

mTOR inhibition improves immune function in the elderly

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Inhibition of the mammalian target of rapamycin (mTOR) pathway extends life span in all species studied to date, and in mice delays the onset of age-related diseases and comorbidities. However, it is unknown if mTOR inhibition affects aging or its consequences in humans. To begin to assess the effects of mTOR inhibition on human aging-related conditions, we evaluated whether the mTOR inhibitor RAD001 ameliorated immunosenescence (the decline in immune function during aging) in elderly volunteers, as assessed by their response to influenza vaccination. RAD001 enhanced the response to the influenza vaccine by about 20% at doses that were relatively well tolerated. RAD001 also reduced the percentage of CD4 and CD8 T lymphocytes expressing the programmed death-1 (PD-1) receptor, which inhibits T cell signaling and is more highly expressed with age. These results raise the possibility that mTOR inhibition may have beneficial effects on immunosenescence in the elderly.

INTRODUCTION

Increasing evidence suggests that aging is a regulated process, and its course can be modified by modulation of signal transduction pathways. Perturbation of particular pathways can extend life span and ameliorate aging-related phenotypes in multiple species, including mammals (1). One of the pathways most clearly linked to aging is the mammalian target of rapamycin (mTOR) pathway. The mTOR inhibitor rapamycin has extended the life span of mice by 9 to 14% even when treatment was initiated late in life (2). Rapamycin also improved a variety of aging-related conditions in old mice, including tendon stiffening, cardiac dysfunction, cognitive decline, and decreased mobility (3, 4). These findings raise the possibility that mTOR inhibitors may have beneficial effects on aging and aging-related conditions in humans.

Clinical studies of aging in humans are difficult owing to the long periods over which aging-related conditions progress. However, an aging-related phenotype that can be studied in a shorter clinical trial time frame is immunosenescence. Immunosenescence is the decline in immune function that occurs in the elderly, leading to an increased susceptibility to infection and a decreased response to vaccination, including influenza vaccination. Adults 65 years of age and older account for 90% of influenza-related deaths in the United States and also have a lower antibody response to influenza vaccination as compared to younger adults (5, 6). The decline in immune function with age is due to an accumulation of immune defects, including a decrease in the ability of hematopoietic stem cells (HSCs) to generate naïve lymphocytes, and an increase in the numbers of “exhausted” programmed death-1 (PD-1)-positive T lymphocytes that have diminished responses to antigenic stimulation (7–9). In elderly mice, 6 weeks of treatment with the mTOR inhibitor rapamycin rejuvenated HSC function, leading to increased production of naïve

lymphocytes, an improved response to influenza vaccination, and extended life span (10). On the basis of these findings, we investigated whether a 6-week treatment with the mTOR inhibitor RAD001, an analog of rapamycin, improved the response to influenza vaccination in elderly volunteers.

RESULTS

A total of 218 elderly volunteers ≥ 65 years of age, without unstable medical conditions, were enrolled in a randomized observer-blind, placebo-controlled trial at nine sites in New Zealand and Australia. Subjects were randomly assigned to receive placebo or one of three doses of oral RAD001: 0.5 mg daily, 5 mg weekly, or 20 mg weekly. Subjects were treated for 6 weeks with the study drug and, after a 2-week drug-free interval, were given a 2012 seasonal influenza vaccine. Antibody titers to the three strains of influenza virus in the influenza vaccine as well as to two heterologous influenza virus strains not contained in the vaccine were measured in serum collected at baseline and 4 weeks after influenza vaccination.

Baseline demographics between the treatment arms were similar (table S1). Of the 218 subjects enrolled, 211 completed the study. Seven subjects withdrew from the study. Five subjects withdrew because of adverse events, one subject withdrew consent, and one subject left the study as a result of a protocol violation.

In general, RAD001 was relatively well tolerated, particularly the 0.5 mg daily and 5 mg weekly dosing regimens. No deaths occurred during the study. Three subjects experienced four serious adverse events that were assessed as unrelated to RAD001. The four serious adverse events were retinal hemorrhage of the left eye with subsequent blindness in a subject with normal platelet counts who had completed a 6-week course of 5 mg weekly RAD001 approximately 6 weeks prior to the onset of these adverse events, severe back pain in a subject treated with placebo, and severe gastroenteritis in a subject treated with placebo. A list of treatment-related adverse events with an incidence of $>2\%$ in any treatment group is provided in Table 1. The most common RAD001-related adverse events were mouth ulcers that were mild in most cases. Overall, subjects who received RAD001 had a similar incidence of severe

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Table 1. Incidence of treatment-related adverse events >2% in any treatment group.

