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Increased Mammalian Target of Rapamycin Complex 2 Signaling Promotes Age-Related Decline in CD4 T Cell Signaling and Function

Eric Perkey,* Diane Fingar,† Richard A. Miller,‡§ and Gonzalo G. Garcia‡

CD4 T cell function declines significantly during aging. Although 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 naive CD4 T cells from young mice, we demonstrated that increased TORC2, but not TORC1, signaling results in age-associated biochemical changes. Furthermore, elevated TORC2 signaling in naive CD4 T cells from young mice leads to in vivo functional declines. The data presented in this article suggest a novel model in which aging increases TORC2 signaling and leads to CD4 T cell defects in old mice. The Journal of Immunology, 2013, 191: 4648–4655.

Data from diverse model organisms suggest that the mammalian target of rapamycin (TOR) is a critical controller of aging (1–3), and rapamycin treatments can extend the life span of mice (4). TOR, a serine/threonine kinase, forms the catalytic core of at least two 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 Ser235 (pS6[235]), a component of the 40 S ribosomal subunit that mediates translation (9). In lymphocytes, TCR/CD28 signaling leads to TORC1 activation via Rheb and AKT phosphorylation at Thr308 (p-AKT[308]) via PDK1 pathway (10); these events regulate CD4 T cell activation and differentiation of naive CD4 T cells into Th1 and Th17 phenotypes (11). In contrast, TORC2 regulates naive CD4 T cell differentiation into a Th2 phenotype (11, 12), and it also regulates actin polymerization (13), cell size (14), Rac-GTPase and RhoA-GTPase activity (15, 16), and protein kinase C (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 TORC2 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 (serum glucocorticoid kinase 1) at Ser422 (pSGK1[422]), and the N-myc downstream-regulated (pNDRG1) gene at Thr346 (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 prosapoptic 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. In this study, 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.

Materials and Methods

Animals, reagents, and rapamycin treatments

H-2(k/k) TCR-Vα1/Vβ3, 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, Saranac Lake, NY). Specific pathogen-free B10.BR and CB6F1 (BALB/c × C57BL/6) mice were purchased from the Charles River...
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Laboratories (Kingston, ND) and from the National Institute of Aging contract colonies at Harlan (Indianapolis, IN), respectively. The genetically hetero-
geneous mouse population (UM-HET3), generated by crossing CB6F1 females with C3D2F1 males (34), was also bred in our facilities. All ex-
periments 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 mo of age; B10.BR or CD4KO adoptive host mice were 2–3 mo 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 (http://www.
sigmaaldrich.com). CFSE, violet blue cell division trackers, and rabbit poly-
clonal anti-HA were purchased from Invitrogen (http://www.invitrogen.com). Flow cytometry Abs to surface CD4 T cells molecules were purchased from Biologend (http://www.biologend.com) or Becton Dickson (www.bdbiosciences.
com). Rabbit polyclonal anti-My and monoclonals anti-Sin1, anti-mTOR, and anti-Rictor were purchased from Millipore (http://www.millipore.com). Rabbit anti–glucose transporter type I (anti-Glut1) was from Epitomics (http://
www.epitomics.com). Protein Abs, phospho-specific rabbit monoclonal
Abs, and rabbit polyclonal anti-Flag Abs were purchased from Cell Signa-
thing (http://www.cellsignal.com), with the exception of pSGK1(422) and
NDRG, which were obtained from Santa Cruz (http://www.scbt.com). Anti-
rabbit FITC-conjugated Abs were obtained from Jackson Immunoresearch
(http://www.jacksonimmuno.com). Plasmids corresponding to the human
pR5K-Rheb-Flag, pR5K-Rictor-HA, pR5K-Sin1-Myc, and pRK5 stocks were
donated by Diane Fingar (Universitously ichigan) and purifi-
ated with Qiagen endotoxin-free purification kits (http://www.qiagen.com).
Rapamycin treatments were performed as previously described (4, 34) by
mixing microencapsulated rapamycin at different doses in Purina 5LG6
chow as described earlier 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 else-
where (30).

