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The Mechanisms of mTOR-mediated Monocyte Recruitment to HLA Class I Antibody-Activated Endothelial Cells

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The Mechanisms of mTOR-mediated Monocyte Recruitment to HLA Class I Antibody-Activated Endothelial Cells

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy in Cellular & Molecular Pathology

by

Sahar Salehi

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ABSTRACT OF THE DISSERTATION

The Mechanisms of mTOR-mediated Monocyte Recruitment to HLA Class I Antibody-Activated Endothelial Cells

by

Sahar Salehi

Doctor of Philosophy in Cellular and Molecular Pathology

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Professor Elaine F. Reed, Chair

Rejection remains a major challenge to successful organ transplantation. Central to the immune response to the allograft are antibodies reactive to donor human leukocyte antigen (HLA) molecules which trigger acute and chronic antibody-mediated rejection (AMR) and contribute to graft loss. HLA class I antibody-induced injury to the allograft endothelium recruits monocytes, which have been implicated as seminal players in the process of allograft rejection. Recent studies suggest that mTOR inhibitors modulate leukocyte recruitment to endothelium in models of inflammation. As such, we investigated the role of mTOR signaling in monocyte recruitment in HLA class I-induced acute AMR. First, we used an *in vitro* system of HLA I-stimulated

human primary endothelial cells pre-treated with mTOR inhibitors and found that mTOR is a key regulator of monocyte binding to the endothelium. This finding was confirmed in an *in vivo* murine model of acute AMR, in which MHC I antibody-induced endothelial injury and monocyte infiltration in the graft were significantly reduced with administration of the mTOR inhibitor rapamycin. Additionally, rapamycin treatment blocked phosphorylation of mTOR proteins downstream of HLA I signaling in endothelium. To elucidate the mechanisms responsible for this process we studied the effects of mTOR inhibition on the expression and function of adhesion molecules on the cell surface of HLA I-stimulated endothelial cells. Monocytes firmly adhere to the endothelium via ICAM-1 engagement. mTOR inhibition in HLA I-stimulated endothelial cells impaired ICAM-1 function and hampered the capacity of endothelium to support firm adhesion of monocytes. Next, we explored the requirement of mTOR signaling in monocyte activation and adherence to HLA I-stimulated endothelium. mTOR modulates monocyte activation and adhesiveness following PSGL-1 crosslinking. To conclude, we found mTOR inhibition dampens endothelial activation in response to HLA I antibodies as well as monocyte activation following PSGL-1 engagement, and prevents monocytic infiltration into cardiac allografts. These findings can be translated to the clinical setting, providing the rationale for the application of mTOR inhibitors for mitigating the risks of AMR.

The dissertation of Sahar Salehi is approved.

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DEDICATION

To my maternal grandmother, who supplied me with an endless number of cockroaches, teaching me that eventually, the way to deal with fears is to crush them.

To my nerves, for not abandoning me these five years.

To the gym, for being my fortress of solitude.

To my family, for being the perfect parallel to my scientific endeavors in reminding me that it's certainly possible to be frustrated by the things you love.

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Chapter 1:
Introduction

1.1 HLA Antibodies in Rejection

Solid organ transplantation is considered the only therapeutic alternative for the treatment of patients with end-stage organ failure. Although new immunosuppressive drugs and increased understanding of the mechanisms of allograft rejection have improved the rates of allograft survival, the percentage of five-year allograft loss remains high at almost 40% [1, 2].

Antibody-mediated graft injury and rejection (AMR), one of the major causes of graft loss, is caused by antibodies directed against polymorphic donor human leukocyte Class I and II antigens (HLA) and is generally considered to have a worse prognosis than cellular rejection. Recent findings indicate that 60% or more incidences of late kidney graft losses are due to antibody-mediated injury. Furthermore, studies show that HLA antibodies significantly correlate with acute and chronic allograft rejection episodes in other solid organ transplantations and portend poor graft outcome[3-6]. In particular, chronically rejecting tissues develop transplant vasculopathy, defined by HLA-induced endothelial dysfunction and intimal thickening of larger vessels.

HLA class I antigens (A, B, and C antigens) are expressed on all nucleated cells, while HLA class II antigens (DP, DQ, and DR) are found on antigen-presenting cells such as dendritic cells, B cells, and activated endothelial cells. Though antibodies can also be directed against non-HLA antigens including such as MHC-class I-related chain A (MICA) antigens, MHC-class I-related chain B (MICB) antigens, [7] HLA-specific antibodies are found in more than 90% of cases of AMR[8, 9]. This thesis focuses predominantly on antibodies against HLA class I with brief discussion of HLA class II antibodies.

The presence and identification of donor-specific HLA antibodies are critical for the diagnosis of AMR and its management. Most occurrences of AMR have been found to be associated with HLA class I antibodies against HLA-A, HLA-B, and HLA class II antibodies directed against HLA-DR and HLA-DQ [2, 8, 10-12]. Notably, recent findings suggest that as much as 60% of DSAs are HLA class II antibodies[13]. Despite their undisputable contributions to AMR and graft pathogenesis, HLA II antibodies are poorly understood. The varied manifestations of the presence of HLA II antibodies in graft recipients and the mechanisms by which they mediate injury would pave the way for new therapeutic strategies.

Donor specific antibodies (DSA) cross-link HLA class I and II molecules on the surface of endothelium and cause a variety of effects, culminating in endothelial cell activation and injury. HLA antibodies mediate injury via two main pathways: Fc-dependent and Fc-independent mechanisms.

In an Fc-dependent manner, HLA antibodies regulate complement activation, resulting in endothelial injury and tissue damage. Additionally complement activation results in enzymatic cleavage of the components C3 and C5, generating the peptides C3a and C5a, both of which are soluble anaphylatoxins. C3a and C5a regulate the immune response by interacting with their receptors, C3aR and C5aR, thus potentiating recruitment of monocytes and neutrophils and alloreactive T cells which can damage the endothelium further. The capacity of alloantibodies to bind C1q and activate the classical complement cascade is based upon their IgG subclass. Antibodies of the IgG1 and IgG3 isotypes have been shown to efficiently activate complement, while the IgG2 and IgG4 subclasses fix complement weakly[14]. However, additional factors, such

as antibody titer and antigen density potentially affect functional deposition of complement products. As such, newly emerging assays and techniques will enhance our ability to characterize the role of HLA antibodies in complement activation.

In addition to the activation of the classical complement cascade, the Fc receptor on HLA antibodies interact with the Fc receptors on monocyte, neutrophils, and natural killer cells to augment their recruitment to the graft and to mediate phagocytosis and antibody dependent cellular cytotoxicity (ADCC). ADCC is mediated by innate immune cells, including natural killer (NK) cells and macrophages, which are activated upon binding Fc portion of the antibody through specific receptors. In this manner, alloantibodies can induce donor cell death[15].

Antibodies can also have an Fc-independent effect on endothelial cells by binding and crosslinking HLA molecules and co-receptors that activate the endothelium and induce functional changes such as upregulating genes involved in basement membrane and vessel remodeling, creating lesions that compromise graft function[3, 14, 16]. HLA I antibodies in particular induce intracellular signaling cascades in graft vascular cells, leading to phenotypic and functional changes. Our group has demonstrated that HLA I crosslinking by antibodies transduces intracellular signals, leading cytoskeletal rearrangement, migration and proliferation of endothelial cells (ECs) [17-19]. Additionally, our group demonstrated that ligation of endothelial HLA I molecules by antibodies results in mobilization of Weibel Palade bodies in a calcium-dependent manner, as demonstrated by increased surface expression of the adhesion molecule P-selectin and release of von Willebrand Factor. Increased expression of P-selectin in turn corresponded with augmented monocyte adherence to HLA I-stimulated endothelium; this effect

was abrogated upon administration of a P-selectin antagonist[20, 21]. Following HLA class I molecule ligation, there is up-regulation of additional genes involved in cell survival, including Akt, Bcl-2, and Bcl-xL. We have shown *in vitro* that anti- HLA I antibodies induce phosphorylation of Src, an increase in expression of basic FGF receptors (bFGFR)[22], as well as additional proteins, leading to endothelial proliferation. Furthermore, our group demonstrated that HLA class I ligation of ECs up-regulates the expression of anti-apoptotic proteins Bcl-2 and Bcl-xL through the phosphatidylinositol-3-kinase (PI3K) and AKT pathways. These findings were subsequently validated in biopsy samples of cardiac transplant recipients, who manifested with increased expression of Bcl-2 and Bcl-xL[23]. Similarly, HLA class II antibodies lead to S6 ribosomal protein (S6RP) phosphorylation via Akt activation[18], which is also seen following HLA class I stimulation of ECs. Immunohistochemical (IHC) analysis of human cardiac allograft biopsies demonstrated phosphorylated S6RP expression in AMR-positive patients manifesting with HLA class II antibodies. No correlation was found in vivo with HLA class I antibodies.

Additional studies are needed to elucidate their full contributions to the pathogenesis of graft rejection. Further investigations into how antibodies cause graft injury and promote acute and chronic rejection would contribute to a deeper understanding of AMR and facilitate improved therapeutics and patient care.

1.2 Leukocytes in Rejection

Anti-donor antibodies targeting allograft tissue can induce inflammatory signals, including cytokines such as IL-8, and activation of the classical complement cascade and generation of anaphylatoxins, which activate the graft vasculature and recruit leukocytes via multiple mechanisms. Activated ECs mobilize pre-formed vesicles called Weibel-Palade bodies (WPb) and up-regulate the adhesion molecule P-selectin, which allows for leukocyte capture from the blood by binding to PSGL-1. While P-selectin leads to increased leukocyte rolling along the endothelial surface and subsequent adherence[20], in order to transmigrate into graft tissue, leukocytes must firmly adhere to endothelial ICAM-1 or VCAM-1 via integrins. Chemokines released from the ECs mediate conformational changes in leukocyte integrin molecules that support binding to ICAM-1. This high affinity interaction mediates arrest of leukocytes on the ECs and begins the process of leukocytic extravasation into the allograft[24].

As key potentiators of the inflammatory immune response, T cells, B cells, monocytes and NK cells propagate injury, contributing to the processes of acute and chronic rejection. Current literature suggests that T cells play a major role in acute and chronic graft dysfunction. Studies have found predominant activation of CD8 T cells in murine allograft rejection[25, 26], which closely resembles the events taking place in humans, where allo-specific CD8 T cells are known to be important for promoting transplant rejection[6]. While T-cell deficient animals fail to reject fully mismatched transplants, adoptive transfer of wild-type T cells into these animals restores incidence of rejection with high numbers of activated effector T cells recruited into the allograft[27]. In patients receiving T cell-depleting therapies, episodes of acute rejection were prevented or reversed and long-term graft outcome was improved[28].

As such, the majority of immunosuppressive endeavors have targeted T-cell activity in allografts. While these drugs demonstrably improve cardiac and renal allograft survival by up to 95% in the first year, acute rejection still occurs in certain cases, and often, allografts culminate in chronic rejection[6].

Recent findings suggest that B cells play a role in cellular rejections, as well as acute and chronic AMR. B cell interaction with activated T helper cells leads to production of antibodies against the graft, thus regulating the allo-immune response. As a response to the growing interest in the role of B cells in rejection, new therapies have emerged to target B cells. Rituximab, an anti-CD20 antibody which depletes B cells, has been used to treat AMR in recipients of renal allografts[29] and has been shown to reduce circulating levels of DSAs in cardiac and renal allograft models[30, 31]. Of note, B cells may additionally contribute to the rejection process in the form of B-cell clusters, as B-cell-specific transcripts have been found in rejecting allografts[32-35]. Given the growing body of evidence implicating leukocytes in graft injury and pathogenesis, it is important to study the effects of other leukocytes in allograft rejection.

The adaptive immune response is essential to the process of rejection; however, accumulating data implicate cells of the innate immune system as important mediators of graft outcome, suggesting that monocytes and macrophages exert detrimental effects. The accumulation of macrophages in the allograft is considered a histological hallmark of antibody-mediated rejection[36, 37], and macrophages are important for the pathogenesis of acute and chronic rejection[38, 39], as well as ischemia reperfusion injury. Macrophages play a multi-faceted role in transplantation. While inflammatory macrophages contribute to initial damage, alternatively

activated macrophages, also known as wound healing macrophages, promote repair following injury to the graft. In in vitro and in vivo models of acute and chronic allograft rejection, depletion of certain macrophage subsets or inhibition of macrophage activation significantly reduced graft injury and rejection. Alternatively, studies of regulatory macrophages suggest a promising potential for this macrophage subset in treatment of graft injury, though the specific mechanisms are yet not clearly understood (reviewed in Chapter 2). Studies suggest that currently used immunosuppressants such as cyclosporine A (CsA), FK506 (calcineurin inhibitors) or mycophenolate mofetil [MMF] exert slight though distinctive effects on innate immune cells[40, 41]. CsA and FK506 have been shown to activate macrophages via inhibition of the calcineurin/NFAT pathway, while Bortezomib, a protease inhibitor inhibits cytokine production by macrophages *in vitro*. As evidence mounts, emphasizing the importance of monocytes and macrophages in allograft rejection and survival, further studies are required to clearly define the mode of action of immunosuppressive agents and their effects on the innate immune system.

1.3 mTOR Inhibitors in Transplantation

Mammalian target of rapamycin (mTOR) is a serine/threonine protein kinase that regulates broad aspects of cellular function such as protein synthesis, cellular metabolism, survival, and growth by initiating gene translation in response to environmental cues[42]. mTOR is present as two distinct complexes in the cell: mTORC1, comprised of mTOR, PRAS40, GβL and raptor, and mTORC2, comprised of mTOR, GβL, Sin1, and rictor. mTORC1 is mainly involved in transcription, CAP-dependent mRNA translation and cell growth, while mTORC2 regulates cell survival, metabolism, and cytoskeletal rearrangements. Our lab found mTOR is a central regulator of HLA I-induced proliferative and survival signaling in endothelial cells of cardiac allografts[17, 19] in models of AMR. Additional experimental evidence suggests that mTOR modulates recruitment of leukocytes by endothelial cells in the context of other disease models[43, 44].

Rapamycin and its analogs (rapalogs) inhibit mTOR indirectly by binding to FKBP12 and prevent formation of both mTORC1 and mTORC2[45]. Traditionally, mTORC1 was considered to be rapamycin sensitive and mTORC2 rapamycin insensitive; however, recent studies show long-term (greater than 6 hours) treatment with rapamycin disrupts mTORC2 formation as well. Commonly used to treat various cancers, these inhibitors have a cytostatic effect on proliferating cells both *in vitro* and *in vivo*[46, 47]. Though mTOR inhibitors yielded early success in renal cancers, they have since been shown to be useful for the treatment of a variety of cancers, including renal, liver, breast and prostate with many clinical trials completed and ongoing[48, 49]. Additionally, rapamycin and its rapalogs have been used to treat mantle cell lymphomas and TSC-related tumors, for which there are limited therapeutic options[42].

mTOR inhibitors are used in a variety of inflammatory disease models. One study reported that implantation of stents coated with the mTOR inhibitor everolimus into atherosclerotic arteries of cholesterol-fed rabbits significantly reduced macrophage content[44]. Concurrent *in vitro* studies demonstrated that treatment of macrophages with everolimus inhibited protein translation and induced cell death, suggesting that plaque-causing macrophages in atherosclerosis can by selectively targeted by mTOR inhibition[50]. In this aspect, mTOR inhibitors may be a promising therapy for treatment of atherosclerotic plaques and coronary artery disease.

The efficacy of mTOR inhibitors in transplantation has been and continues to be explored, driven by the need to limit rejection in allograft recipients. Rapamycin (sirolimus) and its analogue, everolimus, are the two mTOR inhibitors that have been approved for use in human transplantation[51]. While everolimus has been licensed for renal, heart, and liver transplantation, rapamycin is only approved for renal transplantation, though several centers have used it for the treatment of liver transplant recipeints[52]. Recent clinical trials have shown a reduction in the incidence of chronic cardiac allograft rejection when mTOR inhibitors were used[53, 54], adding further promise to the potential of mTOR-targeted therapies.

Immunosuppressive drugs used to treat allograft recipients exhibit toxicity profiles which at times influence graft homeostasis and potentiate the adverse outcomes seen in the long term. While Calcineurin inhibitors (CNIs) demonstrate nephrotoxicity in recipients of renal allografts, mTOR inhibitors have yet to demonstrate clinical side effects that represent a major threat and generally result in low rates of acute rejection and graft failure when compared to CNIs and MMF[52, 55, 56]. mTOR inhibitors have been shown to be effective immunosuppressant agents in

renal and cardiac transplantation in a CNI-free regimen. Evidence from a number of studies suggests that the use of mTOR inhibitors as adjunct therapy to non-CNIs is effective in preventing or attenuating cardiac allograft vasculopathy[57-59], providing the rationale for using mTOR inhibitors as an adjunct or alternative to CNIs. Furthermore, there is evidence suggesting that avoidance of CNIs in favor of mTOR inhibitors reduces some post-transplant infections such as CMV[55, 56].

Given these findings, much attention is being directed to CNI minimization protocols, and conversion from CNI-based to mTOR-inhibitor-based regimens are currently being examined in clinical situations with short-term data suggesting improvement in graft function[60]. A recently completed phase 4 clinical trial (NCT01046045) compared the efficacy of CsA (CNI) versus everolimus treatment in patients with chronic renal allograft rejection, though the results are still awaited.

The growing interest in mTOR inhibitor-based therapies has created a need to determine the mechanisms of action of these drugs. mTOR is known to play a major role in the immune response. As such, mTOR inhibitors have been used to block proliferation of activated T cells in organ transplant recipients. Furthermore, mTOR inhibitors are capable of inducing anergy in naïve T cells and promoting the expansion of regulatory T cells[61]. A recent clinical trial (NCT01014234) explored the effects of rapamycin on regulatory T cells in kidney transplant patients to determine the potential of rapamycin in inducing tolerance after transplantation. In addition to its effects on the adaptive immune response, a central role for mTOR in innate immunity has been recently defined by its ability to limit the production of pro-inflammatory

mediators from myeloid cells[62]. mTOR inhibitors have also been shown to block the maturation of dendritic cells and promote tolerance[61, 63, 64]. An unmet need clearly remains for identifying all mechanisms of mTOR inhibition in the setting of AMR in solid organ transplantation which will have relevance for short and long-term management of patients.

1.4 Conclusion

Advances in the field of transplantation have led to remarkable improvements in short-term graft outcomes among patients in recent years, though long-term graft survival remains a crucial problem. As important mediators of acute and chronic injury, HLA antibodies trigger vascular injury through a multitude of mechanisms. Appropriate therapy requires targeting HLA antibody-induced endothelial activation and abrogating vascular inflammation responsible for promoting vasculopathy, intragraft leukocytic infiltrates, and complement deposition.

Studies conducted by our group implicate mTOR signaling in HLA antibody-mediated endothelial activation and suggest that mTOR inhibition prevents signatures of HLA antibody-induced injury, including endothelial proliferation, migration and leukocyte recruitment[45, 65, 66]. Furthermore, accumulating evidence from clinical trials, as well as *in vitro* and *in vivo* studies suggests that mTOR inhibitors may be effective in preventing and treating aspect of antibody-mediate injury. For this reason, it is crucial to further explore the impact of mTOR inhibition on HLA-antibody-activated graft endothelium and to identify all mechanisms of mTOR inhibition in the setting of transplantation.