	RAD001, 0.5 mg daily (N = 53)	RAD001, 5 mg weekly (N = 53)	RAD001, 20 mg weekly (N = 53)	Placebo, pooled (N = 59)	Total (N = 218)
	<i>n</i> (%)				
Total adverse events	35	46	109	21	211
Subjects with adverse events	22 (41.5)	20 (37.7)	27 (50.9)	12 (20.3)	81 (37.2)
Mouth ulceration	6 (11.3)	2 (3.8)	9 (17.0)	3 (5.1)	20 (9.2)
Headache	0	2 (3.8)	9 (17.0)	1 (1.7)	12 (5.5)
Blood cholesterol increased	2 (3.8)	2 (3.8)	2 (3.8)	0	6 (2.8)
Diarrhea	1 (1.9)	4 (7.5)	1 (1.9)	0	6 (2.8)
Dyspepsia	0	3 (5.7)	2 (3.8)	1 (1.7)	6 (2.8)
Fatigue	0	2 (3.8)	4 (7.5)	0	6 (2.8)
Low-density lipoprotein increased	2 (3.8)	1 (1.9)	2 (3.8)	0	5 (2.3)
Tongue ulceration	3 (5.7)	1 (1.9)	0	1 (1.7)	5 (2.3)
Insomnia	1 (1.9)	2 (3.8)	1 (1.9)	0	4 (1.8)
Dry mouth	0	0	2 (3.8)	1 (1.7)	3 (1.4)
Neutropenia	0	0	3 (5.7)	0	3 (1.4)
Oral pain	0	2 (3.8)	1 (1.9)	0	3 (1.4)
Pruritus	0	2 (3.8)	1 (1.9)	0	3 (1.4)
Conjunctivitis	0	2 (3.8)	0	0	2 (0.9)
Erythema	0	2 (3.8)	0	0	2 (0.9)
Limb discomfort	0	2 (3.8)	0	0	2 (0.9)
Mucosal inflammation	0	0	2 (3.8)	0	2 (0.9)
Paresthesia (oral)	2 (3.8)	0	0	0	2 (0.9)
Stomatitis	0	0	2 (3.8)	0	2 (0.9)
Thrombocytopenia	0	0	2 (3.8)	0	2 (0.9)
Urinary tract infection	0	0	2 (3.8)	0	2 (0.9)

adverse events as those treated with placebo. Only one severe adverse event was assessed as related to RAD001: mouth ulcers in a subject treated with 20 mg weekly RAD001.

The ability of RAD001 to improve immune function in elderly volunteers was evaluated by measuring the serologic response to the 2012 seasonal influenza vaccine. The increase in hemagglutination inhibition (HI) titers per subject and the geometric mean titers (GMTs) to each of the three influenza vaccine strains at 4 weeks after influenza vaccination as compared to baseline are shown in fig. S1 and table S2. The HI titer measures the concentration of antibodies to the hemagglutinin protein of the influenza virus. The HI titer correlates with protection against influenza illness. HI titer values are expressed as geometric means rather than averages because averages may be biased by a few very high titer values. The primary analysis variable was the HI GMT ratio (4 weeks after vaccination/baseline). The study was powered to be able to demonstrate that in at least two of three influenza vaccine strains, there was a ≥ 1.2 -fold GMT ratio increase relative to placebo and a posterior probability of at least 80% that the placebo-corrected GMT ratio exceeded 1.0. This endpoint was chosen because an about 1.2-fold increase in the influenza GMT ratio induced by the MF59 vaccine adjuvant was associated with a decrease in influenza illness (11).

In the intent-to-treat population, the low-dose RAD001 (0.5 mg daily or 5 mg weekly) cohorts, but not the higher-dose (20 mg weekly) cohort, met the primary endpoint of the study (Fig. 1A). Modeling and simulation based on mTOR-mediated phosphorylation of its downstream target S6 kinase (S6K) predicted that the 20 mg weekly dosing regimen inhibited mTOR-mediated S6K phosphorylation almost completely, the 5 mg weekly dosing regimen inhibited S6K phosphorylation by more than 50%, and the 0.5 mg daily dosing regimen inhibited S6K phosphorylation by about 38% over the dosing interval (12). Thus, partial inhibition of mTOR-mediated S6K phosphorylation achieved with a relatively low dose of RAD001 may be as effective as nearly complete inhibition associated with high-dose RAD001 at enhancing the immune response of the elderly volunteers.

In a subgroup analysis, the subset of subjects with low baseline influenza titers ($\leq 1:40$) experienced a greater RAD001-associated increase in titers than did the entire intent-to-treat population (Fig. 1B). These data suggested that RAD001 may have been particularly effective at enhancing the influenza vaccine response of subjects who did not have protective ($>1:40$) titers at baseline and therefore were at greater risk of influenza illness.

Rates of seroconversion 4 weeks after influenza vaccination also were evaluated. Seroconversion was defined as the change from a negative prevaccination titer (that is, HI titer $<1:10$) to a postvaccination HI titer

