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Influence of the Novel ATP-Competitive Dual mTORC1/2 Inhibitor AZD2014 on Immune Cell Populations and Heart Allograft Rejection

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Abstract

Background—Little is known about how new generation adenosine triphosphate (ATP)-competitive mechanistic target of rapamycin (mTOR) kinase inhibitors (TORKinibs) affect immunity and allograft rejection.

Methods—mTOR complex (C) 1 and 2 signaling in dendritic cells (DC) and T cells was analyzed by Western blotting, while immune cell populations in normal and heart allograft recipient mice were analyzed by flow cytometry. Alloreactive T cell proliferation was quantified in MLR; intracellular cytokine production and serum antidonor IgG levels were determined by flow analysis and immunofluorescence staining used to detect IgG in allografts.

Results—The novel TORKinib AZD2014 impaired DC differentiation and T cell proliferation in vitro and depressed immune cells and allospecific T cell responses in vivo. A 9-day course of

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Authorship

DF and AWT designed the experiments; DF, HD, YO, AW, SY, KM, and OY conducted the experiments and analyzed the data; MAR and SCW generated and interpreted tissue immunofluorescence staining data; AV provided critical input on design and interpretation of Ab measurement data; BR provided key input on design and interpretation of flow cytometry data; KM and DMR conducted analyses of regulatory B cells and analyzed data; DF, HD and AWT wrote the manuscript; DF, DMR and AWT revised the manuscript and all authors approved of the final manuscript.

Disclosure

The authors have no conflicts of interest.

AZD2014 (10 mg/kg ip twice daily) or rapamycin (RAPA; 1mg/kg ip daily) prolonged median heart allograft survival time significantly (25 days for AZD2014; 100 days for RAPA; 9.5 days for control). Like RAPA, AZD2014 suppressed graft mononuclear cell infiltration, increased regulatory T cell (Treg) to effector memory T cell (Tem) ratios and reduced T follicular helper (Tfh) and B cells 7 days posttransplant. By 21 days (10 days after drug withdrawal), however, Tfh and B cells and donor-specific IgG1 and IgG2c antibody titers were significantly lower in RAPA-compared with AZD2014-treated mice. Elevated Treg to Tem ratios were maintained after RAPA, but not AZD2014 withdrawal.

Conclusions—Immunomodulatory effects of AZD2014, unlike those of RAPA, were not sustained after drug withdrawal, possibly reflecting distinct pharmacokinetics or/and inhibitory effects of AZD2014 on mTORC2.

Introduction

The mechanistic target of rapamycin (mTOR) is an evolutionarily conserved serine/threonine kinase that drives organelle and cell growth^{1,2} through upregulation of glycolysis to fuel nucleotide, protein and lipid synthesis. mTOR functions as a component of at least 2 distinct multiprotein complexes, mTOR complex 1 (mTORC1) and mTORC2.^{3,4} While both complexes include mLST8 (mammalian lethal with SEC13 protein 8) and DEPTOR (DEP domain-containing mTOR-interacting protein), mTORC1 uniquely associates with Raptor (regulatory associated protein of mTOR) and PRAS40 (proline-rich Akt substrate of 40 kDa). In contrast, mTORC2 associates with Rictor (rapamycin-insensitive companion of mTOR), mSIN1 (mammalian stress-activated protein kinase-interacting protein 1) and Protor (protein observed with Rictor). While mTORC1 has been implicated in regulation of nucleotide and protein synthesis, as well as autophagy, much less is known about the functions of mTORC2. Recently however, mTORC2 has been implicated in regulation of cell growth, proliferation, survival and cytoskeletal organization, as well as sodium handling in the kidney.^{5–7}

There have been important recent advances in understanding of how mTOR complexes regulate immune cell differentiation and function.⁸ Thus, genetic deletion of either mTORC1 or mTORC2 in T cells has revealed that T helper (Th) 1 and Th17 differentiation is selectively regulated by mTORC1, whereas Th2 development is mTORC2-dependent.^{9,10} Furthermore, inhibition of both mTORC1 and mTORC2 favors regulatory T cell (Treg) development more than inhibition of either complex alone. In separate studies, small hairpin RNA vectors targeting Raptor (mTORC1) induce T follicular B helper cell (Tfh) differentiation at the expense of Th1 cells, while Rictor deletion promotes Th1 cells, with minimal effect on Tfh cells.¹¹ While less is known concerning how mTOR impacts B cell function, deletion of Rictor in B cells causes marked deficiencies in mature follicular, marginal zone and B1a B cells with consequent effects on antibody (Ab) responses in vivo.¹²

The immunosuppressive prodrug rapamycin (RAPA) is an allosteric inhibitor of mTOR that mediates its effects indirectly via interaction with the immunophilin FK506 binding protein (FKBP) 12 and formation of a drug-immunophilin complex that directly binds the FKBP-

rapamycin-binding (FRB) domain of mTOR.¹³ While assembly of mTORC1 is RAPA-sensitive, mTORC2 is insensitive to RAPA. Recent studies in yeast demonstrating that the C terminal part of Avo3, a subunit unique to mTORC2, prevents RAPA-FKBP12 from accessing the FRB domain¹⁴ may help explain this phenomenon.

