

Original Article

Resveratrol attenuates stimulated T-cell activation and proliferation: potential therapy against cellular rejection in organ transplantation

Jimmy JH Kang¹, Sabin J Bozso², Dana E Boe¹, David P Al-Adra³, Michael C Moon², Darren H Freed², Jayan Nagendran², Jeevan Nagendran²

¹Faculty of Medicine and Dentistry, University of Alberta, Edmonton, AB, Canada; ²Department of Surgery, Division of Cardiac Surgery, University of Alberta, Edmonton, AB, Canada; ³Department of Surgery, Division of Transplantation, University of Wisconsin School of Medicine and Public Health, Madison, WI, USA

Received August 15, 2020; Accepted November 18, 2020; Epub December 15, 2020; Published December 30, 2020

Abstract: Background: Pharmaceuticals to inhibit mammalian target of rapamycin (mTOR) protein, which plays an integral role in T cell survival and function, have been used to prevent complications associated with organ transplantation. Although studies have individually shown that resveratrol can inhibit mTOR and that inhibiting mTOR leads to attenuated immune function, no studies to date have examined these two functions conjointly under one study. Therefore, we hypothesize that resveratrol will decrease mTOR activation and expression as well as attenuate stimulated T cell activation and proliferation in peripheral blood mononuclear cells (PBMC). Methods and Materials: Human PBMC were isolated and cultured. The cells were pre-treated with resveratrol (50 μ M) overnight (18 hrs) before stimulation. The cells were collected for subsequent biochemical analysis after 1, 3, and 5 days. Additionally, the cells were stained with proliferation dye and cultured for 24 hours in PMA/Ionomycin with resveratrol for flow cytometry analysis. Results: Resveratrol treated stimulated PBMCs displayed a significant decrease in activated phosphorylation of mTOR at days 1, 3, and 5 ($P < 0.0329$). Markers of T cell activation, tumour necrosis factor-alpha (TNF- α) and interferon-gamma (INF- γ), were also significantly reduced along with T cell proliferation following stimulated PBMC resveratrol treatment when compared to vehicle-treated controls ($P < 0.01$). Conclusion: Taken together, our data suggest that resveratrol can decrease the immune response of stimulated T-cells and inhibit the expression and activation of mTOR mediated cellular signalling under the same study setting. Therefore, resveratrol proposes a possible adjunctive therapy option for patients undergoing organ transplantation.

Keywords: Resveratrol, organ transplantation, lymphocytes

Introduction

Organ transplantation in the last 50 years has undergone major improvements. However, they are still met with a major challenge of effectively preventing post-transplant malignancies associated with long term immunosuppressive therapy. The literature provides ample evidence of a 2 to 4-fold increase in the occurrence of malignancy in transplant recipients when compared to the general population [1-9]. Thus, mammalian target of rapamycin (mTOR) inhibitors such as sirolimus and everolimus that have both the immunosuppressive and anticarcinogenic properties have been developed. However, these drugs have been associated with

adverse side effects such as leukopenia, thrombocytopenia, anemia, hypercholesterolemia, hypertriglyceridemia [10, 11] and increased mortality in kidney transplant patients [12]. These side effects led to the search for alternative modes of mTOR inhibition.

mTOR was discovered to play an integral role in cancer cell survival and immune regulation as it acts as a major convergence point for the regulation of anabolic activity. Several studies have shown that mTOR regulates immune cell survival, proliferation, activation, and differentiation [13, 14]. Inhibition of mTOR also induces autophagy making it a viable anticancer drug target [15]. Specifically pertaining to organ

Resveratrol attenuates T-lymphocytes

transplants, inhibition of mTOR was found to preferentially induce CD8⁺ cells to become memory cells which can fight off infection while preventing graft rejection [13]. One of the proposed adjuvant therapies to mTOR inhibitors is resveratrol (3,4',5-trihydroxy-trans-stilbene), found in grape skins, peanuts, and red wine. Resveratrol is a naturally occurring polyphenol with a comparably minute side effect profile to other immunosuppressants and has been recently shown to directly inhibit the activity of mTOR via competitive inhibition at the adenosine triphosphate (ATP) binding site [15]. Although it has been shown that resveratrol can inhibit mTOR, and that inhibition of mTOR leads to enervated immune function, these results have been reported as discrete outcomes. To date, no study has examined these two functions in tandem with one another. Therefore, we aim to measure resveratrol's effects on both the mTOR protein and the T cells. We hypothesize that resveratrol will decrease mTOR activation and expression as well as attenuate stimulated T cell activation and proliferation in peripheral blood mononuclear cells (PBMC).

Materials and methods

Resveratrol

Resveratrol (3,4',5-Trihydroxy-trans-stilbene - Sigma Chemical Co., St Louis Mo.) was suspended in ethanol at a concentration of 1 mM and stored at 4°C for further use.

PBMC isolation and culture

PBMCs were isolated from whole blood of 4 healthy donors. All participants provided consented for the collection of samples and information. The research protocol was approved by local Research Ethics Board. The PBMCs were counted using Trypan Blue exclusion dye and resuspended in an appropriate amount of "Complete Medium" comprised of RPMI 1640 medium containing 10% human serum, 2 mM L-glutamine, 50 IU penicillin/ml, and 50 ml streptomycin/ml (Thermo Fisher Scientific, California) to create a solution containing 1×10^6 cells/ml. PBMCs were stimulated and treated with either 50 μ M/ml of working solution of resveratrol suspended in ethanol or in 50 μ M/ml of ethanol alone to create the resveratrol treated and vehicle treated PBMC groups, respectively. 1 ml of the solution containing

PBMC and 1 ml of the complete medium were aliquoted into Corning™ 12-well cell culturing plate (Fisher Scientific, California). Both groups were left in the 37°C 5% CO₂ incubator for either 1, 3 or 5 days.