1.5 References

1. Lenihan CR, Lockridge JB, Tan JC. A new clinical prediction tool for 5-year kidney transplant outcome. *American Journal of Kidney Diseases*. 2014;63:549-551
2. Everly MJ, Rebellato LM, Haisch CE, Ozawa M, Parker K, Briley KP, Catrou PG, Bolin P, Kendrick WT, Kendrick SA, Harland RC, Terasaki PI. Incidence and impact of de novo donor-specific alloantibody in primary renal allografts. *Transplantation*. 2013;95:410-417
3. Valenzuela NM, Reed EF. The link between major histocompatibility complex antibodies and cell proliferation. *Transplantation Reviews*. 2011;25:154-166
4. Sellares J, de Freitas DG, Mengel M, Reeve J, Einecke G, Sis B, Hidalgo LG, Famulski K, Matas A, Halloran PF. Understanding the causes of kidney transplant failure: The dominant role of antibody-mediated rejection and nonadherence. *American Journal of Transplantation*. 2012;12:388-399
5. Berry GJ, Burke MM, Andersen C, Bruneval P, Fedrigo M, Fishbein MC, Goddard M, Hammond EH, Leone O, Marboe C, Miller D, Neil DL, Rassl D, Revelo MP, Rice A, Rodriguez ER, Stewart S, Tan CD, Winters GL, West L, Mehra MR, Angelini AL. The 2013 international society for heart and lung transplantation working formulation for the standardization of nomenclature in the pathologic diagnosis of antibody-mediated rejection in heart transplantation. *Journal of Heart and Lung Transplantation*. 2013;32:1147-1162
6. Colvin RB, Smith RN. Antibody-mediated organ-allograft rejection. *Nature Reviews Immunology*. 2005;5:807-817

7. Puttarajappa C, Shapiro R, Tan HP. Antibody-mediated rejection in kidney transplantation: A review. *Journal of transplantation*. 2012;2012:193724

8. Dorwal P, Phanish M, Duggal R, Chauhan R, Raina V, Kher V. Chronic active antibody mediated rejection associated with human leukocyte antigen-c*07 antibodies. *Indian journal of nephrology*. 2016;26:63-65

9. Sumitran-Holgersson S, Wilczeck HE, Holgersson J, Soderstrom K. Identification of the nonclassical hla molecules, mica, as targets for humoral immunity associated with irreversible rejection of kidney allografts. *Transplantation*. 2002;74:268-277

10. Lim WH, Chapman JR, Russ GR, Watson N, Holdsworth R, Wong G. Association between human leukocyte antigen (hla)-dq mismatches and rejection and graft loss in kidney transplant recipients. *Nephrology*. 2015;20:16-16

11. Lee H, Min JW, Kim JI, Moon IS, Park KH, Yang CW, Chung BH, Oh EJ. Clinical significance of hla-dq antibodies in the development of chronic antibody-mediated rejection and allograft failure in kidney transplant recipients. *Medicine*. 2016;95:E3094-E3094

12. Zeevi A, Marrari M, Spichty K, Morrell M, Gries C, McDyer J, Pilewski J, Zaldonis D, Bhama J, Shigemura N, Yousem S, Duquesnoy R, D'Cunha J, Bermudez C. Increased frequency of class ii hla-dq donor-specific antibodies is associated with mixed cellular and humoral rejection in lung transplantation. *Journal of Heart and Lung Transplantation*. 2013;32:S76-S76

13. Hidalgo LG, Campbell PM, Sis B, Einecke G, Mengel M, Chang J, Sellares J, Reeve J, Halloran PF. De novo donor-specific antibody at the time of kidney transplant biopsy

associates with microvascular pathology and late graft failure. *American Journal of Transplantation*. 2009;9:2532-2541

14. Valenzuela NM, McNamara JT, Reed EF. Antibody-mediated graft injury: Complement-dependent and complement-independent mechanisms. *Current Opinion in Organ Transplantation*. 2014;19:33-40

15. Thomas KA, Valenzuela NM, Reed EF. The perfect storm: Hla antibodies, complement, fc gamma rs, and endothelium in transplant rejection. *Trends in Molecular Medicine*. 2015;21:319-329

16. Colvin RB. Antibody-mediated renal allograft rejection: Diagnosis and pathogenesis. *Journal of the American Society of Nephrology*. 2007;18:1046-1056

17. Jin YP, Jindra PT, Gong KW, Lepin EJ, Reed EF. Anti-hla class i antibodies activate endothelial cells and promote chronic rejection. *Transplantation*. 2005;79:S19-S21

18. Lepin EJ, Zhang Q, Zhang X, Jindra PT, Hong LS, Ayele P, Peralta MVP, Gjertson DW, Kobashigawa JA, Wallace WD, Fishbein MC, Reed EF. Phosphorylated s6 ribosomal protein: A novel biomarker of antibody-mediated rejection in heart allografts. *American Journal of Transplantation*. 2006;6:1560-1571

19. Jindra PT, Jin YP, Jacamo R, Rozengurt E, Reed EF. Mhc class i and integrin ligation induce erk activation via an mtorc2-dependent pathway. *Biochemical and Biophysical Research Communications*. 2008;369:781-787

20. Valenzuela NM, Hong L, Shen XD, Gao F, Young SH, Rozengurt E, Kupiec-Weglinski JW, Fishbein MC, Reed EF. Blockade of p-selectin is sufficient to reduce mhc i antibody-elicited monocyte recruitment in vitro and in vivo. *American Journal of Transplantation*. 2013;13:299-311

21. Valenzuela NM, Mulder A, Reed EF. Hla class i antibodies trigger increased adherence of monocytes to endothelial cells by eliciting an increase in endothelial p-selectin and, depending on subclass, by engaging fc gamma rs. *Journal of Immunology*. 2013;190:6635-6650

22. Jin YP, Singh RP, Du ZY, Rajasekaran AK, Rozengurt E, Reed EF. Ligation of hla class i molecules on endothelial cells induces phosphorylation of src, paxillin, and focal adhesion kinase in an actin-dependent manner. *Journal of Immunology*. 2002;168:5415-5423

23. Jin YP, Fishbein MC, Said JW, Jindra PT, Rajalingam R, Rozengurt E, Reed EF. Anti-hla class i antibody-mediated activation of the pi3k/akt signaling pathway and induction of bcl-2 and bcl-xl expression in endothelial cells. *Human Immunology*. 2004;65:291-302

24. Ley K, Laudanna C, Cybulsky MI, Nourshargh S. Getting to the site of inflammation: The leukocyte adhesion cascade updated. *Nature Reviews Immunology*. 2007;7:678-689

25. Schnickel GT, Whiting D, Hsieh GR, Yun JJ, Fischbein MP, Fishbein MC, Yao W, Shfizadeh A, Ardehali A. Cd8 lymphocytes are sufficient for the development of chronic rejection. *Transplantation*. 2004;78:1634-1639

26. Ingulli E. Mechanism of cellular rejection in transplantation. *Pediatric Nephrology*. 2010;25:61-74

27. Hara M, Kingsley CI, Niimi M, Read S, Turvey SE, Bushell AR, Morris PJ, Powrie F, Wood KJ. Il-30 is required for regulatory t cells to mediate tolerance to alloantigens in vivo. *Journal of Immunology*. 2001;166:3789-3796

28. Tan HP, Chaudhary A, Humar A, Donaldson J, Basu A, Morgan C, Unruh M, McCauley J, Wu C, Shah N, Randhawa P, Shapiro R. Rejection characteristics of 200 living donor

kidney transplantations using alemtuzumab pretreatment and tacrolimus monotherapy: Mean 5-year follow-up. *American Journal of Transplantation*. 2010;10:394-394

29. DiLillo DJ, Griffiths R, Seshan SV, Magro CM, Ruiz P, Coffman TM, Tedder TF. B lymphocytes differentially influence acute and chronic allograft rejection in mice. *Journal of Immunology*. 2011;186:2643-2654

30. Zarkhin V, Chalasani G, Sarwal MM. The yin and yang of b cells in graft rejection and tolerance. *Transplantation Reviews*. 2010;24:67-78

31. Abe T, Ishii D, Gorbacheva V, Kohei N, Tsue H, Tanaka T, N D, Farichild R. Anti-hucd20 antibody therapy for antibody-mediated rejection of renal allografts in a mouse model. 2014

32. Zarkhin V, Li L, Sarwal M. "To b or not to b?" B-cells and graft rejection. *Transplantation*. 2008;85:1705-1714

33. Zarkhin V, Lovelace PA, Li L, Hsieh SC, Sarwal MM. Phenotypic evaluation of b-cell subsets after rituximab for treatment of acute renal allograft rejection in pediatric recipients. *Transplantation*. 2011;91:1010-1018

34. Kwun J, Knechtle SJ. Overcoming chronic rejection-can it b? *Transplantation*. 2009;88:955-961

35. Thaunat O. Pathophysiologic significance of b-cell clusters in chronically rejected grafts. *Transplantation*. 2011;92:121-126

36. Fishbein MC, Kobashigawa J. Biopsy-negative cardiac transplant rejection: Etiology, diagnosis, and therapy. *Current Opinion in Cardiology*. 2004;19:166-169

37. Fishbein GA, Fishbein MC. Morphologic and immunohistochemical findings in antibody-mediated rejection of the cardiac allograft. *Human Immunology*. 2012;73:1213-1217

38. Zecher D, van Rooijen N, Rothstein DM, Shlomchik WD, Lakkis FG. An innate response to allogeneic nonself mediated by monocytes. *Journal of Immunology*. 2009;183:7810-7816

39. Salehi S, Reed EF. The divergent roles of macrophages in solid organ transplantation. *Current Opinion in Organ Transplantation*. 2015;20:446-453

40. Hirayama M, Azuma E, Nakagawa-Nakazawa A, Kumamoto T, Iwamoto S, Amano K, Tamaki S, Usui E, Komada Y. Interleukin-10 spot-forming cells as a novel biomarker of chronic graft-versus-host disease. *Haematologica-the Hematology Journal*. 2013;98:41-49

41. Weimer R, Mytilineos J, Feustel A, Preiss A, Daniel V, Grimm H, Wiesel M, Opelz G. Mycophenolate mofetil-based immunosuppression and cytokine genotypes: Effects on monokine secretion and antigen presentation in long-term renal transplant recipients. *Transplantation*. 2003;75:2090-2099

42. Zarogoulidis P, Lampaki S, Turner JF, Huang HD, Kakolyris S, Syrigos K, Zarogoulidis K. Mtor pathway: A current, up-to-date mini-review. *Oncology Letters*. 2014;8:2367-2370

43. Farkas S, Hornung M, Sattler C, Guba M, Steinbauer M, Anthuber M, Herfarth H, Schlitt HJ, Geissler EK. Rapamycin decreases leukocyte migration in vivo and effectively reduces experimentally induced chronic colitis. *International Journal of Colorectal Disease*. 2006;21:747-753

44. Baetta R, Granata A, Canavesi M, Ferri N, Arnaboldi L, Bellosta S, Pfister P, Corsini A. Everolimus inhibits monocyte/macrophage migration in vitro and their accumulation in carotid lesions of cholesterol-fed rabbits. *Journal of Pharmacology and Experimental Therapeutics*. 2009;328:419-425

45. Jin YP, Valenzuela NM, Ziegler ME, Rozengurt E, Reed EF. Everolimus inhibits anti-hla i antibody-mediated endothelial cell signaling, migration and proliferation more potently than sirolimus. *American Journal of Transplantation*. 2014;14:806-819

46. Law BK. Rapamycin: An anti-cancer immunosuppressant? *Critical Reviews in Oncology Hematology*. 2005;56:47-60

47. Dufour M, Dormond-Meuwly A, Demartines N, Dormond O. Targeting the mammalian target of rapamycin (mtor) in cancer therapy: Lessons from past and future perspectives. *Cancers*. 2011;3:2478-2500

48. Meric-Bernstam F, Gonzalez-Angulo AM. Targeting the mtor signaling network for cancer therapy. *Journal of Clinical Oncology*. 2009;27:2278-2287

49. Wander SA, Hennessy BT, Slingerland JM. Next-generation mtor inhibitors in clinical oncology: How pathway complexity informs therapeutic strategy. *Journal of Clinical Investigation*. 2011;121:1231-1241

50. Martinet W, Verheye S, De Meyer GRY. Everolimus-induced rntor inhibition selectively depletes macrophages in atherosclerotic plaques by autophagy. *Autophagy*. 2007;3:241-244

51. Macdonald AS. Use of mtor inhibitors in human organ transplantation. *Expert review of clinical immunology*. 2007;3:423-436

52. Klintmalm GB, Nashan B. The role of mtor inhibitors in liver transplantation: Reviewing the evidence. *Journal of transplantation*. 2014;2014:845438

53. Matsuo Y, Cassar A, Yoshino S, Flammer AJ, Li J, Gulati R, Topilsky Y, Raichlin E, Lennon RJ, Lerman LO, Rihal CS, Kushwaha SS, Lerman A. Attenuation of cardiac allograft vasculopathy by sirolimus: Relationship to time interval after head transplantation. *Journal of Heart and Lung Transplantation*. 2013;32:784-791

54. Eisen HJ, Tuzcu EM, Dorent R, Kobashigawa J, Mancini D, Valantine-von Kaeppler HA, Starling RC, Sorensen K, Hummel M, Lind JM, Abeywickrama KH, Bernhardt P, Grp RBS. Everolimus for the prevention of allograft rejection and vasculopathy in cardiac-transplant recipients. *New England Journal of Medicine*. 2003;349:847-858

55. Patel SJ, Dawson KL, Knight RJ, Abdellatif A, Achkar K, Gaber LW, Gaber AO. The role of mtor inhibition in renal transplant immune suppression. *Dialysis & Transplantation*. 2011;40:23-29

56. Hirt SW, Bara C, Barten MJ, Deuse T, Doesch AO, Kaczmarek I, Schulz U, Stypmann J, Haneya A, Lehmkuhl HB. Everolimus in heart transplantation: An update. *Journal of transplantation*. 2013;2013:683964

57. Masetti M, Potena L, Nardozza M, Prestinenzi P, Taglieri N, Saia F, Pece V, Magnani G, Fallani F, Coccolo F, Russo A, Rapezzi C, Grigioni F, Branzi A. Differential effect of everolimus on progression of early and late cardiac allograft vasculopathy in current clinical practice. *American Journal of Transplantation*. 2013;13:1217-1226

58. Kushwaha SS. Mtor inhibitors as primary immunosuppression after heart transplant: Confounding factors in clinical trials. *American Journal of Transplantation*. 2014;14:1958-1959

59. Topilsky Y, Hasin T, Raichlin E, Boilson BA, Schirger JA, Pereira NL, Edwards BS, Clavell AL, Rodeheffer RJ, Frantz RP, Maltais S, Park SJ, Daly RC, Lerman A, Kushwaha SS. Sirolimus as primary immunosuppression attenuates allograft vasculopathy with improved late survival and decreased cardiac events after cardiac transplantation. *Circulation*. 2012;125:708-U153

60. Russ GR. Optimising the use of mtor inhibitors in renal transplantation. *Transplantation research*. 2013;2:S4

61. McMahon G, Weir MR, Li XC, Mandelbrot DA. The evolving role of mtor inhibition in transplantation tolerance. *Journal of the American Society of Nephrology*. 2011;22:408-415

62. Weichhart T, Haidinger M, Katholnig K, Kopecky C, Poglitsch M, Lassnig C, Rosner M, Zlabinger GJ, Hengstschlager M, Muller M, Horl WH, Saemann MD. Inhibition of mtor blocks the anti-inflammatory effects of glucocorticoids in myeloid immune cells. *Blood*. 2011;117:4273-4283

63. Dupont P, Warrens AN. The evolving role of sirolimus in renal transplantation. *Qjm-an International Journal of Medicine*. 2003;96:401-409

64. Levitsky J, Mathew JM, Abecassis M, Tambur A, Leventhal J, Chandrasekaran D, Herrera N, Al-Saden P, Gallon L, Abdul-Nabi A, Yang GY, Kurian SM, Salomon DR, Miller J. Systemic immunoregulatory and proteogenomic effects of tacrolimus to sirolimus conversion in liver transplant recipients. *Hepatology*. 2013;57:239-248

65. Jindra PT, Jin YP, Rozengurt E, Reed EF. Hla class i antibody-mediated endothelial cell proliferation via the mtor pathwayle. *Journal of Immunology*. 2008;180:2357-2366

66. Jindra PT, Hsueh A, Hong L, Gjertson D, Shen XD, Gao F, Dang J, Mischel PS, Baldwin WM, Fishbein MC, Kupiec-Weglinski JW, Reed EF. Anti-mhc class i antibody activation of proliferation and survival signaling in murine cardiac allografts. *Journal of Immunology*. 2008;180:2214-2224

Chapter 2:

The Divergent Roles of Macrophages in Solid Organ Transplantation

2.1 Abstract

Purpose of review: This review summarizes the phenotype and function of macrophages in the context of solid organ transplantation and will focus on fundamental insights into their paradoxical pro-inflammatory versus suppressive function. We will also discuss the therapeutic potential of regulatory macrophages in tolerance induction.

Recent findings: Macrophages are emerging as an essential element of solid organ transplantation. Macrophages are involved in the pathogenesis of ischemia reperfusion injury, as well as both acute and chronic rejection, exacerbating injury through secretion of inflammatory effectors and by amplifying adaptive immune responses. Notably, not all responses associated with macrophages are deleterious to the graft, and graft protection can in fact be conferred by macrophages. This has been attributed to the presence of macrophages with tissue-repair capabilities, as well as the effects of regulatory macrophages.

Summary: The explosion of new information on the role of macrophages in solid organ transplantation has opened up new avenues of research and the possibility of therapeutic intervention. However, the role of myeloid cells in graft rejection, resolution of rejection and tissue repair remains poorly understood. A better understanding of plasticity and regulation of monocyte polarization is vital for the development of new therapies for the treatment of acute and chronic transplant rejection.

Keywords: Macrophage; allograft rejection; acute; chronic, regulatory macrophages

2.2 Introduction

Macrophages and their precursors, monocytes, constitute an essential component of the innate immune system and form the first line of defense against pathogens [1]. Macrophages have the capacity to differentiate into a variety of phenotypes in response to cues from the microenvironment, and it is this notable phenotypic plasticity that governs the expression of the broad range of inducible effectors [2]. In the transplant setting, macrophages can cause allograft injury, tissue remodeling or have immunoregulatory/suppressive effects depending on their state of activation [2-4]. In response to stimuli, infiltrating macrophages differentiate preferentially into "classically activated" or "alternatively-activated" subsets with markedly different functions [3, 4]. Classically activated macrophages, also referred to as M1 macrophages, develop in response to IFN-γ and engagement of Toll-like receptors (TLRs) by microbial products [2, 5]. They generally display a pro-inflammatory phenotype expressing high levels of CD86, iNOS, TNF-α, IL-1 and IL-6 (Figure 1a) [4]. In contrast, exposure to IL-4 or IL-13 leads to the development of "alternatively-activated" or "wound-healing" macrophages, also referred to as M2 macrophages, that display markers of alternative activation including CD206, the scavenger protein CD163, arginase-1, and IL-10 (Figure 1b) [6-8]. The M2 subset of macrophages is not a uniform population and is further subdivided into M2a, M2b, and M2c. Within this subset, M2a macrophages, generally referred to as alternatively activated macrophages, are induced by IL-4 and IL-13, with surface expression of CD163, CD206, CD209, IL-4, FcεR, and Dectin-1. Ligation of macrophage FcRs by IgG complexes coupled with TLR or CD40/CD44 engagement induces a Type II activation [2, 3, 9], which corresponds with an M2b phenotype. M2b macrophages are immunoregulatory and produce high levels of IL-10, IL-1, IL-6 and TNF-α. M2c macrophages are referred to as deactivated macrophages given their role in down-regulation of pro-inflammatory cytokines, as well as tissue repair and remodeling. This macrophage subset is induced by IL-10, TGF-β, and glucocorticoids and in turn produces large amounts of IL-10 and TGF-β with surface expression of CD163, CD206, RAGE and other scavenger receptors [3, 10]. While these M2 variants have been explored in a variety of disease models [11, 12], they have yet to

be characterized in the setting of solid organ transplantation. Regulatory macrophages (M regs), a less well-characterized subtype of macrophages, can suppress T cell function and have been utilized as therapeutic agents in transplantation (Figure 1c) [13, 14]. Regulatory macrophages express iNOS, MHC class II, and PD-L1, though little CD40 or CD86 [15]. M regs are fundamentally distinct and do not express most markers found on M1 or M2 macrophages and have been shown to mitigate acute and chronic inflammation in different disease models [16]. Though regulatory macrophages modulate inflammatory immune responses, these cells do not actively participate in wound healing [15]. Notably, peripheral blood monocytes have been divided into two subsets with distinct function and phenotype. The pro-inflammatory CD14+CD16+ subset exhibits high expression of pro-inflammatory cytokines [17], while the immunosuppressive monocytes are CD14+CD163+ and exhibit immunosuppressive mechanisms including IL-10 production [18]. The role of peripheral blood monocyte subsets in transplantation has been minimally studied, with contradictory findings, and requires further investigation [18, 19].

2.3 Macrophages in Ischemia Reperfusion Injury

Ischemia reperfusion injury (IRI) is a multifactorial process, involving both innate and adaptive immunity, which impacts early and late graft dysfunction [20]. Cells of the innate immune system, particularly macrophages, are key potentiators of IRI, participating in both the early stages of injury and in late stage repair [21-25]. Animal models of IRI show that injury is associated with an influx of macrophages, implicating these innate immune cells in augmentation of ischemic injury [26]. One study demonstrated that knocking out CCR2 , a receptor for monocyte chemo-attractant protein 1 (MCP-1), protected mice from kidney IRI, correlating with reduced macrophage infiltration [27]. In a murine model of liver IRI, blockade of TIM-1 on CD4 cells inhibited T-cell mediated activation of macrophages and mitigated injury [28]. In a clinical study, using the selectin antagonist (rPSGL-1) reduced liver IRI with improved liver function and augmented cytoprotective IL-10, with a reduction in MCP-1, suggesting

inhibition of macrophage infiltration [20]. Notably, while inflammatory macrophages contribute to the initial damage during IRI [21], alternatively activated macrophages promote repair following the injury. As such, Huen et al. showed that macrophages in the setting of kidney IRI can be skewed toward a distinct reparative phenotype which supports tubular proliferation and repair in response to GM-CSF [29, 30]. Similarly, a myeloid-specific PTEN knockout conferred protection from liver IRI by promoting development of M2 macrophages in response to TLR engagement. PTEN deficiency resulted in constitutive activation of the pro-survival PI3K pathway, which regulates macrophage differentiation by upregulating miR-155. This M2 differentiation correlates with a decrease in expression of certain pro-inflammatory mediators and a marked increase in the anti-inflammatory cytokine IL-10 [31]. Moreover, over-expression of macrophage heme-oxygenase-1, an enzyme with anti-inflammatory properties, imposed an anti-inflammatory or M2 phenotype, selectively inhibiting M1 polarization. When adoptively transferred into mice, these macrophages mitigated injury and inflammation caused by ischemia reperfusion [32]. Collectively, these findings point to an instrumental role for macrophages in the pathophysiology of IRI depending on the nature of the macrophage subset during the time course of injury, as M1 macrophages can mediate the inflammatory process at the onset of ischemic injury, while M2 macrophages are involved in post-injury resolution. As IRI is an antigen-independent event, macrophages involved in this process are activated through cytokines and/or engagement of TLRs or other pattern recognition receptors by endogenous ligands generated through cellular damage [33]. Consequently, mice deficient in TLR4 demonstrated reduced IRI after liver transplantation [34], while donor TLR4 was shown to contribute to renal allograft inflammation in humans [35]. A recent study revealed lipocalin-2 (Lcn2), a defense mediator expressed in response to TLR activation, plays a crucial role in cardiac IRI, and neutralization of Lcn2 suppressed M1 macrophage polarization and instead mediated skewing of macrophages toward an M2 phenotype. Additionally, Lcn2 treatment suppressed infiltration of macrophages further limiting IRI [36].

