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Increased TORC2 signaling promotes age-related decline in CD4 T cell signaling and function

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Abstract

CD4 T cell function declines significantly during aging. While the mammalian target of rapamycin (TOR) has been implicated in aging, the roles of the TOR complexes (TORC1, TORC2) in the functional declines of CD4 T cells remain unknown. In this study, we demonstrate that aging increases TORC2 signaling in murine CD4 T cells, a change blocked by long-term exposure to rapamycin, suggesting that functional defects may be the result of enhanced TORC2 function. Using overexpression of Rheb to activate TORC1 and Rictor plus Sin1 to augment TORC2 in naïve CD4 T cells from young mice, we demonstrated that increased TORC2, but not TORC1, signaling results in aging-associated biochemical changes. Furthermore, elevated TORC2 signaling in naïve CD4 T cells from young mice leads to *in vivo* functional declines. The data presented herein suggest a novel model in which aging increases TORC2 signaling and leads to CD4 T cell defects in old mice.

Keywords

T cells; Aging; Rapamycin; mTOR; TRC signaling

Introduction

Data from diverse model organisms suggest that the mammal target of rapamycin (TOR) is a critical controller of aging (1–3), and rapamycin treatments can extend the lifespan of mice (4). TOR, a serine/threonine kinase, forms the catalytic core of at least 2 complexes, TOR complex 1 (TORC1) and TOR complex 2 (TORC2). The role of each complex in aging is not well understood (2). TORC1 is known to respond to nutrient environmental signals responsible for modulating cell growth and stress responses (5). The GTP-binding protein, Ras homolog enriched in brain (Rheb), represents a critical upstream activator of TORC1 (6). Rheb overexpression enhances TORC1 activity (7) and increases phosphorylation of its downstream substrates such as S6K1 (8). In turn, S6K1 enhances the phosphorylation of the ribosomal protein S6 kinase at Ser-235 (pS6₍₂₃₅₎) a component of the 40 S ribosomal subunit that mediates translation (9). In lymphocytes, TCR/CD28 signaling leads to TORC1

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activation via Rheb and AKT phosphorylation at Thr-308 (pAKT₍₃₀₈₎) via PDK1 pathway (10); these events regulate CD4 T cell activation and differentiation of naïve CD4 T cells into Th-1 and Th-17 phenotypes (11). In contrast, TORC2 regulates naïve CD4 T cell differentiation into a Th-2 phenotype (11, 12) and it also regulates actin polymerization (13), cell size (14), Rac-GTPase and RhoA-GTPase activity (15, 16), and PKC function (17), and may also regulate the phosphorylation of Ezrin and Moesin (pERM) cytoskeleton proteins involved in immune synapse formation (18). In addition, inhibition of TORC2 can increase apoptosis (19, 20), suggesting a role for TORC1 in the regulation of lymphocyte survival. TORC2 contains the Rictor and Sin1 scaffolding proteins that are critical for its function (21). The best-characterized phosphorylation substrates of TORC2 are AKT at Ser473 (pAKT₍₄₇₃₎), serum glucocorticoid kinase 1 at Ser-422 (pSGK1₍₄₂₂₎), and the N-myc downstream-regulated (pNDRG1) gene at Thr-346 (pNDRG1₍₃₄₆₎) via SGK1 activation (22).

Aging alters numerous aspects of CD4 T cell function (23, 24). These alterations include cytoskeletal changes and declines in TCR dependent activation (25–29), increased cell size (27), altered RhoA and Rac activity (30), decreased pERM₍₅₅₈₎ expression (30), and decreased proliferation *in vivo* (31). We recently demonstrated that some of these defects are the result of alterations in TCR/CD28 signaling (32). In addition, others have reported that aging decreases CD4 T cell proliferation and alters the expression of the Bcl-2 family of apoptotic proteins, including declines in pro-apoptotic BIM expression (33). However, the molecular mechanism(s) that regulate these changes remain unknown. Because many of these phenotypic changes in lymphocytes from old mice are controlled by TORC2, we postulated that the age-related defects in CD4 T cell function could be the result of alterations in TORC2 function. Here, using *in vitro* and *in vivo* models of CD4 T cell function, we evaluated TORC1 and TORC2 signaling in the context of the age-related functional changes of CD4 T lymphocytes.

Material and Methods

Animals, reagents, and rapamycin treatments

H-2(k/k) TCR-V_{11V}₃ CD4⁺ mice (AND mice) and CD4 knockout (CD4KO) mice on a B10.BR background were bred in our facilities from stock generously provided by Susan Swain and Laura Haynes (Trudeau Institute, NY). Specific pathogen-free B10.BR and CB6F1 (BALB/c × C57BL/6) mice were purchased from the Charles River Laboratories (Kingston, NJ) and from the National Institute of Aging contract colonies at Harlan (Indianapolis, IN), respectively. The genetically heterogeneous mice population (UM-HET) generated by crossing CB6F1 females with C3D2F1 males (34) was also bred in our facilities. All experiments involving mice conformed to institutional and national standards and were approved by the University of Michigan's Committee on Use and Care of Animals. All mice were given free access to food and water. Sentinel animals were examined quarterly for evidence of viral infection; all tests were negative during the course of these studies. Mice found to have splenomegaly or macroscopically visible tumors were not used for the experiments. AND mice were used at 4 months of age; B10.BR or CD4KO adoptive host mice were 2–3 months of age. CB6F1 mice used in the study were either 6–8 (young) or 20–22 (old) months of age. All chemical reagents were purchased from Sigma (www.sigmaldrich.com). Carboxyfluorescein succinimidyl ester (CFSE), violet blue cell division trackers, and rabbit polyclonal anti-HA were purchased from Invitrogen (www.invitrogen.com). Flow cytometry antibodies to surface CD4 T cells molecules were purchased from Biolegends (www.biolegends.com) or Becton Dickson (www.bdbiosciences.com). Rabbit polyclonal anti-Myc, monoclonals anti-Sin1, anti-mTOR and anti-Rictor were purchased from Millipore (www.millipore.com). Rabbit anti-Glut1 was from Epitomics (www.epitomics.com). Protein antibodies, phospho-specific rabbit