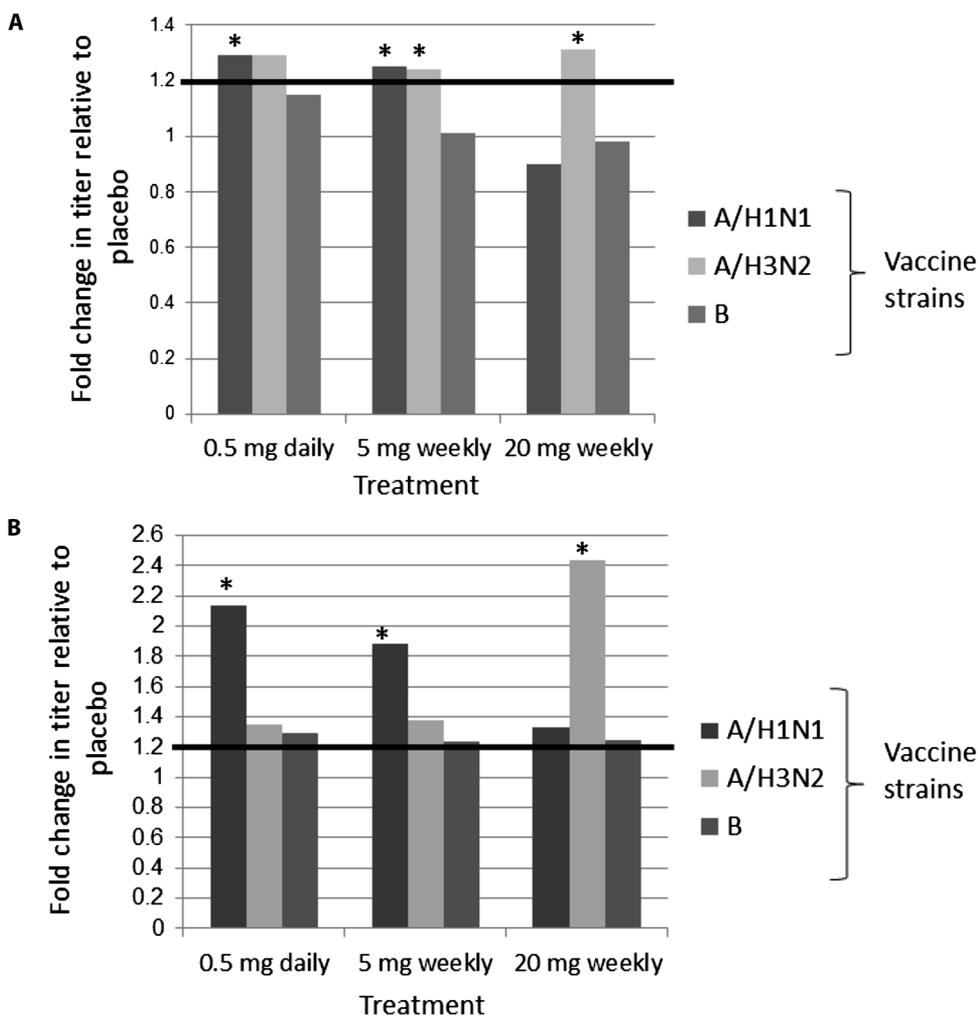


Fig. 1. Increase in antibody titers to influenza vaccine strains in RAD001-treated versus placebo cohorts. (A) Increase above baseline in GMTs to each of the three influenza vaccine strains (H1N1 A/California/07/2009, H3N2 A/Victoria/210/2009, and B/Brisbane/60/2008) 4 weeks after vaccination in RAD001-treated cohorts relative to the placebo cohort. The fold changes in GMTs relative to the placebo cohort are shown for all of the RAD001 dosing cohorts in the intent-to-treat population. The bold black line indicates the 1.2-fold increase in titers relative to placebo that is required for two of three influenza vaccine strains in order to meet the primary endpoint of the study. Asterisks indicate that the increase in GMT relative to placebo exceeds 1.0 with posterior probability of at least 80%. (B) The same data as in (A) are shown for the subset of subjects with baseline influenza titers $\leq 1:40$.

$\geq 1:40$ or at least a fourfold increase from a nonnegative ($\geq 1:10$) prevaccination HI titer. In the intent-to-treat population, seroconversion rates for the H3N2 and B influenza strains were increased in the RAD001-treated cohorts as compared to the placebo cohorts, although the increases did not meet statistical significance (Table 2). In the subpopulation of subjects with baseline influenza titers $\leq 1:40$, RAD001 treatment also increased the rates of seroconversion to the H3N2 and B influenza strains, and these results reached statistical significance for the B strain in the 0.5 mg daily dosing cohort (Table 2).

Current seasonal influenza vaccines often provide inadequate protection against continuously emerging strains of influenza virus that present as variants of previously circulating viruses. However, mice vaccinated against influenza in the presence of the mTOR inhibitor rapamycin, as compared to placebo, developed a broader

serologic response to influenza (13). The broader serologic response included antibodies to conserved epitopes expressed by multiple subtypes of influenza that provided protection against infection with heterologous strains of influenza virus not contained in the vaccine (13). To determine whether RAD001 treatment broadened the serologic response to influenza in the elderly volunteers, we measured HI titers to two heterologous strains of influenza not contained in the seasonal influenza vaccine (A/H1N1 strain A/New Jersey/8/76 and A/H3N2 strain A/Victoria/361/11). The increase in the HI GMT ratio (4 weeks after vaccination/baseline) for the heterologous strains was higher in the RAD001-treated cohorts compared to the placebo cohort (Fig. 2). In addition, seroconversion rates for the heterologous strains were higher in the RAD001-treated cohorts compared to the placebo cohort. The increase in seroconversion rates in the 5 and 20 mg RAD001 weekly dosing cohorts was statistically significant for the H3N2 heterologous strain (Table 3). The H3N2 seroconversion rate for the pooled RAD001 cohorts was 39% versus 20% for the placebo cohort ($P = 0.007$).