To overcome shortcomings of RAPA and its analogues (rapalogs) as therapy for advanced malignancies, new generation adenosine triphosphate (ATP)-competitive mTOR inhibitors (TORKinibs) have been developed. By targeting both mTORC1 and mTORC2, these second generation mTOR inhibitors have been predicted to have more potent antitumor effects. Based on encouraging preclinical results, TORKinibs are being tested in early-phase clinical trials for treatment of advanced solid tumors or multiple myeloma.^{15,16} While much of our understanding of the effects of these TORKinibs stems from studies in oncology, little is known about their influence on immunity or their potential as immunosuppressive agents. Recently, in a limited proof-of-principle study,¹⁷ we showed that the ATP-competitive mTOR inhibitor AZD8055 could suppress T cell proliferation and prolong graft survival in mice. AZD8055 is no longer in clinical development due to frequently reported elevations in transaminases. However, AZD2014 (Vistusertib), a related compound, with a more favorable pharmacokinetic profile,^{18,19} has entered early-phase trials in advanced malignancy.^{19–21} Here, we examined for the first time, the influence of AZD2014 (compared with RAPA) on immune cell populations, allograft rejection and underlying cellular and humoral immunity.

Materials and Methods

Mice

Male C57BL/6J (B6; H2K^b), BALB/c (H2K^d), C3H/HeJ (C3H; H2K^k) and B6.Cg-Tg (Tcr α ,Tcr β)3Ayr/J (referred to as 1H3.1) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). They were maintained under specific pathogen-free conditions in an American Association for the Accreditation of Laboratory Animal Care-accredited facility. All experiments were performed under an Institutional Animal Care and Use Committee-approved protocol, in accordance with NIH guidelines.

DC Generation and Stimulation

DC were generated from freshly-isolated B6 bone marrow (BM) cells, as described.²² On day 2 of culture, either RAPA (LC Laboratories, Woburn, MA) or AZD2014 (Selleck Chemicals, Houston, TX), prediluted in RPMI-1640, was added at the concentration indicated and refreshed on days 4 and 6. Control cultures with no drug administration were set up in parallel. On day 7, cells were collected, counted and CD11c⁺ cells isolated by anti-CD11c immunomagnetic bead purification (Miltenyi Biotec, Auburn, CA). In some experiments, the DC were stimulated for 16–18 h with lipopolysaccharide (LPS) (*Salmonella minnesota*, 10 ng/ml; R595, Alexis Biochemicals, San Diego, CA).

T Cell Stimulation and Proliferation

CD3⁺ T cells were isolated by negative selection and labeled with Violet Proliferation Dye 450 (VPD450; BD Horizon) as per the manufacturer's instructions. They were stimulated (2.10⁵ cells/well) by α CD3/CD28 Dynabeads (Gibco) in 96-well, round-bottom plates in the

presence of either RAPA or AZD2014 at a bead-to-cell ratio of 1:2. Cultures were maintained for 72 h before analysis of cell proliferation by flow cytometry. To assess antidonor reactivity in graft recipients, splenic T cells isolated on day 7 or 21 posttransplant and labeled with VPD450 were stimulated for 5 days with T cell-depleted (Miltenyi) donor (BALB/c) or third-party (C3H) splenocytes (1:1 ratio). Cell proliferation was analyzed by flow cytometry.

Cell Surface and Foxp3 Staining

Lymphocytes ($5\text{--}10 \times 10^6$) were first stained with either Zombie Aqua or Zombie NIR (Biolegend; San Diego, CA) to exclude dead cells. All fluorochrome-conjugated Abs were purchased from eBioscience (San Diego, CA), BD Biosciences (San Jose, CA) or BioLegend and used as described.^{23,24} Cell surface staining of DC was performed as described.²⁵ For Forkhead box p3 (Foxp3) staining, T cells were fixed and permeabilized using Foxp3 Fix Permkit (eBioscience). All data were acquired using an LSR Fortessa flow cytometer and analyzed using FlowJo Version 10.1.

Intracellular Cytokine Staining

These methods are detailed in the Supplemental Methods.