monoclonal antibodies, and rabbit polyclonal anti-Flag antibodies were purchased from Cell Signaling (www.cellsignal.com), with the exception of pSGK1₍₄₂₂₎ and NDRG, which were obtained from Santa Cruz (www.scb.com). Anti-rabbit FITC-conjugated antibodies were obtained from Jackson ImmunoResearch (www.jacksonimmuno.com). Plasmids corresponding to the human pRK5-Rheb-Flag, pRK5-Rictor-HA, pRK5-Sin1-Myc, and pRK5 stocks were generously donated by Diane Fingar (University of Michigan, MI) and purified with Qiagen endotoxin-free purification kits (www.qiagen.com). Rapamycin treatments were performed as previously described (434) by mixing microencapsulated rapamycin at different doses in Purina 5LG6 chow as described in text and given *ad libitum* to the mice. Spleens from the rapamycin-treated mice were harvested and the lymphocytes were purified as previously described (25). Then, lymphocytes were stained for CD4 and CD44 and intracellular staining was performed as described elsewhere (30).

Transient transfection of CD4 T cells, intracellular staining, and western blot analysis

Naïve CD4 cells from the spleen and lymph nodes were obtained by negative selection using the Miltenyi CD4 purification kit II according to the manufacturer's recommendations (www.miltenyibiotec.com). Analysis of a typical preparation showed the cells to be 90% positive for both CD3 and CD4. Then, 10×10^6 CD4 T cells were transfected with the different plasmids by electroporation using the Amaxa Nucleofector mouse CD4 T cell transfection kit (www.biолonza.com) as described elsewhere (35, 36). In each experiment, we performed electroporation of the following constructs: 5 µg of pRK5 (Vector), 5 µg of pRK5-Rheb-Flag (Rh), or a mix of 5 µg of pRK5-Rictor-HA plus 5 µg of pRK5-Sin1-Myc (R+S). Transfection efficiency controls were performed for each experiment using Pmax-green fluorescent protein (GFP) as recommended by the Amaxa Nucleofector systems. Then, cells were cultured in complete RPMI-1640 medium and after 24 hours the dead cells and debris were removed using the lympholyte-M method and the live cells were counted. For each analysis, 10×10^6 CD4 T cells were lysed and the aliquots were analyzed by western blotting using phospho-specific antibodies or total protein. The respective bands were quantified as described elsewhere (30). In other cases, analysis by flow cytometry using intracellular staining was performed as we previously described (30).

Adoptive transfer and analysis of *in vivo* CD4 T cell activation and proliferation

Purified splenic naïve CD4 T cells from young AND mice were transfected with the different plasmids according to the methods described above. Then, electroporated CD4 T cells (V, Rh, and R+S) were labeled with violet blue cell division tracker following the manufacturer's recommendations (www.invitrogen.com). In addition, untransfected CD4 T cells (Controls, C) were labeled with CFSE (31). Then, 1×10^6 CD4 T cells from each of the transfected populations (Vector, Rheb, and R+S) were mixed with 1×10^6 untransfected CD4 T cells (C) and injected into the tail veins of CD4KO host mice. Twenty-four hours later, the mice were primed with 10 µg of PCC in 100 µL of PBS and the spleen and lymph nodes were harvested at the indicated time points. Analysis of the activation and proliferation of the adoptively transferred CD4 T cells was performed using flow cytometry as previously described (31).

Statistical analysis

Unless otherwise indicated, results are presented as mean \pm standard error of the mean (SEM). Statistical significance was assessed using Mann-Whitney tests, with the significance level set at $p = 0.05$.

Results

In vivo rapamycin treatment can prevent age-related changes in CD4 T cells

Rapamycin treatment can prolong the lifespan of lower organisms and mice (3, 37). While the underlying mechanism is not well understood, it has been suggested that inhibition of mTORC1 may be important (38). However, long-term rapamycin treatment can also inhibit TORC2 function (2, 39), and its effects on immune function during aging remain unknown. With increased age, CD4 T cells show decreased pERM₍₅₅₈₎ expression, which is associated with declines in TCR signaling, immune synapse formation, and activation (23, 30). Other laboratories have documented age-related declines in the expression of the pro-apoptotic protein BIM (33). Because these proteins are regulated by TOR, we hypothesized that long-term rapamycin-induced TOR inhibition could prevent some of the age-related changes in CD4 T cells. To test this hypothesis, we treated a genetically heterogeneous population of mice (UM-HET) with rapamycin starting at 9 months of age. The UM-HET mice were given either control chow (Purina 5LG6) or chow containing 3 different doses of rapamycin for 12 months (High: 42 ppm; Middle: 14.7 ppm; Low: 4.7 ppm) as described previously (34). Then, we harvested the spleens from 9-month-old (Young, Y), untreated 22-month-old (Old, O), and rapamycin-treated 22-month-old (O_R) mice and evaluated the effects of rapamycin on the age-related alterations of CD4 T cells using intracellular staining and flow cytometry. Flow cytometric methods were selected because they enable the analysis of the expression of multiple age-related changes (pERM, BIM, pAKT₍₄₇₃₎, and pAKT₍₃₀₈₎) in both naïve and memory CD4 T cells using CD44 and CD62L differentiation markers (40). A typical analysis is shown in Figure 1A. We found that aging decreases the number of CD4 T cells expressing high levels of BIM and pERM₍₅₅₈₎ when compared to CD4 T cells from young mice (first 2 columns of Figure 1A). Interestingly, untreated CD4 T cells from old mice showed an increased number of cells with higher levels of pAKT₍₄₇₃₎, but aging had no effect on pAKT₍₃₀₈₎ (Y vs. O in Figure 1A). All doses of rapamycin led to a decline in the CD4/CD8 T cell ratio and diminished the age-related increases in the proportion of memory cells as indicated by the CD44 and CD62L markers (see Supplemental Figure 1A). These results correspond well with those in the literature demonstrating that rapamycin affects CD4 T cell differentiation (41). However, as shown in Figure 1A, rapamycin treatment led to partial, though not complete, prevention of the declines in BIM and pERM expression and increased pAKT₍₄₇₃₎ in both naïve and memory CD4 T cells, with no effects on pAKT₍₃₀₈₎. To test for statistical significance, we analyzed the data from at least 12 independent experiments (with a minimum of 6 females and 6 males from each group) As shown in Figure 1B, 12-month rapamycin treatment, at 14.7 ppm, seem to prevent some of the BIM declines in both naïve (p = 0.003) and memory (p = 0.02) CD4 T cells. These effects could also be observed with the lower and higher rapamycin doses (see Supplemental Figure 1). Furthermore, rapamycin also appeared to prevent declines in pERM expression in both naïve and memory CD4 T cells. However, statistical significance was achieved only at the high dose (see Supplemental Figure 1). Furthermore, the age-related increase in pAKT₍₄₇₃₎ was significant in both naïve (p = 0.005) and memory (p = 0.04) CD4 T cells (Y vs. O in Figure 1B). Long-term rapamycin treatment can inhibit TORC2 function (2); as expected, we observed that rapamycin treatment prevented the age-related increase in pAKT₍₄₃₇₎ (naïve, p = 0.007; memory, p = 0.001) to near to the levels observed in CD4 T cells from young donors (O vs. O_R in Figure 1B). On the other hand, we found no significant age- or rapamycin treatment-associated changes (example in Figure 1A, full data set not shown) in pAKT₍₃₀₈₎, as predicted by the models showing that pAKT₍₃₀₈₎ is upstream of TOR (42).