2.4 Macrophages in Acute Allograft Rejection

Macrophages were first implicated in rejecting renal allografts over fifty years ago [37]. Macrophage accumulation in the allograft is associated with both acute antibody-mediated rejection (AMR), and acute cell-mediated rejection [38, 39]. In instances of acute and chronic injury [39] in animal models as well as humans [40, 41], macrophages account for 38-60% of infiltrating leukocytes in rejecting organs [42-45]. Notably, a murine model of pancreatic islet grafts provided evidence of direct destruction of islet tissue by macrophages [46]. The presence of CD68+ macrophage infiltrates is associated with diagnosis of acute rejection in human renal allografts [39, 47-49]. Macrophage depletion has led to amelioration of graft injury and a reduction in pathological features of acute rejection in experimental models [40, 44, 50, 51]. Similarly, inhibition of macrophage accumulation and activation in murine cardiac allografts results in abrogation of graft injury and rejection [43, 52].

Macrophages propagate injury in the setting of AMR [53, 54] and are a distinguishing feature of graft pathology in AMR lesions [49]. In fact, one of the most important diagnostic criteria for AMR in cardiac transplantation is the presence of intravascular macrophages in the capillaries of endomyocardial biopsies [55]. In a clinical study, Kirk and colleagues found that there was a high incidence of AMR associated with infiltrating macrophages in renal transplant patients treated with Campath, a T cell-depleting drug [56, 57]. Similar detrimental effects were observed in a lymphocyte-deficient RAG-/- cardiac murine model of acute AMR [53, 54], adding support to the claim that macrophages are sufficient to induce allograft injury. In this study, passive transfer of anti-donor HLA antibodies induced accumulation of intravascular macrophages in heterotopic cardiac allografts, demonstrating pathological features of injury. *In vitro*, P-selectin blockade was shown to prevent antibody-mediated monocyte recruitment to endothelial cells, conferring protection from antibody-induced damage. This was recapitulated in the above-mentioned murine model of acute AMR [53] and has had promising results in IRI [20], with potential for use in AMR in solid organ transplantation. In the setting of AMR, donor specific HLA IgG antibodies have been shown to recruit monocytes via an FcγR-dependent mechanism

[9, 58]. Consequently, eliminating these antibody-FcγR interactions using EndoS, an endoglycosidase that modifies protein glycosylation, and IdeS, an IgG-degrading enzyme, was shown to significantly lessen monocyte recruitment to cardiac endothelium *in vitro* [58].

These combined findings implicate macrophages as an essential determinant in the induction of acute rejection. Though the exact mechanism by which macrophages mediate injury is not fully understood, *in vitro* and *in vivo* studies implicate the production of inflammatory mediators as a central mechanism whereby macrophages contribute to allograft injury [5]. Inside the graft, macrophages release inflammatory mediators such as nitric oxide (iNOS), IL-2, IL-6, IL-12, MCP-1, and TNF-α [40, 44], which activate and damage the microvasculature, recruit leukocytes, and induce donor-specific cytotoxic responses [1]. Studies where macrophages have been depleted, or receptors for leukocyte recruitment antagonized, confirmed the role of macrophage cytokine production and other pro-inflammatory mediators in graft rejection. For instance, chemical macrophage depletion results in a reduction in the severity of acute allograft rejection in rodent models of small bowel transplantation [44, 59]. The reduction in small bowel injury was attributed, in part, to lower expression of inflammatory genes including iNOS, MCP-1 and IL-6, factors associated with M1 macrophages. Blockade of inflammatory cytokines such as TNF-α and iNOS was shown to extend cardiac graft survival, underscoring the importance of macrophage-mediated-inflammation in heart transplant rejection [60, 61]. Similarly, administration of the chemokine receptor antagonist, Met-Rantes, inhibited monocyte adhesion to inflamed endothelium in a rat model of acute cellular renal injury in which monocytes constitute the majority of the infiltrating cells. Correspondingly, the treated animals displayed a decrease in the expression level of several pro-inflammatory cytokines [62, 63]. While M1 macrophages mediate injury, M2 macrophages are generally implicated in injury resolution and tissue remodeling, and therefore, they may promote allograft damage repair; though currently, their role in acute injury remains speculative. Histological studies of murine corneal allografts exhibiting acute rejection revealed the presence of M1 macrophages secreting pro-inflammatory mediators, while M2 macrophages were detected in the animals

that did not reject the transplants [64]. An M1-dominant response was also observed in a rat model of acute renal AMR and in clinical biopsy samples of acutely rejecting kidney allograft recipients [65].

In light of these findings, selective depletion of macrophage subpopulations may be exploited to provide additional insight into the myriad functions of macrophages in the context of acute allograft injury and repair, more specifically targeting M1 macrophages as a therapeutic tactic. Albeit, it might be more prudent to target destructive macrophage subsets for manipulation, such as those skewed toward the M1 phenotype, for manipulation, rather than depletion, as studies suggest that macrophages are plastic and do not remain committed to a single phenotype/activation state [2, 3].

2.5 Macrophages in Chronic Allograft Rejection

Chronic rejection is the leading cause of long-term graft failure. The manifestations of chronic allograft rejection include vasculopathy and chronic vascular lesions, often accompanied by sub-endothelial leukocytes, and proliferation of vascular endothelial and smooth muscle cells [66]. Histological sections of chronically rejecting tissues stain positive for macrophage infiltrates, and macrophage labeling has been explored as a means of detecting chronic rejection prior to the onset of graft dysfunction [67]. Intragraft macrophages are associated with worse outcome in renal, liver, and cardiac transplantation in humans as well as animal models [68-70], and macrophages have been shown to directly cause tissue injury and fibrosis. Case studies focusing on the development of chronic allograft nephropathy have emphasized the pivotal role of macrophages in human biopsies culminating in end-stage renal failure [69, 71, 72]. Interestingly, monocytes have been shown to have altered activation levels, exhibited by enhanced TNF-α production, in patients undergoing chronic renal rejection [73].

As in the case of acute rejection, the current view is that macrophages promote worse graft outcome through the release of inflammatory mediators and regulation of cytokine dynamics. Studies conducted during the course of chronic rejection found up-regulation of MCP-1, RANTES, TNF-α, IFN-γ and iNOS among others, correlating with macrophage activation [74]. Yang et al. used a previously

established rat renal allograft model to target a variety of macrophage-derived and macrophage-activating soluble mediators implicated in chronic graft rejection. Blocking the actions of TNF-α, IL-12, and IFN-γ reduced macrophage-mediated chronic injury [75]. Macrophage participation in chronically rejecting vascularized grafts can be further modulated by blockade of chemokine-chemokine receptor interactions, as administration of Met-RANTES, an agonist to the chemokine receptor CCR5, to transplant recipients has been successful in significantly lessening chronic injury in cardiac and renal grafts [76, 77]. A macrophage-specific inhibitor, gamma lactone, was successfully used to prevent murine chronic renal allograft nephropathy [68] with a correlative reduction in the levels of macrophage-produced inflammatory mediators. As in acute injury, the impact of macrophages in models of chronic rejection has been assessed through depletion strategies, demonstrating attenuation of chronic lesions and vasculopathy [78].

In patients presenting with chronic allograft nephropathy, mRNA levels of PAI-1, a glycoprotein which promotes fibrosis by inhibiting degradation of the extracellular matrix , were found to be increased in macrophages infiltrating the kidney [72]. These findings identify an additional mechanism where macrophages incite chronic rejection by promoting fibrosis. Fibrosis precedes clinical dysfunction of the allograft and the development of progressive fibrosis in turn has been attributed to M2 macrophages in the context of dysregulated inflammation [48]. Though the majority of M2 macrophages, including M2a and M2c macrophages, are generally considered to demonstrate beneficial reparative characteristics, with regard to ongoing injury, sustained activity may result in the continuous production of various wound-healing growth factors, ultimately becoming a pathological process leading to fibrosis [79]. Consequently, M2 macrophages were identified as the dominant macrophage subset found in chronic lesions [6]. Steroids and calcineurin inhibitors, used routinely in transplantation therapy, have been shown to induce CD163+ M2 macrophage polarization, with a correlative increase in mRNA levels of pro-fibrotic cytokines such as TGFβ-1 and connective tissue growth factor, thus promoting development of fibrosis and at times exacerbating rejection [6, 80]. These recent findings link progression of fibrosis to this subset

of macrophages, suggesting that they may serve as a predictive biomarker of chronic rejection and that restricting their activity would serve as a potential therapeutic strategy to protect against macrophage-dependent mechanisms related to fibrosis. Fully understanding the function of the M2 macrophage subset in the setting of chronic rejection requires additional studies.

2.6 Macrophages as a therapeutic agent

Though much attention has been given to the detrimental role of macrophages in organ transplantation, limited studies have ascertained that regulatory macrophages have the potential to prolong allograft survival.

M regs have been used in immunodeficient mice [81], and in non-immunosuppressed recipients of a mismatched heterotopic heart allografts, to ameliorate symptoms of rejection and prolong allograft survival [15]. Furthermore, administration of M regs to porcine recipients of single lung allografts improved graft prognosis [82].

Presently, it is not fully understood how M regs exert their immunosuppressive effects *in vivo*, though it is assumed it is controlled by multiple mechanisms. In principle, M regs could directly regulate and suppress polyclonal T cell proliferation and mediate T cell elimination through an iNOS-dependent mechanism and their ability to down-regulate L-selectin levels on T cells, which ultimately prevents T cell activation [15, 83]. Alternatively, M regs may secrete anti-inflammatory mediators, which help promote tissue repair. Consistent with this idea, the suppressive capacity of M regs has been attributed to IFN-γ-induced iNOS [15, 84], which has recently been implicated in macrophage-mediated immune suppression [15, 85].

From a therapeutic viewpoint, regulatory macrophages with the capacity to quell an aberrant inflammatory response could be used as a pharmacological agent for tolerance induction. A recent study showed that M regs can be generated from peripheral blood monocytes for potential use in solid organ transplantation [86]. Two human recipients of kidney allografts were adoptively transferred with donor-

derived infusions of M regs and weaned to monotherapy [13]. No incidence of acute or chronic rejection has been observed at 5 years. The absence of acute rejection and lack of signs indicative of subclinical rejection suggested a lack of or attenuation of anti-donor reactivity [87]. In these studies, M regs demonstrated graft-protective functions and pre-operative administration of M reg-based therapy was shown to mediate tolerance of the donor allograft. Donor M regs are used instead of recipient M regs, as a study by Riquelme et al. established that the graft-protective effect of M regs is specific to donor cells [15]. The described findings suggest there is a benefit to distinguishing between macrophage subsets present in allograft settings, as depletion of certain subsets of macrophages may prove more beneficial than total macrophage depletion.

Several key clinical concerns remain to be addressed regarding the translation of M reg therapy to clinical transplantation, such as the stability and safety of M regs *in vivo* and the efficacy of M reg usage in a wide and variable population. Some of these questions are now being addressed in the ONE Study consortium in Europe, aimed at determining the efficacy and safety of administering donor-derived M reg preparations to living-donor solid organ transplant recipients as a cellular immunotherapy, with the ultimate goal of reducing the need for conventional immunosuppression (NCT02085629).

2.7 The Effects of Immunosuppressives and therapeutics on Macrophages

Immunosuppressive drugs used routinely for the prevention of allograft rejection have been shown to affect the phenotype and function of macrophages. Macrophages treated with rapamycin, an inhibitor of the serine/threonine kinase mTOR, were impaired in their ability to present antigens and displayed a notable reduction in the expression of CD80 [88]. Rapamycin has also been shown to inhibit production of the inflammatory mediator iNOS in macrophage cell lines [89]. Bortezomib is a protease inhibitor mainly used in the treatment of AMR [90] and has also been found to block T-cell mediated responses [91, 92]. In a murine model of contact hypersensitivity, an inflammatory immune reaction mediated by T cells, Bortezomib treatment resulted in a noted reduction in macrophage infiltration [91].

Furthermore, Bortezomib has been shown to reduce inflammatory cytokine production in macrophages stimulated with LPS *in vitro* [91, 93]. Use of the calcineurin inhibitors CsA and FK506 has been shown to regulate TLR mediated pathways in myeloid cells and lead to macrophage activation by inhibiting the calcineurin/NFAT pathway. Blocking NFAT leads to activation of the downstream NF-κB and MAPK pathways, and to subsequent production of inflammatory mediators including IL-12 and TNF-α [94, 95]. As mentioned previously, calcineurin inhibitors have also been implicated in the promotion of M2 macrophage differentiation , as identified by the marker CD163 [6]. Butyric acid is used for treatment of auto-immune disorders and has been investigated for tolerance induction in allografts [96]. Butyrate treatment of monocytes *in vitro* was found to decrease their phagocytic capabilities and to reduce expression of markers including CD14, CD86 and MHCII [97]. In a separate study, butyrate prevented IL-12 production in human monocytes and promoted production of IL-10 [98], suggesting that it might play a role in the development of anti-inflammatory macrophages.

2.8 Conclusion

Modulation of graft homeostasis involves the interplay between the various subpopulations of macrophages, which can contribute allograft-destructive or protective mechanisms based on their phenotype and function. Though major advances have been made with regard to an improved understanding of the contribution of macrophages to graft outcome, there is a paucity of clinical data and further studies are warranted to establish a comprehensive understanding of their contribution to graft injury, repair and graft acceptance.

Key points

- Based on cues from their microenvironment, macrophages differentiate into inflammatory (M1), wound-healing (M2), or regulatory macrophages all with distinct functions and phenotypes.

- Macrophages generate inflammatory mediators that contribute to ischemia reperfusion injury and acute and chronic allograft rejection.
- Regulatory macrophages are an attractive candidate for use as an adjunct cell-based therapy to suppress allograft rejection in human transplantation.

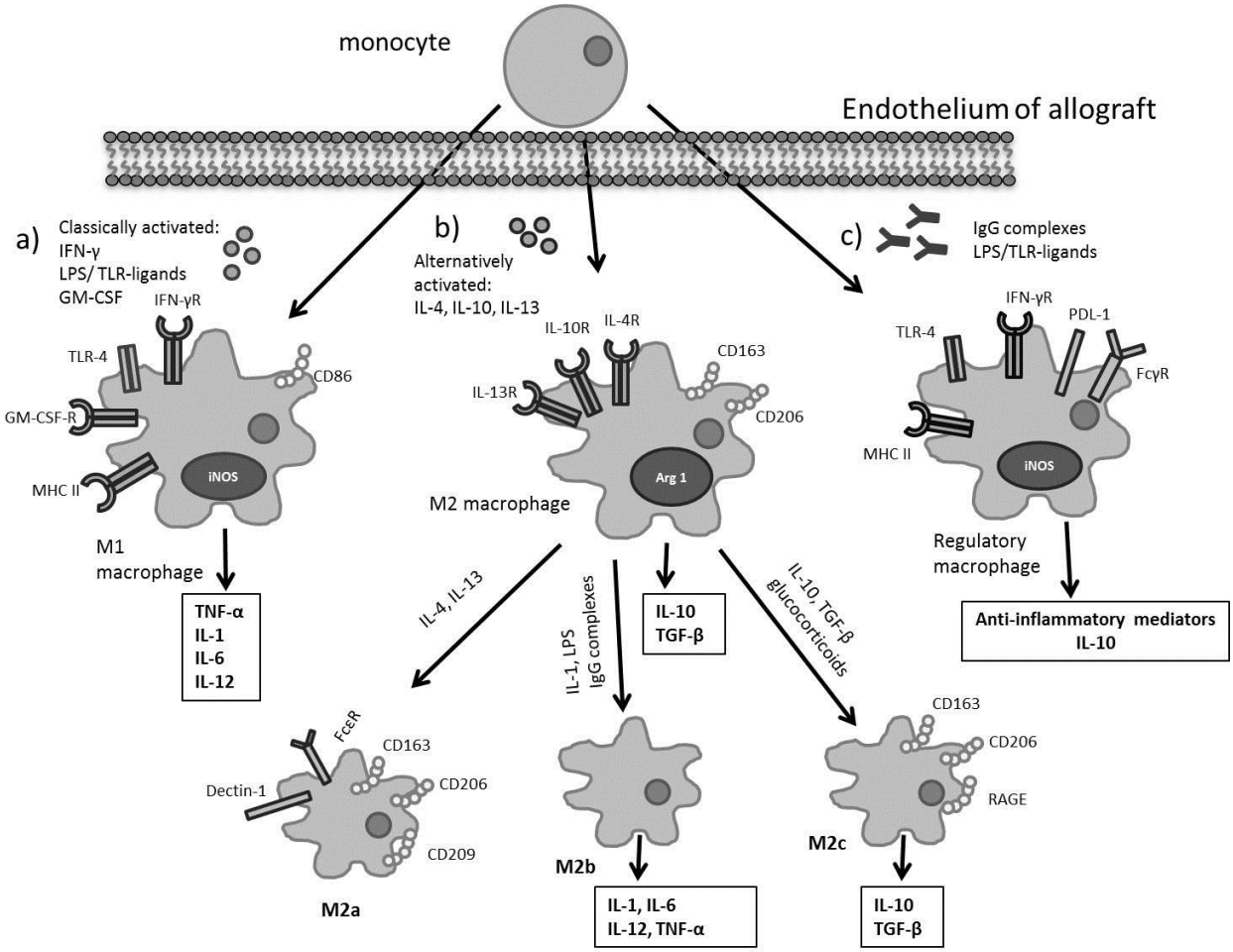


Figure 2-1. Macrophage plasticity and function in the context of allograft rejection.

a) M1 macrophages are classically activated, damage graft endothelium, recruit additional leukocytes, and mediate tissue injury. They are the dominant phenotype in acute rejection and their activity can be modulated by blockade of their activation or the factors they produce. b) M2 macrophages are alternatively activated, mediate tissue repair and injury resolution, and promote fibrosis. This subset is predominantly found in chronically-damaged allografts. c) Mregs are activated in a fashion distinct from the other two subsets. They modulate anti-inflammatory response, have T cell suppressive capacity, and are being investigated for use in cell-based therapy.

2.9 References:

[1] Murphy K. Janeway's Immunobiology. Garland Science, Taylor & Francis Group, LLC; 2012.

[2] Gordon S. Alternative Activation of Macrophages. In: Nature Reviews Immunology; 2003. pp. 23-35.

[3] Martinez FO, Gordon S. The M1 and M2 paradigm of macrophage activation: time for reassessment. F1000prime reports 2014; 6:13.

[4] Sica A, Mantovani A. Macrophage plasticity and polarization: in vivo veritas. Journal of Clinical Investigation 2012; 122:787-795.

[5] Mosser DM. The many faces of macrophage activation. Journal of Leukocyte Biology 2003; 73:209-212.

[6] Ikezumi Y, Suzuki T, Yamada T *et al.* Alternatively activated macrophages in the pathogenesis of chronic kidney allograft injury. In: Edited by: University M. Pediatric Nephrology: 2014.
This study explored the localization of M2 macrophages and their contribution to intersitial fibrosis in the setting of chronic kidney allograft injury.

[7] Koh TJ, DiPietro LA. Inflammation and wound healing: the role of the macrophage. Expert Reviews in Molecular Medicine 2011; 13:12.

[8] Barros MHM, Hauck F, Dreyer JH *et al.* Macrophage Polarisation: an Immunohistochemical Approach for Identifying M1 and M2 Macrophages. Plos One 2013; 8:11.

[9] Valenzuela NM, Mulder A, Reed EF. HLA Class I Antibodies Trigger Increased Adherence of Monocytes to Endothelial Cells by Eliciting an Increase in Endothelial P-Selectin and, Depending on Subclass, by Engaging Fc gamma Rs. Journal of Immunology 2013; 190:6635-6650.

[10] Mantovani A, Sozzani S, Locati M *et al.* Macrophage polarization: tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes. Trends in Immunology 2002; 23:549-555.

[11] Grugan KD, McCabe FL, Kinder M *et al.* Tumor-Associated Macrophages Promote Invasion while Retaining Fc-Dependent Anti-Tumor Function. Journal of Immunology 2012; 189:5457-5466.

[12] Lugo-Villarino G, Verollet C, Maridonneau-Parini I *et al*. Macrophage polarization: convergence point targeted by mycobacterium tuberculosis and HIV. Frontiers in immunology 2011; 2:43.

[13] Broichhausen C, Riquelme P, Geissler EK *et al*. Regulatory macrophages as therapeutic targets and therapeutic agents in solid organ transplantation. Current Opinion in Organ Transplantation 2012; 17:332-342.

[14] Suzuki T, Arumugam P, Sakagami T *et al.* Pulmonary macrophage transplantation therapy. Nature 2014; 514:450-+.

[15] Riquelme P, Tomiuk S, Kammler A *et al.* IFN-gamma-induced iNOS Expression in Mouse Regulatory Macrophages Prolongs Allograft Survival in Fully Immunocompetent Recipients. Molecular Therapy 2013; 21:409-422.

[16] Fleming BD, Mosser DM. Regulatory macrophages: Setting the Threshold for Therapy. European Journal of Immunology 2011; 41:2498-2502.

[17] Ziegler-Heitbrock L. The CD14+CD16+blood monocytes: their role in infection and inflammation. Journal of Leukocyte Biology 2007; 81:584-592.

[18] Sekerkova A, Krepsova E, Brabcova E *et al.* CD14+CD16+and CD14+CD163+monocyte subpopulations in kidney allograft transplantation. Bmc Immunology 2014; 15:10.

[19] Vereyken EJF, Kraaij MD, Baan CC *et al.* A Shift towards Pro-Inflammatory CD16+Monocyte Subsets with Preserved Cytokine Production Potential after Kidney Transplantation. Plos One 2013; 8:10.