Western Blots

These methods are detailed in the Supplemental Methods

Adoptive Transfer of 1H3.1 TCR Tg CD4 T Cells

1H3.1 TCR-tg CD4⁺ T cells (CD90.1⁺V β 6⁺) were purified from spleens of 1H3.1 mice by negative selection, labeled with VPD450 and injected (6×10^6 cells in PBS i.v.) retroorbitally. At 48 h, BALB/c I-E α -derived allopeptide (IE α ₅₂₋₆₈; 500 μ g/kg) (Thermo Scientific, Waltham, MA) was injected i.p. Twenty-four h later, the mice received an i.p. injection of either drug vehicle, RAPA or AZD2014 that was repeated for 72 h until analysis of alloreactive TCR-tg T cell proliferation by flow cytometry.

Heart Transplantation and mTOR Inhibitor Administration

BALB/c to B6 heterotopic heart transplantation was performed as described.²⁶ Graft recipients received a 9-day course of either drug vehicle (i.p. twice daily), RAPA (1mg/kg/day i.p. daily) or AZD2014 (10 mg/kg i.p. twice daily). For in vivo administration, RAPA was dissolved in ethanol and diluted in a vehicle containing PBS with 30% polyethylene glycol and 0.5% Tween 80. AZD2014 was dissolved in 65% cremophor EL in ethanol and diluted in the same vehicle. Vehicle-treated control animals received 65% cremophor EL alone prediluted in the same vehicle. Graft survival was monitored by abdominal palpation; rejection was determined by complete cessation of cardiac contraction and confirmed histologically. Groups of recipients were also euthanized on day 7 or 21 posttransplant for immunological analyses.

Donor-Specific AlloAb Measurement

Antidonator and third party-reactive Ab titers were determined as described.²⁷ Donor (BALB/c) and third party (C3H) thymocytes (2×10^6 cells per round-bottom well) were incubated with 100 μ l of serially diluted serum. Fluorochrome-conjugated rat anti-mouse IgG1, IgG2c and IgG3, as well as biotin-conjugated rat anti-mouse IgG2b Abs were used at 1:50 to 1:100 dilution (BD Biosciences). Cells were washed and analyzed by flow cytometry.

Histology and Immunofluorescence Staining

These methods are detailed in the Supplemental Methods.

Statistical Analyses

Results are expressed as means $\pm$ 1 SD. Significances of differences between means were determined using either the unpaired, 2-tailed Student's *t* test or 1-way ANOVA test. Log-rank tests were used to establish the significances of differences between survival curves (GraphPad Prism; San Diego, CA). $p < 0.05$ was considered significant.

Results

AZD2014 but not RAPA, inhibits both mTORC1 and mTORC2 Signaling

Although previous studies^{19,28} have shown that the ATP-competitive inhibitor AZD2014 inhibits signaling downstream of both mTORC1 and 2, these studies have been conducted using tumor cell lines. To determine the influence of AZD2014 compared with RAPA on mTOR signaling in DC, purified B6 BM-derived myeloid DC were incubated with various concentrations of either mTOR inhibitor for 1 h in the absence or presence of LPS. All in vitro studies were subsequently performed using 11.9 nM RAPA as this dose potentially inhibited phosphorylation of S6K at threonine 389 (mTORC1). Figure 1A shows that 500nM AZD2014 significantly inhibited both 4E-BP1 threonine 37/46 (mTORC1) and Akt Ser473 (mTORC2) phosphorylation in LPS-activated DC compared to LPS-activated control DC. Akt phosphorylation at Ser473 was not significantly affected by any of the concentrations of RAPA tested (11.9, 100, 500 and 1000 nM; data not shown). In contrast, both RAPA and AZD2014 inhibited phosphorylation of S6K at threonine 389 (mTORC1) (data not shown).

AZD2014 Inhibits DC Generation from BM and both DC and T Cell Responses to Stimulation

We next examined the influence of AZD2014 on DC differentiation from BM. DC were generated with GM-CSF and IL-4 in the presence of either AZD2014, RAPA or no drug at concentrations shown to inhibit mTOR signaling. Both agents markedly reduced the yield of DC that could be harvested on 7 day of culture (Figure 1B). Coregulatory molecule (CD86 and B7-H1=programed death ligand 1 [PD-L1]) expression (mean fluorescence intensity; MFI) by these DC was diminished (although CD86 not significantly) compared with that by cells harvested from control cultures and was similarly diminished following LPS stimulation (Figure 1C). Both AZD2014 and RAPA also inhibited the proliferation of

α CD3/CD28-stimulated CD4⁺ and CD8⁺ T cells when added at the start of 3-day cultures (Figure 1D). With respect to both DC differentiation and maturation and T cell proliferative responses RAPA, used at a concentration 40 times lower, was more potent than AZD2014 in achieving these inhibitory effects.

