaccharides and N-terminal signal peptide (19) accumulated in those thymocytes (Fig. 5E, arrow, lanes 5–8, upper panel). These results suggest increased misprocessing of de novo–synthesized TCR α-chains in the absence of rictor. In support of this, there was augmented ubiquitylation of Vα2-chains in OT-1/rictor<sup>+/−</sup> cell lysates (Fig. 5E, lanes 6–8, lower panel). Furthermore, treating rictor-deficient thymocytes with the proteasome inhibitor MG132 enhanced TCRα and TCRβ expression in whole-cell lysates by almost 4-fold compared with only 2-fold for wild-type cells (Fig. 5C, lane 2 versus 4 and data not shown). In contrast, TCR surface expression was unaffected by the addition of MG132 in the cell culture prior to flow cytometric analysis (Supplemental Fig. 2C). Overall, our results support a role for mTORC2 in the processing and maturation of TCR chains.

Next, we used lectin pull-down assays to examine whether defective TCRα glycosylation caused receptor misprocessing in the absence of rictor. The lectin <i>Sambucus nigra</i> agglutinin (SNA) that recognizes terminal sialic acid residues of complex N-glycan moieties (13) bound similar amounts of TCRα from wild-type and rictor-deficient thymocytes (Fig. 5F, lane 5 versus 6, upper panel). Thus, a defect in this late glycan modification could not account for enhanced TCR misprocessing in the absence of rictor. Indeed, SNA did not bind more receptor following MG132 treatment (Fig. 5F, lane 6 versus 8, upper panel) despite a 4-fold increase in TCRα expression (Fig. 5F, lane 2 versus 4, upper panel). We then used <i>Gallocate</i> (GNL) lectin (GNL), which binds to α-1,3-mannose residues that are generated at early steps of N-glycan trimming. GNL bound quite comparable amounts of TCR α-chains from MG132-treated and nontreated wild-type thymocytes (Fig. 5F, lane 5 versus 7, lower panel). In contrast, GNL was ~3-fold more efficient in binding TCRα from inhibitor-treated rictor-deficient cells as compared with nontreated counterparts (Fig. 5F, lane 6

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**FIGURE 4.** Rictor deficiency caused suboptimal TCR signaling. (A) Rictor<sup>+/+</sup> and rictor<sup>+/−</sup> DP thymocytes were stimulated in vitro for the indicated times with 10 μg/ml CD3ε mAb. Phosphorylation of ERK (T202/Y204) as well as the HM of Akt (S473) was analyzed by immunoblotting with the specific phosphorylated Abs and quantitated by densitometry. Membranes were stripped and reblotted with sera against the native form of the respective kinases. Shown is a representative experiment out of three as well as a quantitative plot of fold increase of protein phosphorylation as compared with the nonstimulated kinases from wild-type littermates. (B) Thymocytes were stimulated for 10 min with PMA (16 nM) and lysates were subjected to immunoblotting. Shown are representative blots of three experiments. (C) Thymocytes of three rictor<sup>+/+</sup> and three rictor<sup>+/−</sup> littersmates were harvested and counted, and lysates were resolved by immunoblotting. Shown are representative blots of three experiments. (D) Thymocytes were cultured ex vivo at 37°C for 1–4 d. Following cell staining with annexin V and PI, viability was measured at various time points by flow cytometric analysis (n = 4). Error bars denote SEM. **p < 0.01, ***p < 0.001, as determined by a Student t test.
versus 8, lower panel), indicating a partially defective receptor glycosylation in the absence of rictor that markedly augmented TCRα misprocessing and proteosomal degradation. This defect likely occurs either in the cis- or medial-Golgi during the early steps of protein glycosylation.

**mTORC2 disruption or inhibition deregulates the expression of CD147 in immortalized T cells as well as MEFs**

Considering that CD147 expression was reduced on the surface of rictor-deficient thymocytes (Figs. 2A, 3B), but, contrary to the TCR, this receptor is ubiquitously expressed on proliferating cells (20), we used it as a model to further examine the role of mTORC2 in receptor glycosylation and processing. The level of CD147 was significantly diminished on the surface of SIN1-deficient MEFs (Fig. 6A) as compared with wild-type cells in which mTORC2 is only GNL associated substantially to low-glycosylated CD147 in these cells (Fig. 6C, HG in lane 3), implying that most of the CD147 in these cells contained mannose residues, but not more complex glycan structures. These

