INFECTIOUS DISEASE

TORC1 inhibition enhances immune function and reduces infections in the elderly

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Inhibition of the mechanistic target of rapamycin (mTOR) protein kinase extends life span and ameliorates aging-related pathologies including declining immune function in model organisms. The objective of this phase 2a randomized, placebo-controlled clinical trial was to determine whether low-dose mTOR inhibitor therapy enhanced immune function and decreased infection rates in 264 elderly subjects given the study drugs for 6 weeks. A low-dose combination of a catalytic (BEZ235) plus an allosteric (RAD001) mTOR inhibitor that selectively inhibits target of rapamycin complex 1 (TORC1) downstream of mTOR was safe and was associated with a significant (P = 0.001) decrease in the rate of infections reported by elderly subjects for a year after study drug initiation. In addition, we observed an up-regulation of antiviral gene expression and an improvement in the response to influenza vaccination in this treatment group. Thus, selective TORC1 inhibition has the potential to improve immune function and reduce infections in the elderly.

INTRODUCTION

Aging may be due, in part, to alterations in a discrete set of cell signaling pathways including the mechanistic target of rapamycin (mTOR) pathway (*1*). Inhibition of the mTOR pathway has extended life span in every species studied to date, suggesting that mTOR signaling is an evolutionarily conserved pathway that may regulate aging (*2*). One of the aging-related conditions that improves in old mice treated with mTOR inhibitors is immunosenescence (the decline in immune function that occurs during aging) (*3*). Immunosenescence leads to increased rates of infections including respiratory tract infections that are the fourth leading cause of death in people ≥85 years of age and the eighth leading cause of death in people ≥65 years of age in the United States (*4*, *5*). Therefore, infection-related morbidity and mortality in the elderly may be substantially reduced if mTOR inhibition enhances the ability of the aging immune system to fight infectious pathogens.

mTOR is a protein kinase that signals via two complexes: TORC1 and TORC2. Many of the beneficial effects of mTOR inhibition on aging may be mediated by inhibition of TORC1 (*6*, *7*). In contrast, TORC2 inhibition has been associated with adverse events including hyperglycemia and hypercholesterolemia and with decreased life span in male mice (*7*, *8*). In addition, several long-lived animal models have increased rather than decreased TORC2 activity (*9*). Therefore, optimal mTOR inhibition for the treatment of aging-related conditions such as immunosenescence may be a regimen that inhibits TORC1 without inhibiting TORC2.

Rapalogs such as RAD001 are a class of allosteric mTOR inhibitors that are derivatives of rapamycin and consistently inhibit only S6 kinase (S6K) downstream of TORC1 (*10*). BEZ235 is a dual phosphatidylinositol 3-kinase (PI3K)/mTOR adenosine triphosphate–competitive catalytic site inhibitor that, at high concentrations, inhibits TORC1, TORC2, and PI3K but, at low concentrations (≤20 nM), mainly inhibits S6K phosphorylation and, to a lesser extent, eukaryotic translation initiation factor 4E-binding protein 1 (4EBP1) phosphorylation downstream of TORC1 (*11*–*13*). However, low doses of BEZ235 (and other catalytic mTOR inhibitors) in combination with low doses of RAD001 synergistically inhibit multiple nodes downstream of TORC1 without inhibiting TORC2 activity (*14*, *15*). Thus, the combination of a low-dose rapalog plus a catalytic site mTOR inhibitor may be more efficacious than monotherapy with a rapalog or catalytic site mTOR inhibitor for the treatment of aging-related conditions such as immunosenescence. In a previous study, we demonstrated that treatment with the rapalog RAD001 improved immune function in elderly volunteers as assessed by response to influenza vaccination (*16*). Here, we extended these findings and assessed whether a low-dose combination of a rapalog (RAD001) plus a catalytic site mTOR inhibitor (BEZ235) resulted in a greater improvement in immune function (as assessed by the response to influenza vaccination) in elderly subjects than did monotherapy with either RAD001 or BEZ235. We also investigated whether low-dose mTOR inhibitor treatment was associated with a decrease in total infection rates in the elderly subjects at doses that were safe and well tolerated.

RESULTS

A total of 264 elderly volunteers ≥65 years of age, without unstable medical conditions, were enrolled in a randomized, double-blinded, placebo-controlled trial at 12 clinical sites (Fig. 1). Subjects were assigned randomly to receive one of four oral mTOR inhibitor dosing regimens or a corresponding matching placebo: 0.5 mg of RAD001 once daily, 0.1 mg of RAD001 once daily, 10 mg of BEZ235 once daily, or a combination of 0.1 mg of RAD001 and 10 mg of BEZ235 once daily. The placebo groups were pooled for analysis.

Subjects were treated for 6 weeks with a study drug and, after a 2-week drug-free interval, were given a seasonal influenza vaccine (Fluvax, CSL Biotherapies). Antibody titers to the three strains of influenza virus in the vaccine (A/H1N1, A/H2N3, and B) were measured

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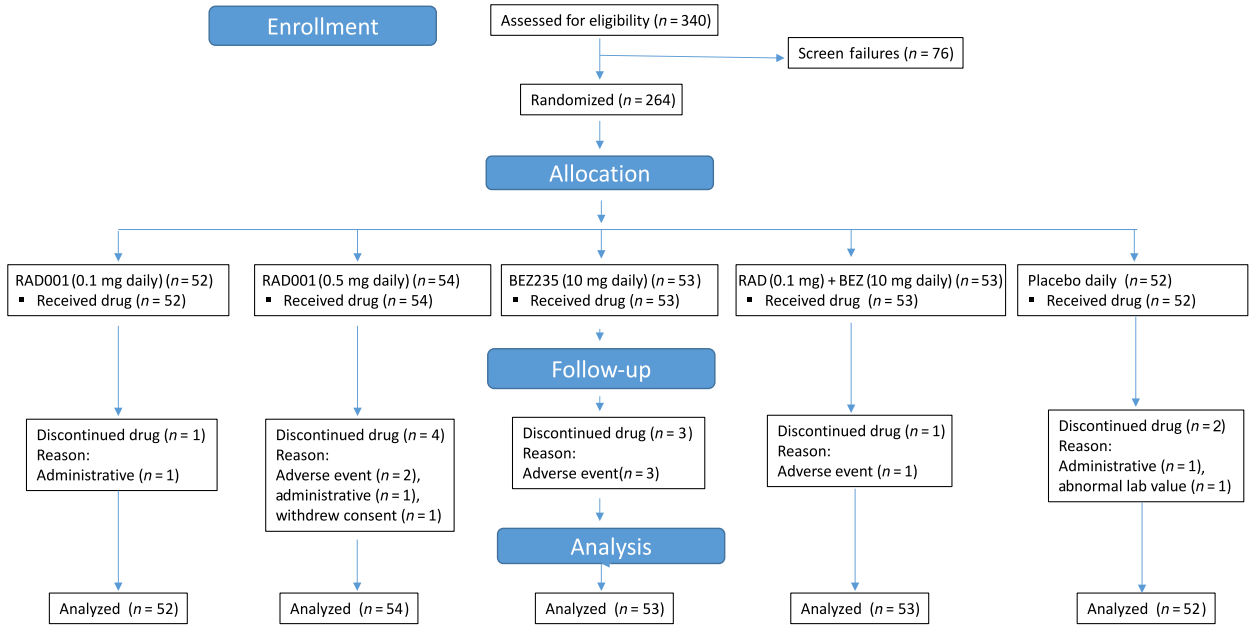