AZD2014 Administration Markedly Reduces Thymocyte Populations, but Enhances the Incidence of Thymic Treg

Naïve B6 mice were injected with either vehicle control, RAPA or AZD2014 for 9 days. As documented previously for RAPA,²⁹ AZD2014 exerted profound effects on the thymus (Figure 2). Thymic size, architecture and cellularity were all altered significantly by AZD2014 (Figure 2A-D). While T cell subsets (CD4⁺CD8⁻, CD4⁻CD8⁺, CD4⁺CD8⁺ cells and CD4⁺CD25⁺Foxp3⁺ Treg) were all reduced by AZD2014 administration, the percentage of Treg within the CD4 compartment was increased significantly by either AZD2014 or RAPA (Figure 2E-H). Thus, as has been described for RAPA,^{30,31} dual mTORC1/2 inhibition with AZD2014 enriched for thymic Treg, indicating that these cells were more resistant to AZD2014 than effector T cells.

AZD2014 Administration Reduces Immune Cell Populations in Secondary Lymphoid Tissue

Concomitant analysis of the spleen after 9 days of drug administration (Figure 3) revealed that all T cell subsets, including Tfh and Treg, were reduced by AZD2014 as well as RAPA (Figure 3A-H). In addition, other immune cells, including B cells, natural killer (NK) cells, NKT cells and CD11c⁺DC, were suppressed (although B cells and NKT cells not significantly) by AZD2014 administration (Figure 3I-L). Overall however, the T cell-depleting effects were more moderate than those observed in the thymus. Furthermore, in contrast to the thymus, Treg enrichment was not observed in naïve mice following either AZD2014 or RAPA administration.

AZD2014 Inhibits Alloreactive CD4 T Cell Proliferation In Vivo

We next determined the influence of AZD2014 on T cell proliferation in response to alloAg stimulation in vivo. 1H3.1 TCR-tg CD4⁺ T cells (CD90.1⁺V β 6⁺) isolated from TEa mice and labeled with VPD450 were adoptively transferred (6x10⁶ i.v.) into wild-type recipient B6 mice. Forty-eight hours later, I-E _{α} (52-68) peptide and subsequently either vehicle, RAPA or AZD2014 were injected i.p. Seventy-two hours thereafter, spleens were harvested and alloreactive tg T cell proliferation assessed by flow cytometry (Figure 4A). Compared to vehicle control-treated mice, those injected with either RAPA (1 mg/kg) or AZD2014 (10 mg/kg bid) showed marked inhibition of alloreactive CD4⁺ T cell proliferation (Figure 4B and C).

AZD2014 Prolongs Vascularized Heart Allograft Survival

Given the foregoing observations, we hypothesized that AZD2014 monotherapy would prolong organ allograft survival. Given the known effects of mTOR inhibitors on wound healing^{32,33} and evidence mTOR may protect against cardiac ischemia-reperfusion injury,^{34,35} we delayed administration of AZD2014 until day 3 posttransplant (Figure 5A).

Administration of AZD2014 for 9 days significantly prolonged graft survival (median survival time [MST] 25 days vs 9.5 days in vehicle-treated controls (Figure 5B), associated with marked reductions in graft mononuclear cell infiltration and tissue injury on days 7 and 21 (Figure 5C). MST with RAPA administration however, was greater than with AZD2014 (100 days; $p=0.05$).

AZD2014 and RAPA Differentially Impact Tfh and Treg Posttransplant

We hypothesized that, while naïve and effector T cells would be profoundly inhibited by AZD2014 in transplant recipients, Treg and Tfh subsets that are known to be less dependent on mTOR-mediated metabolism,^{11,36,37} might be relatively spared. We analyzed splenic CD4, CD8, Treg and Tfh cells 7 and 21 days posttransplant. Seven days posttransplant, AZD2014, like RAPA, reduced absolute numbers of CD4⁺ and CD8⁺ (data not shown), Tfh and Treg (Figure 5D) and increased the mean Treg/Tmem ratio, although not significantly (Figure 5E). By 21 days posttransplant (10 days after drug withdrawal), the degree of T cell suppression was reduced. Absolute numbers of both Tfh and Treg in AZD2014-treated mice were significantly higher compared to 7 days posttransplant. Furthermore, both Tfh and Treg were significantly higher in AZD2014- than in RAPA-treated graft recipients at 21 days (Figure 5D). The elevated ratio of Treg/Tem in the spleen, while preserved in RAPA-treated mice at 21 days, was no longer evident in AZD2014-treated animals (Figure 5E). Thus, although AZD2014 was withdrawn at the same time posttransplant as RAPA, its inhibitory effects on the T cell compartment and its influence on the Treg/Tem ratio were less persistent.