The age-related increase of pAKT₍₄₇₃₎ is not the result of changes in AKT1 expression (example in Figure 2, full data set not shown), instead suggesting enhanced downstream TORC2 signaling. To further test this hypothesis, we measured the phosphorylation levels of downstream targets of TORC2, including pNDRG1₍₃₄₆₎, pAKT₍₄₇₃₎, and pSGK1₍₄₄₂₎ using

western blotting in highly purified CD4 T cells from the spleens of young (6–8 months) and old (22–24 months) CB6F1 mice. Figure 2 shows a typical western blot comparing the phosphorylation status of these proteins and their relative total expression in CD4 T cells from young and old donor mice. In addition, Figure 2 shows the statistical analysis of the relative ratios of at least 6 independent experiments (with a total of 12 young and 6 old mice). We found that aging significantly increases the relative level of pAKT₍₄₇₃₎ ($p = 0.001$) and pNDRG1₍₃₄₆₎ ($p = 0.0003$). We also analyzed pSGK1₍₄₂₂₎ and found a similar 2-fold age-related increase ($p = 0.0002$); however, since this antibody may recognize other substrates in the same molecular weight range (43), we cannot conclude that pSGK1 is affected by aging (data not shown). The age-related increases in TORC2 activity are not the results of changes in the expression levels of TORC2 associated proteins Rictor and Sin1 in purified CD4 T cells from young and old donors (See Supplemental Figure 3). Nevertheless, the results in rapamycin-treated mice, in addition to the flow cytometric analysis of untreated naïve and memory CD4 T cells with regard to pAKT₍₄₇₃₎, suggest that aging enhances TORC2 signaling and function.

***In vitro* functional consequences of enhanced TORC1 or TORC2 signaling in naïve CD4 T cells**

Data from Figure 1 and 2 suggest that aging may increase TORC2 activity; however, these results do not exclude the possibility that TORC1 plays a role in T cell immunosenescence. To differentiate between the roles of TORC2 and TORC1, we purified CD4 T cells from the spleens of young CB6F1 donors (>90% naïve cells). Then, we transfected the cells with constructs capable of activating TORC1 or TORC2 function. We enhanced TORC1 activity using Rheb overexpression (Rh), while we enhanced TORC2 activity by cotransfecting CD4 T cells with Rictor and Sin1 proteins. The latter method was based on data suggesting that using Rictor plus Sin1 can stabilize the TORC2 complex, leading to its activation (21, 44). We selected electroporation as a method of transfection because it results in consistent efficiency between different constructs and multiple experiments (see below). After electroporation, untransfected and transfected CD4 T cells from young donors were incubated for 24 hours. Then, cells were stained for CD4 and a live/dead fixable red stain to identify live cells, followed by intracellular staining against the corresponding protein-tags: a) Flag for Rheb, b) HA for Rictor, and c) Myc for Sin1; analysis was performed using intracellular flow cytometry. A parallel transfection of CD4 T cells with GFP was performed for measuring the daily transfection efficiency as recommended by the electroporation kit manufacturer. We observed a 30–50% transfection efficiency of CD4 T cells for all experiments with approximately 20–30% cell death (Supplemental Figure 2). After removing the dead cells, around 10×10^6 CD4 T cells from each group were lysed and analyzed by western blotting using specific antibodies for pAKT₍₄₇₃₎ and pNDRG₍₃₄₆₎, which are downstream targets of TORC2, and pS6₍₂₃₅₎, which is a downstream target of TORC1. As shown in Figure 3A, compared to control (C) or vector alone (V), Rheb (Rh) overexpression significantly enhanced pS6₍₂₃₅₎ levels ($p = 0.03$) without affecting mTORC2 targets. On the other hand, Rictor plus Sin1 (R+S) overexpression significantly enhanced pAKT₍₄₇₃₎ ($p = 0.01$) and pNDRG1₍₃₄₆₎ ($p = 0.02$) levels without affecting pS6₍₂₃₅₎. These results suggest that we can independently enhance TORC1 and TORC2 activity in naïve CD4 T cells (see Supplemental Figure 3 for all of the western blot results obtained from a typical experiment).