Fig. 1. Flow diagram showing participants and study design. The number of elderly subjects screened (assessed for eligibility), the number of subjects randomized to each treatment arm (allocation), and the number of subjects who discontinued study drug in each treatment arm. The term analyzed indicates the number of subjects in each cohort in the modified intention to treat population.

in serum collected at baseline before study drug administration, after 6 weeks of study drug administration but before vaccination, and 4 weeks after vaccination (table S1). Six weeks of study drug treatment did not significantly change antibody titers to the three influenza vaccine strains. Therefore, the antibody titers obtained after 6 weeks of study drug treatment were used as the baseline prevaccination titer values. The subjects were then followed for about 9 months off study drug (about 1 year after initiation of study drug treatment).

TORC1 inhibitor treatment was safe in elderly subjects

Baseline demographics between the treatment arms were similar (table S2). Of the 264 subjects enrolled, 253 completed the study (Fig. 1). In general, the mTOR inhibitor regimens were well tolerated. No deaths occurred during the study. Twenty six of 264 participants experienced at least one serious adverse event (SAE) during the 12 months they were followed in the study. There were no significant differences in the percentage of subjects experiencing SAEs between treatment groups and placebo: 9.6% in the 0.1 mg of RAD001 daily cohort, 13% in the 0.5 mg of RAD001 daily cohort, 9.4% in the 10 mg of BEZ235 daily cohort, 7.5% in the 0.1 mg of RAD001 + 10 mg of BEZ235 cohort, and 9.6% in the placebo cohort. Only one SAE (syncope in a subject in the placebo cohort) was deemed by an investigator to be related to the study drug. A list of adverse events that occurred in more than 5% of subjects in any treatment group in the safety population (defined as any subject who received at least one partial dose of study drug) is provided in Table 1. Diarrhea was the most frequently reported adverse event that occurred more often in the mTOR inhibitor cohorts than in the placebo group and was of mild severity in most of the cases. Notably, rates of hyperglycemia and hypercholesterolemia (adverse events associated with TORC2 inhibition) were lower in the mTOR inhibitor treatment groups than in the placebo group, suggesting that the mTOR inhibitor treatment regimens were not inhibiting TORC2. Hyperglycemia was reported as an adverse event in 3.8% of subjects in the placebo cohort, in 1.9% of subjects in the 0.1 mg of RAD001 cohort, and in no subjects in the 0.5 mg of RAD001, 10 mg of BEZ235, or 0.1 mg of RAD001 + 10 mg of BEZ235 cohorts. Hypercholesterolemia was reported as an adverse event in 1.9% of subjects in the placebo cohort and in none of the subjects in the RAD001 and BEZ235 monotherapy and combination treatment cohorts.

TORC1 inhibitor treatment improved influenza vaccination response in elderly subjects

The ability of RAD001 and BEZ235 as monotherapies or in combination to improve immune function in elderly volunteers was evaluated by measuring the serologic response to the 2014 seasonal influenza vaccine. The primary end point of the study was a 1.2-fold increase relative to placebo in the hemagglutination inhibition geometric mean titer (GMT) ratio (GMT 4 weeks after vaccination/GMT at baseline) for at least two of three influenza vaccine strains. This end point was chosen because the about 1.2-fold increase in the influenza GMT ratio induced by the MF-59 vaccine adjuvant was associated with a decrease in influenza illness (*17*). For each influenza strain, GMT titer ratios were analyzed with a normal Bayesian repeated measures regression model with noninformative flat priors (a noninformative flat prior assigns equal likelihood to all possible values of the parameter; therefore, the results depend only on the data as observed, with no prior information assumed).

In the modified intent-to-treat population (those subjects who received at least one full dose of study drug), only the combination low-dose RAD001 (0.1 mg daily) + BEZ235 (10 mg daily) met the primary end point of the study and resulted in a greater than 20% increase in the influenza GMT ratio for all three influenza vaccine strains (Fig. 2). RAD001 monotherapy (0.1 or 0.5 mg daily) resulted in a greater than 20% increase in influenza GMT ratio for one of three influenza vaccine strains. BEZ235 monotherapy did not result

Table 1. Adverse events (AEs) that occurred in >5% of elderly subjects in any treatment group during the 1-year study. Ordered by total frequency. Under one treatment, a participant with multiple occurrences of an AE is counted only once in the AE category.