PBMC stimulation

For Western blot and flow cytometry, PBMCs were stimulated with Phorbol Myristate Acetate (PMA) at a concentration of 50 ng/mL and Ionomycin at a concentration of 1 μ g/mL (eBioscience, California) in RPMI 1640 Complete Medium. Both groups were cultured in a 37°C 5% CO₂ incubator for 24 hours. The cells were centrifuged at 1200 g at 4°C for 10 minutes, washed with phosphate buffered saline (PBS) and centrifuged again. The pellet was resuspended in RPMI 1640 Complete Medium and split into a vehicle and resveratrol treated group for Western blot and flow cytometry. Following the appropriate culture times (1, 3 or 5 days), the cells centrifuged, and the pellets were stored at -80°C for protein analysis using the Western blot technique. The unstimulated cells underwent the same protocol and the supernatant containing the cytokines were stored at -80°C for ELISA analysis.

Proliferation assay

PBMCs were isolated as described above at a concentration of 1×10^6 cells/ml and incubated in the 0.5 μ M Cell Trace Violet stain solution for 20 min. Unbound dye was absorbed by the addition of 5 times the volume of RPMI 1640 and incubated for 5 min. Cells were centrifuged and resuspended in the Complete Medium. Aliquots of stained cells were then distributed as outlined below. After 5-days of culture, cells were stained with fluorescent antibodies against CD3⁺, CD4⁺, and CD8⁺. Analysis of proliferation was performed on a single representative sample using flow cytometry with a 405 nm excitation and a 450/40 bandpass emission filter. PerCP-Cy5.5 was used as a live/dead fixable red stain. Proliferation was identified by a reduction in CellTrace Violet signal intensity, with each peak representing a duplication in cell number.

Protein analysis

Cells from each group that were extracted after 1, 3, and 5 days of incubation at 37°C in a 5%

CO₂ incubator underwent Western blot analysis for p-mTOR and mTOR. Pellets were resuspended in 30 μ L RIPA lysis buffer with sodium orthovanadate (10 μ L/mL), protease inhibitor (15 μ L/mL), PMSF (10 μ L/mL), and phosphatase inhibitor (10 μ L/mL). Protein concentration was determined using the Bradford assay (Bio-Rad Laboratories, California). Samples were adjusted to 35 μ g total protein with Laemmli 6X reducing sample buffer, then heated for 5 minutes at 95°C. The samples were loaded onto a Mini-PROTEAN TGX Precast Gel, 4-15% gradient for SDS-PAGE; then transferred to PVDF membranes at 4°C for 12 hours using a Tris-glycine refrigerated transfer buffer with 8% methanol (Bio-Rad Laboratories, California). Blots were blocked with 5% BSA in TBS-T (10 mM Tris, 150 mM NaCl, pH 8.0, 0.5% Tween 20) for 2 hours, incubated overnight with the appropriate primary antibody at 4°C, thoroughly washed with TBS-T, and subsequently incubated for 90 minutes in the appropriate peroxidase-conjugated secondary antibodies. The following dilutions and antibodies were used: 1:1000 dilution of rabbit p-mTOR (Ser. 2448 Cat. No. 2971), mTOR (Cat. No. 2972) Ab, (Cell Signaling Technology, Danvers, MA). 1:2000 dilution of HRP linked anti-rabbit antibody (Cat. No. 7074) was used as the secondary antibody (Cell Signaling Technology, Danvers, MA). Detection was performed using the ECL detection system (Carestream Health, Rochester, NY). Luminescence was visualized by autoradiography. The intensities of the bands were evaluated by densitometric scanning using a Personal Densitometer SI with Image J software (Laboratory for Optical and Computational Instrumentation, University of Wisconsin).

Quantification of cytokines

The supernatant containing cytokines that were stored in -80°C freezer was thawed and underwent enzyme-linked immunosorbent assay (ELISA) analysis according to the manufacturer's recommended protocol for human tumor necrosis factor- α (TNF- α) DuoSet ELISA Kit (Cat. No. DY210-05) and Human interferon gamma (INF- γ) DuoSet ELISA kit (Cat. No. DY285B-05) (R&D Systems, Minneapolis). Absorbance reading at 450 nm and 540 nm were performed using Synergy H4 hybrid reader (Biotek, Winooski, Vermont).

Statistical analysis

All data are expressed as mean \pm standard deviations. Differences among groups were assessed using one-way analysis of variance (ANOVA) followed by Fisher's least significant difference *posthoc* test, and statistical significance was taken at $P < 0.05$. Prism 8 GraphPad (SanDiego, CA, USA) was used for statistical analysis.

Results

Resveratrol treatment of stimulated PBMC cells leads to inhibition of mTOR expression and activation

The latest evidence on resveratrol's inhibition of mTOR revealed a direct inhibitory mechanism on mTOR via ATP competition [15]. Thus, we intended to identify the effects of 50 μ M/ml of resveratrol on the PBMC cells' mTOR expression and phosphorylation in a time-dependent manner. PMA and Ionomycin stimulated PBMC cells from the resveratrol and vehicle-treated groups were extracted after 1, 3, and 5 days of incubation and analyzed for mTOR expression and activation using western blot. There was an overall qualitative decrease in the expression of stimulated phosphorylated mTOR in the resveratrol treated group versus vehicle-treated group at days 1, 3, and 5 (**Figure 1**). Additionally, densitometry analysis of phosphorylated mTOR/total mTOR levels was examined for the quantitative analysis of mTOR expression. We observed a statistically significant reduction in the phosphorylation of mTOR for resveratrol treated group versus vehicle-treated group at day 1 (0.946 ± 0.041 vs 0.513 ± 0.172 , $P = 0.0027$) (**Figure 1A**), day 3 (0.744 ± 0.150 vs 0.370 ± 0.150 , $P = 0.0124$) (**Figure 1B**), and day 5 (0.491 ± 0.182 vs 0.230 ± 0.053 , $P = 0.0329$) (**Figure 1C**). Complete western blot and densitometric analysis can be found in [Supplementary Figure 1](#). Taken together, these results suggest that resveratrol directly attenuates the expression and activation of mTOR proteins that contribute to T cell differentiation, activation, and function [13, 16-21].