To explore the mechanism by which RAD001 enhanced immune function in elderly volunteers, we performed immunophenotyping on peripheral blood mononuclear cell (PBMC) samples obtained from subjects at baseline, after 6 weeks of drug treatment, and 4 weeks after influenza vaccination (that is, 6 weeks after study drug discontinuation). Although the percentage of most PBMC subsets did not differ between the RAD001-treated and placebo cohorts, the pooled RAD001 cohort had a significantly lower percentage of PD-1-positive CD4 ($P = 0.03$) and

CD8 T cells ($P = 0.008$) compared to the placebo cohort after 6 weeks of study drug treatment (Fig. 3).

DISCUSSION

The data presented here suggest that the mTOR inhibitor RAD001 ameliorated the age-related decline in immunological function of human elderly volunteers as assessed by response to influenza vaccination, and that this amelioration was obtained with an acceptable risk/benefit balance. In a study of elderly mice, treatment with the mTOR inhibitor rapamycin for 6 weeks not only enhanced the response to influenza vaccination but also extended life span (10). Although that was a small preclinical study that needs to be replicated, those

Table 2. Percentages of subjects with seroconversion to influenza 4 weeks after vaccination.

	Placebo (N = 54)	0.5 mg (N = 48)	5 mg (N = 49)	20 mg (N = 48)
Intent-to-treat population (%)				
H1N1	24	27	27	17
H3N2	17	27	24	25
B	17	27	22	19
Subjects with baseline titers ≤40 (%)				
H1N1	40	42	45	36
H3N2	42	64	53	71
B	16	40*	33	28

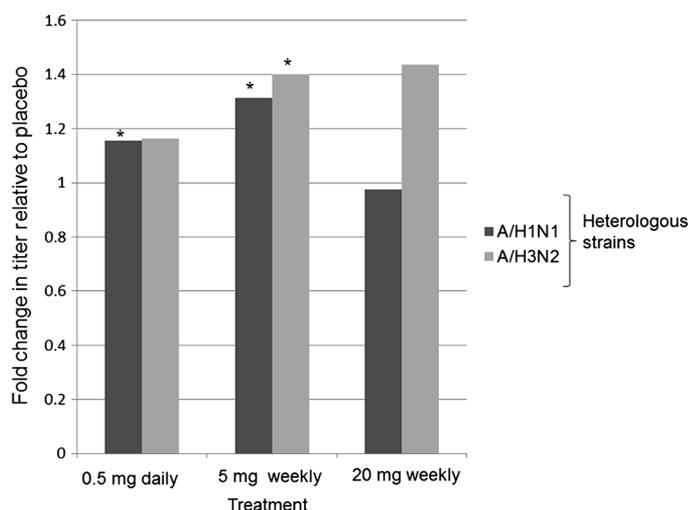


Fig. 2. Increase in antibody titers to heterologous influenza strains in RAD001-treated versus placebo cohorts. Increase above baseline in the GMTs to two heterologous influenza strains (A/H1N1 strain A/New Jersey/8/76 and A/H3N2 strain A/Victoria/361/11) that were not contained in the seasonal influenza vaccine for RAD001-treated relative to placebo cohorts, 4 weeks after vaccination. Asterisks indicate that the increase in GMT relative to placebo exceeded 1.0 with a posterior probability of at least 80%.

Table 3. Percentages of subjects who seroconverted to heterologous strains of influenza 4 weeks after receiving the seasonal influenza vaccine.

	Placebo, pooled (%)	RAD001, 0.5 mg daily (%)	RAD001, 5 mg weekly (%)	RAD001, 20 mg weekly (%)
A/H1N1 strain: A/New Jersey/8/76	7	17	16	8
A/H3N2 strain: A/Victoria/361/11	20	38	39*	40*

*Odds ratio for seroconversion between RAD001-treated and placebo cohorts that was significantly different from 1.0 (two-sided *P* value <0.05 obtained by logistic regression with treatment as fixed effect).

results raise the possibility that amelioration of immunosenescence may be a marker of a broader effect on aging-related phenotypes. It remains to be determined whether RAD001 ameliorates additional aging-related conditions beyond a diminished vaccination response in elderly humans.

The mechanism by which RAD001 enhanced the vaccination response in the elderly volunteers warrants further investigation. Given that RAD001 dosing was discontinued 2 weeks before vaccination, the immune-enhancing effects of RAD001 likely were mediated by changes in a relevant cell population that persisted after drug discontinuation. Here, RAD001 treatment decreased the percentage of PD-1-positive CD4 and CD8 T cells compared to placebo. PD-1-positive CD4 and CD8 T cells accumulate with age and have diminished responses to antigen stimulation because PD-1 inhibits T cell receptor-induced T cell proliferation, cytokine production, and cytolytic function (8). PD-1 expression is induced by T cell signaling and remains high in the setting of persistent antigen stimulation, including chronic viral infection. It is possible that RAD001 reduced chronic immune activation in