	RAD001 (0.1 mg) *N* = 52 (%)	RAD001 (0.5 mg) *N* = 54 (%)	BEZ235 (10 mg) *N* = 53 (%)	RAD001 (0.1 mg) + BEZ235 (10 mg) *N* = 53 (%)	Placebo, pooled *N* = 52 (%)	Total *N* = 264 (%)
Total AEs	320	406	346	319	401	1792
Patients with AEs	50 (96.2)	52 (96.3)	52 (98.1)	51 (96.2)	51 (98.1)	256 (97.0)
Upper respiratory tract infection	11 (21.2)	12 (22.2)	11 (20.8)	14 (26.4)	16 (30.8)	64 (24.2)
Headache	7 (13.5)	16 (29.6)	13 (24.5)	11 (20.8)	9 (17.3)	56 (21.2)
Nasopharyngitis	14 (26.9)	13 (24.1)	6 (11.3)	7 (13.2)	10 (19.2)	50 (18.9)
Diarrhea	10 (19.2)	12 (22.2)	10 (18.9)	9 (17.0)	4 (7.7)	45 (17.0)
Cough	7 (13.5)	7 (13.0)	6 (11.3)	5 (9.4)	10 (19.2)	35 (13.3)
Fatigue	6 (11.5)	4 (7.4)	8 (15.1)	4 (7.5)	6 (11.5)	28 (10.6)
Oropharyngeal pain	8 (15.4)	2 (3.7)	8 (15.1)	1 (1.9)	7 (13.5)	26 (9.8)
Arthralgia	4 (7.7)	7 (13.0)	6 (11.3)	3 (5.7)	5 (9.6)	25 (9.5)
Fall	4 (7.7)	3 (5.6)	6 (11.3)	7 (13.2)	5 (9.6)	25 (9.5)
Nausea	4 (7.7)	6 (11.1)	4 (7.5)	7 (13.2)	4 (7.7)	25 (9.5)
Rhinitis	3 (5.8)	4 (7.4)	3 (5.7)	9 (17.0)	6 (11.5)	25 (9.5)
Dizziness	5 (9.6)	5 (9.3)	6 (11.3)	3 (5.7)	4 (7.7)	23 (8.7)
Back pain	5 (9.6)	0	8 (15.1)	2 (3.8)	5 (9.6)	20 (7.6)
Contusion	5 (9.6)	2 (3.7)	2 (3.8)	7 (13.2)	4 (7.7)	20 (7.6)
Oral herpes	5 (9.6)	2 (3.7)	7 (13.2)	3 (5.7)	3 (5.8)	20 (7.6)
Urinary tract infection	5 (9.6)	5 (9.3)	2 (3.8)	0	7 (13.5)	19 (7.2)
Lower respiratory tract infection	3 (5.8)	3 (5.6)	1 (1.9)	5 (9.4)	6 (11.5)	18 (6.8)
Gastroenteritis	3 (5.8)	4 (7.4)	3 (5.7)	5 (9.4)	2 (3.8)	17 (6.4)
Mouth ulceration	5 (9.6)	2 (3.7)	2 (3.8)	6 (11.3)	2 (3.8)	17 (6.4)
Muscle strain	3 (5.8)	4 (7.4)	2 (3.8)	3 (5.7)	5 (9.6)	17 (6.4)
Constipation	3 (5.8)	6 (11.1)	4 (7.5)	1 (1.9)	2 (3.8)	16 (6.1)
Bronchitis	3 (5.8)	3 (5.6)	2 (3.8)	3 (5.7)	3 (5.8)	14 (5.3)
Laceration	4 (7.7)	2 (3.7)	2 (3.8)	3 (5.7)	3 (5.8)	14 (5.3)
Musculoskeletal pain	1 (1.9)	8 (14.8)	1 (1.9)	4 (7.5)	0	14 (5.3)
Pain in extremity	1 (1.9)	3 (5.6)	3 (5.7)	2 (3.8)	5 (9.6)	14 (5.3)

in an increase in influenza GMT ratios for any of the three influenza vaccine strains. These results suggest that a combination of a low-dose allosteric mTOR inhibitor (RAD001) plus a catalytic mTOR inhibitor (BEZ235) resulted in a greater improvement in the response to influenza vaccination than did RAD001 or BEZ235 monotherapy.

Combinations of low doses of catalytic and allosteric mTOR inhibitors have been reported to inhibit synergistically multiple nodes downstream of TORC1 without inhibiting TORC2 (*14*). Although currently available assays are not sufficiently sensitive to measure TORC1 activity in human peripheral blood samples, concentrations of RAD001 and BEZ235 in the blood of elderly subjects treated with 0.1 mg of RAD001 (about 0.8 nM Cmax) and 10 mg of BEZ235 (about 14 nM Cmax) in the current trial have been shown to synergistically inhibit the phosphorylation of both S6K and 4EBP1 downstream of TORC1 ex vivo (*15*). To confirm in vivo that the combination of low-dose RAD001 + BEZ235 is associated with more complete TORC1 inhibition than low-dose RAD001 or BEZ235 monotherapy, we measured phosphorylation of the TORC1 substrates S6K, S6, and 4EBP1 in the livers of rats treated for 7 days with the dose equivalent of 0.1 mg of RAD001, 0.5 mg of RAD001, 10 mg of BEZ235, or a combination of 0.1 mg of RAD001 and 10 mg of BEZ235 (Fig. 3). All mTOR inhibitor dosing regimens except the dose equivalent of 0.1 mg of RAD001 significantly ($P < 0.05$) inhibited the phosphorylation of S6 (Fig. 3). However, only BEZ235 alone or in combination with RAD001 inhibited the phosphorylation of 4EBP1 (Fig. 3B). Moreover, the only mTOR inhibitor dosing regimen that significantly ($P < 0.05$) inhibited all three nodes downstream of TORC1 was the combination of RAD001 and BEZ235 (Fig. 3B). These data suggest that treatment of elderly subjects with a combination of low-dose RAD001 + BEZ235 may result in greater improvement in influenza vaccination response than treatment with RAD001 or BEZ235 monotherapy due to more complete TORC1 inhibition achieved with the combination.