endoglycosidase H, the low-glycosylated CD147 from SIN1−/− MEFs further collapsed into a <30-kDa polypeptide (Fig. 6B, lane 2 versus 5). This faster migrating protein corresponded to the fully deglycosylated form of CD147, as revealed by treatment with the glycosidase peptide-N-glycosidase F (Fig. 6B, lane 4) and OT-1/rictorT−/− mice and treated with vehicle or MG132. Lysates were subjected to SNA-agarose or GNL-agarose pull-down assay followed by immunoblotting. The means and SEM of fold changes as compared with TCR expressions in untreated wild-type cells (lane 1) indicated below each blot. Shown are representative blots of five independent experiments. (D) Thymocytes from 6-wk-old wild-type and rictor-deficient OT-1/Rag-2−/− littermates were stained for CD4, CD8α, Vα2 (TCRα), and Vβ5 (TCRβ) followed by flow cytometric analysis of Vo2 expression (mean fluorescence intensity [MFI]) on the surface of DP and CD8 SP cells (n = 3). (E) OT-1/rictorT−/− and OT-1/rictorT−/− thymocytes were subjected to pulse-chase labeling. Labeled immunoprecipitated TCR α-chains were visualized by autoradiography, whereas the amounts and ubiquitylation of immunoprecipitated TCRα (Ubi-TCRα) were assessed by immunoblotting. The arrow indicates the faster migrating TCRα band. Molecular masses (kDa) are indicated on the right. Shown are a representative of four experiments as well as quantitative plots (means ± SEM) of fold changes of the faster migrating TCRα (lower band) and TCR ubiquitylation as compared with wild-type cells at the start of the pulse-chase (lane 1). The radioactive input was identical for all samples (not shown). mk, mock immunoprecipitation. (F) Thymocytes were harvested from OT-1/Rag-2−/−/rictorT−/+ and OT-1/Rag-2−/−/rictorT−/− mice and treated with vehicle or MG132. Lysates were subjected to SNA-agarose or GNL-agarose pull-down assay followed by immunoblotting. The means and SEM of fold changes as compared with TCR expressions in untreated wild-type cells (lane 1) are indicated below each blot. Shown is a representative experiment out of three. Error bars denote SEM. **p < 0.01, ***p < 0.001, as determined by a Student t test.

**FIGURE 5.** Rictor deficiency caused defective processing of αβTCR. (A) RNA was extracted from wild-type (+/+ ) and rictor-deficient (−/− ) thymocytes and the mRNA expression of TCRα, TCRβ, CD3e, and CD127 was analyzed by RT-PCR and normalized to the expression of β-actin using ImageJ software. The means and SEM of CD127 fold changes as compared with the expression in wild-type cells are indicated. Shown is a representative experiment out of three. mk, mock amplification. (B) Thymocytes were cultured ex vivo in the presence of 100 μM cycloheximide for the indicated times and the amount of TCR was assessed by flow cytometric analysis and plotted relative to the expression on the surface of wild-type cells (+/+ ) at the start of the experiment (100%). T1/2, 50% reduction of TCR surface expression (n = 3). (C) Thymocytes were treated with or without MG132 and total protein expression was analyzed in whole-cell lysates. The means and SEM of fold changes as compared with protein expressions in untreated wild-type cells (lane 1) are indicated below each blot. Shown are representative blots of five independent experiments. (D) Thymocytes from 6-wk-old wild-type and rictor-deficient OT-1/Rag-2−/− littersmates were stained for CD4, CD8α, Vα2 (TCRα), and Vβ5 (TCRβ) followed by flow cytometric analysis of Vo2 expression (mean fluorescence intensity [MFI]) on the surface of DP and CD8 SP cells (n = 3). (E) OT-1/rictorT−/− and OT-1/rictorT−/− thymocytes were subjected to pulse-chase labeling. Labeled immunoprecipitated TCR α-chains were visualized by autoradiography, whereas the amounts and ubiquitylation of immunoprecipitated TCRα (Ubi-TCRα) were assessed by immunoblotting. The arrow indicates the faster migrating TCRα band. Molecular masses (kDa) are indicated on the right. Shown are a representative of four experiments as well as quantitative plots (means ± SEM) of fold changes of the faster migrating TCRα (lower band) and TCR ubiquitylation as compared with wild-type cells at the start of the pulse-chase (lane 1). The radioactive input was identical for all samples (not shown). mk, mock immunoprecipitation. (F) Thymocytes were harvested from OT-1/Rag-2−/−/rictorT−/+ and OT-1/Rag-2−/−/rictorT−/− mice and treated with vehicle or MG132. Lysates were subjected to SNA-agarose or GNL-agarose pull-down assay followed by immunoblotting. The means and SEM of fold changes as compared with TCR expressions in untreated wild-type cells (lane 1) are indicated below each blot. Shown is a representative experiment out of three. Error bars denote SEM. **p < 0.01, ***p < 0.001, as determined by a Student t test.
results indicate a partial CD147 glycosylation defect in absence of SIN1 at an early step of glycan maturation. Additionally, the results concur with our findings documenting a defective TCR glycosylation in rictor-ablated thymocytes and they further support an involvement of mTORC2 in the early steps of receptor glycosylation and processing.