Resveratrol mediated inhibition of mTOR is associated with decreased cytokine production in stimulated PBMC

Previous studies on resveratrol's role in human immune cell function have demonstrated sup-

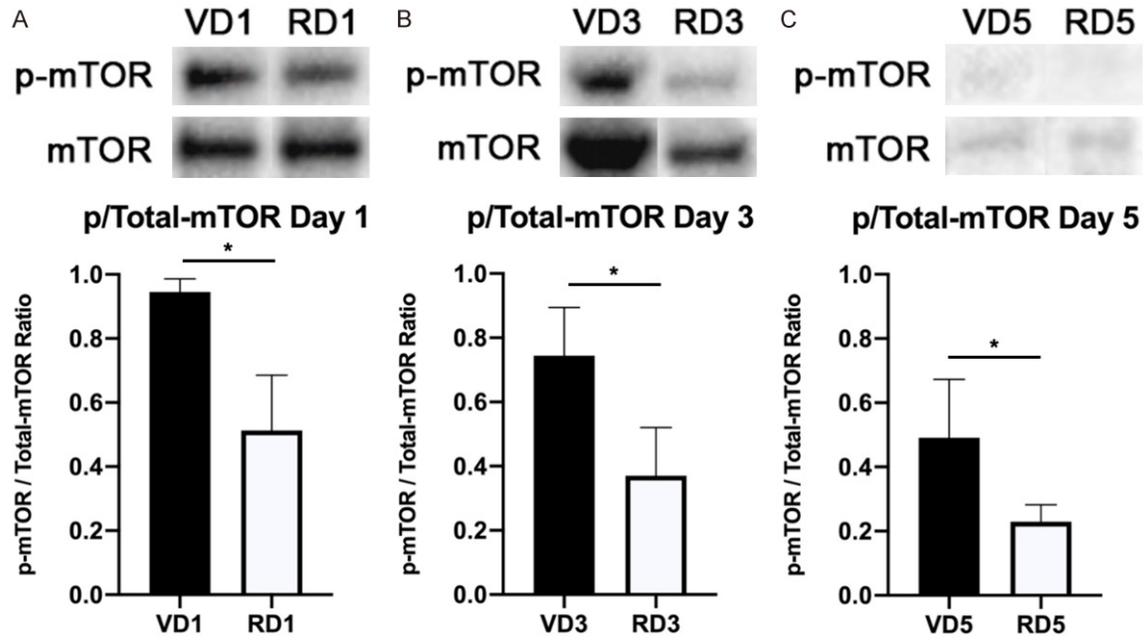


Figure 1. Representative western blot for phosphorylated mTOR and total mTOR protein and densitometric comparison of the phosphorylated mTOR/total mTOR ratio (n = 4) for (A) VD1 (Vehicle Day 1) and RD1 (Resveratrol Day 1), (B) VD3 (Vehicle Day 3) and RD3 (Resveratrol Day 3), (C) VD5 (Vehicle Day 5) and RD5 (Resveratrol Day 5). *P < 0.02 versus control.

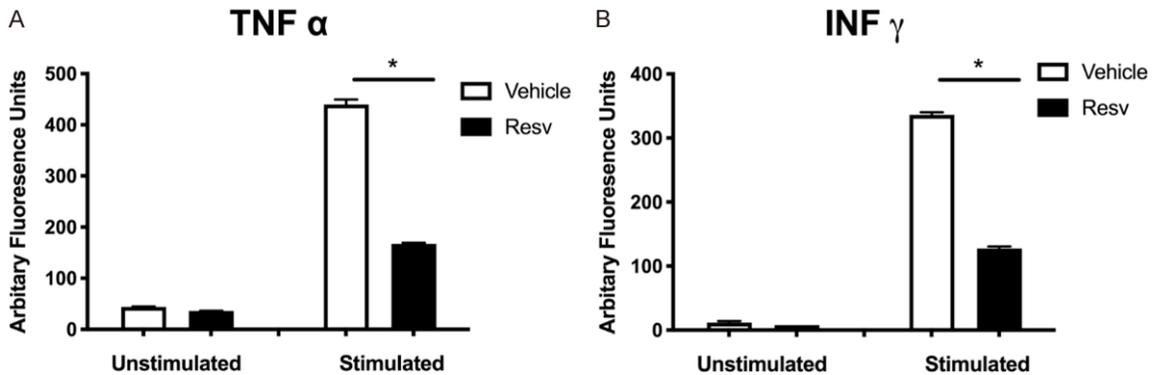


Figure 2. Effects of resveratrol on (A) TNF-α and (B) INF-γ cytokine expression under unstimulated and stimulated conditions. Data are expressed as means ± SD. *P < 0.001 versus control vehicle group.

pression of cytokines such as INF-γ, IL2, IL4 from CD4⁺ and CD8⁺ T cells as well as cytotoxic lymphocytes and natural killer cells *in vitro* at high concentrations [22]. With the inhibition of mTOR proteins contributing to altered T cell differentiation, activation, and function [13, 16-20], we hypothesized that the downstream effect of mTOR inhibition would result in decreased cytokines produced by T lymphocytes. In unstimulated PBMC groups, tumour necrosis factor-α (TNF-α) levels (Figure 2A) were comparable between resveratrol treated and vehicle-

treated control groups (35.85 ± 0.62 versus 43.79 ± 0.86 arbitrary fluorescence units). Interferon-gamma (INF-γ) levels (Figure 2B) were also comparable between resveratrol treated and vehicle-treated control groups (7.79 ± 0.09 versus 11.74 ± 2.58 arbitrary fluorescence units). However, when PBMCs were stimulated, resveratrol treated cells displayed a significant decrease in TNF-α (Figure 2A) when compared to vehicle controls (167.24 ± 2.32 versus 439.68 ± 9.62 arbitrary fluorescence units, P < 0.001). Similarly, INF-γ levels

(**Figure 2B**) in stimulated PBMCs showed a significant decrease in the resveratrol treated group when compared to vehicle controls (127.46 ± 2.90 versus 336.14 ± 4.18 arbitrary fluorescence units, $P < 0.001$). Taken together, our data suggest that resveratrol can blunt both the T cell activation and cytokine production ostensibly through inhibition of the mTOR protein pathway.