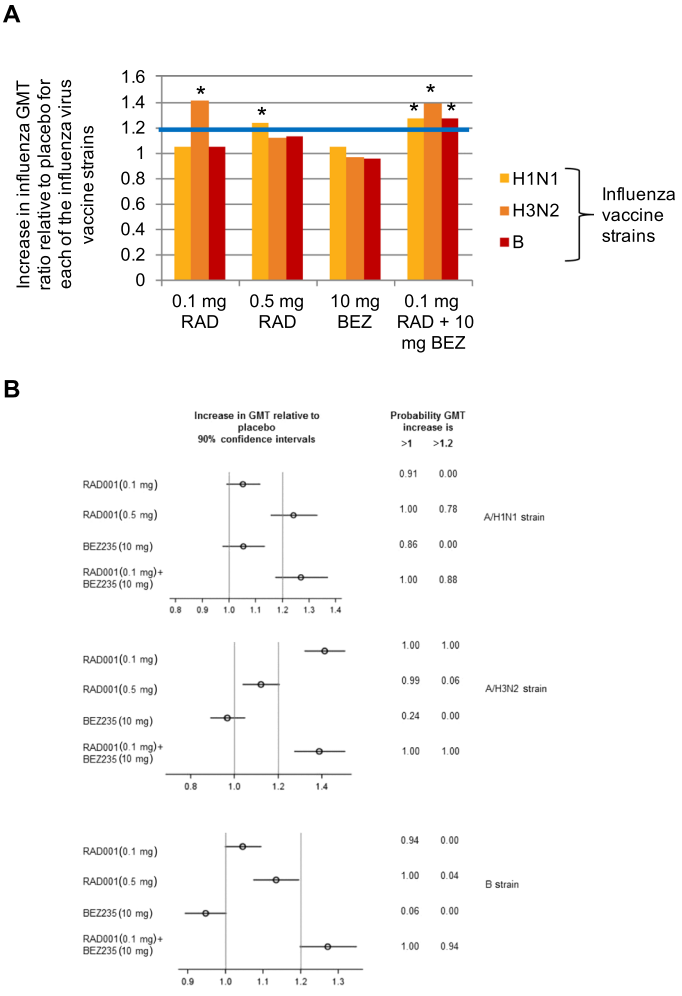

Fig. 2. Increase in antibody titers to influenza virus vaccine strains in mTOR inhibitor treatment groups relative to the placebo group. (**A**) Increase in the ratio (4 weeks after vaccination: baseline) in GMT for each of the three influenza virus vaccine strains in elderly subjects treated with RAD001, BEZ235, RAD001 + BEZ235, or placebo. The three influenza virus vaccine strains used were as follows: A/H1N1 (A/California/7/2009), A/H3N2 (A/Texas/50/2012), and B (B/Massachusetts/2/2012). The blue line indicates the 20% increase in GMT ratios relative to placebo that was required for two of the three influenza virus vaccine strains to meet the primary end point of the study. Asterisks indicate that the probability that the increase in GMT ratio relative to placebo exceeded 1.0 is 100%. (**B**) Forest plots of the data presented in (A) including 90% confidence intervals and probability that the GMT ratio compared to placebo is >1 or >1.2.

TORC1 inhibitor treatment decreased infection rates in elderly subjects

As an additional evaluation of immune function, the overall infection rate in each treatment group was prespecified as a study end point and assessed by having subjects record any infections they experienced during the year after the initiation of study drug treatment in a diary that was reviewed by study investigators at each study visit. In addition, infections were captured through phone calls between site staff and subjects that occurred weekly during the 6 weeks the subjects were on study drug and then monthly for the remainder of the trial. The largest and most statistically significant decrease ($P = 0.001$ versus placebo) in the fitted annualized rate of infections reported by subjects was in the RAD001 + BEZ235 combination treatment group (1.49 infections per person per year; 95% confidence interval, 1.19 to 1.86) as compared to the placebo group (2.41 infections per person per year; 95% confidence interval, 2.00 to 2.90; Fig. 4A). The BEZ235 monotherapy treatment group also had a statistically significant ($P = 0.008$ versus placebo) reduction in the annualized rate of infections reported by subjects (1.61 infections per person per year; 95% confidence interval, 1.28 to 2.03; Fig. 4A). There was a trend toward a reduction in infection rates in both RAD001 monotherapy treatment groups, but the reductions were not statistically significant.

Most of the infections reported during the trial were respiratory tract infections. To determine whether a reduction in respiratory tract infections contributed to the reduction in total infections reported in the BEZ235 and RAD001 + BEZ235 treatment groups, the annualized rate of respiratory tract infections reported in these two treatment groups relative to placebo was assessed as a post hoc analysis. Both BEZ235 monotherapy ($P = 0.008$) and BEZ235 + RAD001 combination therapy ($P = 0.01$) were associated with a significant reduction as compared to placebo in the annualized rate of respiratory tract infections reported by subjects (Fig. 4B).

Given that the combination of low-dose RAD001 + BEZ235 was the only mTOR inhibitor dosing regimen associated both with a significant ($P = 0.001$) decrease in infections and an improvement in the response to three of three influenza vaccine strains, subsequent mechanistic studies focused on this treatment group. To explore whether the combination of low-dose RAD001 + BEZ235 enhanced immune function by decreasing systemic inflammation, we measured several inflammatory cytokines in serum obtained from subjects at baseline and after 6 weeks of either placebo or RAD001 + BEZ235 treatment. There were no significant differences in serum concentrations of interleukin-6 (IL-6), interferon-γ (IFN-γ), tumor necrosis factor–α (TNFα), or IL-18 in the RAD001 + BEZ235 treatment group compared to the placebo group (fig. S1).

To assess other possible molecular mechanisms underlying the enhanced immune function of the RAD001 + BEZ235 combination, we conducted mRNA sequencing analysis of whole blood from subjects at baseline and after 6 weeks of either placebo or RAD001 + BEZ235 treatment. Whole-blood gene expression data revealed a statistically significant ($P < 10^{-15}$) up-regulation of pathways related to IFN signaling (Table 2 and fig. S2). Some of the genes whose expression was most highly up-regulated in the enriched pathways were a subset of type 1 IFN–induced genes that play a critical role in the immune response to viruses (Table 2) (*18*). A complete list of significantly ($P < 10^{-10}$) enriched pathways after RAD001 + BEZ235 treatment is provided in table S3. These findings raise the possibility that up-regulation of a subset of type 1 IFN–induced genes by a low-dose combination of RAD001 + BEZ235 may be one mechanism contributing to enhanced immune function and reduced infection rates in the elderly.

DISCUSSION

The current study demonstrates that a combination of low-dose RAD001 + BEZ235 was associated with a significant ($P = 0.001$) decrease in the annualized rate of infections reported by elderly subjects and an increase in the humoral response to influenza vaccination. These findings suggest that low-dose RAD001 + BEZ235 may lead to a clinically relevant enhancement of immune function in the elderly. In addition, our findings suggest that synergistic inhibition of multiple nodes downstream of TORC1 with a combination of low-dose RAD001 + BEZ235 may result in greater improvement