To analyze whether enhanced TORC1 and TORC2 signaling affects the levels of BIM and pERM, we performed similar experiments as described in Figure 3. However, to facilitate the measurement of multiple TORC downstream targets in the same samples, we performed intracellular staining as described in Figure 1. A typical flow cytometric analysis of untransfected (C) and transfected groups (V, Rh, and R+S) is shown in Figure 4A. As

expected, untransfected or vector-alone CD4 T cells from young mice expressed high levels of BIM and pERM and had relatively low levels of pNDRG1₍₃₄₆₎ and pS6₍₂₃₅₎. Furthermore, Rheb overexpression (Rh) increased pS6₍₂₃₅₎ levels but did not affect those of BIM or pERM (Figure 4A, middle panels). In contrast, overexpression of Rictor plus Sin1 decreased the number of CD4 T cells expressing BIM and pERM while enhancing pNDRG1₍₃₄₆₎ levels (Figure 4A, lower panels). In addition, none of the constructs appeared to affect pAKT₍₃₀₈₎ (data not shown). To test whether these changes were statistically significant, we performed a series of 6 independent experiments as shown in Figure 4B. The data confirmed that vector and Rheb overexpression did not affect BIM or pERM levels. However, enhanced mTORC2 significantly reduced BIM ($p < 0.0001$) and pERM ($p < 0.0001$) levels and increased pNDRG1₍₃₄₆₎ ($p = 0.03$) levels without affecting pS6₍₂₃₅₎. In this data set, we found that Rheb or Rictor plus Sin1 overexpression has no significant effect on the expression of endogenous ERM, NDRG1, and S6 proteins (data not shown), suggesting that the above changes are the result of alterations in the phosphorylation pattern. These overall findings suggest that age-related increases in TORC2 signaling lead to declines in pERM and BIM expression, as is found with aging, and support a model in which inhibition of TORC2 (shown in Figure 1) is responsible for preventing age-related declines in CD4 T cell function in rapamycin-treated mice.

***In vivo* functional consequences of enhanced TORC1 or TORC2 signaling in the activation and proliferation of naïve CD4 T cells**

To test the hypothesis that enhanced TORC2 or TORC1 signaling affects *in vivo* CD4 T cell from young mice function in a manner that is consistent with the response of CD4 T cells from old mice (31, 45, 46), we used an *in vivo* model of immune function based on adoptive transfer of transgenic naïve CD4 T cells that recognize a specific sequence on the PCC protein (AND mice) into syngeneic CD4KO mice as previously described (31). In this model, *in vivo* CD4 T cell expansion is dependent on PCC priming, and the rate of CD4 T cell proliferation significantly declines with aging as measured by cell division trackers and flow cytometric analysis. To test the hypothesis that elevated TORC2 activity may be responsible for declines in CD4 T cell function, we purified naïve CD4 T cells from the spleens of young AND mice (4 months), and aliquots of 5×10^6 CD4 T cells were labeled with a CFSE cell division tracker. In addition, aliquots of 20×10^6 CD4 T cells were labeled with a violet cell division tracer. After labeling, approximately 6×10^6 violet-labeled CD4 T cells were electroporated with each of the constructs (vector, Rh, and R+S). Then, 2×10^6 live CD4 T cells from each transfection group were mixed with 1×10^6 untransfected cells (CFSE controls, C) and adoptively transferred to CD4KO young hosts (2–4 months). This dual labeling of untransfected (CFSE) and transfected (Violet) CD4 T cells transferred to a single host minimizes the effects of host variation and enables analysis between multiple hosts. Twenty-four hours after adoptive transfer, each host mouse was primed with 10 μ g of PCC in PBS. Forty-eight hours later, the spleen and lymph nodes were harvested and the proliferation of the adoptively transferred CD4 T cells was analyzed by flow cytometry as previously described (31). Figure 5A shows a typical profile of CD4 T cell division for the untransfected or vector-alone groups, where approximately 25% of the CD4 T cells in the lymph nodes and 55% in the spleen have not yet divided. These cell division profiles correspond well with previous literature (31, 32). Rheb overexpression does not appear to affect proliferation relative to untransfected or vector-alone groups. In contrast, overexpression of Rictor plus Sin1 increased the number of non-dividing CD4 T cells (50% in the lymph nodes; 75% in the spleen). To test whether these declines were statistically significant, we performed a series of 3 independent experiments with at least 2 host mice receiving cells from each of the transfection groups (control/vector, control/Rh, control/R+S) per experiment. As shown in Figure 5B, we found no effect of Rheb overexpression. These results suggest that enhanced TORC1 signaling does not seem to affect cell

proliferation under our experimental conditions. However, overexpression of Rictor plus Sin1 leads to a significant (30–40%) increase in the number of non-dividing CD4 T cells in the lymph nodes ($p = 0.004$) and spleen ($p = 0.002$) relative to controls in the same host. These reductions in proliferation are almost identical to the age-related declines in proliferation that we previously reported using the same *in vivo* model (31). These results suggest a model in which an age-related increase in TORC2 signaling leads to declines in CD4 T cell function. Furthermore, we previously demonstrated that aging decreases the expression of ICOS on CD4 T cells (32), a surface protein involved in the interactions with B cells and dendritic cells (DCs) during immune responses (47). As shown in Figure 5B, Rictor plus Sin1 overexpression leads to significant decline in total ICOS expression in the lymph nodes ($p < 0.001$) and spleen ($p = 0.002$) (for a typical flow cytometric analysis see Supplemental Figure 4A). We also found that most of the decreased ICOS expression occurred in the non-dividing population, suggesting that enhanced TORC2 signaling blocked CD4 T cell activation from young mice, as we previously described in CD4 T cells from old mice (32). It is possible that overexpression of Rictor and Sin1 may result in an unresponsive or anergic state in CD4 T cells. To test this possibility, we performed similar experiments as shown in Figure 5 while increasing the PCC priming dose to 100 μg . Under this strong stimulus, the control CD4 T cells underwent numerous rounds of cell division (See Supplemental Figure 4B). The proliferation differences between the control and Rictor plus Sin1-overexpressing cells did not reach statistical significance, suggesting that the CD4 T cells were not anergic, but rather that increased TORC2 signaling led to TCR signaling alterations that required a higher threshold of TCR activation, thus causing declines in TCR or CD28 signals observed during aging (48). Analysis of cotransfection with GFP or intracellular stains and the HA or Myc tag (Supplemental Figure 4C and D) suggested no differences in the efficiency of transfection and tag expression compared to that described for our *in vitro* experiments using the CB6F1 model (Supplemental Figure 2), indicating that similar alterations of TORC1 and TORC2 activity are likely to have occurred in the *in vivo* transfer system using AND mice.