AZD2014 and RAPA Affect Both T Cell Cytokine Production and Antidonor T Cell Proliferation Posttransplant

To assess the influence of AZD2014 on host T cell function, splenocytes were harvested 7 days posttransplant and stimulated ex vivo with PMA and ionomycin. T cell production of IFN γ , TNF α and IL-2 was then assessed by mAb staining and flow cytometry. As shown in Figure 6A and B, IFN γ expression by CD4 T cells was reduced significantly in AZD2014-, as in RAPA-treated mice, while CD8 T cell expression of IFN γ was also inhibited, although not significantly. By contrast, effects on TNF α (data not shown) and IL-2 expression by T cells were less pronounced. We also determined the influence of AZD2014 on antidonor T cell proliferative responses. The inhibitory effects of AZD2014 on ex vivo MLR were less pronounced than those of RAPA at days 7 and 21 posttransplant (Figure 6C).

AZD2014 and RAPA Differentially Affect Total B Cells, IL-10⁺ B Cells and Donor-Specific Ab Levels in Graft Recipients

Given the elevations in absolute numbers of Tfh observed in spleens of graft recipients after withdrawal of AZD2014 compared with RAPA treatment (day 21; see Figure 5D), we hypothesized that this might have significant consequences on downstream mediators of graft rejection ie, total B cells, IL-10⁺ B cells and donor-specific Ab (DSA). Consistent with their inhibitory effects on B cells in naïve mice, both RAPA and AZD2014 suppressed absolute numbers of B cells in graft recipient spleens 7 days posttransplant. While there was a relative sparing of IL-10⁺ B cells (B cells with regulatory function^{38–40}) in response to RAPA (Figure 7A), this effect was not observed with AZD2014. Furthermore, by 21 days

posttransplant (10 days after drug withdrawal), while total numbers of B cells increased compared with day 7, the incidence of IL-10⁺ B cells did not, irrespective of which mTOR inhibitor was administered.

We also examined IgG deposition in the graft and DSA levels in the serum of AZD2014-treated graft recipients. At 21 days posttransplant, while marked IgG deposition was evident in AZD2014-treated mice, only minimal deposition was detected in RAPA-treated animals (Figure 7B). In this heart transplant model, serum DSA levels generally peak 3–6 weeks posttransplant (17). Twenty-one days posttransplant titers of IgG1 were significantly lower in RAPA-treated compared with AZD2014-treated graft recipients that had titers of IgG1 and IgG2b like vehicle control-treated graft recipients (Figure 7C). Even at 100 days posttransplant, IgG2c titers remained lower in transplant recipients treated with RAPA, whereas IgG1 titers were similar between the 2 mTOR inhibitor-treated groups. No significant effects on IgG2b levels were detected.

Discussion

Targeting of ATP and of the purinergic system⁴¹ offer potential benefits in allograft rejection and tolerance. Recently, we showed¹⁷ that AZD8055, a novel, specific ATP-competitive mTORC1/2 dual inhibitor⁴² could suppress T cell proliferation and prolong mouse heart allograft survival. Despite encouraging antitumor activity in preclinical models,⁴³ due to high turnover in human hepatocytes and inconsistent rodent pharmacokinetics,¹⁸ development of this agent has been discontinued and interest focused on the more potent related mTOR inhibitor AZD2014. This molecule has superior/consistent rodent pharmacokinetics, low turnover in human hepatocytes¹⁸ and has been well-tolerated in phase I trials in solid tumor patients.^{21,44} Thus we considered it appropriate to study AZD2014 in order to expand understanding of how novel ATP competitive dual mTOR inhibitors impact cellular and humoral alloimmunity (compared to rapalogs). In particular, based on previous rodent studies showing an important role for mTORC2 in B cell maturation, homeostasis and Ab production, we hypothesized that AZD2014 might have (unique) advantages over RAPA in terms of inhibitory effects on B cells, circulating DSA levels and Ab deposition in the graft,- variables that we did not examine in our AZD8055 study.¹⁷