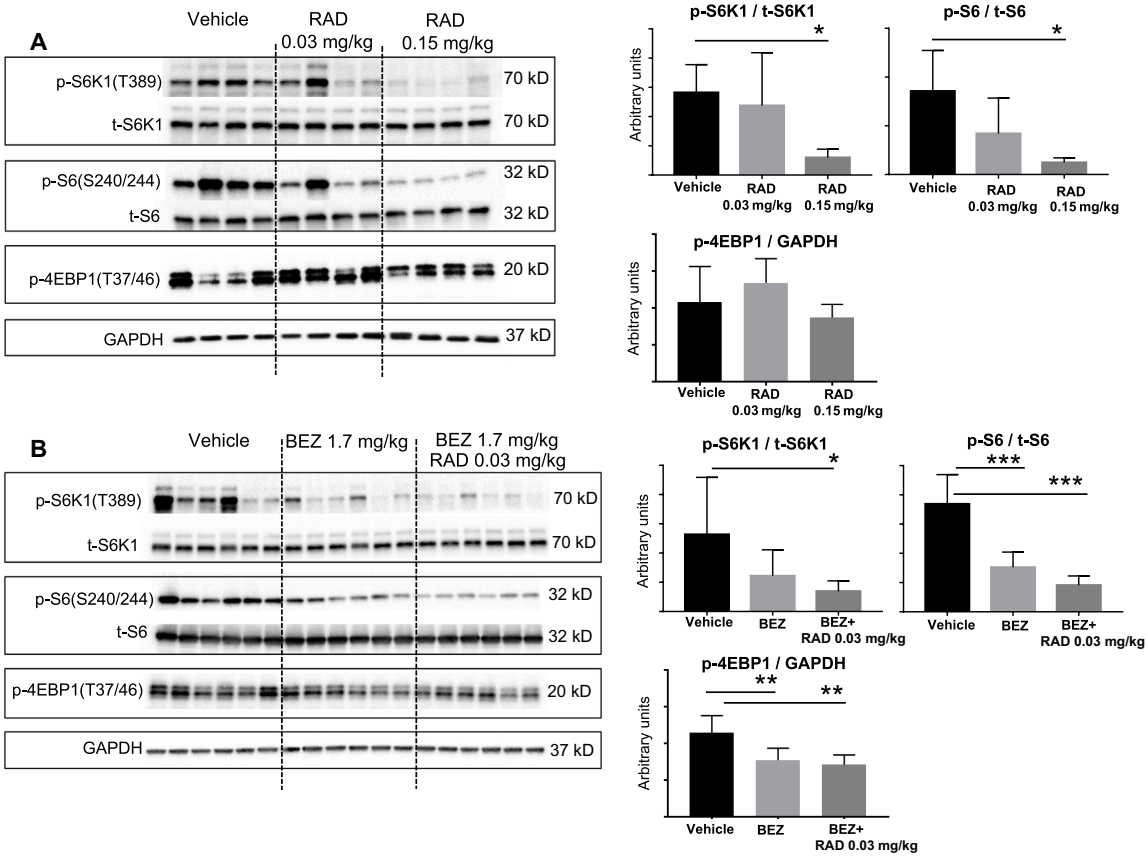

Fig. 3. Low doses of RAD001 and BEZ235 inhibit TORC1. Western blots for phosphorylated (p) and total (t) protein amounts for S6K1, S6, and 4EBP1 in rat livers after 7 days of drug treatment. (**A**) Rats were treated daily for 7 days with RAD001 (RAD) at the dose equivalent of 0.1 mg (0.03 mg/kg) or 0.5 mg (0.15 mg/kg) in humans. (**B**) Rats were treated daily for 7 days with BEZ235 (BEZ) given at the dose equivalent of 10 mg (1.7 mg/kg) in humans alone or in combination with the dose equivalent of RAD001 0.1 mg (0.03 mg/kg). Tissues were collected 4 hours after the last drug dose. Left: Each lane in the immunoblots represents liver tissue from one rat. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is shown as a loading control. Right: The amounts of p-S6K1(T389) and p-S6(Ser240/244) on the immunoblots were quantified relative to their respective total protein amounts by densitometry. Amounts of p-4EBP1 (T37/46) were quantified relative to GAPDH. *Y* axes represent arbitrary units. For each group, $n = 4$ to 6 rats. Data are mean ± SD. Data were analyzed with a one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison tests, where means from all groups were compared to the vehicle-treated group. In (A), $^{*}P = 0.048$ and $^{*}P = 0.018$ for p-S6K1 and p-S6, respectively. In (B), $^{*}P = 0.015$ for p-S6K1, $^{**}P \leq 0.005$ for p-4EBP1, and $^{***}P \leq 0.001$ for p-S6.

in immune function in elderly subjects than low-dose RAD001 or BEZ235 monotherapy, which are associated with less inhibition of nodes downstream of TORC1.

Limitations of our study include assessment of infections based on self-reporting, which may have led to misclassification. However, rates of misclassification are likely to be similar in all the treatment arms because the study was blinded and placebo-controlled, and a previous study found that elderly subjects were able to accurately self-report infections (*19*). In addition, the improvement in influenza vaccination response and up-regulation of antiviral gene expression provide mechanistic support to the clinical finding of decreased infection rates in elderly subjects treated with TORC1 inhibitors. Another limitation of our study was that peripheral blood mononuclear cell samples were not obtained from subjects for ex vivo assessments of immune function including inhibition of virus replication in infected cells. Ex vivo assessments of immune function in future clinical studies will help to further elucidate the mechanisms responsible for the reduction in infection rates.

The results of the current study suggest that the pharmacodynamic effects of mTOR inhibitors are dose-dependent. The doses of RAD001 used in this study are 3- to 100-fold lower than the doses of RAD001 approved for use in organ transplant and oncology patients. Similarly, the dose of BEZ235 used in the current study is 120-fold lower than the maximum tolerated dose established in oncology patients. At higher doses, mTOR inhibitors suppress T cell proliferation and have been associated with increased rates of infection. In contrast, results of this study suggest that very low doses of mTOR inhibitors that partially inhibit TORC1 activity enhance immune function and decrease infection rates in the elderly.

The mechanisms underlying the decrease in infection rates and enhancement in influenza vaccination response in elderly subjects treated with low doses of mTOR inhibitors are likely to be multifactorial. In a previous study in elderly subjects, we demonstrated that mTOR inhibition with RAD001 monotherapy decreased the percentage of exhausted programmed cell death protein 1 (PD1)–positive T cells that had a defective response to antigen (*16*). Here, we demonstrate that the combination of RAD001 + BEZ235 also up-regulated a subset of IFN-stimulated genes that play a critical role in the innate immune response to pathogens, particularly viruses. There was no change in