Finally, because the expression of CD147 has been found to be elevated in a number of tumors, including T cell lymphoid leukemia (21, 22), we examined whether its levels on the surface of the T cell line, Jurkat, correlated with augmented mTORC2 activity. Compared to both nontransformed peripheral T cells and thymocytes, Jurkat cells bear a tremendously higher phosphorylation of mTOR and the HM of Akt (Fig. 6D, lane 2 versus 3 and 4) that was associated with a dramatic increase of CD147 surface expression (Fig. 6E). Indeed, knocking down rictor (Fig. 6D, lane 1 versus 2) or blocking the activity of mTORC2 (Supplemental Fig. 2D) significantly reduced the expression of CD147 on the surface of Jurkat cells. Thus, besides controlling the amounts of surface receptors, mTORC2 would optimize the transduction of signals required for cell fate decisions. Among the many receptors relevant for early thymocyte development, the reduced pre-TCR/TCR levels (Fig. 2E) could account for decreased DN4 proliferation (Fig. 3A), and together with the decreased Notch and CD147 expression (Figs. 2A, 2D, 3B) could delay maturation to the DP stage, even in presence of increased expression of CD127 (Figs. 2C, 5A). Additionally, diminished TCR as well as CD4 and CD8 expression on DP thymocytes (Fig. 2B, 2E, 2F) generated suboptimal signaling (Fig. 4A), leading to delayed activation and maturation to the SP stage (Fig. 3C). Furthermore, reduced TCR as well as CD4 or CD8 levels (Supplemental Fig. 2A) could impact homeostasis of peripheral rictor-deficient T cells, even in the presence of increased levels of cytokine receptors such as CD127, and explain in part some of the changes in peripheral T cell differentiation previously reported (5). Thus, overall abnormal expression of relevant receptors could account for most of the T cell–specific developmental defects that we have documented here upon rictor deletion.

Discussion
Our findings of a partial block of thymocyte development at the DN3 stage concur with previous reports documenting the disruption of mTORC2 in T cells (6, 7). Importantly, we reveal for the first time, to our knowledge, that mTORC2 could control cell responses by regulating the levels of critical plasma membrane receptors that drive specific stages of cell development or proliferation. By controlling the amounts of surface receptors, mTORC2 would optimize the transduction of signals required for cell fate decisions. Among the many receptors relevant for early thymocyte development, the reduced pre-TCR/TCR levels (Fig. 2E) could account for decreased DN4 proliferation (Fig. 3A), and together with the decreased Notch and CD147 expression (Figs. 2A, 2D, 3B) could delay maturation to the DP stage, even in presence of increased expression of CD127 (Figs. 2C, 5A). Additionally, diminished TCR as well as CD4 and CD8 expression on DP thymocytes (Fig. 2B, 2E, 2F) generated suboptimal signaling (Fig. 4A), leading to delayed activation and maturation to the SP stage (Fig. 3C). Furthermore, reduced TCR as well as CD4 or CD8 levels (Supplemental Fig. 2A) could impact homeostasis of peripheral rictor-deficient T cells, even in the presence of increased levels of cytokine receptors such as CD127, and explain in part some of the changes in peripheral T cell differentiation previously reported (5). Thus, overall abnormal expression of relevant receptors could account for most of the T cell–specific developmental defects that we have documented here upon rictor deletion.