[20] Busuttil RW, Lipshutz GS, Kupiec-Weglinski JW *et al.* rPSGL-Ig for Improvement of Early Liver Allograft Function: A Double-Blind, Placebo-Controlled, Single-Center Phase II Study. American Journal of Transplantation 2011; 11:786-797.

[21] Ysebaert DK, De Greef KE, Vercauteren SR *et al.* Identification and kinetics of leukocytes after severe ischaemia/reperfusion renal injury. Nephrology Dialysis Transplantation 2000; 15:1562-1574.

[22] Takada M, Nadeau KC, Shaw GD *et al.* The cytokine-adhesion molecule cascade in ischemia/reperfusion injury of the rat kidney - Inhibition by a soluble P-selectin ligand. Journal of Clinical Investigation 1997; 99:2682-2690.

[23] Persy VP, Verhulst A, Ysebaert DK *et al.* Reduced postischemic macrophage infiltration and interstitial fibrosis in osteopontin knockout mice. Kidney International 2003; 63:543-553.

[24] Day YJ, Huang LP, McDuffie MJ *et al.* Renal protection from ischemia mediated by A(2A) adenosine receptors on bone marrow-derived cells. Journal of Clinical Investigation 2003; 112:883-891.

[25] Lemay S, Rabb H, Postler G *et al*. Prominent and sustained up-regulation of gp130-signaling cytokines and of the chemokine MIP-2 in murine renal ischemia-reperfusion injury. Transplantation 2000; 69:959-963.

[26] Jo SK, Sung SA, Cho WY *et al.* Macrophages contribute to the initiation of ischaemic acute renal failure in rats. Nephrology Dialysis Transplantation 2006; 21:1231-1239.

[27] Furuichi K, Wada T, Iwata Y *et al.* CCR2 signaling contributes to ischemia-reperfusion injury in kidney. Journal of the American Society of Nephrology 2003; 14:2503-2515.

[28] Zhang Y, Ji H, Shen X *et al.* Targeting TIM-1 on CD4 T Cells Depresses Macrophage Activation and Overcomes Ischemia-Reperfusion Injury in Mouse Orthotopic Liver Transplantation. American Journal of Transplantation 2013; 13:56-66.

[29] Huen SC, Huynh L, Marlier A *et al.* GM-CSF Promotes Macrophage Alternative Activation after Renal Ischemia/Reperfusion Injury. In: J Am Soc Nephrol; 2014.

This study identifies GM-CSF as a novel factor produced by renal tubular cells that can skew macrophages toward a reparative phenotype that supports tubular proliferation after renal ischemic reperfusion injury.

[30] Huen SC, Cantley LG. Macrophage-mediated injury and repair after ischemic kidney injury. Pediatric Nephrology 2015; 30:199-209.

[31] Yue S, Rao JH, Zhu JJ *et al.* Myeloid PTEN Deficiency Protects Livers from Ischemia Reperfusion Injury by Facilitating M2 Macrophage Differentiation. Journal of Immunology 2014; 192:5343-5353. The novel findings from this study highlight the important role of PTEN in regulating macrophage polarization and the tissue inflammatory immune response in heptic ischemia reperfusion injury.

[32] Huang J, Shen X-D, Yue S *et al.* Adoptive transfer of heme oxygenase-1 (HO-1)-modified macrophages rescues the nuclear factor erythroid 2-related factor (Nrf2) antiinflammatory phenotype in liver ischemia/reperfusion injury. Molecular medicine (Cambridge, Mass.) 2014; 20:448-455.

[33] Zhai Y, Petrowsky H, Hong JC *et al.* Ischaemia-reperfusion injury, in liver transplantation-from bench to bedside. Nature Reviews Gastroenterology & Hepatology 2013; 10:79-89.

[34] Shen XD, Ke B, Zhai Y *et al.* Absence of toll-like receptor 4 (TLR4) signaling in the donor organ reduces ischemia and reperfusion injury in a murine liver transplantation model. Liver Transplantation 2007; 13:1435-1443.

[35] Kruger B, Krick S, Dhillon N *et al.* Donor Toll-like receptor 4 contributes to ischemia and reperfusion injury following human kidney transplantation. Proceedings of the National Academy of Sciences of the United States of America 2009; 106:3390-3395.

[36] Cheng L, Xing H, Mao X *et al.* Lipocalin-2 Promotes M1 Macrophages Polarization in a Mouse Cardiac Ischaemia-Reperfusion Injury Model. Scandinavian Journal of Immunology 2015; 81:31-38.

[37] Brent L, Brown J, Medawar PB. Skin transplantation immunity in relation to hypersensitivity. Lancet 1958; 2:561-564.

[38] Fishbein MC, Kobashigawa J. Biopsy-negative cardiac transplant rejection: etiology, diagnosis, and therapy. Current Opinion in Cardiology 2004; 19:166-169.

[39] Tinckam KJ, Djurdjev O, Magil AB. Glomerular monocytes predict worse outcomes after acute renal allograft rejection independent of C4d status. Kidney International 2005; 68:1866-1874.

[40] Wyburn KR, Jose MD, Wu HL *et al.* The role of macrophages in allograft rejection. Transplantation 2005; 80:1641-1647.

[41] Strom TB, Tilney NL, Carpenter CB *et al*. Identity and Cytotoxic Capacity of Cells Infiltrating Renal Allograft. New England Journal of Medicine 1975; 292:1257-1263.

[42] Hancock WW, Thomson NM, Atkins RC. Composition of interstitial cellular infiltrate identified by monoclocal antibodies in renal biopsies of rejecting human renal allografts. Transplantation 1983; 35:458-463.

[43] Abe T, Su CA, Iida S *et al.* Graft-Derived CCL2 Increases Graft Injury During Antibody-Mediated Rejection of Cardiac Allografts. American Journal of Transplantation 2014; 14:1753-1764.

This study explores the role of graft derived monocyte chemoattractant protein-1 in AMR and the contribution of intragraft macrophages to cardiac AMR.

[44] Schafer N, Tahara K, von Websky M *et al.* Role of resident macrophages in the immunologic response and smooth muscle dysfunction during acute allograft rejection after intestinal transplantation. Transplant International 2008; 21:778-791.

[45] Schmidt A, Sucke J, Fuchs-Moll G *et al.* Macrophages in experimental rat lung isografts and allografts: infiltration and proliferation in situ. Journal of Leukocyte Biology 2007; 81:186-194.

[46] Yi SN, Hawthorne WJ, Lehnert AM *et al.* T cell-activated macrophages are capable of both recognition and rejection of pancreatic islet xenografts. Journal of Immunology 2003; 170:2750-2758.

[47] Girlanda R, Kleiner DE, Duan Z *et al.* Monocyte infiltration and kidney allograft dysfunction during acute rejection. American Journal of Transplantation 2008; 8:600-607.

[48] Toki D, Zhang W, Hor KLM *et al.* The Role of Macrophages in the Development of Human Renal Allograft Fibrosis in the First Year After Transplantation. American Journal of Transplantation 2014; 14:2126-2136.

This study investigated the role of macrophages in human renal allograft fibrosis. Human renal biopsies with macrophage infiltrates showed subclinical alloimmune inflammation, tubular injury and fibrosis.

[49] Fishbein GA, Fishbein MC. Morphologic and immunohistochemical findings in antibody-mediated rejection of the cardiac allograft. Human Immunology 2012; 73:1213-1217.

[50] Qi F, Adair A, Ferenbach D *et al.* Depletion of Cells of Monocyte Lineage Prevents Loss of Renal Microvasculature in Murine Kidney Transplantation. Transplantation 2008; 86:1267-1274.

[51] Jose MD, Ikezumi Y, van Rooijen N *et al.* Macrophages act as effectors of tissue damage in acute renal allograft rejection. Transplantation 2003; 76:1015-1022.

[52] Takeiri M, Tachibana M, Kaneda A *et al.* Inhibition of macrophage activation and suppression of graft rejection by DTCM-glutarimide, a novel piperidine derived from the antibiotic 9-methylstreptimidone. Inflammation Research 2011; 60:879-888.

[53] Valenzuela NM, Hong L, Shen XD *et al.* Blockade of P-Selectin Is Sufficient to Reduce MHC I Antibody-Elicited Monocyte Recruitment In Vitro and In Vivo. American Journal of Transplantation 2013; 13:299-311.

[54] Jindra PT, Hsueh A, Hong L *et al.* Anti-MHC class I antibody activation of proliferation and survival signaling in murine cardiac allografts. Journal of Immunology 2008; 180:2214-2224.

[55] Berry GJ, Burke MM, Andersen C *et al.* The 2013 International Society for Heart and Lung Transplantation Working Formulation for the standardization of nomenclature in the pathologic diagnosis of antibody-mediated rejection in heart transplantation. Journal of Heart and Lung Transplantation 2013; 32:1147-1162.

[56] Agarwal A, Shen LY, Kirk AD. The role of alemtuzumab in facilitating maintenance immunosuppression minimization following solid organ transplantation. Transplant Immunology 2008; 20:6-11.

[57] Kirk AD, Mannon RB, Kleiner DE *et al.* Results from a human renal allograft tolerance trial evaluating T-cell depletion with alemtuzumab combined with deoxyspergualin. Transplantation 2005; 80:1051-1059.

[58] Valenzuela NM, Trinh KR, Mulder A *et al.* Monocyte Recruitment by HLA IgG-Activated Endothelium: The Relationship Between IgG Subclass and FcgammaRIIa Polymorphisms. American Journal of Transplantation: 2015. p. 17.

This is the first study to explore the effects of HLA IgG modulation on FcγR-mediated monocyte recruitment. The findings from this study highlight HLA IgG antibodies as a target for therapy in AMR rejection.

[59] Fryer J, Grant D, Jiang JF *et al.* Influence of macrophage depletion on bacterial translocation and rejection in small bowel transplantation. Transplantation 1996; 62:553-559.

[60] Imagawa DK, Millis JM, Seu P *et al.* The role of tumor necrosis factor in allograft rejection. Evidence that anti-TNF antibody therapy prolongs allograft survival in rats with acute rejection. Transplantation 1991; 51:57-62.

[61] Roza AM, Cooper M, Pieper G *et al.* NOX 100, a nitric oxide scavenger, enhances cardiac allograft survival and promotes long-term graft acceptance. Transplantation 2000; 69:227-231.

[62] Grone HJ, Weber C, Weber KSC *et al.* Met-RANTES reduces vascular and tubular damage during acute renal transplant rejection: blocking monocyte arrest and recruitment. Faseb Journal 1999; 13:1371-1383.

[63] Pattison J, Nelson PJ, Huie P *et al.* RANTES chemokine expression in cell-mediated transplant rejection of the kidney. Lancet 1994; 343:209-211.

[64] Oh JY, Lee HJ, Ko AY *et al.* Analysis of Macrophage Phenotype in Rejected Corneal Allografts. Investigative Ophthalmology & Visual Science 2013; 54:7779-7784.

[65] Huang G, Wilson NA, Reese SR *et al.* Characterization of Transfusion- Elicited Acute Antibody-Mediated Rejection in a Rat Model of Kidney Transplantation. American Journal of Transplantation 2014; 14:1061-1072.

[66] Hutchinson IV. Cardiac allograft vasculopathy - the cellular attack. Zeitschrift Fur Kardiologie 2000; 89:16-20.

[67] Beckmann N, Cannet C, Fringeli-Tanner M *et al.* Macrophage labeling by SPIO as an early marker of allograft chronic rejection in a rat model of kidney transplantation. Magnetic Resonance in Medicine 2003; 49:459-467.

[68] Azuma H, Nadeau KC, Ishibashi M *et al*. Prevention of functional, structural, and molecular changes of chronic rejection of rat renal allografts by a specific macrophage inhibitor. Transplantation 1995; 60:1577-1582.

[69] Pilmore HL, Painter DM, Bishop GA *et al.* Early up-regulation of macrophages and myofibroblasts - A new marker for development of chronic renal allograft rejection. Transplantation 2000; 69:2658-2662.

[70] Miyagawa-Hayashino A, Tsuruyama T, Haga H *et al.* Arteriopathy in chronic allograft rejection in liver transplantation. Liver Transplantation 2004; 10:513-519.

[71] Croker BP, Clapp WL, Shamat A *et al.* Macrophages and chronic renal allograft nephropathy. Kidney International 1996; 50:S42-S49.

[72] Revelo MP, Federspiel C, Helderman H *et al*. Chronic allograft nephropathy: expression and localization of PAI-1 and PPAR-gamma. Nephrology Dialysis Transplantation 2005; 20:2812-2819.

[73] Heidenreich S, Lang D, Tepel M *et al*. Monocyte activation for enhanced tumour necrosis factor-alpha and interleukin 6 production during chronic renal allograft rejection. Transplant immunology 1994; 2:35-40.

[74] Nadeau KC, Azuma H, Tilney NL. Sequential cytokine dynamics in chronic rejection of rat renal allografts: role for cytokines RANTES and MCP-1. Proceedings of the National Academy of Sciences of the United States of America 1995; 92:8729-8733.

[75] Yang J, Reutzel-Selke A, Steier C *et al.* Targeting of macrophage activity by adenovirus-mediated intragraft overexpression of TNFRp55-Ig, IL-12p40, and vIL-10 ameliorates adenovirus-mediated chronic graft injury, whereas stimulation of macrophages by overexpression of IFN-gamma accelerates chronic graft injury in a rat renal allograft model. Journal of the American Society of Nephrology 2003; 14:214-225.

[76] Yun JJ, Whiting D, Fischbein MP *et al.* Combined blockade of the chemokine receptors CCR1 and CCR5 attenuates chronic rejection. Circulation 2004; 109:932-937.

[77] Song EW, Zou HQ, Yao YS *et al.* Early application of Met-RANTES ameliorates chronic allograft nephropathy. Kidney International 2002; 61:676-685.

[78] Kitchens WH, Chase CM, Uehara S *et al.* Macrophage depletion suppresses cardiac allograft vasculopathy in mice. American Journal of Transplantation 2007; 7:2675-2682.

[79] Ricardo SD, van Goor H, Eddy AA. Macrophage diversity in renal injury and repair. Journal of Clinical Investigation 2008; 118:3522-3530.

[80] Ikezumi Y, Suzuki T, Karasawa T *et al.* Identification of alternatively activated macrophages in new-onset paediatric and adult immunoglobulin A nephropathy: potential role in mesangial matrix expansion. Histopathology 2011; 58:198-210.

[81] Broichhausen C, Riquelme P, Ahrens N *et al.* In question: the scientific value of preclinical safety pharmacology and toxicology studies with cell-based therapies In: Molecular Therapy — Methods & Clinical Development: 2014.

This study explores the pharmacokinetics and safety of M regs in a murine model and questions the justification of using M regs in human patients.

[82] Warnecke G, Hutchinson JA, Riquelme P *et al.* Postoperative intravenous infusion of donor-derived transplant acceptance-inducing cells as an adjunct immunosuppressive therapy in a porcine pulmonary allograft model. Transplant International 2009; 22:332-341.

[83] Hanson EM, Clements VK, Sinha P *et al.* Myeloid-Derived Suppressor Cells Down-Regulate L-Selectin Expression on CD4(+) and CD8(+) T Cells. Journal of Immunology 2009; 183:937-944.

[84] Brem-Exner BG, Sattler C, Hutchinson JA *et al.* Macrophages driven to a novel state of activation have anti-inflammatory properties in mice. Journal of Immunology 2008; 180:335-349.

[85] Arakawa Y, Qin J, Chou HS *et al.* Cotransplantation With Myeloid-Derived Suppressor Cells Protects Cell Transplants: A Crucial Role of Inducible Nitric Oxide Synthase. Transplantation 2014; 97:740-747.

[86] Hutchinson JA, Riquelme P, Geissler EK *et al*. Human Regulatory Macrophages. In: Suppression and Regulation of Immune Responses: Methods and Protocols. Edited by: Cuturi MC, Anegon I. Totowa: Humana Press Inc; 2011. pp. 181-192.

[87] Hutchinson JA, Riquelme P, Tomiuk S *et al.* Immunological consequences and trafficking of human regulatory macrophages administered to renal transplant recipients. Transplant International 2011; 24:219-219.

[88] Shen H, Li L, Zhao Y. Effects of immunosuppressive drugs on phenotypes and function of differentiated macrophages. Jiepou Xuebao 2011; 42:345-349.

[89] Attur MG, Patel R, Thakker G *et al.* Differential anti-inflammatory effects of immunosuppressive drugs: Cyclosporin, rapamycin and FK-506 on inducible nitric oxide synthase, nitric oxide, cyclooxygenase-2 and PGE(2) production. Inflammation Research 2000; 49:20-26.

[90] Ejaz NS, Alloway RR, Halleck F *et al.* Review of Bortezomib Treatment of Antibody-Mediated Rejection in Renal Transplantation. Antioxidants & Redox Signaling 2014; 21:2401-2418.

[91] Yanaba K, Yoshizaki A, Muroi E *et al.* The proteasome inhibitor bortezomib inhibits T cell-dependent inflammatory responses. Journal of Leukocyte Biology 2010; 88:117-122.

[92] Everly MJ, Everly JJ, Susskind B *et al.* Bortezomib Provides Effective Therapy for Antibody- and Cell-Mediated Acute Rejection. Transplantation 2008; 86:1754-1761.

[93] Han SH, Kim JS, Woo JH *et al.* The Effect of Bortezomib on Expression of Inflammatory Cytokines and Survival in a Murine Sepsis Model Induced by Cecal Ligation and Puncture. Yonsei Medical Journal 2015; 56:112-123.

This is the first study to determine that Bortezomib treatment reduces production of inflammatory mediators by macrophages. This finding provides additional insight into the mechanism of Bortezomib and indicates a novel application for its use in allograft rejection.

[94] Kang YJ, Kusler B, Otsuka M *et al.* Calcineurin negatively regulates TLR-Mediated activation pathways. Journal of Immunology 2007; 179:4598-4607.

[95] Fric J, Zelante T, Wong AYW *et al.* NFAT control of innate immunity. Blood 2012; 120:1380-1389.

[96] Bohmig GA, Krieger PM, Saemann MD *et al.* Stable prodrugs of n-butyric acid: suppression of T cell alloresponses in vitro and prolongation of heart allograft survival in a fully allogeneic rat strain combination. Transplant Immunology 1999; 7:221-227.

[97] Millard AL, Mertes PM, Ittelet D *et al.* Butyrate affects differentiation, maturation and function of human monocyte-derived dendritic cells and macrophages. Clinical and Experimental Immunology 2002; 130:245-255.

[98] Saemann MD, Bohmig GA, Osterreicher CH *et al.* Anti-inflammatory effects of sodium butyrate on human monocytes: potent inhibition of IL-12 and up-regulation of IL-10 production. Faseb Journal 2000; 14:2380-2382.

Chapter 3:

mTOR Inhibits HLA Class I Ab-mediated Endothelial Activation and Monocyte Recruitment in an ICAM1-Dependent Manner in Model of Acute Cardiac Rejection

3.1 Abstract

Background-Antibody-mediated rejection (AMR) resulting in allograft vasculopathy remains a major obstacle for long-term patient and cardiac transplant survival. mTOR inhibitors have proven efficacy to reduce intimal hyperplasia of the transplanted organ, yet the mechanisms underlying the capacity of Rapalogs to inhibit vasculopathy have not been fully elucidated.

Methods and Results-We explored the underlying molecular mechanisms of HLA class I (HLA-I) antibody-induced AMR and the role of the mTOR signaling network in endothelial-monocyte interactions. We discovered a previously undescribed regulatory property of mTOR in endothelial cells (ECs) via abrogation of HLA I antibody-induced circulating monocyte recruitment *in vitro*. We confirmed these findings in a murine model of cardiac AMR in which recipients of fully-MHC mismatched donor cardiac allografts were administered donor-specific major histocompatibility complex class I (MHC-I) antibodies, manifesting in endothelial injury and intravascular macrophage accumulation. mTOR inhibition significantly reduced macrophage infiltration and vascular injury *in vivo*. Furthermore, HLA I ligation of ECs induces ICAM-1-mediated firm adhesion of monocytes in an mTOR-dependent manner.

Conclusions-Our results suggest that mTOR inhibition dampens activation of endothelial cell signaling pathways in response to HLA-I antibodies and prevents macrophage infiltration into cardiac allografts, and may prove effective in the treatment of acute AMR. These findings indicate a novel application for the use of sirolimus and everolimus in patients manifesting with AMR, to target endothelial-monocyte interactions.

Key words: cell adhesion molecules, endothelium, inflammation, rejection, transplantation

3.2 Introduction

Cardiac transplantation remains the most effective treatment for patients with end-stage heart failure. Advances in immunosuppression and patient management have increased the rate of one-year cardiac graft survival to nearly 90%. However, the development of antibody-mediated rejection (AMR) persists as a major issue limiting long-term patient and graft survival. AMR is caused by the binding of donor specific human leukocyte antigen (HLA) antibodies (HLA-DSAs) to the mismatched donor HLA class I and class II antigens expressed on the endothelium of the graft. DSA binding activates the endothelium resulting in microvascular inflammation with a characteristic histologic phenotype of mononuclear cell infiltration, and the presence of intravascular macrophages, with or without complement deposition(Colvin and Smith, 2005). Chronic exposure of the allograft to DSA leads to the development of coronary allograft vasculopathy (CAV), a manifestation of chronic antibody-mediated rejection, resulting in graft dysfunction, allograft loss and patient death (Amico et al., 2009; Ho et al., 2011; Willicombe et al., 2011). Therefore, efforts to disrupt antibody-mediated microvascular inflammation and accompanying leukocyte recruitment may prevent CAV and extend the life of the transplant recipient. Recent studies support this concept as depletion of NK cells significantly reduced neointimal formation in a murine model of chronic AMR. Furthermore, depletion of monocytes reduces inflammation and development of rejection (Salehi and Reed, 2015).