Resveratrol mediated inhibition of mTOR is accompanied by decreased T cell proliferation

mTOR1-mTOR2 (mTOR complex 1 - mTOR complex 2) have been shown to be key regulators in controlling the anabolic processes necessary for the activation and the effector functions of immune cells including protein synthesis, cell growth, morphology change, and proliferation (3,4). Following this, we examined if resveratrol could suppress the CD3⁺CD4⁺ T-lymphocyte and CD3⁺CD8⁺ T lymphocyte proliferation under flow cytometry. Resveratrol treated T cell proliferation assay showed significantly reduced CD4⁺ and CD8⁺ T-lymphocyte proliferation (**Figure 3B**) when compared to the vehicle-treated T lymphocyte proliferation assay (**Figure 3A**) where it displayed increased proliferation of T lymphocytes indicated by peaks with fold fractional decrease corresponding to each proliferation of T cells (**Figure 3Ae, 3Af**). Taken together, our data suggest that resveratrol can reduce the CD4⁺ and CD8⁺ T lymphocyte proliferation under stimulation.

Discussion

The activation of the innate immune system is both metabolically demanding and highly anabolic in nature. Myeloid and lymphoid cells contain mTORC1-mTORC2 to control the anabolic processes necessary for their activation and effector functions, including protein synthesis, cell growth, morphology change, and proliferation [14, 23]. Thus, our group postulates that the inhibition of mTOR results in the vitiation of immune cell metabolism, and necessarily immune cell function. Recently Park et al. have demonstrated that resveratrol can directly inhibit the activity of mTOR proteins via docking onto the mTOR ATP binding pocket, negating the need for inhibition of upstream regulators. Their research proposed that the benefits of resveratrol induced mTOR inhibition is derived from the mTOR-dependent autophagy

induction and reduction of cancer cell viability [15]. This study was pivotal as it provided new evidence on the direct molecular targets of resveratrol, whereas previous studies have relied primarily on the involvement of upstream regulators such as Sirtuin 1 (SIRT1) (NAD⁺-dependent deacetylase) [24-28], AMP-activated Kinase (AMPK) [29-34], phosphodiesterase (PDE) [35], Phosphoinositide 3-kinase (PI3K) [23], and DEP domain-containing mTOR-interacting protein (DEPTOR) [36] as the mechanism behind mTOR inhibition. Notwithstanding this previous research, the exact mechanism and molecular targets of resveratrol remain complex and incompletely elucidated.

Our purpose was to investigate resveratrol's ability to inhibit the expression and activation of mTOR and its ability to attenuate the activation and proliferation of T lymphocytes in tandem. Using stimulated PBMCs isolated from human whole blood, our results demonstrate both a qualitative decrease in the mTOR protein expression as well as a significant reduction in the mTOR phosphorylation under densitometry analysis. TNF- α and INF- γ cytokine production and T cell proliferation are also significantly decreased with resveratrol treatment when compared to vehicle-treated controls. This data endorses resveratrol's ability to further enhance the immunosuppressive and anticarcinogenic effects of conventional mTOR inhibitors. Several previous studies have examined resveratrol's effect on the mTOR pathway using glioma and various cancer cells [22-25] and others have independently examined the human immune response to resveratrol using T cells and Sirt1 protein [26]. However, to date our study is the first to examine both resveratrol's effect on mTOR inhibition in addition to the downstream immune response of human PBMC cells to the polyphenol, initiating consolidation of the link between the two processes.

Our results showing a simultaneous decrease in the expression and activation of mTOR with resveratrol after 5 days (**Figure 1C**) should be noted, as this result runs contrary to the effects of traditional mTOR1 inhibitors such as sirolimus. Sirolimus' incomplete suppressive effect on mTOR1 has been paradoxically met with an abrogation of the feedback inhibition resulting in the activation of protein kinase B (AKT) within 72 hours, which lies upstream to