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

Naive 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 (http://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 dif-
ferent plasmids by electroporation using the Amaza Nucleofector mouse
CD4 cell transfection kit (http://www.lonza.com) as described elsewhere
(35, 36). In each experiment, we performed electroporation of the following
constructs: 5 μg pR5K vector [V], 5 μg pR5K-Rheb-Flag (Rh), or a mix of
5 μg pR5K-Rictor-HA plus 5 μg pR5K-Sin1-Myc. Transfection efficiency
controls were performed for each experiment using Pmax-GFP as re-
commended by the Amaza Nucleofector systems. Then, cells were cultured
in complete RPMI 1640 medium, and after 24 h in the dead cells and
debri 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 Abs 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 naive CD4 T cells from young AND mice were transfected
with the different plasmids according to the methods described earlier.
Then, electroporated CD4 T cells (V, Rh, and Rictor plus Sin1 [R+S]) were
labeled with violet blue cell division tracker following the manufacturer’s
recommendations (http://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 (V, Rh, and R+S) were
mixed with 1 × 10^6 untransfected CD4 T cells (C) and injected into the
tail veins of C4DKO host mice. Twenty-four hours later, the mice were
prized with 10 μg pigeon cytochrome C (PCC) in 100 μl PBS, and the spleen
and lymphoid organs were harvested at the indicated time points after
analysis. 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 ± SEM. Sta-
tistical significance was assessed using Mann–Whitney U 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 life span of lower organisms and
mice (3, 37). Although 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 associ-
ated with declines in TCR signaling, immune synapse formation,
and activation (23, 30). Other laboratories have documented age-
related declines in the expression of the proapoptotic protein BIM (33).
Because these proteins are regulated by TOR, we hypothe-
sized 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-HET3) with rapamycin starting at 9 mo of age. The
UM-HET3 mice were given either control chow (Purina 5LG6) or
chow containing three different doses of rapamycin for 12 mo (high:
42 ppm; middle: 14.7 ppm; low: 4.7 ppm) as described previously
(34). Then, we harvested the spleens from 9-mo-old (young [Y]),
untreated 22-mo-old (old [O]), and rapamycin-treated 22-mo-old
(OS) 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 naive
and memory CD4 T cells using CD44 and CD62L differentiation
markers (40). A typical analysis is shown in Fig. 1A. We found
that aging decreases the number of CD4 T cells expressing high
levels of BIM and pERM(558) when compared with CD4 T cells
from young mice (Fig. 1A, first two columns). Interestingly,
untreated CD4 T cells from old mice showed more cells with higher
levels of pAKT(473), but aging had no effect on pAKT(308) (Fig.
1A, Y versus O). All doses of rapamycin led to a decline in the
expression of CD4CD8 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 Fig. 1A). These results
correspond well with those in the literature demonstrating that
rapamycin affects CD4 T cell differentiation (41). However, as
shown in Fig. 1A, rapamycin treatment led to partial, although not
complete, prevention of the declines in BIM and pERM expres-
sion, and increased pAKT(473) in both naive 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 Fig. 1B, 12-mo rapamycin treatment, at 14.7
ppm, seems to prevent some of the BIM declines in both naive
(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 Fig. 1). Furthermore, rapamycin also appeared
to prevent declines in pERM expression in both naive and memory
CD4 T cells. However, statistical significance was achieved only at
the high dose (see Supplemental Fig. 1). Furthermore, the age-related
increase in pAKT(473) was significant in both naive (p = 0.005)
and memory (p = 0.04) CD4 T cells (Fig. 1B, Y versus O). Long-term
rapamycin treatment can inhibit TORC2 function (2); as expected,
we observed that rapamycin treatment prevented the age-related increase in pAKT(473) (naive: \( p = 0.007 \), memory: \( p = 0.001 \)) to near to the levels observed in CD4 T cells from young donors (Fig. 1B, O versus O\(_{R}\)). In contrast, we found no significant age- or rapamycin treatment–associated changes (example in Fig. 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 Fig. 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(345), pAKT(473), and pSGK1(442), using Western blotting in highly purified CD4 T cells from the spleens of young (6–8 mo) and old (22–24 mo) CB6F1 mice. Fig. 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, Fig. 2 shows the statistical analysis of the relative ratios of at least 6 independent experiments, including a total of 12 young and 8 old mice. Values represent the mean ± SEM. Asterisks indicate statistically significant age-related changes relative to CD4 T cell controls from young mice (Y).