Enhanced TORC2 function decreases TCR signaling, leading to declines in CD4 T cell activation

We have reported that CD4 T cells from old mice exhibit defects in CD28 signaling that include the inability to enhance pAKT₍₃₀₈₎ levels and the *de novo* expression of Glut1 and CD69 (32). The decreased ICOS expression shown in Supplemental Figure 4 suggest that enhanced TORC2 signaling may lead to declines in CD4 T cell activation and CD28 signaling from young mice similar to those observed in CD4 T cells from old mice. To test this hypothesis, we performed experiments similar to those of Figure 5, except that the analysis of the CD4 T cells was performed at 24 hours after priming and thus before cell division had occurred. Control, vector alone-, or R+S-transfected CD4 T cells from the spleen and lymph nodes were analyzed for pAKT₍₃₀₈₎, Glut1, and CD69 levels as previously described (32). We excluded Rheb from this analysis because Rheb overexpression does not affect ICOS expression or proliferation, suggesting no effect on CD4 T cell activation (Figure 5). Figure 6A shows a typical analysis in which host mouse priming led to enhanced pAKT₍₃₀₈₎ levels and the *de novo* expression of Glut1 and CD69 in CD4 T cells, as we previously reported (32). In contrast, adoptively transferred CD4 T cells transfected with Rictor and Sin1 (R+S) showed defects in the antigen-dependent changes in pAKT₍₃₀₈₎, Glut1, and CD69 levels. To test whether these differences were statistically significant, we performed a series of 3 experiments. As shown in Figure 6B, we found that enhancing TORC2 signaling could significantly inhibit the expression of CD69 (lymph nodes, $p = 0.006$; spleen, $p = 0.01$), Glut1 (lymph nodes, $p = 0.002$; spleen, $p < 0.001$), and pAKT₍₃₀₈₎ (lymph nodes, $p = 0.03$; spleen, $p < 0.001$). These results suggest that enhanced TORC2 signaling leads to defects in the CD28 signaling pathways that mimic the effects of aging

(32) and support a model in which TORC2 is a key mediator in the age-related decline of CD4 T cell function in mice.

Discussion

In lymphocytes, tissue-specific TORC1 and TORC2 knockout mice show defects in CD4 T cell differentiation (11). Elimination of TORC2 function in cell lines has indicated that TORC2 regulates several important signaling pathways involved in cytoskeletal organization and apoptosis, including PKC function, transcription factor localization (49), and CD28-PI3K-AKT signaling pathways (perhaps by regulating phosphatase expression) (50, 51). However, the functional consequences of elevated TORC1 and TORC2 activity and their roles in aging remain unknown (52), with the exception of the association with cancer (53). We have previously shown that CD4 T lymphocytes exhibit alterations in the cytoskeleton, in TCR signaling, and in immune synapse formation that are associated with decreased pERM expression (29) and defects in CD28 signaling (32). Other investigators have reported declines in BIM expression (33). We hypothesized that some of these changes are modulated by central mechanism(s) such as TOR signaling. We tested this hypothesis by treating a genetically heterogeneous population of mice (UM-HET) with different doses of rapamycin, including a dose (14.7 ppm) already known to extend life (4). As expected, rapamycin prevented CD4 T cell differentiation (Supplemental Figure 1; (54), and these effects may be the result of TORC1 and/or TORC2 inhibition. There is evidence that rapamycin inhibition of TORC2 may be detrimental to some tissues and may impair glucose homeostasis (39). In contrast, our rapamycin data suggest that TOR inhibition can prevent key age-related changes in naïve and memory CD4 T cells, including increased pAKT₍₄₃₇₎ and pNDRG levels as well as declines in pERM and BIM expression (Figures 1 and 2). However, because mice were treated systematically with rapamycin it is still unclear whether these changes are due to direct effects on CD4 T cells or on other cell types such as APCs. Materials from rapamycin-treated and control mice were taken from mice included in a separate study designed to evaluate potential effects of this drug on mouse lifespan (34). One weakness of the protocol was the lack of control mice fed with microencapsulated mock alone. We think it is highly unlikely the material used for encapsulation (for composition see www.patent.ipexl.com/20120064143) could, without incorporated rapamycin, either extend life span or prevent age-related changes in lymphocytes, but we cannot at this point exclude the hypothesis that some of the effects we attribute to rapamycin might be caused by the encapsulation vehicle itself. We also note a recent report, using C57BL/6J mice, which suggested that rapamycin treatment could reverse some of the age-related changes in the immune system, including increases in CD44 high CD4 T cells from old mice (55), and also documenting age-independent effects in male C57BL/6 mice. Our data (see Figure S1) suggest similar conclusions: rapamycin prevented some of the age-related changes in CD4 T cells (specifically changes in CD44 high cells), but also produced effects on outcomes not usually altered by aging, such as the significant decline in CD4 T cell number.

In the other hand, our findings suggest that selective inhibition of TORC2 may have beneficial effects on preventing the age-related decline of immune function. This model could be tested once a specific inhibitor of TORC2 function becomes available that can be administered *in vivo* to old mice to reverse the age-related decline of immune function.