Resveratrol attenuates T-lymphocytes

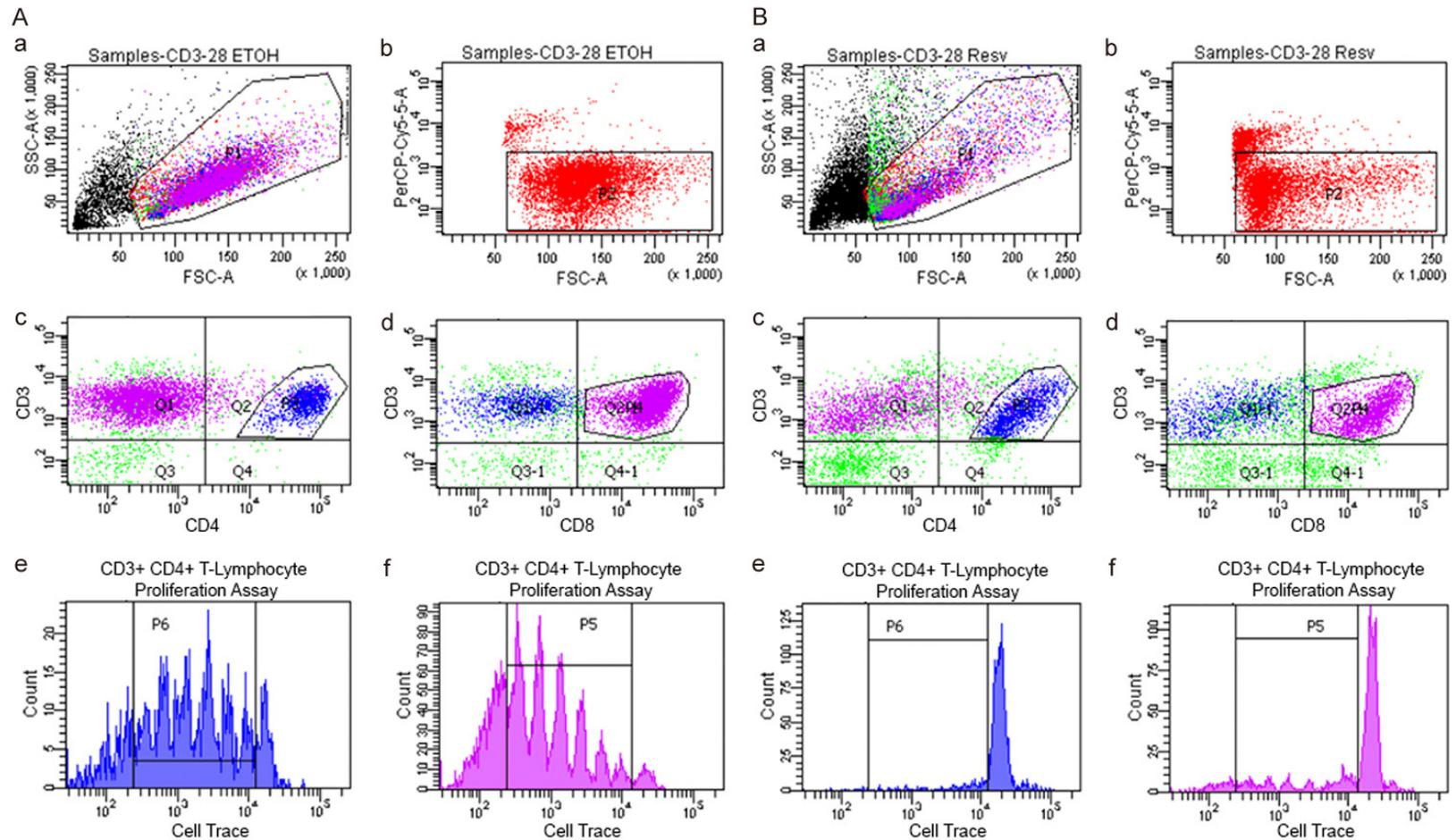


Figure 3. CD3⁺CD4⁺ and CD3⁺CD8⁺ T-lymphocyte proliferation analysis under flow cytometry after 3 days of incubation of (A) vehicle treated control and (B) resveratrol treated samples.

mTOR1 and potentiates its activation [41]. Additionally, AKT inhibition appears to be substrate dependant and does not globally inhibit the effects of mTORC1 and has negligible efficacy for inhibiting the action of mTORC2 [14]. Consequently, sirolimus has produced undistinguished therapeutic outcomes, and a new generation of more specific mTOR inhibitors have begun to enter clinical trials [42]. Herein resveratrol shows promise as one of these adjuvant therapies to inhibit both mTOR directly and its dependent signalling axes, as our results have shown it to continually decrease mTOR expression after 5 days without interfering in the AKT feedback loop.

Over the past decade, comprehensive studies of the interplay between the mTOR protein pathway and T cell activation have been conducted and they firmly establish a complex role of mTOR contributing to T cell differentiation, activation, and function [13, 16-20]. mTOR is activated when naïve CD4⁺ and CD8⁺ T cells undergo antigen recognition and this is believed to program their differentiation into distinct lineages, specifically into Th1, Th2, Th17, iTreg [17]. Hitherto it was shown that mTOR deficient CD4⁺ T cells under stimulatory conditions could not differentiate into Th1, Th17, or Th2 effector cells although the T-cell receptors were intact [43]. Additionally, mTORC1 inhibition in macrophages leads to increased autophagy, and a decrease in inflammatory cytokine production [14, 27]. This intrinsic relationship between mTOR and T cell demonstrated in the literature reinforce resveratrol's inhibition of mTOR functionality as playing an integral role in the decreased T cell differentiation and TNF- α and INF- γ cytokine production.

Reduced TNF- α and INF- γ proinflammatory cytokine production in our stimulated resveratrol treated group (**Figure 2A, 2B**) show similar results to studies examining the attenuation of immune activation using resveratrol treatment. However, these studies have focused on resveratrol's effects on upstream regulators and pathways as opposed to its unmediated effect on mTOR. Zou et al. have discovered that expression and activation of Sirt1 protein enhance resveratrol's inhibition of CD4⁺ activation [26], and Falchetti et al. have implied that their findings of decreased IL2, INF- γ , IL4 cytokines by CD4, CD8 cells were mediated by res-

veratrol's effects on the NF- κ B pathway, which controls the expression of many cytokines in the immune response [21]. Our results of decreased TNF- α and INF- γ cytokine production and reduced T cell proliferation corroborate this prior research regarding the attenuating effect of resveratrol on human cellular immunity.

Regulatory T cells have been suggested to have an advantage during mTOR inhibition [44, 45]. This is congruent with the theory that mTOR modulates immune function via metabolic regulation as regulatory T cells have significantly lower metabolic demand. Regulatory T cells inhibit cytokine production and T cell proliferation, leading to an overall suppression of the adaptive immune response [13]. However, it appears that both mTORC1 and mTORC2 need to be inhibited to generate this response [46], making an inhibitor of the mTOR ATP binding site found in both mTORC1 and mTORC2 essential. Therefore, resveratrol's inhibition of both complexes may be a valuable way to suppress the adaptive immune response in select patients requiring tolerable long-term immunotherapy.

This study is not without limitations. The dose of resveratrol used for the study was based on a dose-dependent study of resveratrol on TH17 cell response that has shown significantly decreased mTOR activation at 50 μ M/ml vs 25 μ M/ml [47]. However, the experimental dose is recorded to be higher than the realistic dose of resveratrol and appropriate clinical dosage of resveratrol is yet to be elucidated. Further dose dependent studies of resveratrol on T cells and mTOR should be conducted to translate the experimental dosage to clinical use. The experimental corroboration of a causative relationship between mTOR and T cell activation was out of the scope of this initial study but has been previously reported in the literature. Selective deletion of mTOR proteins and measurement of the resulting differential T cell response in animal models is be a future enterprise to better understand and strengthen this proposed causal relationship. Furthermore, measuring various T cell differentiation using different T helper cells may help identify the specific differentiation pathways that are affected by resveratrol. Lastly, analyzing other cytokines such as IL2 and IL4 that are impact-

ed by PMA ionomycin stimulation would build a more complete picture as to the proinflammatory cytokines expression affected by resveratrol.