Here we show for the first time, that AZD2014 exerts multiple effects on immune cell populations, thus (i) inhibiting myeloid DC generation and T cell proliferation in vitro, (ii) markedly depleting thymocytes in vivo, although enriching for thymic Treg and (iii) reducing innate and adaptive immune cell populations (DC, NK cells, NKT cells, T cell subsets and B cells) in secondary lymphoid tissue. The greater potency of RAPA observed is consistent with a reported IC₅₀ for mTOR of 0.1nM and 2.8nM for RAPA and AZD2014, respectively. We know from others' work that doses of 7.5–15 mg/kg of AZD2014 administered to mice by oral gavage produced a C_{max} from 1 to 10 μmol/L in plasma.²⁰ Based on these observations, one would expect a dose of 10 mg/kg of AZD2014 administered intraperitoneally to produce peak plasma and intracellular drug concentrations above 2000nM (2 μmol/L). Furthermore, in the first-in-human pharmacokinetic study of AZD2014, the maximum tolerated dose was 50 mg twice daily taken orally.⁴⁴ Using a formula for dose translation based on body surface area, a dose of 10mg/kg delivered to

mice is equivalent to the maximum tolerated dose in humans (based on a body weight of 60 kg).⁴⁵ Taken together, these known pharmacokinetic properties of AZD2014, justified administering 10 mg/kg twice daily, a dose 20-fold higher than RAPA, in the current study. AZD2014 significantly prolonged MHC-mismatched organ allograft survival, while inhibiting effector T cell responses to donor, reducing Tfh cells and B cells, but exhibiting little effect on DSA production. Significantly, 10 days following drug withdrawal, both Tfh cell numbers and DSA (IgG1, IgG2b and IgG2c) titers in AZD2014-treated graft recipients were higher than in RAPA-treated recipients and did not differ significantly from levels in vehicle-treated controls.

We observed severe thymic atrophy and profound reduction in absolute thymocyte numbers, with an increased incidence of CD4⁺CD25⁺Foxp3⁺ Treg in AZD2014-treated mice. In contrast, relative Treg enrichment was not seen in the spleen in response to either mTOR inhibitor. These results are consistent with effects of RAPA in lymphocyte-replete mice.⁴⁶ In these latter studies, administration of RAPA inhibited homeostatic and alloAg-induced Treg proliferation in the spleen and lymph nodes (the thymus was not examined). Furthermore, Treg and conventional T cells were depleted similarly. By contrast, in a separate study, 2 weeks of RAPA administration enriched for Treg in both the thymus and spleen.⁴⁷

Interestingly, Tfh cells, a subset of T cells that stimulates germinal center (GC) B cells and Ab class switching, was also suppressed by AZD2014 or RAPA during the drug administration period. Although previous studies¹¹ have suggested that Tfh, like Treg, utilize less glycolysis, mitochondrial respiration and as a consequence, mTOR function than Th1 cells, AZD2014 and RAPA both suppressed Tfh effectively. This is consistent with recent data⁴⁸ demonstrating that glucose metabolism and both mTORC1 and mTORC2 signaling are essential for Tfh differentiation and GC reaction in Peyer's patches. In the latter study, mTORC2 was uniquely required for normal Tfh cell localization in PP, expression of Forkhead box protein 01 (Fox01) targets and nuclear exclusion of Fox01 upon inducible T cell costimulator ligation. By contrast, in the present investigation, 10 days after drug withdrawal, repopulation of the T cell compartment was more robust in graft recipients given AZD2014 than those given RAPA. Furthermore, statistically significant differences in the number of Tfh (and to a lesser extent Treg) were clearly evident. One possible explanation for these differences is that reversal of both mTORC1 and mTORC2 inhibition upon AZD2014 withdrawal may trigger a greater proliferative response in T cells (particularly in Tfh) than reversal of selective mTORC1 inhibition after RAPA withdrawal.

Given the importance of humoral immunity in long-term allograft survival, the influence of mTOR inhibitors on B cells and subsequent Ab production has been the subject of considerable recent interest. RAPA can strongly inhibit B cell responses and Ig production in vitro.⁴⁹ We found that while RAPA suppressed the total number of host B cells posttransplant, it simultaneously preserved the incidence of IL-10⁺ B cells and suppressed titers of circulating DSA (IgG1 and IgG2c) and IgG deposition within the graft. Others have shown that RAPA increases graft-infiltrating IL-10⁺ transforming growth factor β ⁺ Breg in a mouse tracheal transplant model.⁵⁰ Thus, based on these preclinical data, one might expect RAPA to exert suppressive effects on human pathogenic complement-fixing IgG1 and IgG3 DSA generation posttransplant.⁵¹ Paradoxically however, compared to standard calcineurin

inhibitor-based immunosuppression, several retrospective analyses have documented a higher incidence of de novo DSA in kidney transplant patients treated with mTOR inhibitors.^{5,52,53}

Using a mouse lymphocytic choriomeningitis virus model with modulation of mTOR signaling by RNAi, B cells were found recently to be the primary cell targets of RAPA, with reduced Tfh formation due to lower GC B cell responses.⁵⁴ The differential effects of RAPA and AZD2014 on IL-10⁺ B cells is another interesting finding that warrants further investigation. In contrast to RAPA, while AZD2014 was also able to suppress total B cells, it did not preserve IL-10⁺ B cells or prevent alloAb generation and deposition.