The rapamycin data and enhancement of TORC2 signaling in old mice (Figures 1 and 2) do not directly address the question of which TOR complex is involved in the age-associated decline of immune function. To test which TOR complex is involved, we transfected primary naïve CD4 T cells from young mice with Rheb to activate TORC1 signaling, or with Rictor plus Sin1 to activate TORC2 signaling (Figures 3 and 4 and Supplemental

Figures 2 and 3). Then, we measured the effects on pERM and BIM expression. As shown in Figure 4, enhancing TORC2, but not TORC1, signaling leads to significant declines in BIM and pERM. Single transfections with Sin1 or Rictor do not alter pERM or BIM expression in naïve CD4 T cells from young donors (data not shown). In addition, Sin1 transfection alone does not increase the phosphorylation of downstream targets of TORC2 (data not shown). On the other hand, transfection with Rictor alone induces some changes in pAKT and pNDRG, but these are inconsistent and do not reach statically significance (data not shown). The results suggest that activation of TORC2 may require the overexpression of both proteins. This is consistent with the literature suggesting that Rictor and Sin1 form a complex that associates with and stabilizes TORC2 leading to its activation (21, 44). The overall results suggest that some aspects of the age-related changes in CD4 T cells are regulated by TORC2, and support a model in which aging increases TORC2 activity, leading to changes in the function of CD4 T cells from old mice. This model may help to explain and expand our knowledge of how inhibition of each of these TOR complexes by rapamycin prevents some aspects of the age-related decline of immune function in CD4 T cells and other cell types (56–58) and extend life. Furthermore, it would be interesting to establish which specific TORC2 pathways are involved in the age-related changes in CD4 T cells, including alterations in PKC localization (59, 60), Rac and Rho-A signaling (30), phosphatases (61), as well as the regulation of transcription factors that may affect apoptosis and proliferation (62). However, the complexity of TORC2 signaling would require the use of complementary approaches, including microarray analysis and signaling pathway knockouts to test which specific mechanism(s) is involved. Such results would provide important clues as to how aging affects T lymphocytes.

In addition, we tested the biological significance of enhanced TORC2 signaling by studying *in vivo* CD4 T cell activation and proliferation. In this regard, using an *in vivo* model of adoptive transfer, we demonstrated that specific aspects of CD28 signaling in naïve CD4 T cells from old mice are defective, including defects in enhancing pAKT₍₃₀₈₎, Glut1, CD69, and ICOS expression (32). These defects lead to decreased CD4 T cell proliferation (31) that has been linked to declines in immune function (24) such as CD4 T cell-dependent antibody production (32, 63). We used this *in vivo* model of CD4 T cell function to test whether enhancement of TORC1 or TORC2 signaling may lead to similar defects in proliferation, CD28 signaling, and activation. As is shown in Figure 5, we found that enhancing TORC1 signaling does not seem to decrease the proliferation of naïve CD4 T cells from young mice. In contrast, as shown in Figures 5 and 6, enhancing TORC2 signaling leads to significant (30–40%) declines in the proliferation, CD28 signaling, and activation of naïve CD4 T cells. These decreases resemble those that we described in CD4 T cells of old mice, suggesting the involvement of TORC2 signaling in the decline of CD4 T cell function. A more extensive analysis of other markers of CD4 T cell activation will be needed to confirm this observation. In the other hand, because TOR is involved in lymphocyte differentiation and cytokine production (11), it is likely that enhancing TORC2 signaling may lead to changes in cytokine production. It would be interesting to further pursue this hypothesis by analyzing how enhancing TORC2 signaling might result in a pattern of cytokine expression that also resembles the aging phenotype of CD4 T cells (24), i.e., declines in IL-2 and defective memory formation (64). Prolonged activation of TOR can lead to a feedback loop which inactivates upstream activators of TOR (65). These may include elements of CD28 signaling pathways that affect TOR function in CD4 T cells. In principle, these might involve upstream TORC2-dependent regulation of PDK1/PI3K pathways or PTEN phosphatases (65), or other mechanisms still to be investigated. Unfortunately, the transient nature of Rictor and Sin1 expression in CD4 T cells after proliferation may preclude some of these experiments and analyses.

It would be interesting to evaluate whether TORC2 function is also altered in humans, particularly in CD4 T cells that show declines or defects in CD28 expression (66). We have shown that defects in CD28 signaling of murine CD4 T cells are similar to those observed in human CD4 T cells (32); thus, it would be interesting to test this hypothesis in CD28-deficient human CD4 T cells using our model. Furthermore, most of our work made use of CD4 T cells from young donors, and it would therefore be important to test the hypothesis that modulating TORC2 activity would also improve function of aged T cells. It is also possible, however, that manipulation of Rictor or Sin1 levels could alter aspects of T cell development and function (67–69) in addition to acute effects on T cell activation. Work along these lines to see if manipulation of TORC2 activity can prevent, or reverse, immune dysfunction is a major goal of our future studies. Nevertheless, the overall results presented herein suggest a novel model in which age-associated enhanced TORC2 signaling leads to defects of lymphocyte function in mice and likely humans as well.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

TOR	mammalian target of rapamycin
TORC1	mammalian target of rapamycin complex 1
TORC2	mammalian target of rapamycin complex 2
Rh	Rheb
R+S	Rictor plus Sin-1
Glut-1	glucose transporter type I

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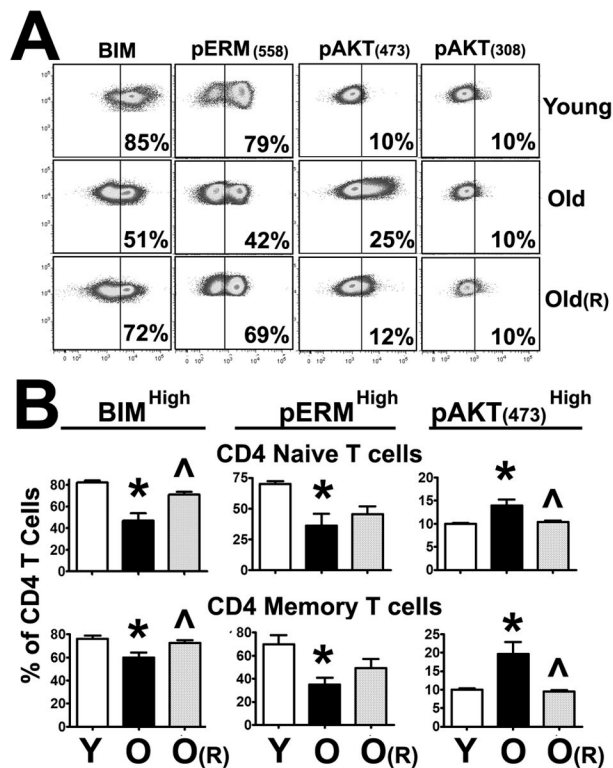