In addition to their well-known role in antigen presentation, HLA class I (HLA-I) molecules transduce signals that elicit functional alterations in endothelial cells (ECs). HLA-I crosslinking induces cytoskeletal remodeling, proliferation and migration of ECs (Li et al., 2011; Narayanan

et al., 2006; Valenzuela and Reed, 2011). HLA-I signaling also triggers rapid mobilization of Weibel Palade Bodies, induction of P-selectin and the recruitment of myeloid cells and neutrophils to the endothelium (Valenzuela et al., 2013a; Valenzuela et al., 2013c; Yamakuchi et al., 2007). A critical pathway regulating HLA-I-mediated signaling in ECs is the mammalian target of rapamycin (mTOR). Blocking mTOR activity has been shown to prevent HLA-I antibody-mediated EC proliferation and migration, suggesting that rapalogs may be beneficial in preventing graft injury by HLA antibodies(Jin et al., 2014; Jindra et al., 2008b; Jindra et al., 2008c). Pretreatment of endothelial cells with rapamycin inhibits TNF-induced expression of the adhesion molecule VCAM-1 on ECs and reduced the capacity of TNF-treated ECs to capture T cells under physiologic flow conditions(Wang et al., 2014). Furthermore, rapamycin effectively reduced leukocyte binding and extravasation in submucosal venules thereby reducing inflammation in a murine model of colitis(Farkas et al., 2006). mTOR inhibition in monocytes regulates cell surface expression of UPAR which influences surface proteolysis, matrix interactions, and signaling and migration of monocytes and other leukocytes(Mahoney et al., 2001). Inhibition of mTOR by rapamycin was also found to abolish ROCK-1 synthesis in macrophages resulting in an inhibition of chemotaxis and phagocytosis (Fox et al., 2007).

While mTOR inhibitors are widely used for the treatment of a variety of diseases (Meng and Zheng, 2015), including prevention of allograft rejection (Andreassen et al., 2015; Qiu et al., 2015), the mechanism by which inhibition of the mTOR signaling pathway reduces development of CAV(Eisen et al., 2013; Eisen et al., 2003; Kobashigawa et al., 1998) is not fully understood. Here we demonstrate that HLA-I signaling in ECs mediates activation of RhoA, MAPK and ERM proteins downstream of mTOR causing subsequent ICAM-1 clustering on the endothelial

cell surface, a necessary step for myeloid cell adhesion and extravasation. Furthermore, we show that mTOR inhibition reduces myeloid cell burden in heart allografts undergoing AMR. We conclude that HLA-I/mTOR-dependent signaling mechanisms trigger clustering of endothelial ICAM-1 into a membrane structure that provides a platform for stable monocyte firm arrest and transendothelial cell migration.

3.3 Results

3.3.1 Inhibition of mTOR in endothelial cells significantly reduces HLA I antibody-induced monocyte recruitment

mTOR has been shown to be required for endothelial activation following HLA class I ligation by antibody, therefore, we sought to determine if mTOR inhibition can mitigate features of HLA class I-induced AMR by abrogating recruitment of cell-line and primary monocytes to endothelial cells in culture. We found that pre-treatment of HLA I Ab-activated human aortic endothelial cells (HAECs) with mTOR inhibitors rapamycin (rapa) and everolimus (RAD) reduced adherence of the monocytic cell line MM6 to the endothelium by approximately 1.5 fold compared to non-mTOR inhibited activated ECs **(Fig. 1A).** Additionally, we demonstrated pharmacological inhibition of mTOR blocks HLA I-induced recruitment of primary monocytes to activated endothelium (**Fig. 1B)**. Pre-treatment of ECs with BAPTA-AM, a Ca^{2+} chelator, was used as a positive control to confirm inhibition of monocyte adherence, as we have previously demonstrated (Valenzuela et al., 2013b).

To establish ideal dosages for mTOR inhibitor use *in vitro*, ECs were pre-treated with varying doses of the pharmacological mTOR inhibitors rapamycin and everolimus (RAD), followed by stimulation with pan HLA-I antibodies (**Suppl. Fig. 1** ; (Jin et al., 2014)). Ideal dosage was defined as the minimum dosage at which we observed equal measures of response as higher dosages. Fc interactions between monocytes and HLA Abs that would result in activation of complement were precluded via incubation with human IgG (Valenzuela et al., 2013c).

We confirmed that stimulation of ECs with HLA I Abs or the classical agonist thrombin results in significant monocyte adherence compared to untreated ECs. Using an antibody against

integrin β3 (ITGB3) had no effect **(Figure 1A, 1B).** ECs were activated through two different pan HLA I Abs (HLA I Ab (mIgG2a) and HLA I Ab (mIgG1), targeting different epitopes of the same molecule, to demonstrate reproducibility of our findings. We also observed inhibition of monocyte adherence to HLA I-stimulated ECs when endothelial mTOR was inhibited for 2 h (**Figure 1C**). Rapamycin/everolimus pre-treatment for 2 hours has been shown to inhibit mTORC1 formation, whereas prolonged exposure (greater than 6 hours), prevents formation of mTORC2 andmTORC1. The use of two different time points (2 and 24 h) allowed us to assess the relative contributions of mTORC1 and mTORC2 (respectively) to monocyte recruitment, suggesting that both mTOR complexes are involved in regulating endothelial recruitment of monocytes following HLA class I engagement.

Representative images illustrate fluorescently labeled MM6 adhere to ECs with HLA I Ab stimulation; this is prevented with rapamycin pre-treatment **(Figure 1D).**

To determine which specific mTOR signaling complex regulates monocyte adherence to HLA I-stimulated ECs, we used gene-specific siRNA to knock down main components of each complex, raptor for mTORC1 and rictor for mTORC2. The efficiency of the silencing protocol is displayed by Western blot **(supplemental figure 2).**

Static adherence assays following siRNA knockdown of raptor and rictor revealed commensurate inhibition of monocyte binding efficiency **(Figure 1E)**. We determined that although silencing of mTORC1 and mTORC2 separately inhibits monocyte adherence to almost control levels, blockade of complete mTOR signaling using mTOR siRNA demonstrated a more robust inhibition of monocyte adherence, suggesting a synergistic effect. In summary, these experiments point to a regulatory role for mTOR in monocyte recruitment to EC.

3.3.2 In vivo blockade of mTOR ameliorates HLA-I antibody-mediated acute AMR

Having established that mTOR inhibition *in vitro* blocks monocyte adherence to HLA I-activated endothelium, we sought to further explore the role of mTOR signaling in monocyte recruitment during AMR by i*n vivo* administration of the mTOR inhibitor rapamycin to transplanted mice undergoing cardiac AMR. We used a previously established murine model of acute antibody mediated injury (Jindra et al., 2008a; Valenzuela et al., 2013a) which demonstrates features analogous to human AMR, including endothelial swelling and injury, intravascular macrophage infiltrates, and complement deposition **(Figure 2A).**

H&E analysis revealed MHC I Ab treatment resulted in AMR pathology as demonstrated by plump and prominent endothelium, and myocardial injury, as demonstrated by shrunken myocytes and disrupted myocardial architecture. In the group that received rapamycin therapy concurrently with anti-donor MHC I Abs, there was significant amelioration of graft pathology. Specifically, endothelial swelling was more subtle and ECs appeared flat and inconspicuous, with less damage to the myocardium, which is similar to what was observed in both isotype control Ab-treated groups **(Figure 2B, E)**. Next, we determined the effects of rapamycin on monocyte recruitment to MHC-I activated murine cardiac allografts by Mac-2 staining. In the group which received anti-donor MHCI antibodies, cellular infiltrates were found between the myocardium in a linear arrangement, suggesting intracapillary macrophage infiltration. By comparison, in the rapamycin + MHC I Ab-treated group, the myocardium showed a significant decrease in macrophage infiltrates **(Figure 2C, F)**. Rapamycin did not have any effect on complement deposition induced by anti-donor MHC I Ab **(Figure 2D, G).** This is consistent with our studies demonstrating that complement deposition is dependent upon endothelial surface HLA molecule density and HLA antibody titer(Thomas et al., 2015a; Thomas et al., 2015b),

neither of which has been shown to be regulated by mTOR signaling to date. The data indicated that administration of rapamycin *in vivo* correlates with a reduction in MHC-induced graft pathology.

3.3.3 mTOR regulates adhesion molecule function but not expression

We proceeded to determine the underlying mechanisms of mTOR regulation of HLA I-Ab-mediated AMR. We previously found that HLA I ligation triggers Weibel Palade body (WPb) exocytosis and subsequent P-selectin expression on the surface of ECs, which is sufficient to induce increased adherence of MM6 (Valenzuela et al., 2013b) to HLA I-stimulated ECs. To probe the impact of mTOR inhibition on HLA I-Ab induced WPb exocytosis, we measured endothelial P-selectin expression and Von Willebrand Factor (vWF) release. Stimulation of ECs with the known mitogen thrombin or HLA-I Ab induced rapid WPb exocytosis as measured by P-selectin upregulation **(Figure 3A)** and vWF release **(Figure 3B),** while exposure to ITGβ3 Ab did not. Notably, pre-treatment of ECs with rapamycin or everolimus (RAD) for 24 h **(Fig. 3A and B)** and 2 h (data not shown) did not affect HLA-I Ab-induced WPb release, as neither P-selectin expression nor vWF secretion were modulated. As expected, pre-treatment of ECs with BAPTA-AM significantly inhibited HLA I Ab-triggered WPb exocytosis.

These experiments excluded the involvement of the adhesion molecule P-selectin in mTOR-mediated HLA-I signaling. Because rapamycin is known to directly inhibit protein synthesis (Huo et al., 2011; Raught et al., 2001), as a confirmatory experiment to rule out non-specific inhibition of protein synthesis, we determined the effect of rapamycin on basal expression of the adhesion molecules E-Selectin (not shown), VCAM-1 (not shown) and ICAM-1, all of which are

involved in the leukocyte adhesion cascade (Ley et al., 2007). Flow cytometric analysis of HLA I-activated and mTOR-inhibited ECs indicated no change in surface expression levels of these adhesion molecules **(Figure 3C and data not shown).**

Having determined mTOR inhibition does not alter the expression of adhesion molecules, we next explored the effects of mTOR inhibition on the functionality of these adhesion molecules. Ibidi flow-based adhesion assays use shear stress and allow for high-resolution visualization of the interactions between the endothelium and monocytes and are, for this reason, a more accurate physiological representation of the process of leukocyte adhesion. Using this technology, we observed that the rolling behavior of monocytes on rapamycin pre-treated and HLA-I-stimulated ECs was unaffected; however, the ability of ECs to support firm adhesion of tethered monocytes was impaired compared to HLA-I-activated ECs that received no inhibitor treatment. When ECs were treated with an ICAM-1 blocking antibody, the same pattern of monocyte rolling without firm adhesion was observed **(Figure 3D)**. This would suggest that rapamycin affects ICAM-1-mediated firm adhesion of monocytes to the endothelium. **Figure 3E** further stratifies the speed of monocytes, noting there was no difference in the monocytes that are considered fast rolling and those not interacting with the ECs (10-50, 50-250 ɥm/s). There was however, a significant decrease in the number of monocytes that are firmly adherent at 0-2 ɥm/s and a corresponding increase in the percentage of monocytes that are slow rolling at a speed of 2-10 ɥm/s. Supplemental videos 1-3 display monocytes firmly adhering to HLA I-treated ECs (Suppl. Vid 2). With rapamycin pre-treatment of ECs, monocytes are unable to adhere firmly, and instead, can be visualized slowly rolling along the endothelium (Suppl. Vid 3).

3.3.4 HLA I-induced ERM phosphorylation is impaired under mTOR inhibition in ECs

We proceeded to elucidate the molecular pathways by which mTOR regulates ICAM-1 function.

As the ezrin/radixin/moesin (ERM) proteins have been shown to be required for ICAM-1 function (Heiska et al., 1998; Ivetic and Ridley, 2004), we first determined the effect of HLA I Ab-stimulation on the phosphorylation of ERM. Pre-treatment of ECs with HLA I Ab increased the levels of phosphorylated ERM compared to controls levels. Increased p-ERM levels following HLA I stimulation in ECs was abrogated upon mTOR inhibition with rapamycin and everolimus (RAD) **(Figure 4A and B).** To ensure that these data were not generated through off-target drug effects and to confirm efficient mTOR inhibition, we analyzed p-mTOR, p-P70S6K, p-AKT, p-ERK, and p-S6RP, which are indicative of class I-mediated activation of the mTOR signaling pathway (Jin et al., 2014) **.** The expression of these phosphorylated proteins was increased following HLA I Ab stimulation and inhibited with rapamycin and everolimus (RAD) pre-treatment **(Figure 4A and B).** Next, to test the association of p-ERM with ICAM-1, we co-immunoprecipiated HLA I-stimulated ECs with an antibody against ICAM-1 and blotted for p-ERM, noting an increase in the levels of p-ERM compared to control levels, confirming its association with ICAM-1. We found a corresponding lack of ICAM-1-associated p-ERM upon mTOR inhibition by comparison, validating the finding that mTOR signaling downstream of HLA I engagement regulates ERM phosphorylation **(Figure 4C and D).** To confirm that the effect of rapamycin and everolimus (RAD) was mediated by inhibition of mTOR in the ECs and to investigate the role of mTORC1 and mTORC2 in HLA I-mediated ERM phosphorylation, we used siRNA as before (Figure 1E) to knock down the mTOR pathway partially (raptor and rictor siRNA) or fully (mTOR siRNA). Our results indicated that both mTOR complexes are involved,

as phosphorylation of ERM is partially inhibited following both raptor (mTORC1) and rictor (mTORC2) knockdown **(Figure 4E and F).** We observed complete inhibition of HLA I-induced ERM phosphorylation with total knockdown of mTOR signaling using mTOR siRNA, suggesting a synergistic effect between the two mTOR complexes. These experiments establish that mTOR activation following HLA I engagement on the surface of EC mediates phosphorylation of the ERM proteins, which then associate with ICAM-1.

3.3.5 mTOR regulates HLA I-induced ERM phosphorylation through the RhoA and MAPK pathway

We proceeded to further characterize the impact of mTOR inhibition on HLA I signaling in ECs. RhoA has been shown to play an important role in monocyte adhesion to EC (Ishibashi et al., 2003; Wojciak-Stothard et al., 1999). Studies support that endothelial cell activation can lead to Rho-induced ERM phosphorylation, which is central to ICAM-1 function (Millan and Ridley, 2005). Furthermore, in certain cell types, Rho has been shown to signal through the MAPK and PCK pathways in order to phosphorylate ERM. To examine the signaling relationship between RhoA and mTOR, we used a commercially available Rho inhibitor, as well as a ROCK inhibitor (Y-27632), an ERK inhibitor (U0126) to target the MAPK pathway, and a PKC inhibitor (Ro-31-7549), to assess the involvement of the Rho, MAPK, and PKC pathways in mTOR-mediated ERM phosphorylation following class I signaling.

To measure RhoA activation, we determined phosphorylation levels of myosin light chain (MLC) and myosin phosphatase target subunit 1 (MYPT1), as both proteins are phosphorylated downstream of RhoA activation and myosin phosphatase has been shown to regulate the interaction of actin and myosin in response to Rho signaling. We found that following HLA I

stimulation of ECs, there was a significant increase in phosphorylated levels of MYPT1 and MLC compared to control levels **(Figure 5).** Pharmacological mTOR inhibition blocked HLA I-mediated phosphorylation of MYPT1 and MLC, suggesting that mTOR signaling is upstream of RhoA signaling. As expected, rapamycin or everolimus (RAD) pre-treatment blocked HLA I-mediated phosphorylation of downstream mediators of the mTOR pathway including p-mTOR, p-P70S6K, p-AKT, p-ERK and p-S6RP **(Figure 5).** Notably, inhibition of the Rho pathway using a ROCK inhibitor (Y-27632) and a direct Rho inhibitor (C3 transferase) blocked HLA I-induced phosphorylation of MYPT1 and MLC, as well as ERM. However, we saw no inhibition of the mTOR pathway as there was no change in the phosphorylation state of mTOR and most of its downstream effector proteins **(Figure 5).** Another intriguing finding is that pre-treatment of HLA I-activated ECs with the ERK inhibitor U0126 resulted in partial inhibition of p-ERM, as well as p-MYPT1 and p-MLC, suggesting that the MAPK pathway might be involved in mTOR regulation of ERM phosphorylation. Our data suggests that PKC is not involved in this process as there was no modulation of HLA I-induced phosphorylated proteins. Collectively, this data suggests that following HLA class I engagement, mTOR signals through the RhoA pathway and partially through the MAPK pathway to mediate ERM phosphorylation in ECs.

3.3.6 In vivo blockade of mTOR prevents HLA-I antibody-activation of endothelium

The aforementioned findings implied that mTOR inhibition dampens HLA I signaling in ECs by blockading ERM phosphorylation. In an attempt to determine the impact of this signaling pathway in AMR *in vivo*, we tested the inhibitory effects of rapamycin on the murine endothelium by immunohistochemical staining for p-ERK, p-AKT, p-P70S6K, which are

downstream effectors of the mTOR pathway following HLA class I engagement, as well as p-ERM. In mice treated with anti-donor MHC I Ab, there was robust and diffuse staining of p-ERK **(Figure 6A, E)** and p-AKT **(Figure 6B, F)** compared to the control Ab-treated groups. ERK and AKT are phosphorylated downstream of mTORC2 signaling, indicating activation of this complex following MHC I antibody-treatment *in vivo*. In the rapamycin + MHC I Ab-treated group, phosphorylation of both ERK and AKT was significantly abrogated, implying blockade of mTORC2 signaling. We also observed a significant increase in the levels of the mTORC1 downstream protein p-P70S6K **(Figure 6C, G)** in mice that received MHC I Ab administration compared to control mice. This effect was blocked with rapamycin administration. Next, we confirmed mTOR-mediated reduction of HLA I-induced p-ERM *in vivo*. P-ERM staining was significantly increased in MHC I-Ab-treated mice compared with their rapamycin-treated counterparts. **(Figure 6D, H).** Although MHC I-Ab-treated mice demonstrated a significant increase in p-ERM levels compared to control groups, it should be noted that IHC-staining of control Ab-treated mice demonstrated basal expression levels of p-ERM, which was not observed in control Ab-treated human cells in culture. This finding can be attributed to activation of the murine endothelium due to surgery, or a slight difference in endothelial activity *in vivo* versus *in vitro*. In summary, these findings indicate that rapamycin inhibits MHC I antibody-elicited mTOR activation and signaling in graft endothelium *in vivo.*

3.3.7 mTOR inhibition of HLA-I stimulated EC impairs ICAM-1 clustering as demonstrated by confocal microscopy

Next, we sought to validate that disruption of ERM phosphorylation does in fact prevent the ability of ICAM-1 to cluster. Following activation of ECs, ICAM-1 concentrates at the apical membrane of ECs and clusters, allowing it to engage in interactions with monocytes from the blood stream (Ley et al., 2007). We investigated the capacity of HLA I stimulation to mediate ICAM-1 clustering and determined if mTOR inhibition blocks this clustering activity in ECs **(Figure 7).** In untreated cells and cells treated with ITGβ3 Ab, there is diffuse distribution of ICAM-1 throughout ECs. In ECs treated with the positive stimulant TNF-α or HLA I Ab, ICAM-1 is shown to be clustered at the margins of ECs **(Figure 7A)**. This correlates with a decrease in the mean number of particles and a corresponding increase in particle size **(Figure 7B and C)**, both quantitative measures indicating an increase in the number of clustered ICAM-1 molecules on the surface of ECs. With rapamycin pre-treatment, HLA I-induced ICAM-1 accumulation and clustering is disrupted, demonstrating a diffuse pattern similar to control cells. There is a corresponding increase in the average number of particles and a decrease in particle size. We also observed disruption of the cytoskeletal stress-fiber architecture with rapamycin pre-treatment, which has been suggested by different reports. These findings suggest that upon mTOR inhibition and blockade of ERM phosphorylation, HLA I Ab-mediated ICAM-1 clustering and ICAM-1 function is disrupted.

3.4 Discussion

Solid organ transplantation continues to progress with the improvement of immunosuppressive therapeutics, corresponding with improved graft function. Among the immunosuppressive medications used for patient treatment, mTOR inhibitors are considered one of four categories of maintenance suppression and used as such in the clinic, primarily credited with suppressing T cell activation (Duhart et al., 2015; Geissler et al., 2016; Lien, 2015; Neff et al., 2003). In a human-mouse chimeric model of allograft rejection, rapamycin pre-treatment of the allograft resulted in reduced infiltration of allogeneic T cells into the artery (Wang et al., 2013). Many current targeted therapies aim to block the adaptive immune response in allograft recipients; however, results of the present study highlight the importance of targeting the innate immune system, specifically macrophages, as well. Macrophages profoundly influence various aspects of transplantation(Salehi and Reed, 2015) and represent an important component in the process culminating in acute antibody-mediated cardiac rejection(Fishbein and Fishbein, 2012). Therefore, targeting this aspect of the pathway provides an effective means of alleviating incidences of rejection. (The mTOR signaling pathway has also been implicated in regulating leukocyte recruitment to the inflamed endothelium. mTOR controls myeloid cell recruitment by affecting both the endothelium and monocytes via distinct mechanisms (Farkas et al., 2006; Fox et al., 2007; Mahoney et al., 2001).)