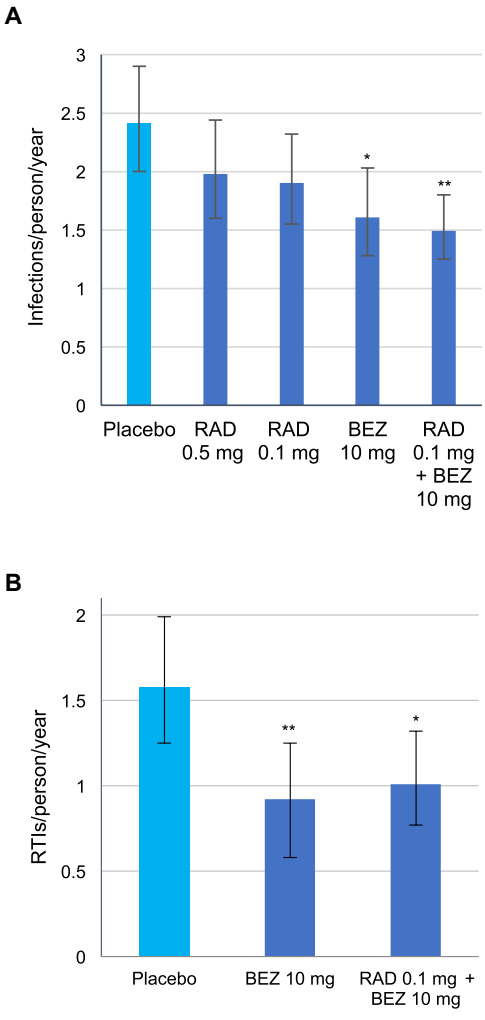

Fig. 4. TORC1 inhibition decreases infection rates in the elderly. (**A**) Fitted annual rates of infections reported per person per year in the 0.1 mg of RAD001, 0.5 mg of RAD001, 10 mg of BEZ235, 0.1 mg of RAD001 + 10 mg of BEZ235, or placebo groups. *P = 0.008, **P = 0.001 versus placebo. (**B**) Fitted annual rates of respiratory tract infections (RTIs) reported per person per year in the placebo group and in the BEZ235 monotherapy and BEZ235 + RAD001 combination treatment groups. *P = 0.01, **P = 0.008 versus placebo. In both figures, error bars indicate 95% confidence intervals as determined by Poisson regression modeling.

Table 2. Pathways and genes up-regulated after RAD001 + BEZ235 treatment as determined by gene expression analysis of whole blood.

Pathway	Mean FC of genes in pathway	*P* value	Up-regulated genes*
IFN α/β signaling	0.08	$10^{-21.8}$	*IFI27, IFIT2, IFIT1, IFIT3, MX1, OAS3, ISG15*
IFN signaling	0.04	$10^{-36.7}$	*IFI27, IFIT2, IFIT1, IFIT3, MX1, OAS3, HERC5, ISG15*
Cytokine signaling in immune system	0.02	$10^{-43.5}$	*IFI27, IFIT2, IFIT1, IFIT3, MX1, OAS3, HERC5, ISG15*

*Listed up-regulated genes are those determined to be outliers by the Tukey method of outlier detection.

IFN-γ concentrations in serum after RAD001 + BEZ235 therapy (fig. S1), suggesting that RAD001 + BEZ235 may be up-regulating signaling pathways downstream of IFNs. One possibility is that TORC1 inhibition by low-dose RAD001 + BEZ235 decreases cholesterol synthesis within cells due to decreased activation of SREBP2 (*6*). Decreased cholesterol biosynthesis after SREBP2 knockdown has been previously shown to increase expression of a subset of antiviral IFN-stimulated genes (similar to the IFN-stimulated genes up-regulated after RAD001 + BEZ235 treatment) and protect against viral infection (*20*). The magnitude of IFN-stimulated gene up-regulation in whole blood after RAD001 + BEZ235 treatment was small (an average increase of 17.8% for genes defined as up-regulated in Table 2). A smaller increase in IFN-stimulated gene expression may be sufficient to enhance immune function in the elderly while avoiding the undesirable adverse events that occur in patients treated with recombinant IFN, in whom much higher IFN-stimulated gene induction is observed (*21*).

Surprisingly, both BEZ235 and the combination of RAD001 + BEZ235 were associated with a significant (P = 0.008 for BEZ235 and P = 0.001 for RAD001 + BEZ235) reduction in infections in the elderly treated group for a year despite the fact that the study drug was discontinued after 6 weeks of treatment. These results suggest that mTOR inhibitor therapy may lead to persistent improvements in immune function after drug discontinuation. In future studies, it will be important to determine how long up-regulation of antiviral gene expression persists after discontinuation of TORC1 inhibitor therapy. Persistent beneficial effects of short courses of mTOR inhibitor therapy also have been demonstrated in mice. Specifically, 6 to 12 weeks of the mTOR inhibitor rapamycin have been shown to extend life span in elderly mice (*3*, *22*). Of interest, BEZ235 monotherapy was associated with a significant (P = 0.008) reduction in infection rates but not an improvement in influenza vaccination response in the current study. These findings suggest that partial TORC1 inhibition with BEZ235 alone or in combination with RAD001 may be sufficient to enhance IFN-induced innate antiviral immunity and thereby decrease infection rates. Broader TORC1 inhibition with the combination treatment of BEZ235 + RAD001 may be necessary to enhance adaptive immunity required for the humoral response to influenza vaccination.

The decrease in infection rates seen in the elderly subjects in our study is of clinical relevance given that infections, particularly respiratory tract infections, are one of the leading causes of death in the elderly (*5*, *6*, *23*). Given that hundreds of different viral serotypes cause respiratory tract infections, drugs that enhance the ability of the immune system to fight many different viruses by up-regulating antiviral genes may be more effective for the treatment of respiratory tract infections than drugs targeting specific viruses. Moreover, the immune system is necessary not only to fight infectious pathogens but also for cancer immunosurveillance and for the clearance of senescent cells that contribute to organ dysfunction during aging. Therefore, therapies that enhance immune function, including potentially BEZ235 monotherapy or RAD001 + BEZ235 combination treatment, may have pleiotropic health benefits in the elderly.

MATERIALS AND METHODS

Study design

From December 2013 to April 2015, 264 elderly volunteers ≥65 years of age without unstable underlying medical diseases were enrolled in a randomized, double-blind, placebo-controlled trial at 12 clinical sites. The primary objective of the study was to determine whether RAD001 alone or in combination with BEZ235 enhanced immune function in the elderly as assessed by response to influenza vaccination. Exclusion criteria at screening included hemoglobin (<10.0 g/dl), white blood cell count (<3500/mm^3), neutrophil count (<2000/mm^3), platelet count (<125,000/mm^3), type 1 or uncontrolled type II 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, impaired renal or liver function, 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 or RAD001 + BEZ235 to corresponding placebo of 4:1 in each treatment arm. The treatment arms were as follows: 0.1 mg of RAD001 daily or matching placebo, 0.5 mg of RAD001 daily or matching placebo, 10 mg of BEZ235 daily or matching placebo, or a combination of 0.1 mg of RAD001 + 10 mg of BEZ235 daily or matching placebo. The treatment duration for all cohorts was 6 weeks during which time subjects underwent safety evaluations in the clinic every 2 weeks.

After completing the 6-week course of study drug, subjects had a 2-week drug-free interval and were then administered a 2014 seasonal influenza vaccination (Fluvax, CSL Biotherapies) containing the strains A/H1N1 (A/California/7/2009), A/H3N2 (A/Texas/50/2012), and B (B/Massachusetts/2/2012). Four weeks after administration of the influenza vaccine, subjects had serum collected for influenza titer measurements. Subjects were contacted monthly by telephone and had subsequent follow-up clinic visits at months 8, 9, and 12 after initiation of study drug treatment. The study was conducted in accordance with the principles of Good Clinical Practice and was approved by the appropriate ethics committees and regulatory agencies. Informed consent was obtained from all subjects. The clinical trial registration number on the Australian New Zealand Clinical Trial Registry was ACTRN12613001351707.