Figure 1. Long-term *in vivo* rapamycin treatment can prevent age-related changes in CD4 T cells
A) Spleen cells from young (Y), old (O), or rapamycin-treated (for 12 months) old mice (O_R) were stained for CD4 and CD44 surface markers and intracellular staining for pERM, BIM, pAKT₍₄₇₃₎, pAKT₍₃₀₈₎ was performed. Then, gated resting naïve CD4+CD44-Low and memory CD4+CD44-High cells were used to evaluate the effects of age and rapamycin. The vertical line shows the threshold used to distinguish high expression of BIM, pERM, and pAKT₍₄₇₃₎ cells for the calculations illustrated in the panel. **B)** Quantification of the age-related changes in at least 6 experiments, including a total of 12 young, 12 old, and 12 rapamycin-treated mice. The values represent the mean ± SEM. The (*) indicates statistically significant age-related changes relative to the CD4 T cells controls from young mice, while the (^) represents statistically significant effects of rapamycin preventing the age-related changes relative to CD4 T cells from old mice.

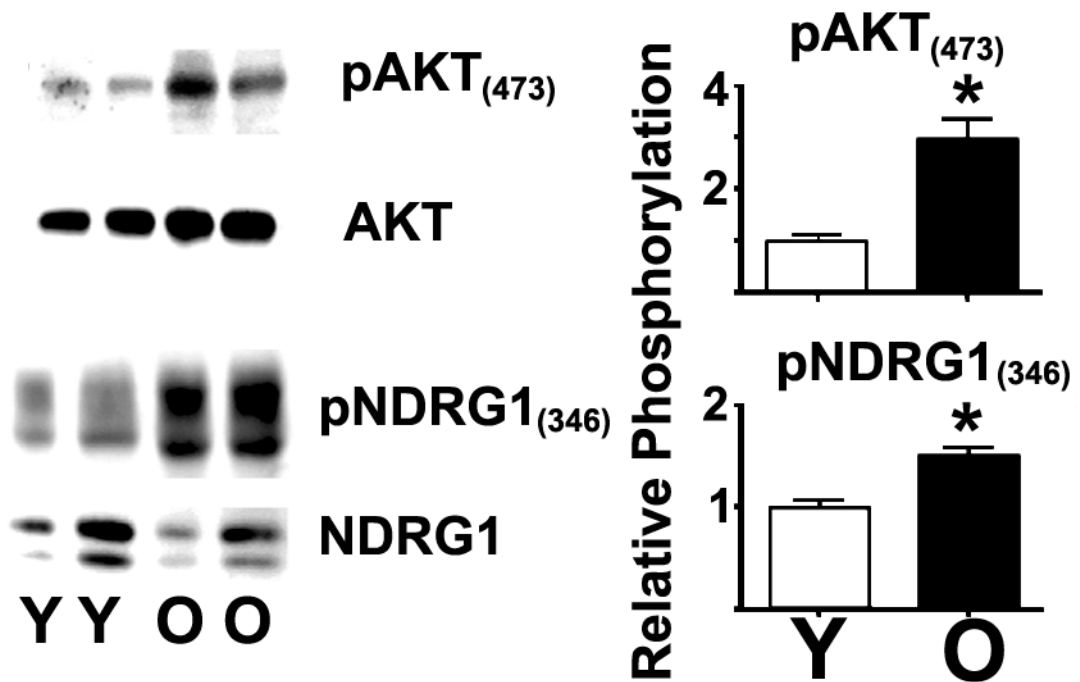


Figure 2. Phosphorylation of downstream TORC2 signaling molecules increases with age in CD4 T cells

A) Representative western blot analysis of the pAKT₍₄₇₃₎ and pNDRG1₍₃₄₅₎ levels and the expression levels of AKT and NDRG1 in resting CD4 T cells from the spleens of young (Y) or old (O) CB6F1 mice. **B)** Quantification of the age-related changes from 8 independent experiments, including a total of 12 young and 8 old mice. The values represent the mean \pm SEM, and (*) indicates statistically significant age-related changes relative to CD4 T cell controls from young mice (Y).

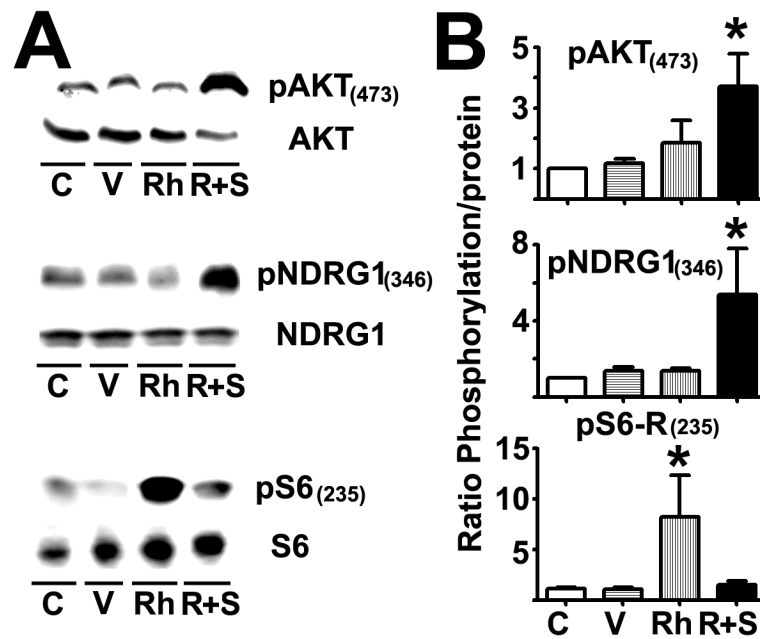


Figure 3. Transfection with Rheb and cotransfection with Rictor plus Sin1 can upregulate mTORC1 and mTORC2 signaling, respectively

A) CD4 T cells from young CB6F1 mice remained untransfected (C) or were transfected with vector (V), Rheb (Rh), or Rictor and Sin1 (R+S) as described in the Methods section. Live cells were lysed and the aliquots were analyzed by western blotting using pAKT₍₄₇₃₎, pNDRG1₍₃₄₆₎, and pS6-R₍₂₃₅₎ antibodies or the respective total proteins. **B)** Quantification of the relative phosphorylation levels from 4 independent experiments. The values represent the mean \pm SEM, and (*) indicates statistically significant changes with respect to controls (C).