How mTORC2 can regulate receptor surface expression remains to be elucidated, but our findings suggest that this complex accounted for the observed defects rather than a global change in mTOR signaling. Indeed, whereas mTORC1 signaling involving S6K and S6 was not diminished in rictor-deficient thymocytes (Fig. 4C, Supplemental Fig. 2B), autophagy was not impaired...
either by the ablation of rictor as indicated by normal LC3-II levels and ULK1 phosphorylation (Fig. 4C). Instead, specific mTORC2 targets and downstream effectors such as the AGC kinases, Akt, and protein kinase C α/β, as well as the Akt-effector Foxo1/3, have defective phosphorylation and signaling in rictor-deficient thymocytes (Fig. 4A–C). Whereas the transcription factor Foxo1 has previously been reported to control the expression of CD127 (23, 24), mTORC2 could contribute to this transcriptional mechanism by regulating the phosphorylation of Akt. Therefore, defective phosphorylation of Akt and Foxo1 could account for the augmented transcription and surface expression of CD127 that we observe in rictor<sup>T<sup>+/−</sup></sup> mice (Figs. 2C, 5A). Notably, although required for T cell ontogeny as well as peripheral homeostasis (25), enhanced CD127 was not able to compensate for the developmental defects observed in rictor-deficient thymocytes. In contrast, and although the mechanism would remain to be investigated, CD127 might have contributed to preserve the viability of rictor<sup>T<sup>+/−</sup></sup> cells (Fig. 4D) even in presence of markedly defective Akt signaling (Fig. 4A–C). Nonetheless, most receptors we examined were decreased, not increased, on the surface of rictor-deficient thymocytes, indicating that mTORC2 could impinge on various molecular mechanisms that would each specifically modulate the levels of particular membrane receptors. For instance, although CD127 was increased on the surface of rictor-deficient cells, it might also potentially be affected by a similar posttranslational defect as the rest of the receptors examined. Indeed, an almost 2-fold–augmented CD127 transcript in rictor<sup>T<sup>+/−</sup></sup> cells (Fig. 4A) resulted in only an ∼30% increase in receptor surface level (Fig. 2C), implying that some CD127 was lost after transcription. Furthermore, as compared with wild-type cells, CD69 was less upregulated on the surface of rictor-deficient DP thymocytes in response to both anti–CD3ε and PMA (Fig. 3D), despite the latter treatment triggering full activation of ERK (Fig. 4B), a signaling cascade required for maximal expression of CD69 (17). Interestingly, the kinetics of ERK activation were quite different in anti–CD3ε-stimulated rictor<sup>T<sup>+/−</sup></sup> and rictor<sup>T<sup>+/+</sup></sup> thymocytes (Fig. 4A). A fast and somewhat transient ERK phosphorylation was observed in wild-type cells, whereas the activation of this kinase was slow but sustained in the absence of rictor (Fig. 4A, right panel). This result was reminiscent of the signature ERK kinetics that we have documented previously in response to ligands inducing either differentiation or death of activated DP thymocytes (12). Whether the abrogation of rictor affects thymic selection, owing to reduced TCR expression and ensuing signaling defects, remains to be investigated further.

Disruption of mTORC2 also led to diminished receptor expression in peripheral T cells (Supplemental Fig. 2A). Although rictor deficiency has been documented to affect Th cell differentiation (26), TCR surface expression was not explicitly accounted for in this study or in any other report published so far discussing T cell–targeted rictor or AGC kinase mouse models, including Akt-null mice (27–29). It would thus also be interesting to determine whether altered amounts of TCR and/or cytokine receptors critical for the generation of Th1 and Th2 account, in part, for the defects reported in the literature by Lee et al. (26). Nevertheless, although we cannot exclude that several mTORC2-dependent AGC kinases cooperate to control receptor expression, our new findings support a role for mTORC2 in the processing of nascent receptor polypeptides and more precisely during their early glycosylation steps. Notably, the loss of rictor decreased the expression of N-glycosylated α- and β-chains of the TCR, but not CD3ε, the nonglycosylated component of the TCR/CD3 complex (Fig. 5C and data not shown). CD147, another highly glycosylated receptor, had decreased expression in DN4 and CD8-ISP thymocytes as well as MEFs and was misprocessed in the latter cells. Interestingly, CD147 has been recently implicated in the maturation of lactate transporters at the plasma membrane of proliferative cells undergoing enhanced glycolysis (30, 31). Thus, defective CD147 surface expression in absence of rictor could possibly impact cell division owing to its role in glycolytic metabolism and, consequently, protein glycosylation. Notably, altered N-glycosylation of growth factor receptors has previously been correlated with deregulated cell proliferation as well as defective Akt signaling (32, 33). Whether Akt could also modulate glycan maturation in the Golgi is a possibility considering previously documented involvement of this kinase in controlling indirectly glycosylation via the ER enzyme ENTPD5 (32). There is also accumulating evidence that mTORC2 plays a role in metabolic and biosynthetic pathways (34, 35). Whether it is involved in the generation of metabolic intermediates crucial for the process of protein glycosylation remains to be investigated. Finally, because altered surface expression of mitogenic receptors plays a role in defective proliferation or differentiation of a number of cells, our studies provide a rationale for specifically targeting mTORC2 in developing new treatments to prevent the onset or progression of various diseases, including malignancies and T cell–derived disorders.

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Disclosures
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References

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