In summary, our study demonstrates that resveratrol significantly inhibits the mTOR protein expression and activation, significantly reduces the production of proinflammatory cytokines TNF- α and INF- γ , and inhibits the proliferation of stimulated T-cells. This study further establishes resveratrol's numerous inhibitory effects on T-cell immune response through mTOR, likely through a direct inhibition of the mTOR protein. These results, taken with resveratrol's minimal side-effect profile, strongly endorse its potential to serve as a novel combination therapy for solid organ transplantation in the future.

Disclosure of conflict of interest

None.

Abbreviations

mTOR, Mammalian Target of Rapamycin; RESV, Resveratrol; PBMC, Peripheral Blood Mononuclear Cells; PMA, Phorbol Myristate Acetate; TNF- α , Tumour Necrosis Factor-Alpha; INF- γ , Interferon-gamma; ELISA, Enzyme-Linked Immunosorbent Assay; mTOR1, mTOR complex 1; mTOR2, mTOR complex 2; AKT, Protein Kinase B; SIRT, Sirtuin 1; AMPK, AMP-Activated Kinase; PDE, Phosphodiesterase; PI3K, Phosphoinositide 3-Kinase; DEPTOR, DEP Domain-Containing mTOR-interacting protein; VD1, Vehicle Day 1; RD1, Resveratrol Day 1; VD3, Vehicle Day 3; RD3, Resveratrol Day 3; VD5, Vehicle Day 5; RD5, Resveratrol Day 5.

Address correspondence to: Jeevan Nagendran, Department of Surgery, Division of Cardiac Surgery, University of Alberta, 4-108A Li Ka Shing Health Research Centre, 8602-112 Street, Edmonton, AB T6G 2E1, Canada. Tel: 780-492-7605; Fax: 780-492-6037; E-mail: jeevan@ualberta.ca

References

[1] Yanik EL, Clarke CA, Snyder JJ, Pfeiffer RM and Engels EA. Variation in cancer incidence among patients with ESRD during kidney function and nonfunction intervals. *J Am Soc Nephrol* 2016; 27: 1495-1504.

- [2] Wisgerhof HC, van der Geest, Lydia GM, de Fijter JW, Haasnoot GW, Claas FH, le Cessie S, Willemze R and Bouwes Bavinck JN. Incidence of cancer in kidney-transplant recipients: a long-term cohort study in a single center. *Cancer Epidemiol* 2011; 35: 105-111.
- [3] Adami J, Gäbel H, Lindelöf B, Ekström K, Rydh B, Glimelius B, Ekblom A, Adami H and Granath F. Cancer risk following organ transplantation: a nationwide cohort study in Sweden. *Br J Cancer* 2003; 89: 1221-1227.
- [4] Collett D, Mumford L, Banner NR, Neuberger J and Watson C. Comparison of the incidence of malignancy in recipients of different types of organ: a UK Registry audit. *Am J Transplant* 2010; 10: 1889-1896.
- [5] de Fijter JW. Cancer and mTOR inhibitors in transplant recipients. *Transplantation* 2017; 101: 45-55.
- [6] Gutierrez-Dalmau A and Campistol JM. Immunosuppressive therapy and malignancy in organ transplant recipients: a systematic review. *Drugs* 2007; 67: 1167-1198.
- [7] Kirklin JK, Naftel DC, Bourge RC, McGiffin DC, Hill JA, Rodeheffer RJ, Jaski BE, Hauptman PJ, Weston M and White-Williams C. Evolving trends in risk profiles and causes of death after heart transplantation: a ten-year multi-institutional study. *J Thorac Cardiovasc Surg* 2003; 125: 881-890.
- [8] Vajdic CM, McDonald SP, McCredie MR, van Leeuwen MT, Stewart JH, Law M, Chapman JR, Webster AC, Kaldor JM and Grulich AE. Cancer incidence before and after kidney transplantation. *JAMA* 2006; 296: 2823-2831.
- [9] Villeneuve PJ, Schaubel DE, Fenton SS, Shepherd FA, Jiang Y and Mao Y. Cancer incidence among Canadian kidney transplant recipients. *Am J Transplant* 2007; 7: 941-948.
- [10] Brattström C, Wilczek HE, Tydén G, Böttiger Y, Säwe J and Groth CG. Hypertriglyceridemia in renal transplant recipients treated with sirolimus. *Transplant Proc* 1998; 30: 3950-3951.
- [11] Euvrard S, Morelon E, Rostaing L, Goffin E, Brocard A, Tromme I, Broeders N, del Marmol V, Chatelet V, Domp Martin A, Kessler M, Serra AL, Hofbauer GF, Pouteil-Noble C, Campistol JM, Kanitakis J, Roux AS, Decullier E and Dantal J. Sirolimus and secondary skin-cancer prevention in kidney transplantation. *N Engl J Med* 2012; 367: 329-339.
- [12] Knöll GA, Kokolo MB, Mallick R, Beck A, Buenaventura CD, Ducharme R, Barsoum R, Bernasconi C, Blydt-Hansen TD, Ekberg H, Felipe CR, Firth J, Gallon L, Gelens M, Glotz D, Gossmann J, Guba M, Morsy AA, Salgo R, Scheuermann EH, Tedesco-Silva H, Vitko S, Watson C and Fergusson DA. Effect of sirolimus on ma-