We observed a 30–50% transfection efficiency of CD4 T cells for all constructs (Fig. 1B, O versus O\(_{R}\)). In contrast, we found no significant age- or rapamycin treatment–associated changes (example in Fig. 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 Fig. 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(345), pAKT(473), and pSGK1(442), using Western blotting in highly purified CD4 T cells from the spleens of young (6–8 mo) and old (22–24 mo) CB6F1 mice. Fig. 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, Fig. 2 shows the statistical analysis of the relative ratios of at least 6 independent experiments, including a total of 12 young and 8 old mice. We found that aging significantly increases the relative level of pAKT(473) (\( p = 0.001 \)) and pNDRG1(345) (\( p = 0.0003 \)). We also analyzed pSGK1(442) and found a similar 2-fold age-related increase (\( p = 0.0002 \)); however, because this Ab may recognize other substrates in the same m.w. 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 Fig. 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.

Data from Figs. 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), whereas we enhanced TORC2 activity by cotransferring CD4 T cells with Rictor and Sin1 proteins. The latter method was based on data suggesting that using R+S 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 later). After electroporation, untransfected and transfected CD4 T cells from young donors were incubated for 24 h. 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: 1) Flag for Rheb, 2) HA for Rictor, and 3) 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 ~20–30% cell death (Supplemental Fig. 2). After removing the dead cells, around 10 \( \times 10^6 \) CD4 T cells from each group were lysed and analyzed by Western blotting using specific Abs for pAKT(473) and pNDRG1(346), which are downstream targets of TORC2, and pS6(235), which is a downstream target of TORC1. As shown in Fig. 3A, compared with C or V, Rheb (Rh) overexpression significantly enhanced pS6(235) levels (\( p = 0.03 \)) without affecting mTORC2 targets. In contrast, 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

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

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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. Values represent the mean ± SEM. Asterisks indicate statistically significant age-related changes relative to CD4 T cell controls from young mice (Y).
TORC2 activity in naive CD4 T cells (see Supplemental Fig. 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 Fig. 3. However, to facilitate the measurement of multiple TORC downstream targets in the same samples, we performed intracellular staining as described in Fig. 1. A typical flow cytometric analysis of untransfected (C) and transfected groups (V, Rh, and R+S) is shown in Fig. 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 (Fig. 4A, middle panels). In contrast, overexpression of R+S decreased the number of CD4 T cells expressing BIM and pERM, while enhancing pNDRG1 (346) levels (Fig. 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 six independent experiments as shown in Fig. 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 R+S overexpression has no significant effect on the expression of endogenous ERM, NDRG1, and S6 proteins (data not shown), suggesting that the earlier 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 Fig. 1) is responsible for preventing age-related declines in CD4 T cell function in rapamycin-treated mice.

**FIGURE 3.** Transfection with Rheb and cotransfection with R+S can upregulate mTORC1 and mTORC2 signaling, respectively. (A) CD4 T cells from young CB6F1 mice remained untransfected (C) or were transfected with V, Rheb (Rh), or R+S as described in *Materials and Methods*. Live cells were lysed and the aliquots were analyzed by Western blotting using pAKT (473), pNDRG1 (346), and pS6-Rib(Ribo)(235) Abs or the respective total proteins. (B) Quantification of the relative phosphorylation levels from four independent experiments. Values represent the mean ± SEM. Asterisks indicate statistically significant changes with respect to controls (C).