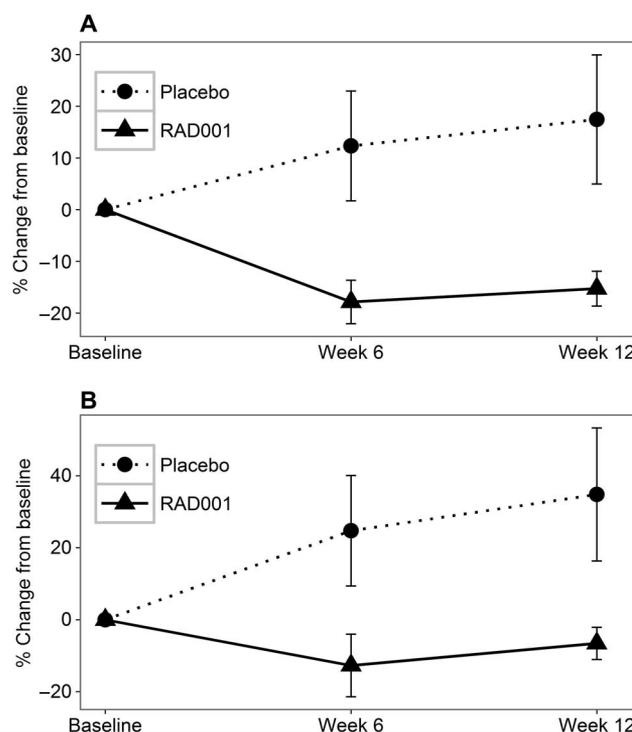


Fig. 3. Decrease in percent of PD-1-positive CD4 and CD8 T cells after RAD001 treatment. The percent of PD-1-positive CD4 and CD8 T cells was determined by fluorescence-activated cell sorting analysis of PBMC samples at baseline, after 6 weeks of drug treatment (week 6), 6 weeks after study drug discontinuation, and 4 weeks after influenza vaccination (week 12). (A) There was a significant decrease of 30.2% in PD-1-positive CD4 T cells at week 6 in the pooled RAD001-treated cohort (*n* = 84) compared to the placebo cohort (*n* = 25) [*P* = 0.03 (*q* = 0.13)]. The decrease in PD-1-positive CD4 T cells at week 12 in the pooled RAD001-treated cohort compared to the placebo cohort was 32.7% [*P* = 0.05 (*q* = 0.19)]. (B) There was a significant decrease of 37.4% in PD-1-positive CD8 T cells at week 6 in the pooled RAD001-treated cohort (*n* = 84) compared to the placebo cohort (*n* = 25) [*P* = 0.008 (*q* = 0.07)]. The decrease in PD-1-positive CD8 T cells at week 12 in the pooled RAD001-treated cohort compared to the placebo cohort was 41.4% [*P* = 0.066 (*q* = 0.21)].

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elderly volunteers and thereby led to a decrease in PD-1 expression. RAD001 also may have inhibited PD-1 expression directly, as has been reported for the immunophilin cyclosporin A (14). A RAD001-associated decrease in the percentage of PD-1-positive CD4 and CD8 T cells may contribute to enhanced immune function and improve the quality of T cell responses to antigenic stimulation in the elderly. This is consistent with previous studies showing that mTOR inhibition improved the quality of memory CD8 T cell responses to vaccination in mice and primates (15). In our study, there was an increase in the percentage of PD-1-positive T cells over time in the placebo cohort but not in the pooled RAD001 cohort. At week 12 (4 weeks after vaccination), the increase in the placebo cohort may have been due in part to influenza vaccination because influenza virus has been shown to increase PD-1-positive T cells (16). In aged mice, mTOR inhibition also has been shown to increase the number of HSCs, leading to increased production of naïve lymphocytes (10). However, in this study, we did not detect significant differences in the percentage of naïve lymphocytes in the RAD001-treated versus placebo cohorts.

Our results also suggest that mTOR inhibition broadened the serologic response of elderly volunteers to influenza vaccination and increased antibody titers to heterologous strains of influenza not contained in the seasonal influenza vaccine. The mechanism by which RAD001 broadened the serologic response to heterologous strains of influenza virus remains to be determined. In a previous study in which mice were dosed with rapamycin for 28 days beginning 1 day before influenza infection, the broadened serologic response to heterologous influenza strains may have been due in part to inhibition of B cell class switching by rapamycin (13). In contrast, we did not find evidence that RAD001 altered B cell class switching in our elderly subjects who had discontinued RAD001 2 weeks before influenza vaccination (fig. S2). Although the underlying mechanism requires further elucidation, an increased serologic response to heterologous influenza strains may confer enhanced protection against influenza illness.

The effect of RAD001 on influenza antibody titers was comparable to the effect of the MF59 vaccine adjuvant, which is approved for enhancing the response of the elderly to influenza vaccination (17). Therefore, RAD001-driven enhancement of the antibody response to influenza vaccination may translate into clinical benefit as has been demonstrated for MF59-adjuvanted influenza vaccine in the elderly (11). However, RAD001 also is used to suppress the immune response to prevent rejection of transplanted organs. These seemingly paradoxical findings raise the possibility that the immunomodulatory effects of mTOR inhibitors may be dependent on age, disease, and/or antigen (18). Notably, mTOR activity is increased in a variety of tissues including HSCs in animal models of aging (10, 19). Thus, down-regulating mTOR activity to levels seen in young tissues, as opposed to more complete suppression of mTOR activity, may be required to obtain clinical benefit in aging-related conditions.