We found that mTOR regulates endothelial cell function, specifically, that mTOR inhibition modulates the capacity of HLA I-activated ECs to recruit and support monocyte adherence and infiltration. Endothelial cells pre-treated with pharmacoloigcal mTOR inhibitors (rapamycin and RAD001) prior to HLA I stimulation were rendered non-adhesive following HLA I stimulation

and failed to support monocyte adherence. This finding was further confirmed by using siRNA to knockdown the mTOR signaling network, suggesting that mTORC1 and mTORC2 are involved in mTOR-mediated monocyte adherence to HLA I-activated EC.

To determine the physiological relevance of our data, we tested the effects of mTOR inhibition in a clinically-relevant murine model of acute antibody-mediated injury and we found an EC-specific effect of rapamycin that perturbs rejection and ameliorates graft function. Allografts of mice receiving daily rapamycin concurrently with biweekly administration of anti-donor MHC I antibodies (H-2K^{d} + H-2D^{d}) demonstrated significantly reduced vascular injury and monocytic infiltration compared to anti-donor Ab-treated mice that did not receive rapamycin treatment. Furthermore, we demonstrated an endothelium-specific effect of rapamycin on the allografts as indicated by dampened mTOR signaling. Thus, we propose that rapamycin treatment of allograft recipients would be expected to provide protective effects by inhibiting activation of the endothelium and subsequent monocyte infiltration.

The *in vivo* findings compelled us to explore the mechanisms underlying mTOR-mediated monocyte recruitment to HLA I antibody-activated endothelium. We tested the requirement for mTOR signaling in adhesion molecule expression. While Wang et al. found mTORC2 inhibition by rapamycin inhibits VCAM1 expression by TNF-α-activated EC (Wang et al., 2013), we did not find any regulation of adhesion molecules, including VCAM-1, E-selectin, P-selectin, or ICAM-1 by mTOR inhibited, HLA-I-activated ECs. Mechanistically, mTOR blockade using pharmacological inhibitors did not alter endothelial expression of these adhesion molecules. Considering the importance of monocyte recruitment and migration in human health and disease

(Gerhardt and Ley, 2015; Salehi and Reed, 2015), we used a live microscopy approach that allows monocytes to be observed with high resolution as they are perfused across endothelial cells. Ibidi flow-based adhesion assays use shear stress and are, for this reason, a more accurate physiological representation of the process of leukocyte adhesion. Using this technology, we discovered that mTOR inhibition impairs the ability of EC to support firm adhesion of tethered monocytes by affecting late stage ICAM-1 clustering.

We provide the first evidence, to our knowledge, that this is due to mTOR-mediated regulation of cytoskeletal proteins involved in firm adhesion of monocytes. We found that pharmacological mTOR inhibition of HLA-I Ab-activated ECs led to a significant reduction in the phosphorylation status of ERM proteins, which have been shown, in various cell types, to be critical for the association of ICAM-1 with the cytoskeleton, and are required for ICAM-1 clustering and function (2007; Barreiro et al., 2002).

Phosphorylated ERM attaches to the cytoplasmic tail of ICAM-1 on its N-terminus, and to the cell cytoskeleton on its C-terminus, creating a physical bridge which mediates clustering of ICAM-1 on the surface of the cell, which consequently is required for the cell to support firm adhesion(2007; Petit and Thiery, 2000).

We also report that HLA-I mediated monocyte recruitment depends on the activity of Rho-GTPases in addition to mTOR . RhoA has been shown to play an important role in monocyte adhesion to EC (Wojciak-Stothard et al., 1999) and studies support that endothelial cell activation can lead to Rho-induced ERM phosphorylation and complex formation, which is central to ICAM-1 clustering (Kluger, 2004; Petit and Thiery, 2000).RhoA activity is in turn has

been shown to be dependent on mTORC1, which mediates synthesis of RhoA at the translational level, as well as mTORC2, which regulates the GTPase activity of RhoA (Barilli et al., 2008; Wojciak-Stothard et al., 1999). We demonstrated that mTOR functions upstream of RhoA in HLA I-mediated signaling and signals through the RhoA pathway to regulate ERM phosphorylation and ICAM-1-mediated firm adhesion.

Lastly, using confocal microscopy, we observed that mTOR inhibition of ECs by rapamycin abrogates HLA-I-mediated ICAM-1 clustering. This suggests that the ability of ECs to support ICAM-1 clustering and subsequent firm adhesion of monocytes is impaired as this clustering event is essential for firm adherence of monocytes.

Taken together, our data support a model in which mTOR inhibition of HLA I-stimulated endothelium affects the recruitment and adherence of monocytes. Our findings demonstrate a novel mechanism by which mTOR inhibitors contribute to graft maintenance and suggest that pre-exposure of graft endothelium to rapamycin could potentially reduce immune-mediated rejection.

3.5 Methods

Antibodies and Chemicals

Pan-HLA antibodies were used as they are well-characterized and recognize monomorphic epitopes on all HLA class I antigens (Tran et al., 2001). Pan-HLA I antibody W6/32 (mIgG2a) was obtained from BioXCell (West Lebanon, NH) Pan-HLA antibodies MEM-81 and MEM-147 (mIgG1) were purchased from Abcam (Cambridge, MA). Human anti-integrin αVβ3 mIgG1 (R&D; Minneapolis, MN) was used as negative control as indicated. Polyclonal sheep antibody to P-selectin (R&D; Minneapolis, MN) was used. Calcium inhibitor BAPTA-AM was from Calbiochem (Billerica, MA). Anti-human ICAM-1 (CD54) antibody for Flow Cytometric analysis, shear-based adhesion assays, immunoprecipitation, and confocal microscopy was obtained from R&D (Minneapolis, MN).

Everolimus (RAD001) was synthesized at Novartis Pharma AG (Mission Viejo, CA) for biomedical research. Rapamycin was purchased from LC Laboratories (Woburn, MA). The purity of both rapamycin and everolimus was >97% by high-performance liquid chromatography. U0126 was purchased from EMD Millipore Corporation (Billerica, MA). Polyclonal Abs against phospho-mTOR (Ser2448), phospho-S6K (Thr389), phospho-S6RP (Ser235/236), phospho-Akt (Ser473), phospho-p44/42 MAPK (ERK1/2) (Thr202/Tyr204), phospho-MYPT1 (Thr696), phosphor-Ezrin (Thr567)/Radixin (Thr564)/Moesin(Thr558), were purchased from Cell Signaling Technology (Danvers, MA). Rabbit polyclonal Abs against mTOR, Raptor, and Rictor were purchased from Millipore/Upstate (Billerica, MA). Goat anti-rabbit horseradish peroxidase (HRP) and goat anti-mouse HRP Abs were obtained from Santa

Cruz Biotechnology (Santa Cruz, CA). Vybrant™ CFDA-SE Cell Tracer Kit was purchased from Invitrogen (Carlsbad, CA).

Human Cells

Use of all human samples and cells was approved by the UCLA Institutional Review Board (IRB#10-001689-CR-00003 and IRB#00-01-023) (IBC # 197.13.0-f) and all donors gave written informed consent.

Cell Culture

Primary human aortic endothelial cells (HAECs) were isolated from the aortic rings of deceased donor hearts (H126 or F1153) as previously described, or obtained from Clonetics (lot number: EC5555) and cultured as described previously (Jin et al., 2007; Jin et al., 2002; Jin et al., 2014). Briefly, HAECs were seeded at confluence on 0.1 % gelatin-coated tissue culture plates in complete medium containing 20% heat-inactivated fetal bovine serum (FBS), antibiotics (P/S) (both from Invitrogen Life Technologies), heparin (Sigma-Alrich) and endothelial cell growth serum (ECGS) (Fisher Scientific. All experiments were repeated with early-passage endothelial cells from 2-3 different donors to rule out donor-specific responses.

siRNA knockdown of mTOR, raptor, and rictor

siRNAs were designed as previously described (Jindra et al., 2008c) and purchased from Dharmacon. HAECs were cultured at 50-70% confluence, maintained in M199 complete medium, and transfected with siRNA in antibiotic-free medium as previously described (Jin et

al., 2007). Cells were placed in 800 µl of M199 medium along with 200 µl of transfection solution (Opti-MEM medium), Mirus transfection reagent, and siRNA (100 nM).After 4-6 h of transfection, 1 ml of M199 containing twice the amount of FBS, ECGS, and heparin were added, and experiments were conducted 48 h after transfection.

Primary human monocytes

Primary human monocytes were isolated from the peripheral blood of multiple healthy volunteers, using MACS pan-monocyte negative selection kit (Miltenyi Biotec, San Diego, CA) as previously described (Valenzuela et al., 2013c). Whole blood was obtained from healthy volunteers in accordance with the University of California, Los Angeles Institutional Review Board. Monocytes were enriched from peripheral blood using Ficoll-Paque density centrifugation (GE Healthcare), followed by MACS pan-monocyte negative selection kit (Miltenyi Biotec, San Diego, CA), according to the manufacturer's protocol.

Static monocyte adhesion assay

Adherence of monocytic cells to endothelium under static conditions was measured as previously described (Valenzuela et al., 2013b; Valenzuela et al., 2013d). ECs were pre-treated with pharmacological inhibitors or siRNA for the indicated amount of time, then stimulated with control antibody anti-human integrin αVβ3, thrombin, or antibody against HLA class I in M199 with 2% FBS (assay medium) for the indicated time. Polyclonal human IgG (20ug/mL) was used to block FcɣR interactions. Primary monocytes or monocytic cells MM6 were fluorescently labeled with 2 mM CFSE (Vybrant Cell Tracer, Invitrogen) in HBSS with Ca^{2+} and Mg^{2+} for 10 min at 37°C, then washed. Monocytes were added at 2 x 10^5 cells/well in a 24-well plate for 20

min at 37°C. The co-culture was washed twice with M199 and once with HBSS, and fixed with freshly prepared 4% paraformaldehyde. Images of adherent monocytes were acquired by fluorescence microscopy on a Nikon Eclipse Ti, in 8–10 4x objective fields for each condition. The number of adherent cells in each field was quantified using CellProfiler (Massachusetts Institute of Technology).

Shear-based monocyte adhesion assay

Monocyte adhesion to endothelial monolayers was conducted under laminar flow conditions using a closed perfusion system (ibidi) as previously described (Valenzuela et al., 2015). ECs (10^5 cells/cm^2) were seeded in gelatin coated flow chamber slides (μ-slide $I^{0.8}$ Luer) and cultured for 24 h. ECs were pre-treated with the mTOR inhibitor rapamycin at 30nM for 24 h or ICAM-1 blocking antibody at 20ug/mL for 30 mins. ECs were then stimulated with control antibody anti-human integrin αVβ3 for 30 mins, 10ng/mL TNF-α for 4-6 h, or antibody against HLA class I for 30 mins in M199 with 2% FBS (assay medium) at 37°C. CFSE labeled MM6 were resuspended in RPMI 5% FBS at 6.25 x 10^5/mL, and added to the syringe of a white perfusion set. Monocytes were perfused over the endothelium at 1 dynes /cm^2 and recorded in real time on a live cell fluorescence microscope (Nikon Eclipse Ti). Data were analyzed using ImarisTrack software version 7.6.2 (BitPlane; Zurich, Switzerland).

Cell-based P-Selectin ELISA

Cell surface expression of P-selectin was measured on adherent ECs by cell-based ELISA as previously described (Valenzuela et al., 2013a; Valenzuela et al., 2015). ECs were grown to confluence a 96-well plate and pre-treated with rapamycin at 30nM/ Everolimus(RAD) 10nM for

2 or 24 h. ECs were then stimulated with thrombin, control or HLA I Abs in M199 with 2% FBS for 30 mins. Cells were washed once with PBS and fixed in freshly prepared 2% paraformaldehyde in PBS for 5 min at room temperature and blocked with 5% BSA in PBS. Sheep anti–P-selectin (R&D Systems) was added at 1 mg/ml for 2 h. Wells were washed three times with PBS and donkey anti-sheep HRP (Millipore) was diluted in PBS and added at 1:1000 for 2 h at room temperature. After washing, tetramethylbenzidine substrate was added and Optical density (A650) was read on a SpectraMax plate reader (Molecular Devices).

Measurement of the von Willebrand Factor

Von Willebrand Factor was measured as previously described (Valenzuela et al., 2013a). ECs were grown to confluence and pre-treated with rapamycin at 30nM/ Everolimus(RAD) 10nM for 2 or 24 h. ECs were then stimulated with thrombin, control or HLA I Abs in M199 with 2% FBS for 30 mins. Supernatant was collected and von Willebrand factor (vWF) was measured using an ELISA kit (Helena Laboratories) according to the manufacturer's protocol.

Cell surface expression of adhesion molecules by Flow cytometric analysis

Cell surface expression of ICAM-1, E-selectin or VCAM-1 was measured by flow cytometry. ECs were treated with inhibitors and stimuli for the indicated times in assay medium and detached using Accutase (Innovative Cell; San Diego, CA) in order to preserve epitopes (Mutin et al., 1996). Cells were assayed on an LSRFortessa flow cytometer (BD Biosciences).

Preparation of Cell Lysates and Western blot

Low-passage HAECs were seeded in dishes coated with 0.1% gelatin. At 70-80% confluence, cells were pretreated with or without mTOR inhibitors rapamycin 30nM/Everolimus(RAD) 10nM or 100nM siRNA, then stimulated with HLA I Abs or Integrin αVβ3, and cell lysates for Western blot were prepared as described previously (Jin et al., 2002). Immunoprecipation experiment was conducted using the anti-human ICAM-1 (CD54) antibody described above. The phosphorylated bands were scanned and densitometry analysis was performed with Image J software (NIH, Bethesda, MD).

Animal Studies

Male B6.129S7-Rag1^{tm1Mom} (B6.RAG1$^{-/-}$, H-2^{b}) aged 8-10 weeks, and male BALB/c (H-2^{d}) mice aged 7–10 weeks old were obtained from The Jackson Laboratory and raised under pathogen-free conditions in the Animal Facilities (DLAM) at University of California (Los Angeles, CA; UCLA). All studies were approved by the UCLA Animal Research Committee and treatments and care were conducted in strict accordance with the Guidelines for Animal Care.

Murine Heterotopic heart transplantation

BALB/c (H-2^{d}) hearts were heterotopically transplanted into B6. RAG1$^{-/-}$ (H-2^{b}) recipients as previously described (Jindra et al., 2008a; Valenzuela et al., 2013a). Briefly, under isoflurane inhalational anesthesia, the donor aorta was anastomosed to the recipient abdominal aorta, and the donor pulmonary artery was joined to the recipient inferior vena cava. Beginning one day

post-transplantation, grafted mice received intraperitoneal (i.p.) injection of rapamycin at 1 mg/kg/day. Rapamycin was dissolved in ethanol and diluted in Dulbecco's PBS. 3 days post-transplant, grafted mice were injected with 30 ug of sterile, azide free isotype control antibody (clone MOPC-173, Biolegend; San Diego, CA) or donor-specific anti-MHC I antibodies (anti-H-2K^{d} mIgG2a, clone SF1-1.1; anti-H-2D^{d} mIgG2a, clone 34-2-12, Biolegend; San Diego, CA) diluted in sterile saline by tail vein. Graft function was monitored by abdominal palpation daily.

Histopathological grading and immunohistochemistry

After 30 days of treatment, mice were anesthetized by isofluorane, donor and native hearts were obtained and fixed in 10% buffered formalin, embedded in paraffin, and sectioned (4-5 μm). Hematoxylin and eosin (H&E) staining was performed. For immunohistochemistry, normal heat-inactivated goat serum was used for blocking. Appropriate primary antibodies specific to ERK ½ Thr202/Tyr204, AKT Ser473, P70S6K Thr421/Ser424, or Ezrin Thr567/Radixin Thr564/Moesin Thr558 were used. Macrophage staining was performed using rat anti-mouseMac2 Ab from Cedarlane as previously described (Jindra et al., 2008a). C4d antibody was a gift from Dr. William Baldwin. Images were viewed under an Olympus BX50 microscope and recorded using an Olympus DP21 camera. To evaluate the histologic features of the allografts, all slides were interpreted and scored by a single pathologist who was blinded to the experimental group. For H & E, microvascular changes, consisting of increased prominence and number of intravascular cells, and expansion of capillaries were graded 0–3 (0, negative; 1, focal; 2, multifocal; 3, diffuse) as previously described (Lones et al., 1995). The presence of Mac2-positive intravascular macrophages was scored on a scale of 0–3 (0, no staining; 1, focal staining; 2, multifocal staining; 3, diffuse staining). Positive ECs staining in capillaries and intramyocardial

arteries and veins for ERK ½ Thr202/Tyr204, AKT Ser473, P70S6K Thr421/Ser424, or Ezrin Thr567/Radixin Thr564/Moesin Thr558 was scored on a scale of 0–3 (0, no staining; 1, focal staining; 2, multifocal staining; 3, diffuse staining).

I-CAM localization and clustering

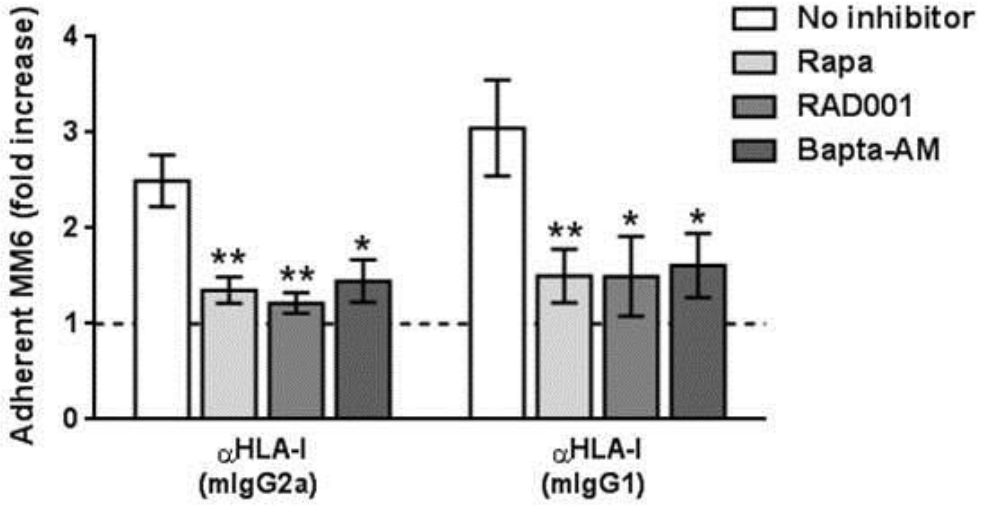
Cellular localization and clustering analysis of ICAM-1 expression on ECs was evaluated and quantified using three-dimensional analysis of confocal laser scanning microscopy (CLSM) images as previously described(Sosa et al., 2013; Sosa et al., 2015) with the following modifications. CLSM images were acquired on a Leica TCS-SP5 inverted Acousto Optical Beam Splitter (AOBS) confocal microscope (Leica Microsystems; Buffalo Grove, IL). Z-stacks of either all channels merged or the red channel only were converted to maximum intensity projections (MIPs) in LAS X software version 1.9 (Leica Microsystems, Buffalo Grove, IL) and loaded as tiffs into FIJI (Schindelin et al., 2012), an image processing package distribution of ImageJ2(Schindelin et al., 2015) (NIH, Bethesda, MD). Data shown are total counts of particles or average particle size obtained for each MIP of red channel only using FIJI's particle analysis algorithm and normalized to the number of cells. MIPs are shown for each image stack for either all channels merged or red channel only.

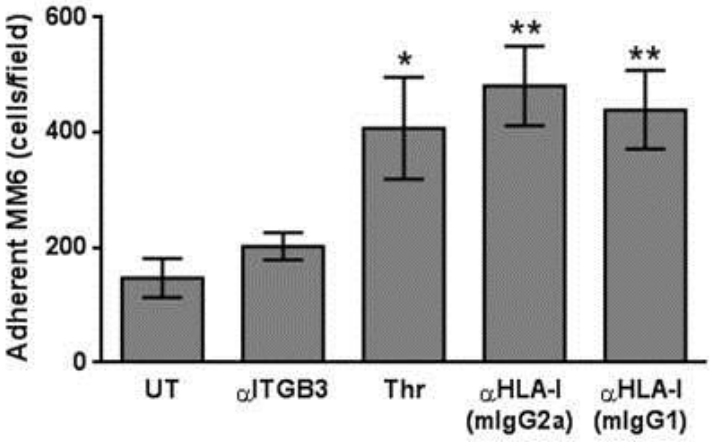
Statistical Analysis

Differences between groups were determined using one or two-way ANOVA using Prism v5 software (GraphPad Software Inc., La Jolla, CA). p values of <0.05 were considered statistically

significant. Histological grading was compared by two-way ANOVA using GraphPad Prism Software (La Jolla, CA). All data are expressed as mean ±SEM, unless otherwise specified.