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

To test the hypothesis that enhanced TORC1 or TORC2 signaling affects the in vivo function of CD4 T cells from young mice 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 naive 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 naive CD4 T cells from the spleens of young AND mice (4 mo), and aliquots of 5 × 10⁶ CD4 T cells were labeled with a CFSE cell division tracker. In addition, aliquots of 20 × 10⁶ CD4 T cells were labeled with a violet cell division tracker. After labeling, ~6 × 10⁶ violet-labeled CD4 T cells were electroporated with each of the constructs (vector, Rh, and R+S). Then, 2 × 10⁶ live CD4 T cells from each transfection group were mixed with 1 × 10⁶ untransfected cells (CFSE controls, C) and adoptively transferred to CD4KO young hosts (2–4 mo). 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 among multiple hosts. Twenty-four hours after adoptive transfer, each host mouse was primed with 10 µg 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). Fig. 5A shows a typical profile of CD4 T cell division for the untransfected or vector-alone groups, where ~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 R+S increased the number of nondividing 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 three independent experiments with at least two host mice receiving cells from each of the transfection groups (control/vector, control/Rh, control/R+S) per experiment. As shown in Fig. 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 R+S leads to a significant (30–40%) increase in the number of nondividing 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 during immune responses (47). As shown in Fig. 5B, R+S 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 Fig. 4A). We also found that most of the decreased ICOS expression occurred in the nondividing 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 Fig. 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 Fig. 4B). The proliferation differences between the control and R+S-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 Fig. 4C, 4D) suggested no differences in the efficiency of transfection and tag expression compared with that described for our in vitro experiments using the CB6F1 model (Supplemental Fig. 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 Fig. 4 suggests 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 Fig. 5, except that the analysis of the CD4 T cells was performed at 24 h 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 (Fig. 5). Fig. 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 R+S showed defects in the Ag-dependent changes in pAKT(308), Glut1, and CD69 levels. To test whether these differences were statistically significant, we performed a series of three experiments. As shown in Fig. 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 KO 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 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-HET3) with different doses of rapamycin data suggest that TOR inhibition can prevent key age-related changes in naive and memory CD4 T cells, including in —pERK and BIM expression (Figs. 1, 2). However, because mice were treated systemically 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 life span (34). One weakness of the protocol was the lack of control mice fed with microencapsulated mock alone. We 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/6j mice. Our data (see Supplemental Fig. 1) 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 contrast, 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 (Figs. 1, 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 naive CD4 T cells from young mice with Rheb to activate TORC1 signaling, or with R+S to activate TORC2 signaling (Figs. 3, 4, Supplemental Figs. 2, 3). Then, we measured the effects on pERM and BIM expression. As shown in Fig. 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 naive 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). In contrast, transfection with Rictor alone induces some changes in pAKT and pNDRG, but these are inconsistent and do not reach statistical 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 extends 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 KOs 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 naive 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 Ab 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 shown in Fig. 5, we found that enhancing TORC1 signaling does not seem to decrease the proliferation of naive CD4 T cells from young mice. In contrast, as shown in Figs. 5 and 6, enhancing TORC2 signaling leads to significant (30–40%) declines in the proliferation, CD28 signaling, and activation of naive 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 contrast, 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), that is, declines in IL-2 and defective memory formation (64). Prolonged activation of TOR can lead to a feedback loop that 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/PDK3 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 aging 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 CD4 T cells from aged human donors (32); thus, it would be interesting to test this hypothesis in CD4-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 those observed in CD4 T cells from aged human donors (32); in contrast, 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), that is, declines in IL-2 and defective memory formation (64). Prolonged activation of TOR can lead to a feedback loop that 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/PDK3 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.

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Disclosures

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References


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