The limitations of our study include the relatively small sample size for a human influenza vaccination study, which limits statistical power. In addition, evaluation of immune function was limited to assessment of vaccination response and immunophenotyping and did not include assessments of cell-mediated immune function. Finally, we did not evaluate the effect of mTOR inhibitors on vaccination responses in a young cohort, so we do not know if mTOR inhibitors improve immune function in both the young and the old. However, the RAD001-

associated decrease in the percentage of PD-1-positive T cells is likely to benefit immune function more in the elderly than in the young because PD-1-positive T cells accumulate with age. Finally, this was an exploratory proof-of-concept study that used a liberal preplanned threshold for significance (80% posterior probability of an effect over placebo). The hypotheses generated concerning the immune-enhancing effects of mTOR inhibitors need to be verified with additional studies.

The safety profile of mTOR inhibitors such as RAD001 in the treatment of aging-related indications has been of concern. The toxicity of RAD001 at doses used in oncology or organ transplantation results in adverse effects such as stomatitis, diarrhea, nausea, cytopenias, hyperlipidemia, and hyperglycemia, which would be unacceptable for many aging-related indications. However, many of these adverse events are related to the pre-dose (trough) concentrations of RAD001 in blood. The RAD001 dosing regimens used in this study were chosen to minimize trough concentrations of the drug. In addition, the limited 6-week course of treatment decreased the risk of adverse events. Thus, our findings suggest that the dosing regimens used in this study may have an acceptable risk/benefit for treating some conditions of the elderly. Nonetheless, significant numbers of subjects in our study developed mouth ulcers even when dosed as low as 0.5 mg RAD001 daily, although most mouth ulcers were transient and of mild severity. Development of mTOR inhibitors with cleaner safety profiles than the currently available rapalogs may provide better therapeutic options for boosting immune responses in the elderly in the future.

MATERIALS AND METHODS

Study design

From December 2011 to April 2012, 218 elderly volunteers ≥ 65 years of age without unstable underlying medical diseases were enrolled in a randomized, observer-blind, placebo-controlled trial at nine sites in New Zealand and Australia. The objective of the study was to determine if RAD001 enhanced immune function in the elderly as assessed by response to influenza vaccination. Response to influenza was assessed by measuring HI titers to the three influenza strains in the 2012 influenza vaccine at baseline and 4 weeks after vaccination. Exclusion criteria at screening included hemoglobin < 9.0 g/dl, white blood cell count $< 3500/\text{mm}^3$, neutrophil count $< 2000/\text{mm}^3$, or platelet count $< 125,000/\text{mm}^3$; uncontrolled diabetes; unstable ischemic heart disease; clinically significant underlying pulmonary disease; history of an immunodeficiency or receiving immunosuppressive therapy; history of coagulopathy or medical condition requiring long-term anticoagulation; estimated glomerular filtration rate < 30 ml/min; and presence of severe uncontrolled hypercholesterolemia (> 350 mg/dl, 9.1 mM) or hypertriglyceridemia (> 500 mg/dl, 5.6 mM).

The subjects were randomized to treatment arms, using a validated automated randomization system with a ratio of RAD001 to placebo of 5:2 in each treatment arm. The treatment arms were as follows: 0.5 mg RAD001 daily or placebo, 5 mg RAD001 weekly or placebo, or 20 mg RAD001 weekly or placebo. The trial was observer-blind because the placebo in the RAD001 0.5 mg daily and 20 mg weekly cohorts differed slightly from the RAD001 tablets in those cohorts. The study personnel evaluating the subjects did not see the study medication and therefore were fully blinded. The treatment duration for all cohorts was 6 weeks, during which time subjects underwent safety evaluations in the clinic every 2 weeks. After the subjects had been dosed for

4 weeks, RAD001 steady-state levels were measured pre-dose and at 1 hour post-dose. After the subjects completed the 6-week course of study drug, they were given a 2-week drug-free break and then were given a 2012 seasonal influenza vaccination (Agrrippal, Novartis Vaccines and Diagnostics) containing the strains H1N1 A/California/07/2009, H3N2 A/Victoria/210/2009, and B/Brisbane/60/2008. Four weeks after influenza vaccination, serum was collected from the subjects for influenza titer measurements. Antibody titers to the three influenza vaccine strains as well as to two heterologous strains (A/H1N1 strain A/New Jersey/8/76 and A/H3N2 strain A/Victoria/361/11) were measured by standard HI assay (20). Levels of immunoglobulin G (IgG) and IgM specific for the A/H1N1/California/07/2009 were measured in serum samples taken before and 4 weeks after influenza vaccination by the Gyrolab technology as described previously (21). Results were expressed as fluorescence intensity. All subjects provided written informed consent. The study was conducted in accordance with the principles of Good Clinical Practice and was approved by the appropriate ethics committees and regulatory agencies.

Safety

Adverse event assessment and blood collection for hematologic and biochemical safety assessments were performed during study visits. Adverse event information was also collected in diaries that subjects filled out at home during the 6 weeks they were on study drug. Data on all adverse events were collected from the time of informed consent until 30 days after the last study visit. Events were classified by the investigators as mild, moderate, or severe.

Immunophenotyping

PBMCs were isolated from whole blood collected at three time points: baseline, after 6 weeks of study drug treatment, and at the end of study when subjects had been off study drug for 6 weeks and 4 weeks after influenza vaccination. Immunophenotyping was performed at the Human Immune Monitoring Center at Stanford University, Stanford, CA, as described previously (22). Seventy-six PBMC subsets were analyzed by flow cytometry with eight-color lyophilized immunophenotyping panels (BD Lyoplate, BD Biosciences). PBMC samples with viability $\geq 80\%$ and yield of 2×10^6 cells/ml or more were included in the analysis.