Figure 3-1a


No inhibitor
Rapa
RAD001
Bapta-AM
Adherent MM6 (fold increase)
0
1
2
3
4
**
**
*
**
*
*
αHLA-I (mIgG2a)
αHLA-I (mIgG1)


Adherent MM6 (cells/field)
0
200
400
600
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**
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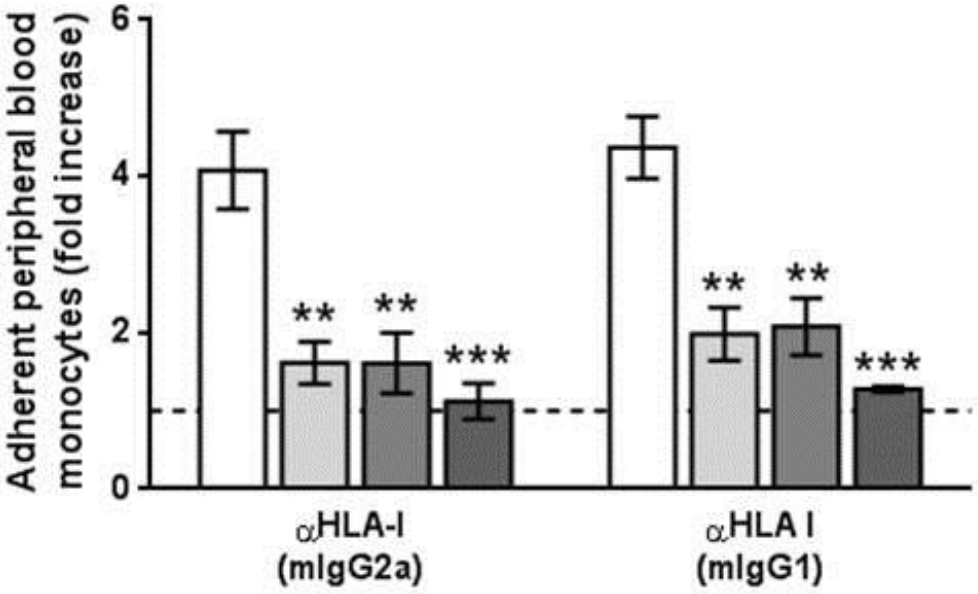
αITGB3
Thr
αHLA-I (mIgG2a)
αHLA-I (mIgG1)

Figure 3-1b


Adherent peripheral blood monocytes (fold increase)
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4
2
0
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**
***
**
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αHLA-I (mIgG2a)
αHLA I (mIgG1)

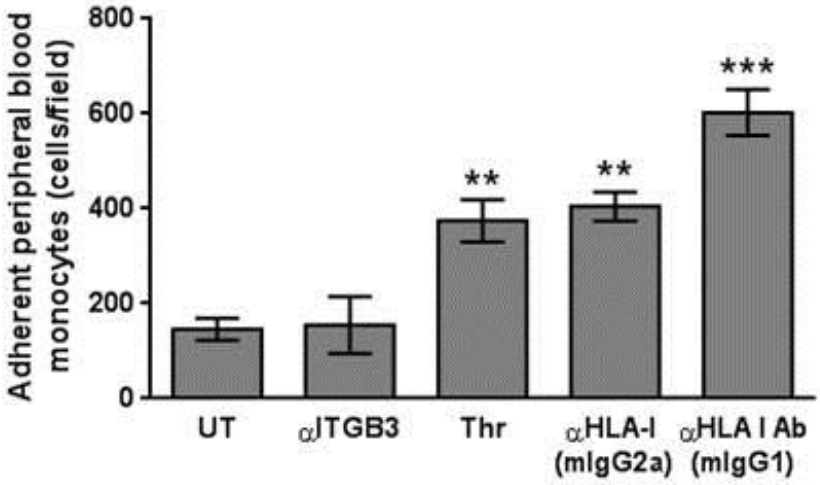
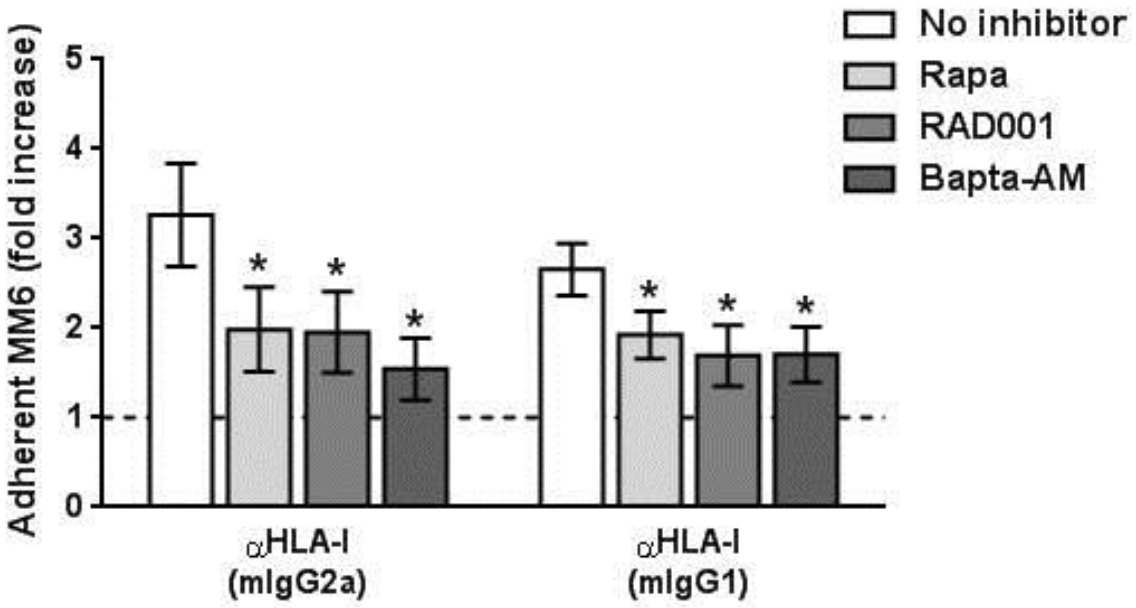

Adherent peripheral blood monocytes (cells/field)
800
600
400
200
0
**
**
***
UT
αITGB3
Thr
αHLA-I (mIgG2a)
αHLA I Ab (mIgG1)

Figure 3-1c


No inhibitor
Rapa
RAD001
Bapta-AM
Adherent MM6 (fold increase)
0
1
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αHLA-I (mIgG2a)
αHLA-I (mIgG1)

Figure 3-1d

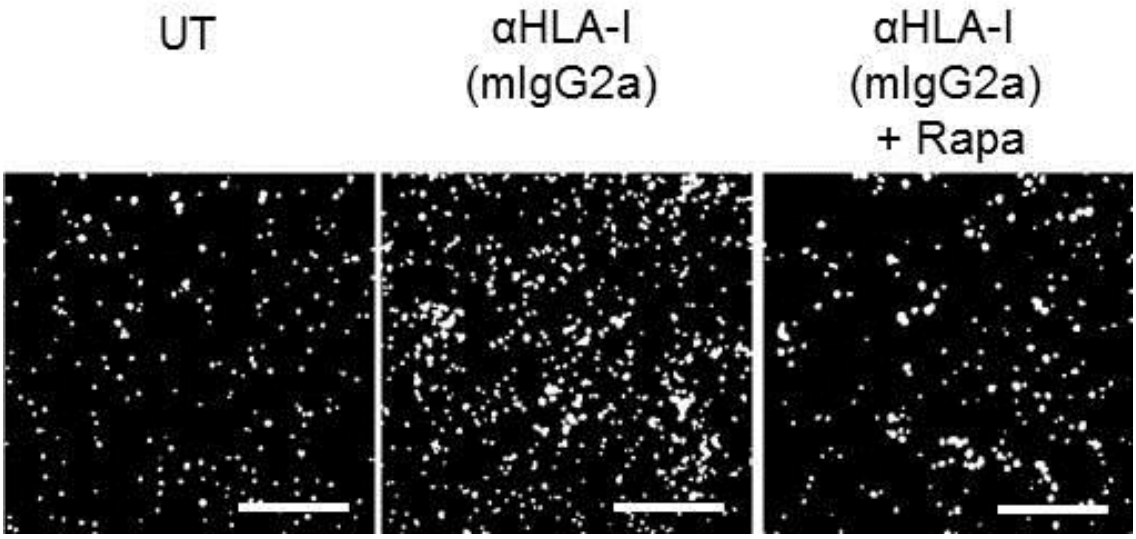


Figure 3-1e

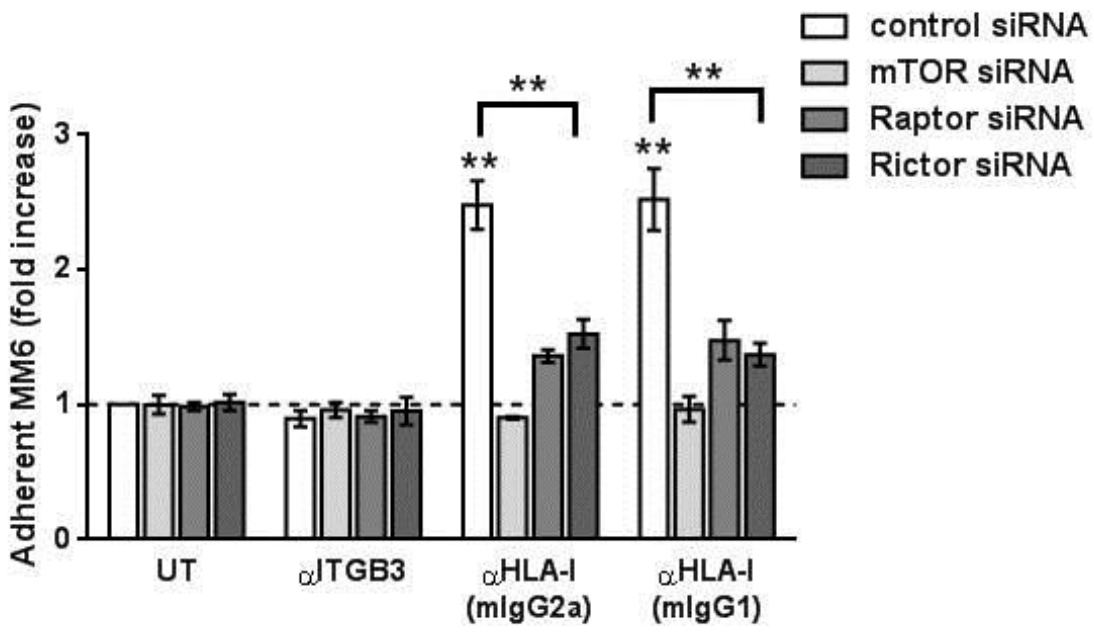


Figure 1. Monocyte adherence to HLA-class I Ab-stimulated endothelium is blocked when the mTOR pathway is inhibited in EC. Primary human aortic endothelium (EC, donor H126, 3F1153, or X127) were either untreated, pretreated with Rapamycin at 30nM or RAD001 at 10nM for 24 **(A)** or 2 **(C)** hours, or BAPTA-AM at 10uM for 30 mins. EC were then stimulated with control antibody against Integrin B3, HLA I intact antibody (W6/32 mIgG2a or MEM-147 mIgG1) at lg/mL or thrombin (Thr) at 1 U/mL diluted in M199 supplemented with 5% FBS for 5 min. Fluorescently labeled Mono Mac 6 **(A,C)** or human monocytes enriched from peripheral blood **(B)** were incubated with soluble human IgG to block FcgammaRs. Monocytes were fluorescently labeled with CFSE, resuspended in RPMI supplemented with 5% FBS, and were added at a ratio of 2–3 monocytes to 1 endothelial cell and allowed to adhere for 20 min. After removal of non-adherent monocytes by washing, adherent monocytes were counted by fluorescence microscopy and automated software (CellProfiler) in 8-10 fields per condition as

previously described [40]. Results from one representative experiment are shown **(A, B lower panels).** Bar graph represents mean fold increase in the number of adherent Mono Mac 6 **(A,C)** or primary monocytes **(B)** from multiple ($n \geq 3$) independent experiments ± SEM. *, $p<0.05$; ** $p<0.01$ versus untreated. *$p<0.05$, **$p<0.01$ versus comparing no inhibitor to inhibitor. **(D)** Representative 4x brightfield micrographs illustrate fluorescently labeled monocytic cells adherent to the endothelial monolayer. scale bars=100 ųm **(E)** Monocyte adherence to HLA-I-stimulated endothelium is blocked when the mTOR pathway is knocked-down in EC using siRNA directed against mTOR, raptor (mTORC1), or rictor (mTORC2). Experiments performed as in (A,B), with data expressed as mean fold increase in the number of adherent MM6 ± SEM.

Figure 3-2a

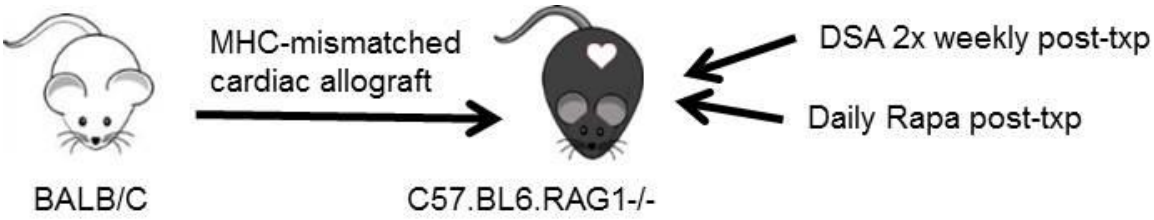

MHC-mismatched
cardiac allograft
DSA 2x weekly post-txp
Daily Rapa post-txp
BALB/C
C57.BL6.RAG1-/-

Figure 3-2b

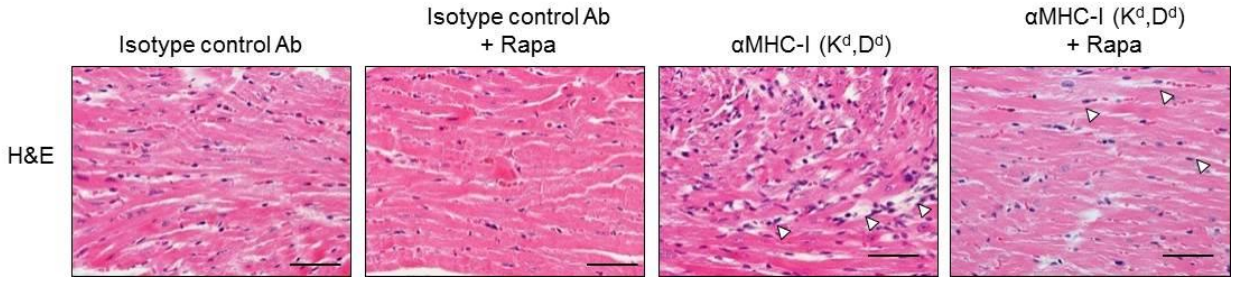

Isotype control Ab
Isotype control Ab
+ Rapa
αMHC-I (Kd,Dd)
αMHC-I (Kd,Dd)
+ Rapa
H&E

Figure 3-2c

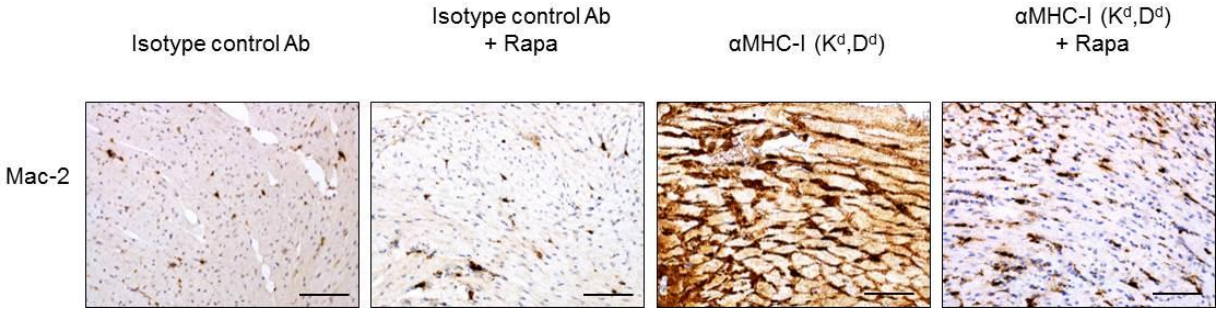

Isotype control Ab
Isotype control Ab + Rapa
αMHC-I (Kd,Dd)
αMHC-I (Kd,Dd) + Rapa
Mac-2

Figure 3-2d

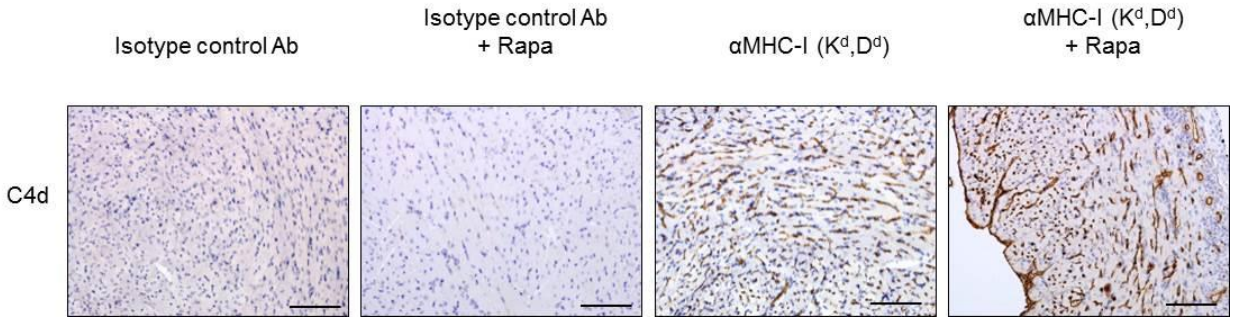

Isotype control Ab
Isotype control Ab + Rapa
αMHC-I (Kd,Dd)
αMHC-I (Kd,Dd) + Rapa
C4d

Figure 3-2e

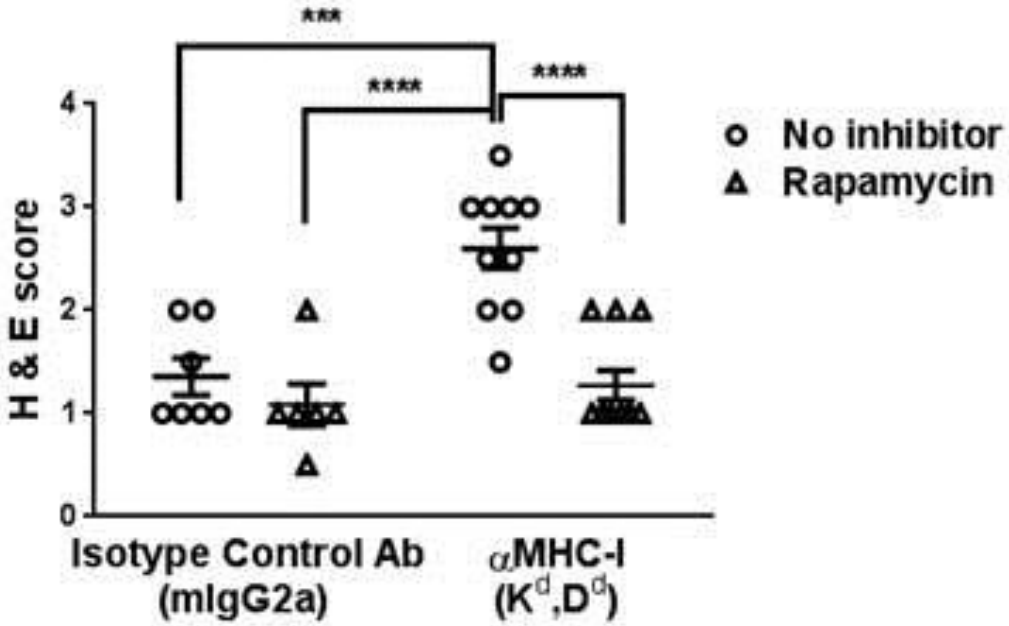

***
****
****
H & E score
4
3
2
1
0
No inhibitor
Rapamycin
Isotype Control Ab
(mIgG2a)
αMHC-I
(K^d,D^d)

Figure 3-2f

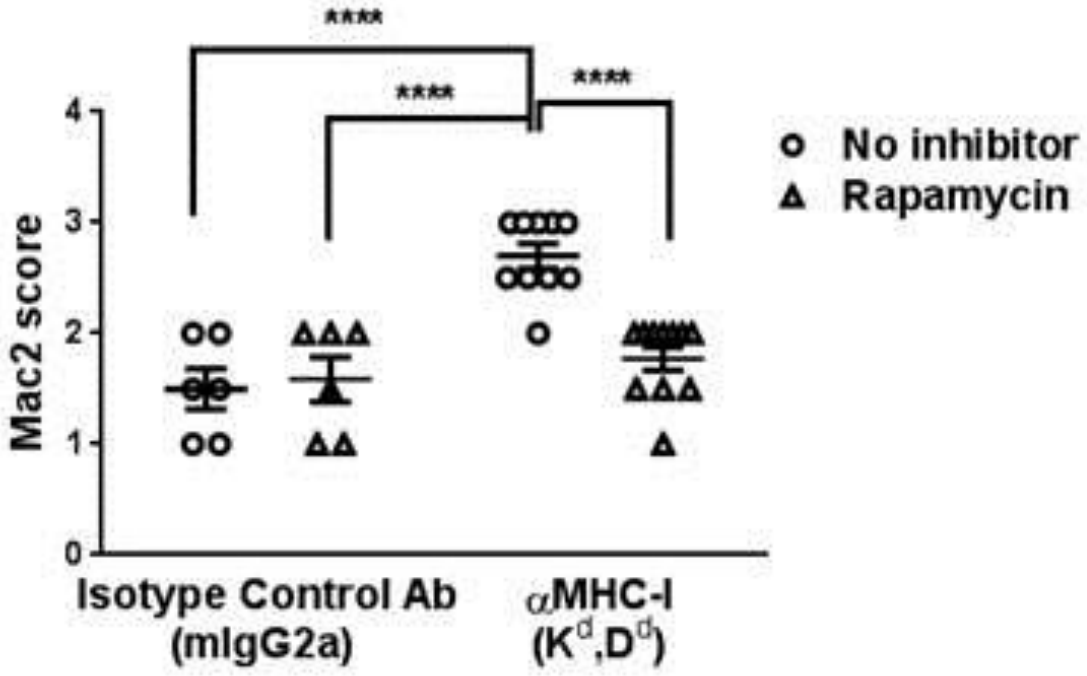

****
****
****
Mac2 score
4
3
2
1
0
No inhibitor
Rapamycin
Isotype Control Ab
(mIgG2a)
αMHC-I
(Kd,Dd)

Figure 3-2g

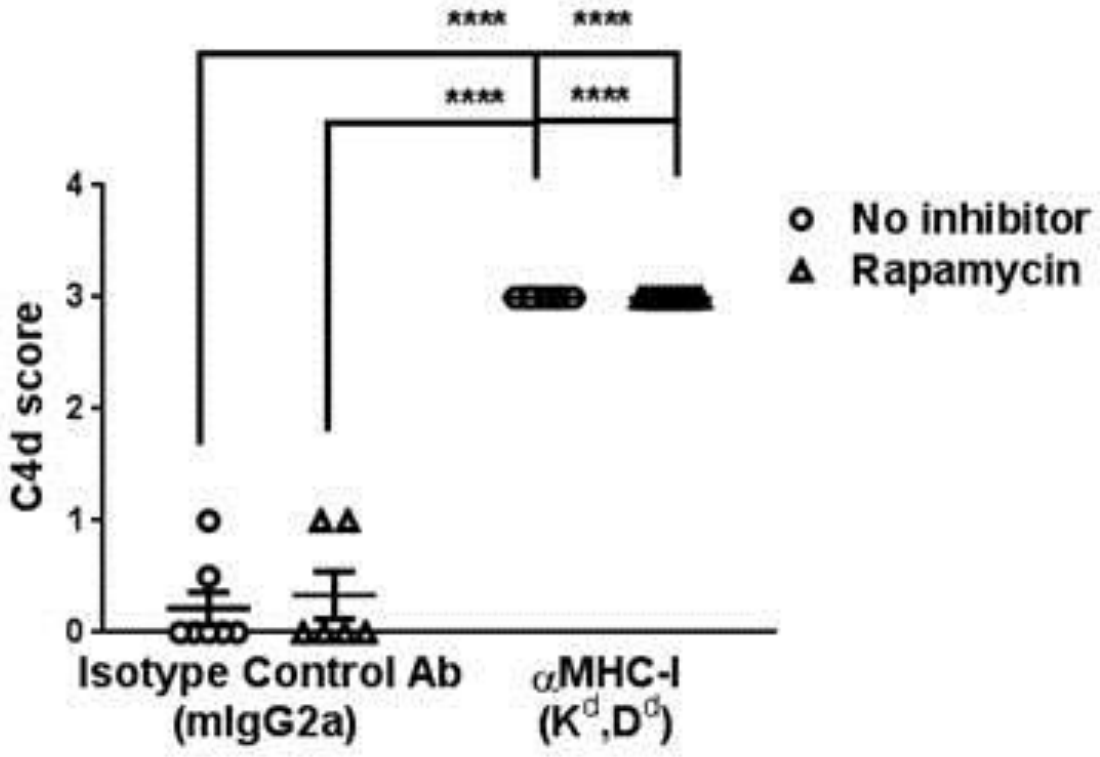


Figure 2. Rapamycin treatment ameliorates acute injury: blocks monocyte recruitment in a murine cardiac allograft.