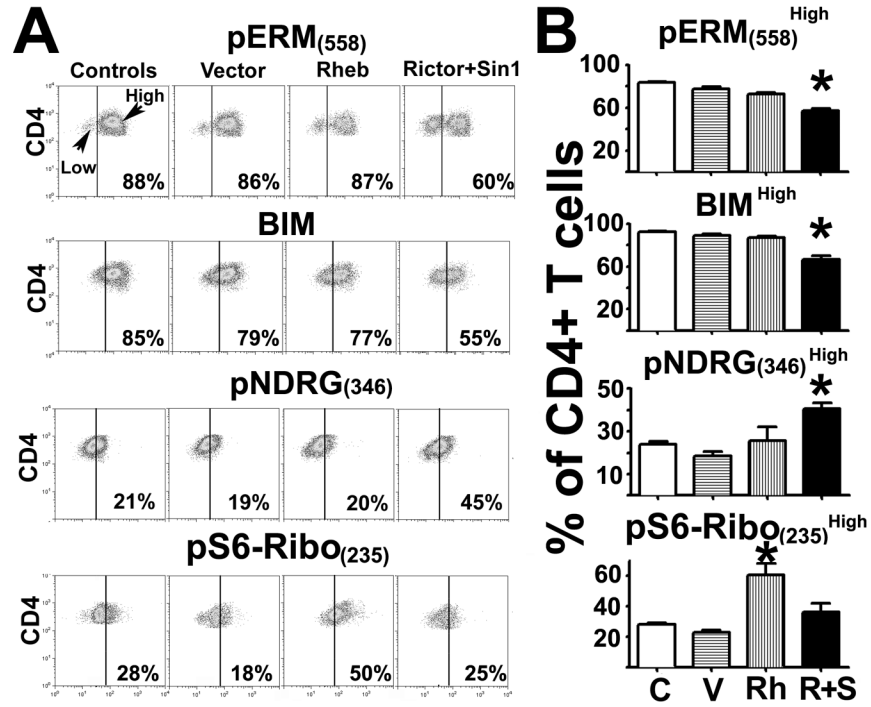


Figure 4. Upregulation of TORC2, but not TORC1, signaling leads to declines in BIM and pERM expression

A) Representative flow cytometric analysis measuring pERM₍₅₅₈₎, BIM, pNDRG₍₃₄₆₎, and pSR-R₍₂₃₆₎ levels in gated live CD4 T cells either untransfected (C) or transfected with vector, Rheb, or Rictor plus Sin1. The vertical line represents the threshold used to distinguish between high or low levels of the respective phosphoproteins and protein expression used for quantification. **B)** Quantification of the relative expression levels from 8 independent experiments. The values represent the mean ± SEM, and (*) indicates statistically significant changes with respect to controls.

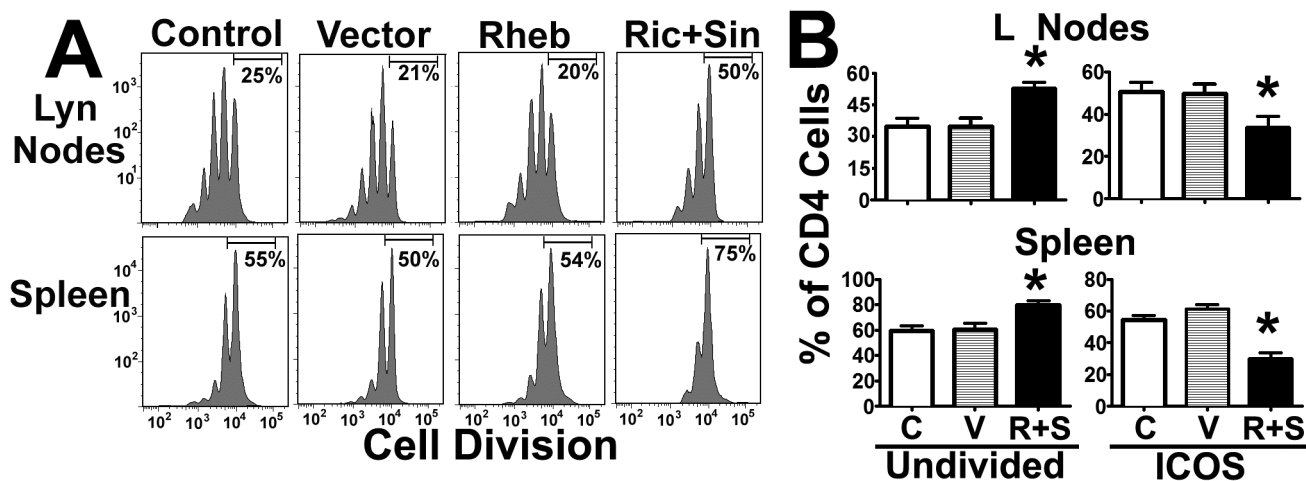


Figure 5. Upregulation of TORC2, but not TORC1, signaling leads to *in vivo* declines in CD4 T cell proliferation and ICOS expression in response to antigen priming

A) CD4 T cells from young AND mice were stained with CFSE or violet cell division tracker, either untransfected (Control, C) or transfected with vector (V), Rheb (Rh), or Rictor plus Sin1 (R+S), and were adoptively transferred into CD4KO host mice as described in the Methods section. Forty-eight hours after priming, the spleens and lymph nodes were collected and cellular proliferation and ICOS expression were analyzed by flow cytometry. The histogram shows a representative experiment of each group with the percentages of non-dividing CD4 T cells. **B)** Quantification of the relative proliferation and expression of ICOS from 3 independent experiments. The values represent the mean \pm SEM, and (*) indicates statistically significant changes with respect to controls.