To determine which immunophenotype changes differed between the treated and placebo groups, within-patient cell count ratios for each measured phenotype were calculated between baseline and week 6 of study drug treatment and between baseline and the end of study (week 12). The ratios were log-transformed and analyzed by analysis of covariance at each time point to detect a difference between the pooled treatment and placebo groups. For this analysis, all three RAD001 dosing groups were combined. Before the analysis, low-viability samples were removed, and we did not conduct missing data imputation in treatment effect analysis. Therefore, if a patient had missing phenotype data at baseline, week 6, or week 12, this patient did not contribute to the analysis of this phenotype for the affected time point.

In the end, we conducted 152 tests in 76 phenotypes to compare the pooled treatment effect against the placebo effect. Stratified false discovery rate (FDR) control methodology was implemented to control the occurrence of false positives associated with multiple testing yet provide considerably better power (23, 24). We took the cell type group as the stratification factor and conducted FDR (q value) calculation within each stratum respectively. We rejected all null hypotheses

at 0.05 significance level and q value less than 20%. This can be interpreted as rejecting only those hypotheses with P values less than 0.05 and less than 20% probability that each observed significant result is due to multiple testing.

Statistical analysis

The primary analysis of GMT ratios was done using a normal Bayesian regression model with noninformative priors. This model was fitted to each antibody titer on the log scale. The primary outcome in each model was the day 84 (4 weeks after vaccination) measurement. The day 63 (1 week after vaccination) measurement was included in the outcome vector. The model was fitted using SAS 9.2 PROC mixed with the prior statement. The covariance structure of the matrix was considered as unstructured (option type = UN). A flat prior was used. For the secondary analysis of seroconversion rates, logistic regression was used. The intent-to-treat population was defined as all subjects who received at least one full dose of the study drug and who had no major protocol deviations affecting efficacy data. Of the total of 218 subjects enrolled in the study, 199 were in the intent-to-treat population.

SUPPLEMENTARY MATERIALS

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Table S1. Demographic and baseline characteristics of the study subjects.

Table S2. HI GMTs for each influenza vaccine strain at baseline and at 4 weeks after influenza vaccination.

Fig. S1. Individual subject HI titer ratios (4 weeks after vaccination/baseline) per cohort for the three influenza vaccine strains and two heterologous influenza strains.

Fig. S2. Increase in anti-H1N1 influenza IgG and IgM after influenza vaccination.

REFERENCES AND NOTES

1. S. C. Johnson, G. M. Martin, P. S. Rabinovitch, M. Kaeberlein, Preserving youth: Does rapamycin deliver? *Sci. Transl. Med.* **5**, 211fs40 (2013).
2. D. E. Harrison, R. Strong, Z. D. Sharp, J. F. Nelson, C. M. Astle, K. Flurkey, N. L. Nadon, J. E. Wilkinson, K. Frenkel, C. S. Carter, M. Pahor, M. A. Javors, E. Fernandez, R. A. Miller, Rapamycin fed late in life extends lifespan in genetically heterogeneous mice. *Nature* **460**, 392–395 (2009).
3. J. E. Wilkinson, L. Burmeister, S. V. Brooks, C. C. Chan, S. Friedline, D. E. Harrison, J. F. Hejtmancik, N. Nadon, R. Strong, L. K. Wood, M. A. Woodward, R. A. Miller, Rapamycin slows aging in mice. *Aging Cell* **11**, 675–682 (2012).
4. J. M. Flynn, M. N. O'Leary, C. A. Zambataro, E. C. Academia, M. P. Presley, B. J. Garrett, A. Zykovich, S. D. Mooney, R. Strong, C. J. Rosen, P. Kapahi, M. D. Nelson, B. K. Kennedy, S. Melov, Late-life rapamycin treatment reverses age-related heart dysfunction. *Aging Cell* **12**, 851–862 (2013).
5. W. W. Thompson, D. K. Shay, E. Weintraub, L. Brammer, N. Cox, L. J. Anderson, K. Fukuda, Mortality associated with influenza and respiratory syncytial virus in the United States. *JAMA* **289**, 179–186 (2003).
6. K. Goodwin, C. Viboud, L. Simonsen, Antibody response to influenza vaccination in the elderly: A quantitative review. *Vaccine* **24**, 1159–1169 (2006).
7. D. Boraschi, M. T. Aguado, C. Dutel, J. Goronzy, J. Louis, B. Grubeck-Loebenstein, R. Rappuoli, G. Del Giudice, The gracefully aging immune system. *Sci. Transl. Med.* **5**, 185ps8 (2013).
8. C. S. Lages, I. Lewkowich, A. Sproles, M. Wills-Karp, C. Chougnet, Partial restoration of T cell function in aged mice by in vitro blockade of the PD-1/PD-L1 pathway. *Aging Cell* **9**, 785–798 (2010).
9. K. Shimatani, Y. Nakashima, M. Hattori, Y. Hamazaki, N. Minato, PD-1⁺ memory phenotype CD4⁺ T cells expressing C/EBP α underlie T cell immunodepression in senescence and leukemia. *Proc. Natl. Acad. Sci. U.S.A.* **106**, 15807–15812 (2009).
10. C. Chen, Y. Liu, Y. Liu, P. Zheng, mTOR regulation and therapeutic rejuvenation of aging hematopoietic stem cells. *Sci. Signal.* **2**, ra75 (2009).
11. A. Iob, G. Brianti, E. Zamparo, T. Gallo, Evidence of increased clinical protection of an MF59-adjuvant influenza vaccine compared to a non-adjuvant vaccine among elderly residents of long-term care facilities in Italy. *Epidemiol. Infect.* **133**, 687–693 (2005).