Infection rates

Study participants recorded the type, onset, duration, and treatment of any infection experienced during the study in a diary that they completed at home. The diary was brought to each study visit, and the information was recorded in electronic case report forms (eCRF). In addition, information about infections was obtained during phone calls between the sites and participants that occurred weekly during the 6 weeks subjects were on study drug and then monthly for the remainder of the trial. During the phone calls, the site staff inquired whether the subjects had experienced any infections since their last study visit or phone call from the site. Infections reported during the phone calls were reconciled with the data collected in the infection diaries and recorded in the eCRF. Subject reporting was used to determine infection rates rather than relying on laboratory confirmation because the most common infections that occur in community dwellers are upper respiratory tract infections. Upper respiratory tract infections are caused by hundreds of different viral serotypes many of which cannot be laboratory-confirmed. In addition, a previous study found that elderly subjects are able to accurately report when they have symptoms of respiratory tract infections (*19*).

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 the infection diaries that subjects filled out at home throughout the study. 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.

Animal maintenance, treatment, and tissue collection

All procedures involving animals were approved by the Institutional Animal Care and Use Committee of the Novartis Institutes for Biomedical Research, Cambridge, MA, USA. All animals were maintained under temperature and light-controlled conditions (22°C, 12-hour light/12-hour dark cycle: lights on at 0600/lights off at 1800) with ad libitum access to food and water. Male Sprague-Dawley rats aged 21 to 22 months were obtained from Envigo, housed singly, and maintained on a 2014 Teklad Global 14% Protein diet (Envigo) ad libitum. Rats were acclimated for about 4 weeks before experiments commenced. Rats were treated per os with a vehicle, RAD001, BEZ235, or a combination of RAD001 + BEZ235 once a day for 7 days. RAD001 (Novartis) was formulated as a custom-made microemulsion preconcentrate at 2% (w/w) and diluted to a final concentration in water, supplemented with 0.25% methylcellulose. BEZ235 was formulated in water with microemulsion preconcentrate and 0.25% methylcellulose. Microemulsion preconcentrate (adequate to a dose) diluted in water with 0.25% methylcellulose served as a vehicle control. Four hours after the last dose, rats were anesthetized with 3.5% isoflurane and killed by exsanguination and thoracotomy. Liver tissue was collected and immediately frozen in liquid nitrogen.

Protein extraction and immunoblotting

For protein extraction, liver tissues were pulverized in liquid nitrogen by mortar and pestle, 30 to 50 mg of powdered tissue was homogenized in MSD Tris Lysis Buffer (Meso Scale Discovery), supplemented with complete EDTA-free protease inhibitor and PhosSTOP phosphatase inhibitor tablets (Roche), and centrifuged at 13,000*g* for 20 min at 4°C. The resultant supernatant was used for immunoblotting. Protein was quantified with a BCA protein assay (Thermo Fisher Scientific). Samples were resolved on 4 to 20% Criterion TGX Precast Midi Protein gels (Bio-Rad) and transferred onto nitrocellulose membranes (Bio-Rad) using a Trans Turbo Blot system (Bio-Rad). Immunoblotting was performed with antibodies to p-ribosomal protein S6 (Ser^{240}/^{244}; #5364), t-ribosomal protein S6 (#2217), p-S6K1(Thr^{389}; #9205), t-S6K1 (#9202), and p4EBP1(Thr^{37}/^{46}; #2855), all from Cell Signaling Technologies (all 1:1000 in TBS-T with 5% bovine serum albumin). The "p" and "t" prefixes signify "phosphorylated" and "total" forms, respectively. Horseradish peroxidase–conjugated secondary antibodies against rabbit (#7074) were from Cell Signaling Technologies. GAPDH was detected with an anti-GAPDH antibody (#5174, Cell Signaling Technologies) and was used as a loading control. The chemiluminescence signal was generated using the SuperSignal West Femto Enhanced Chemiluminescent Substrate (#34095, Thermo Fisher Scientific) or the Western Lightning Plus-ECL Enhanced Chemiluminescence Substrate (NEL103001EA, Perkin Elmer) and was captured using the ChemiDoc MP Imaging System (Bio-Rad). Resultant digital

images were converted into a tagged image file format (TIFF) and quantified using ImageJ software.

Serum cytokine analysis

Concentrations of IL-6, IFN-γ, and TNFα were measured in serum samples obtained from subjects at baseline and after 6 weeks of study drug treatment. Cytokine concentrations were measured at SGS-Cephac Europe with an Electro-chemiluminescence immunoassay using a human Proinflammatory Panel 1 V-plex kit from Meso Scale Discovery. IL-18 concentrations were measured at SGS-Cephac Europe by sandwich enzyme-linked immunosorbent assay using two different monoclonal antibodies against two different epitopes of human IL-18.

RNA collection and sequencing

Total RNA was purified from peripheral venous blood collected in PAXgene tubes, and the quality and yield of the isolated RNA assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies). RNA sequencing (RNA-seq) libraries were prepared from the RNA using the Illumina TruSeq Stranded Total RNA with the Ribo-Zero Globin Kit. This kit removed ribosomal RNA and globin mRNA in a single step. The libraries generated were sequenced using an Illumina HiSeq2500 platform in paired-end mode to a read length of 2 × 76 base pairs (bp). Images from the instrument were processed using the manufacturer's software to generate FASTQ sequence files. Read quality was assessed by running FastQC (version 0.10) on the FASTQ files. Sequencing reads showed excellent quality, with a mean Phred score higher than 30 for all base positions. An average of 59.5 million 2 × 76–bp paired-end reads per sample were mapped to the *Homo sapiens* genome (version GRCh38), and RefSeq UCSC was used for human gene and transcript annotation. An in-house gene quantification pipeline was used to determine the number of counts mapping to each gene (*24*). On average, 94.3% of the total reads were mapped to the genome or the transcripts, and 46.3% were mapped to expressed sequences. More specifically, 34.7% of the total reads were aligned unambiguously (for example, uniquely) to a single expressed gene. Samples with fewer than 8 million expressed reads were removed from further analysis, as were three samples demonstrating unexpected strand imbalance. Any gene with an average expression of less than one count per million in any treatment/time condition was considered undetectable and removed from further analysis. Count data for remaining genes were then normalized in R Statistical Computing Software (v3.1.3) using the voom normalization procedure (*25*).