The distinctions we observed between RAPA and AZD2014 in prolongation of allograft survival, as well as the greater durability of immunosuppression mediated by RAPA following its withdrawal, may reflect pharmacologic differences and also differences in the reversibility of the effects of these agents on T cells. Based on our previous pharmacokinetic studies comparing AZD8055 and RAPA17, coupled with the small body of pharmacologic literature on AZD2014, we hypothesize that differences in tissue and cellular penetration, mTOR affinity and elimination half-life could account for the differential effects on alloimmune reactivity and graft survival. Alternatively, RAPA might uniquely affect several non-mTORC1 pathways important in T cell function. It is also possible that dual inhibition (mTORC1 and mTORC2) might be less immunosuppressive than mTORC1 inhibition in certain immune cells. Analysis of cell signaling pathways in allografts and/or lymphoid tissue at various times after drug withdrawal would allow dissection of the molecular differences between these 2 mTOR inhibitors. Nonetheless, a favorable toxicity profile of ATP-competitive dual mTORC1/2 inhibition would justify testing these novel agents with other immunosuppressive strategies (ie costimulation blockade or calcineurin inhibition) in organ transplantation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

Ab	antibody
Ag	antigen
ATP	adenosine triphosphate
DC	dendritic cell(s)
DSA	donor-specific antibody

Foxp3	forkhead box p3
mTOR(C)	mechanistic target of rapamycin (complex)
RAPA	rapamycin
Tmem	memory T cell
Treg	regulatory T cell
TORKinib	target of rapamycin kinase inhibitor
Tfh	follicular helper T cell
Th	helper T cell
VPD450	violet proliferation dye 450

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Figure 1.

Differential effects of AZD2014 and RAPA on mTOR signaling and inhibition of DC generation and T cell proliferation in vitro. **(A)** CD11c⁺DC generated from B6 mouse bone marrow (BM) cells, as described in the Materials and Methods, were incubated for 1 hour with either RAPA or AZD2014, then for an additional hour in the absence or presence of LPS (100ng/ml). The cells were washed, lysed and proteins resolved by SDS-PAGE. The influence of RAPA or AZD2014 on signaling downstream of mTORC1 (4EBP1 threonine 37/46) and mTORC2 (Akt S473) was analyzed by Western blot. Relative expression is plotted for n=3 independent experiments per condition. **(B)** DC were generated in the presence of GM-CSF and IL-4 and exposed to either RAPA or AZD2014 (AZD), starting on day 2 of culture. On day 7 of culture, the DC were enumerated (total number of cells per culture dish is shown). **(C)** On Day 7 of culture, control (drug vehicle-treated) CD11c⁺ DC and CD11c⁺ DC exposed to either RAPA or AZD2014 were treated with or without LPS (100ng/ml) for 16–18 hours, washed with PBS, stained and analyzed by flow cytometry. Quantitation of CD86, B7-H1 (= programmed death ligand-1) and MHC class II (I-A^b) expression (relative MFI) across multiple experiments is shown. **(D)** Bulk T cells were isolated by negative selection from B6 mouse spleen and labeled with VPD450, as described in the Materials and Methods. They were stimulated with αCD3/CD28 Dynabeads (1:2) in 96-well, round-bottom plates for 72 hours in the presence of RAPA or AZD2014 added at the start of culture. CD4 and CD8 T cell proliferation was assessed by flow cytometry. Proliferating cells were identified as CD4⁺VPD450^{lo} or CD8⁺VPD450^{lo} and % proliferation relative to the total parent population determined. Data are from n=3 independent experiments *p<0.05; **p<0.01; ***p<0.001.

Figure 2.

AZD2014 administration induces thymocyte depletion and enriches for thymic Treg. AZD2014 (AZD; 10mg/kg bid i.p.), RAPA (1mg/kg i.p.) or drug vehicle was administered daily for 9 days to naïve B6 mice. Thereafter, the mice were euthanized, thymi excised, weighed and processed for histology, or total thymocytes isolated, counted and analyzed by mAb staining and flow cytometry. **(A)** and **(B)** Both AZD2014 and RAPA induced marked thymic involution with profound cortical and medullary atrophy. **(C)** The total number of thymocytes as well as **(D)** double-positive (CD4⁺CD8⁺) T cells and **(E)** and **(F)**, single positive CD4⁺ and CD8⁺ T cells were markedly depleted by both AZD2014 or RAPA. **(G)** Although the incidence of Treg (CD4⁺CD25⁺Foxp3⁺) increased significantly in thymi of both AZD2014- and RAPA-treated mice **(H)** the absolute number of Treg diminished in these groups. Data are from 3 animals per condition. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.