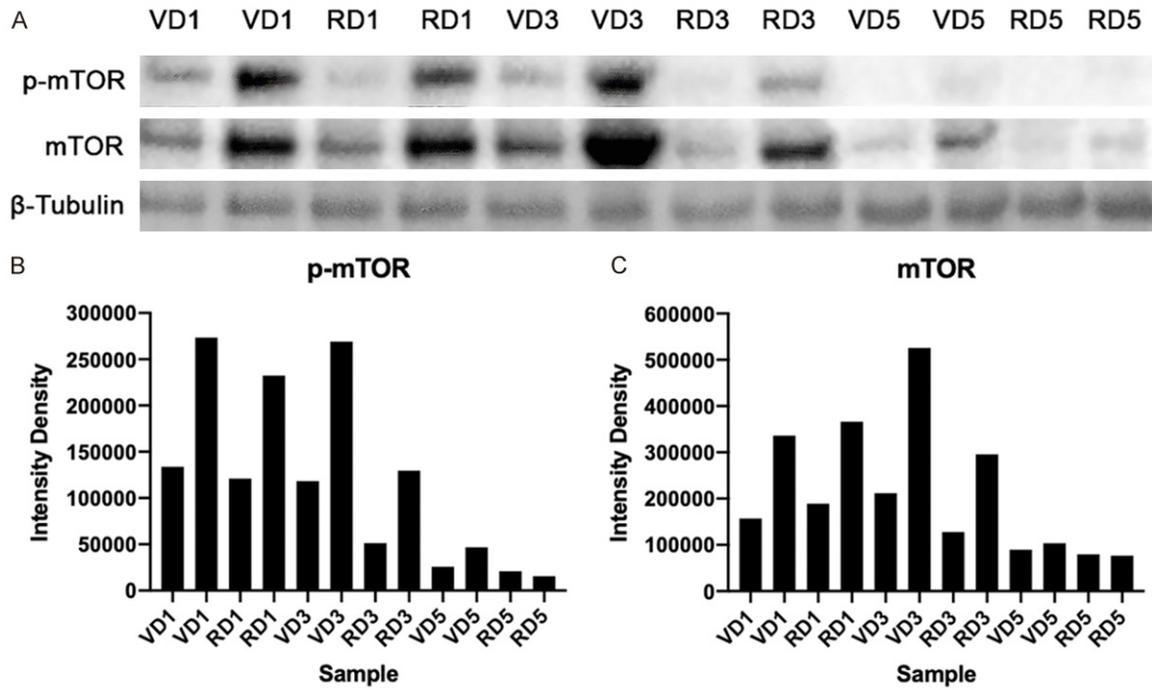
Resveratrol attenuates T-lymphocytes

- lignancy and survival after kidney transplantation: systematic review and meta-analysis of individual patient data. *BMJ* 2014; 349: g6679.
- [13] Powell JD, Pollizzi KN, Heikamp EB and Horton MR. Regulation of immune responses by mTOR. *Annu Rev Immunol* 2012; 30: 39-68.
- [14] Weichhart T, Hengstschläger M and Linke M. Regulation of innate immune cell function by mTOR. *Nat Rev Immunol* 2015; 15: 599-614.
- [15] Park D, Jeong H, Lee MN, Koh A, Kwon O, Yang YR, Noh J, Suh PG, Park H and Ryu SH. Resveratrol induces autophagy by directly inhibiting mTOR through ATP competition. *Sci Rep* 2016; 6: 21772.
- [16] Pollizzi KN and Powell JD. Regulation of T cells by mTOR: the known knowns and the known unknowns. *Trends Immunol* 2015; 36: 13-20.
- [17] Chi H. Regulation and function of mTOR signalling in T cell fate decisions. *Nat Rev Immunol* 2012; 12: 325-338.
- [18] Waickman AT and Powell JD. mTOR, metabolism, and the regulation of T-cell differentiation and function. *Immunol Rev* 2012; 249: 43-58.
- [19] Waickman AT and Powell JD. Mammalian target of rapamycin integrates diverse inputs to guide the outcome of antigen recognition in T Cells. *J Immunol* 2012; 188: 4721-4729.
- [20] Pollizzi KN and Powell JD. Integrating canonical and metabolic signalling programmes in the regulation of T cell responses. *Nat Rev Immunol* 2014; 14: 435-446.
- [21] Falchetti R, Fuggetta MP, Lanzilli G, Tricarico M and Ravagnan G. Effects of resveratrol on human immune cell function. *Life Sci* 2001; 70: 81-96.
- [22] Alayev A, Salamon RS, Sun Y, Schwartz NS, Li C, Yu JJ and Holz MK. Effects of combining rapamycin and resveratrol on apoptosis and growth of TSC2-deficient xenograft tumors. *Am J Respir Cell Mol Biol* 2015; 53: 637-646.
- [23] Alayev A, Berger SM, Kramer MY, Schwartz NS and Holz MK. The combination of rapamycin and resveratrol blocks autophagy and induces apoptosis in breast cancer cells. *J Cell Biochem* 2015; 116: 450-457.
- [24] Alayev A, Salamon RS, Schwartz NS, Berman AY, Wiener SL and Holz MK. Combination of rapamycin and resveratrol for treatment of bladder cancer. *J Cell Physiol* 2017; 232: 436-446.
- [25] Jiang H, Shang X, Wu H, Gautam SC, Al-Holou S, Li C, Kuo J, Zhang L and Chopp M. Resveratrol downregulates PI3K/Akt/mTOR signaling pathways in human U251 glioma cells. *J Exp Ther Oncol* 2009; 8: 25-33.
- [26] Zou T, Yang Y, Xia F, Huang A, Gao X, Fang D, Xiong S and Zhang J. Resveratrol Inhibits CD4+ T cell activation by enhancing the expression and activity of Sirt1. *PLoS One* 2013; 8: e75139.
- [27] Widlund AL, Baur JA and Vang O. mTOR: more targets of resveratrol? *Expert Rev Mol Med* 2013; 15: e10.
- [28] Huber JL, McBurney MW, Distefano PS and McDonagh T. SIRT1-independent mechanisms of the putative sirtuin enzyme activators SRT1720 and SRT2183. *Future Med Chem* 2010; 2: 1751-1759.
- [29] Pacholec M, Bleasdale JE, Chrnyk B, Cunningham D, Flynn D, Garofalo RS, Griffith D, Griffor M, Loulakis P, Pabst B, Qiu X, Stockman B, Thanabal V, Varghese A, Ward J, Withka J and Ahn K. SRT1720, SRT2183, SRT1460, and resveratrol are not direct activators of SIRT1. *J Biol Chem* 2010; 285: 8340-8351.
- [30] Borra MT, Smith BC and Denu JM. Mechanism of human SIRT1 activation by resveratrol. *J Biol Chem* 2005; 280: 17187-17195.
- [31] Kaeberlein M, McDonagh T, Heltweg B, Hixon J, Westman EA, Caldwell SD, Napper A, Curtis R, DiStefano PS, Fields S, Bedalov A and Kennedy BK. Substrate-specific activation of sirtuins by resveratrol. *J Biol Chem* 2005; 280: 17038-17045.
- [32] Howitz KT, Bitterman KJ, Cohen HY, Lamming DW, Lavu S, Wood JG, Zipkin RE, Chung P, Kisielewski A, Zhang L, Scherer B and Sinclair DA. Small molecule activators of sirtuins extend *Saccharomyces cerevisiae* lifespan. *Nature* 2003; 425: 191-196.
- [33] Hawley SA, Ross FA, Chevtzoff C, Green KA, Evans A, Fogarty S, Towler MC, Brown LJ, Ogunbayo OA, Evans AM and Hardie DG. Use of cells expressing gamma subunit variants to identify diverse mechanisms of AMPK activation. *Cell Metab* 2010; 11: 554-565.
- [34] Zheng J and Ramirez VD. Inhibition of mitochondrial proton FOF1-ATPase/ATP synthase by polyphenolic phytochemicals. *Br J Pharmacol* 2000; 130: 1115-1123.
- [35] Cantó C, Jiang LQ, Deshmukh AS, Matakis C, Coste A, Lagouge M, Zierath JR and Auwerx J. Interdependence of AMPK and SIRT1 for metabolic adaptation to fasting and exercise in skeletal muscle. *Cell Metab* 2010; 11: 213-219.
- [36] Hou X, Xu S, Maitland-Toolan KA, Sato K, Jiang B, Ido Y, Lan F, Walsh K, Wierzbicki M, Verbeuren TJ, Cohen RA and Zang M. SIRT1 regulates hepatocyte lipid metabolism through activating AMP-activated protein kinase. *J Biol Chem* 2008; 283: 20015-20026.
- [37] Breen DM, Sanli T, Giacca A and Tsiani E. Stimulation of muscle cell glucose uptake by resveratrol through sirtuins and AMPK. *Biochem Biophys Res Commun* 2008; 374: 117-122.
- [38] Um J, Park S, Kang H, Yang S, Foretz M, McBurney MW, Kim MK, Viollet B and Chung JH. AMP-activated protein kinase-deficient mice are re-