Differential expression analysis

Principal component analysis of the voom-normalized expression measurements indicated that the first principal component (PC1) reflected multiple aspects of sample processing (RNA extraction batch, yield of mRNA, etc.). Thus, the value of this principle component was included as an adjustment for technical variation in subsequent analysis for both treatment arms.

For each gene, the following linear mixed model was fit to the voom-normalized expression data, adjusting for relevant covariates [age, gender, and body mass index (BMI)] and PC1 using SAS v9.3 software. Because RNA extraction, sequencing, and analysis of samples from subjects receiving RAD001 + BEZ235 was done several months after the samples from subject receiving placebo, separate linear models were fit to the data from each treatment group.

```
proc mixed data = exprsData anovaf;
class SubjID Day Trt Gender;
model log2exprs = Day PC1 Age Gender BMI/solution DDFM = KenwardRoger;
repeated Day/type = un subject = SubjID;
by gene;
```

where log2exprs refers to the voom-normalized expression measurement, Day refers to the time point the sample was taken (pre- or postdose), PC1 refers to the value of PC1 for that sample, Age refers to the age of the subject, Gender refers to the sex of the subject, BMI refers to the body mass index of the subject, and SubjID refers to the ID of the subject. Differential expression fold changes (FCs) and *P* values were obtained using the SAS estimate statement. This analysis resulted in gene expression response signatures for each of the treatment arms (RAD + BEZ and placebo). These gene expression signatures consisted of the post- versus pretreatment FC and *P* values for each treatment.

Pathway enrichment analysis

For this analysis, up- or down-regulated skew of genes in defined biological pathways within each treatment response signature was assessed. Canonical pathways (c2.cp.v5.0.entrez.gmt) were downloaded from the canonical signatures database (http://software.broadinstitute.org/gsea/msigdb/). Treatment response signatures were defined as described in the "Differential expression analysis" section. For each pathway and treatment response signature, a weighted Kolmogorov-Smirnov test was performed as previously described (*26*). Genes were considered up-regulated if their fold change was determined to be an outlier using the Tukey method of outlier detection. Specifically, an up-regulated gene met the following criteria: $\log_2$ (FC post- versus pretreatment) greater than Q3 + 3*(Q3 – Q1), where Q3 is the upper (that is, third) quartile, and Q1 is the lower (that is, first) quartile of $\log_2$ (FC) for each treatment.

Statistical analysis of influenza vaccination response and infection rates

The modified intention to treat population was defined as all subjects who received at least one full dose of study drug and who had no major protocol deviations affecting efficacy data. Two hundred forty-four of the total of 264 subjects enrolled in the study were in the modified intention to treat population.

The primary analysis of GMT ratios was done using a normal Bayesian repeated measures regression model with noninformative flat priors. This model was fitted to each antibody titer on the log scale with the primary outcome in each model being the day 84 measurement relative to baseline; the day 63 measurement relative to baseline was included in the outcome vector. The log transformation is to give normality, and differences on the log scale give ratios when back-transformed. The mixed model takes account of the within-subject correlation between repeated antibody titer measurements. The covariance structure of the matrix was considered as variance components structure (option type = VC). The model was fitted using SAS 9.3 PROC MIXED with the PRIOR FLAT statement. PRIOR FLAT specifies a prior density equal to 1 everywhere, making the likelihood function the posterior. Each model includes terms for the baseline titer, visit, treatment group, and appropriate interaction terms such as log_baseline*visit and treatment*visit. The Bayesian-based sample size calculation for primary efficacy, as measured by the GMT ratio active/placebo at day 84, aims for the following criteria to be met for at least two of three influenza strains: (i) 80% level of proof that the GMT ratio is >1 (statistical significance) and (ii) 50% level of proof that the GMT ratio is >1.2 (clinical significance).

The sample size or power for a given scenario is calculated through simulation of trial data under trivariate normal likelihoods centered at 0 ($\log_{10}$ 1) for placebo and 0.176 ($\log_{10}$ 1.5) for mTOR inhibitor treatment, with SDs estimated from a previous Novartis vaccine study of 0.5, 0.6, and 0.63 for the three antibody titers on the log scale and zero correlation between titers to different vaccine strains. For each simulated trial, the posterior distribution of the GMT ratio for each antibody titer was approximated by sampling from the posterior distributions of the GMTs in the two arms (on the log scale). Noninformative priors were used for all parameters. The probability of meeting the above criteria in at least two of three vaccine strains is 77.3% with 40 subjects receiving mTOR inhibitor treatment and 40 receiving placebo.

Poisson regression modeling of infection counts per patient was done. This adjusts predicted rates per treatment group for length of time each patient was in the study. The results reported were found to be robust to assuming Poisson data because when the negative binomial was used as an alternative distribution of counts, the results for treatment were very similar.

SUPPLEMENTARY MATERIALS

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Fig. S1. Changes in serum cytokine concentrations.

Fig. S2. RAD001 + BEZ235 combination treatment but not placebo treatment leads to up-regulation of IFN-induced gene expression.

Table S1. Summary of hemagglutination inhibition titers by influenza virus strain for each treatment over time.

Table S2. Demographic and baseline characteristics of the study subjects.

Table S3. Full list of pathways and genes up-regulated after RAD001 + BEZ235 treatment as assessed by gene expression analysis of whole blood.

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L.B.K. was a board member of resTORbio. J.B.M. and D.G. are co-inventors on the following patents: patent #PCT/US2014/06540, "mTOR Inhibitors for Enhancing the Immune Response"; and patent #PCT/IB2016/052980, "Pharmaceutical Combination of Everolimus with Dactolisib." J.B.M. and M.M. are co-inventors on patent #US20180161319, "Methods of Enhancing Immune Response." H.-U.P.H. is paid for statistical consulting to Novartis as an external trial statistician. **Data and materials availability:** All data are in the paper or in the Supplementary Materials. Materials disclosed herein may be made available upon request under a material transfer agreement. Please send material requests to J.B.M. at jmannick@restorbio.com.

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TORC1 inhibition enhances immune function and reduces infections in the elderly

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Dialing down TORC1 dials up immunity

Aging may be regulated by a discrete set of intracellular proteins including the mechanistic target of rapamycin (mTOR) kinase. mTOR functions within two multiprotein complexes called TORC1 and TORC2. Inhibition of TORC1 has extended life span in every species studied to date and ameliorated multiple aging-related pathologies including declining immune function. Mannick *et al*. now show that low-dose TORC1 inhibitor therapy in elderly humans decreased the incidence of all infections, improved influenza vaccination responses, and up-regulated antiviral immunity. Thus, targeting the TORC1 pathway that regulates aging may have clinical benefits for elderly humans including improvement in immune function and decreased infection rates.