Figure 3.

AZD2014 administration suppresses immune cell populations in secondary lymphoid tissue but does not enrich for Treg. AZD2014 (AZD), RAPA or drug vehicle was administered daily to naïve male B6 mice for 9 days. Thereafter, mice were euthanized, spleens excised, weighed and either processed for histology or total splenocytes isolated, counted and analyzed by mAb staining and flow cytometry. (A) and (B) Representative spleen histology and spleen weights from drug vehicle-, AZD2014- or RAPA-treated mice. The cellularity of both the red and white pulp was depleted markedly in drug-treated compared with vehicle-treated mice. (C) The mean total number of splenocytes, as well as (D–H) the absolute numbers of CD4, CD8, Treg (CD4⁺CD25⁺Foxp3⁺) and Tfh cells (CD4⁺PD1^{hi}CXCR5^{hi}) were reduced by both AZD2014 and RAPA. F, Although the incidence of Treg in the CD4 T cell compartment was either unaffected (AZD2014) or reduced (RAPA) (G) both drugs reduced the absolute number of Treg. (I–L) AZD2014 and RAPA suppressed the mean absolute numbers of other immune cells in the spleen, including CD11c⁺DC, B cells and NK cells. NKT cells were not affected significantly. Data are from 3 animals per condition. *p<0.05; **p<0.01; ***p<0.001.

Figure 4.

AZD2014 inhibits alloreactive T cell proliferation in vivo. **(A)** The protocol for BALB/c I-Ea-derived allopeptide, drug vehicle, RAPA or AZD2014 (AZD) administration following adoptive transfer of VPD450-labeled 1H3.1 TCR-transgenic (tg) CD4⁺ T cells (CD90.1⁺Vβ6⁺) is shown. All mice, other than the “no peptide” group, received allopeptide 48 hours after adoptive transfer of cells. **(B)** Gating strategy for determining percent T cell proliferation. Adoptively transferred tg T cells were identified in the spleen by flow cytometry as CD3⁺CD4⁺CD90.1⁺Vβ6⁺ cells. Then, VPD450^{-lo} cells were gated and percent proliferation determined. **(C)** Percent proliferation 6 days after adoptive transfer of alloreactive tg T cells. Data are from 3–5 animals per condition and n=2 experiments were performed. **p<0.01, ***p<0.0001.

Figure 5.

AZD2014 prolongs heart allograft survival. **(A)** The protocol used for AZD2014 (AZD) or RAPA administration following heart transplantation. Starting on day 3 posttransplant, a 9-day course of either AZD2014 (10mg/kg i.p. twice daily), RAPA (1 mg/kg i.p. daily), or drug vehicle was administered. **(B)** Actuarial graft survival curves; numbers of transplanted mice in each group are shown in parentheses; **(C)** Histological appearance of heart allografts (H & E stain; horizontal bar indicates 200 μ m); **(D-E)** T cell populations (Tfh and Treg) in the spleen and Treg:Tem ratios 7 days and 21 days posttransplant. n=3 graft recipients per group *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.

Figure 6.

AZD2014 inhibits alloreactive T cell proliferation and IFN γ production by host T cells. On day 7 posttransplant, splenocytes from graft recipients were stimulated with PMA and ionomycin and T cell intracellular cytokine expression (IFN γ ; IL-2) assessed by flow cytometry. **(A)** Cytokine production by T cells from representative mice in each group; **(B)** Means + SD from n=2–5 animals per group. **(C)** Antidonor and antithird party CD4⁺ T cell proliferative responses in 5 day ex vivo MLR cultures. *p<0.05; **p<0.01.

Figure 7.

Influence of AZD2014 on B cell responses and DSA production. **(A)** Total as well as IL-10⁺ B cells were determined on day 7 and day 21 posttransplant in host spleens by mAb staining and flow cytometry. **(B)** Antibody deposition in the heart allografts was examined by immunofluorescence at 7 and 21 days posttransplant. Total IgG is shown in green, CD31 (a marker of endothelial cells) is shown in white, actin is shown in red and nuclei are stained with hoescht (blue). A naïve B6 heart is shown for comparison. Horizontal bars indicate 207µm. **(C)** Donor-specific alloAb (DSA) (IgG1, IgG2c and IgG2b) in serum 21 and 100 days posttransplant was quantified by flow cytometry. n=3–5 animals per group *p<0.05; ***p<0.001.