Resveratrol attenuates T-lymphocytes

- sistant to the metabolic effects of resveratrol. *Diabetes* 2010; 59: 554-563.
- [39] Park S, Ahmad F, Philp A, Baar K, Williams T, Luo H, Ke H, Rehmann H, Taussig R, Brown AL, Kim MK, Beaven MA, Burgin AB, Manganiello V and Chung JH. Resveratrol ameliorates aging-related metabolic phenotypes by inhibiting cAMP phosphodiesterases. *Cell* 2012; 148: 421-433.
- [40] Liu M, Wilk SA, Wang A, Zhou L, Wang R, Ogawa W, Deng C, Dong LQ and Liu F. Resveratrol inhibits mTOR signaling by promoting the interaction between mTOR and DEPTOR. *J Biol Chem* 2010; 285: 36387-36394.
- [41] O'Reilly KE, Rojo F, She Q, Solit D, Mills GB, Smith D, Lane H, Hofmann F, Hicklin DJ, Ludwig DL, Baselga J and Rosen N. mTOR inhibition induces upstream receptor tyrosine kinase signaling and activates Akt. *Cancer Res* 2006; 66: 1500-1508.
- [42] Benjamin D, Colombi M, Moroni C and Hall MN. Rapamycin passes the torch: a new generation of mTOR inhibitors. *Nat Rev Drug Discov* 2011; 10: 868-880.
- [43] Delgoffe GM, Kole TP, Zheng Y, Zarek PE, Matthews KL, Xiao B, Worley PF, Kozma SC and Powell JD. The mTOR kinase differentially regulates effector and regulatory T cell lineage commitment. *Immunity* 2009; 30: 832-844.
- [44] Zeiser R, Leveson-Gower DB, Zambricki EA, Kambham N, Beilhack A, Loh J, Hou J and Negrin RS. Differential impact of mammalian target of rapamycin inhibition on CD4+CD25+ Foxp3+ regulatory T cells compared with conventional CD4+ T cells. *Blood* 2008; 111: 453-462.
- [45] Strauss L, Czystowska M, Szajnik M, Mandapathil M and Whiteside TL. Differential responses of human regulatory T cells (Treg) and effector T cells to rapamycin. *PLoS One* 2009; 4: e5994.
- [46] Delgoffe GM, Pollizzi KN, Waickman AT, Heikamp E, Meyers DJ, Horton MR, Xiao B, Worley PF and Powell JD. The kinase mTOR regulates the differentiation of helper T cells through the selective activation of signaling by mTORC1 and mTORC2. *Nat Immunol* 2011; 12: 295-303.
- [47] Doh KC, Kim B, Kim KW, Chung BH and Yang CW. Effects of resveratrol on Th17 cell-related immune responses under tacrolimus-based immunosuppression. *BMC Complement Altern Med* 2019; 19: 54.

Resveratrol attenuates T-lymphocytes



Supplementary Figure 1. Representative western blot of phosphorylated mTOR and total mTOR and β -tubulin for vehicle and resveratrol treated PBMC at Days 1, 3, and 5 (A). Grayscale analysis of p-mTor (B) and mTor (C).