12. C. Tanaka, T. O'Reilly, J. M. Kovarik, N. Shand, K. Hazell, I. Judson, E. Raymond, S. Zumstein-Mecker, C. Stephan, A. Boulay, M. Hattenberger, G. Thomas, H. A. Lane. Identifying optimal biologic doses of everolimus (RAD001) in patients with cancer based on the modeling of preclinical and clinical pharmacokinetic and pharmacodynamic data. *J. Clin. Oncol.* **26**, 1596–1602 (2008).
13. R. Keating, T. Hertz, M. Wehenkel, T. L. Harris, B. A. Edwards, J. L. McClaren, S. A. Brown, S. Surman, Z. S. Wilson, P. Bradley, J. Hurwitz, H. Chi, P. C. Doherty, P. G. Thomas, M. A. McGargill. The kinase mTOR modulates the antibody responses to provide cross-protective immunity to lethal infection with influenza virus. *Nat. Immunol.* **14**, 1266–1276 (2013).
14. K. J. Oestreich, H. Yoon, R. Ahmed, J. M. Boss, NFATc1 regulates PD-1 expression upon T cell activation. *J. Immunol.* **181**, 4832–4839 (2008).
15. K. Araki, A. P. Turner, V. O. Shaffer, S. Gangappa, S. A. Keller, M. F. Bachmann, C. P. Larsen, R. Ahmed, mTOR regulates memory CD8 T-cell differentiation. *Nature* **460**, 108–112 (2009).
16. J. J. Erickson, P. Gilchuk, A. K. Hastings, S. J. Tollefson, M. Johnson, M. B. Downing, K. L. Boyd, J. E. Johnson, A. S. Kim, S. Joyce, J. V. Williams. Viral acute lower respiratory infections impair CD8⁺ T cells through PD-1. *J. Clin. Invest.* **122**, 2967–2982 (2012).
17. A. Podda, The adjuvanted influenza vaccines with novel adjuvants: Experience with the MF59-adjuvanted vaccine. *Vaccine* **19**, 2673–2680 (2001).
18. I. R. Ferrer, M. E. Wagener, J. M. Robertson, A. P. Turner, K. Araki, R. Ahmed, A. D. Kirk, C. P. Larsen, M. L. Ford. Cutting edge: Rapamycin augments pathogen-specific but not graft-reactive CD8⁺ T cell responses. *J. Immunol.* **185**, 2004–2008 (2010).
19. M. Barns, C. Gondro, R. L. Tellam, H. G. Radley-Crabb, M. D. Grounds, T. Shavlakadze. Molecular analyses provide insight into mechanisms underlying sarcopenia and myofibre denervation in old skeletal muscles of mice. *Int. J. Biochem. Cell Biol.* **53**, 174–185 (2014).
20. Centers for Disease Control and Prevention, Haemagglutination inhibition, in *Concepts and Procedures for Laboratory-Based Influenza Surveillance*, A. P. Kendal, M. S. Pereira, J. J. Skehel, Eds. (Centers for Disease Control and Prevention, Atlanta, GA, 2010) pp. B17–B35.
21. F. Spensieri, E. Borgogni, L. Zedda, M. Bardelli, F. Buricchi, G. Volpini, E. Fragapane, S. Tavarini, O. Finco, R. Rappuoli, G. Del Giudice, G. Galli, F. Castellino. Human circulating influenza-CD4⁺ ICOS1⁺IL-21⁺ T cells expand after vaccination, exert helper function, and predict antibody responses. *Proc. Natl. Acad. Sci. U.S.A.* **110**, 14330–14335 (2013).
22. H. T. Maecker, J. P. McCoy, R. Nussenblatt. Standardizing immunophenotyping for the Human Immunology Project. *Nat. Rev. Immunol.* **12**, 191–200 (2012).
23. Y. Benjamini, Y. Hochberg. Controlling the false discovery rate: A practical and powerful approach to multiple testing. *J. R. Statist. Soc. B* **57**, 289–300 (1995).
24. L. Sun, R. V. Craiu, A. D. Paterson, S. B. Bull. Stratified false discovery control for large-scale hypothesis testing with application to genome-wide association studies. *Genet. Epidemiol.* **30**, 519–530 (2006).

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Editor's Summary

mTOR and Human Aging

Inhibition of mTOR signaling extends life span and delays the onset of aging-related diseases in all species studied to date. These findings suggest that the mTOR pathway regulates aging. However, it is unknown if mTOR inhibition has beneficial effects on aging in humans. To begin to address this question, Mannick *et al.* evaluated the effects of the mTOR inhibitor RAD001 on the decline in immune function that occurs during aging in humans. Their findings suggest that RAD001 improved immune function in elderly volunteers as assessed by response to influenza vaccination. It remains to be determined whether mTOR inhibition improves additional aging-related conditions in humans.

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