Treatment with anti-donor MHC I Ab induces histological features of AMR. Concurrent treatment with rapamycin at 1 mg/kg/day abrogates AMR. **(A)** BALB/c cardiac allografts were transplanted into B6.RAG1-/- recipients and passively transfused with 30 ug MHC I (anti-H-2K^{d} + anti-H-2D^{d}) Ab or isotype control mIgG Ab and grafts were examined on day 30. **(B,E)** Microvascular abnormalities were observed by H&E staining in MHC I-Ab-treated group. Arrows indicate prominent nuclei of cells in distended capillaries (MHC I Ab treatment) not seen in rapamycin + MHC Ab-treated group or both groups that received isotype control mIgG Ab where arrows indicate flat and thin nuclei in collapsed capillaries on day 30. **(C,F)** Intravascular

macrophages were assessed by MAC2 immunoperoxidase staining in all recipients at day 30; representative images are displayed. **(D,G)** Complement deposition was measured by C4d deposition in all recipients on day 30. **(B,C,D)** Images are presented at 200X magnification. Images were viewed with an Olympus BX50 microscope and recorded with an Olympus DP21 camera. All stains were scored by a pathologist blinded to treatment groups. (mean ± SEM; n ≥6). *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. **(B)** scale bars=50 ɥm **(C,D)** scale bars=100 ɥm

Figure 3-3a

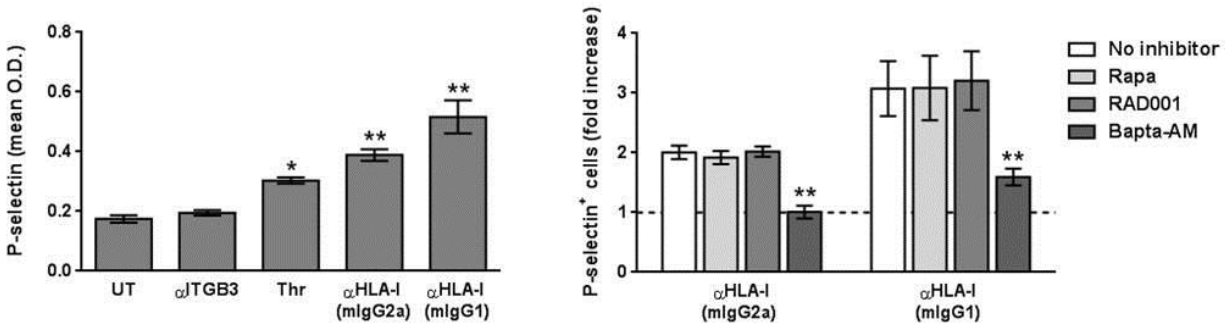
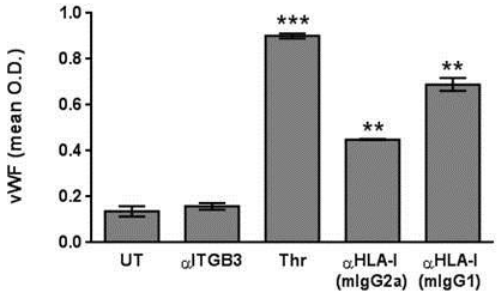

P-selectin (mean O.D.)
0.8
0.6
0.4
0.2
0.0
UT
αITGB3
Thr
αHLA-I (mIgG2a)
αHLA-I (mIgG1)
*
**
**
P-selectin+ cells (fold increase)
4
3
2
1
0
αHLA-I (mIgG2a)
αHLA-I (mIgG1)
**
**
No inhibitor
Rapa
RAD001
Bapta-AM

Figure 3-3b


vWF (mean O.D.)
1.0
0.8
0.6
0.4
0.2
0.0
***
**
**
UT
αITGB3
Thr
αHLA-I (mIgG2a)
αHLA-I (mIgG1)

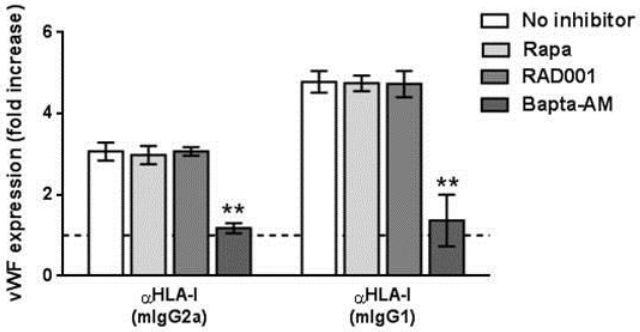
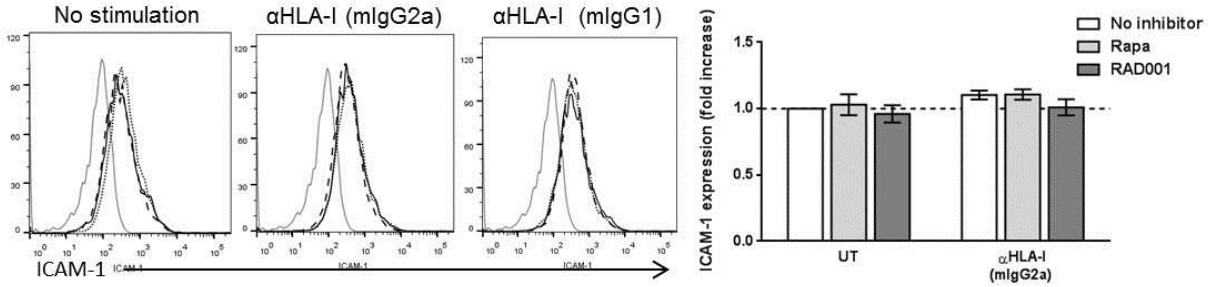

vWF expression (fold increase)
6
4
2
0
No inhibitor
Rapa
RAD001
Bapta-AM
**
**
αHLA-I (mIgG2a)
αHLA-I (mIgG1)

Figure 3-3c



Gray: Secondary alone

Dotted black: Stimulation + Rapamycin

Solid black: Stimulation alone

Dashed black: Stimulation + RAD001

Figure 3-3d

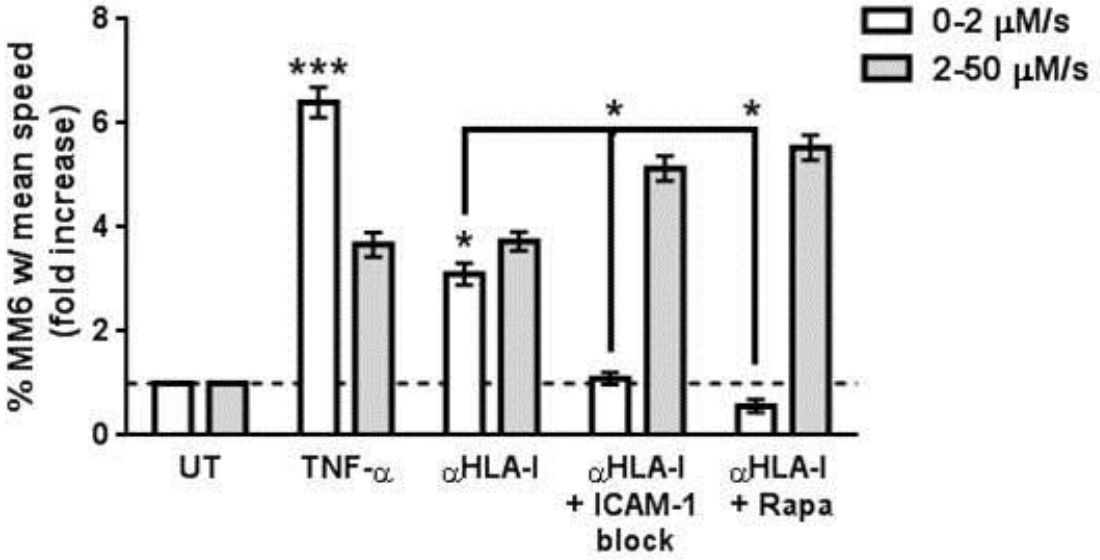

0-2 μM/s
2-50 μM/s
% MM6 w/ mean speed (fold increase)
8
6
4
2
0
***
*
*
*
UT
TNF-α
αHLA-I
αHLA-I + ICAM-1 block
αHLA-I + Rapa

Figure 3-3e

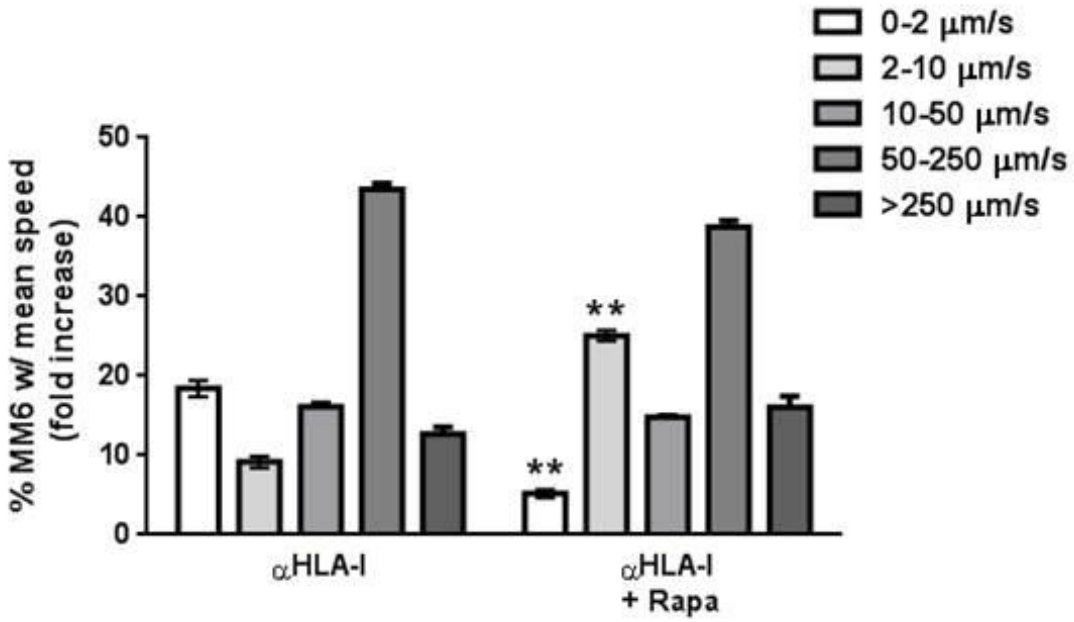


Figure 3. mTOR inhibition of EC does not alter surface expression levels of adhesion molecules; mTOR inhibition alters ICAM-1 induced firm adhesion of monocytes to HLA-Ab stimulated EC.

HAECs were either untreated, pretreated with Rapamycin at 30nM or RAD001 at 10nM for 24 hours, or BAPTA-AM at 10uM for 30 mins. EC were then stimulated with control antibody against Integrin B3, HLA I intact antibody (W6/32 or MEM-147) at lg/mL or thrombin at 1 U/mL diluted in M199 supplemented with 5% FBS for 5 min. **(A)** Expression of P-selectin was detected by cell-based ELISA. Cell surface P-selectin was stained on unpermeabilized cells with sheep anti–P-selectin (CD62P) followed by anti–sheep HRP, and detected by adding tetramethylbenzidine substrate. **(A, Left panel)** Results from one representative experiment showing the average optical density (OD) from duplicate or triplicate measurements +/- SEM for each condition. **(A, Right panel)** Bar graph shows mean fold increase in P-selectin positive cells from multiple independent experiments (n >= 3). * $p<0.05$, ** $p<0.01$ versus untreated. **(B)**

Supernatant was collected from the P-selectin ELISA experiments and vWF was measured by ELISA. **(B, Left panel)** Results from one representative experiment showing the average optical density (OD) from duplicate or triplicate measurements +/- SEM for each condition. Bar graph shows mean fold increase in vWF from multiple independent experiments (n >= 2). * $p<0.05$, ** $p<0.01$ versus untreated. No statistically significant difference was found between the groups with and without mTOR inhibition by two-way ANOVA for (A) and (B).

(C) mTOR inhibition has no effect on ICAM-1 expression as measured by flow-cytometry. Following indicated treatments, HAECs were detached using Accutase. Cell surface ICAM-1 was stained with conjugated anti-ICAM-1-APC and analyzed by flow cytometry. Bar graph shows mean fold increase in ICAM-1 positive cells from multiple independent experiments (n>=2) for each condition. No statistically significant difference was found between the groups with and without mTOR inhibition by two-way ANOVA.

(D,E) Monocytes were perfused at 1 dynes/cm^2 over an endothelial monolayer pre-treated with rapamycin at 30nM for 24 h, ICAM-1 blocking antibody at 20ug/mL for 30 mins and stimulated with HLA I Ab at 1 mg/mL for 30 mins. Three 5-10s videos were collected in real-time for each condition. The mean velocity (um/sec) of each monocyte over video duration was determined using ImarisTrack software (Bitplane). Results are presented as the percent of total monocytes observed in the 5 s video with each indicated speed (i.e. 2–50 um/s). Firmly adherent monocytes were defined as those with a speed of =<2 um/s over 5 s Slow rolling monocytes have a mean velocity of =< 50 um/s, while fast rolling monocytes moved at >50 um/s. *, $p<0.05$; ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$ versus untreated. *$p<0.05$, **$p<0.01$; ; *** $p<0.001$; **** $p<0.0001$ versus comparing no inhibitor to inhibitor. For 4Dand 4E: bars were compared to untreated monocytes of the same speed. Not significant if not indicated.

Figure 3-4a

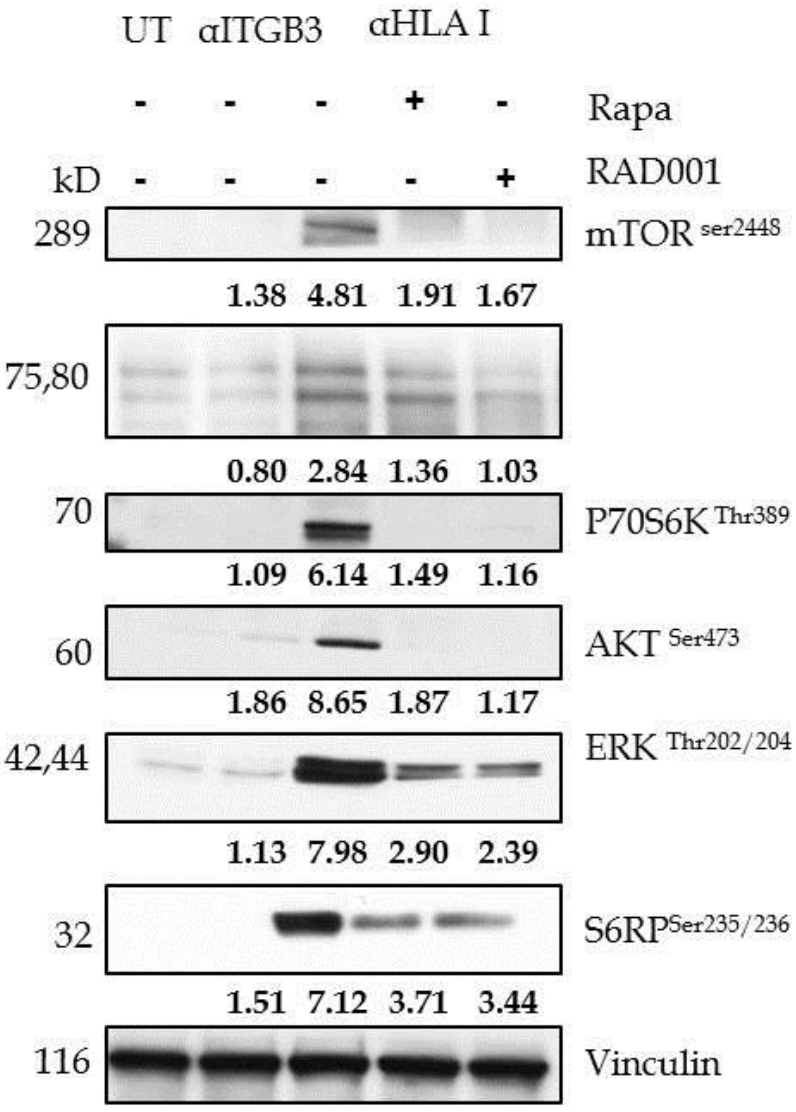

UT
αITGB3
αHLA I
- - - + -
Rapa
kD - - - - +
RAD001
289
mTOR ser2448
1.38 4.81 1.91 1.67
75,80
0.80 2.84 1.36 1.03
70
P70S6K Thr389
1.09 6.14 1.49 1.16
60
AKT Ser473
1.86 8.65 1.87 1.17
42,44
ERK Thr202/204
1.13 7.98 2.90 2.39
32
S6RP Ser235/236
1.51 7.12 3.71 3.44
116
Vinculin

Figure 3-4b

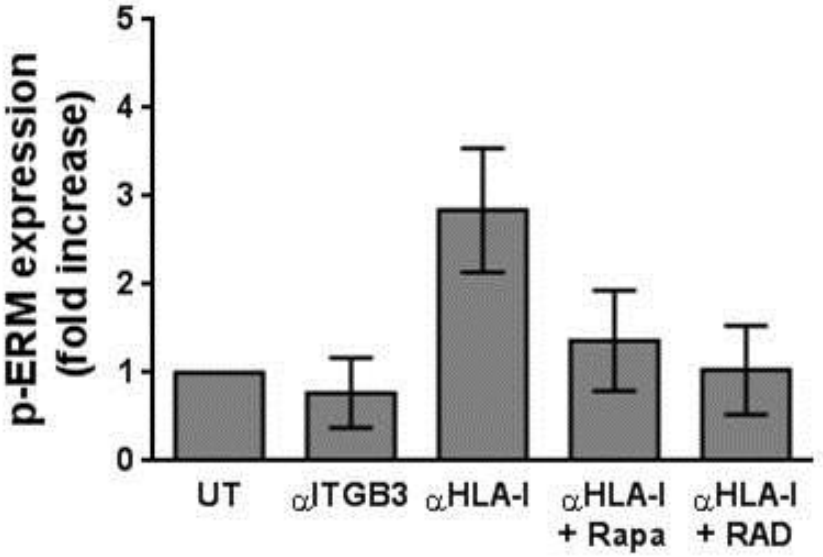

p-ERM expression
(fold increase)
5
4
3
2
1
0
UT
αITGB3
αHLA-I
αHLA-I
+ Rapa
αHLA-I
+ RAD

Figure 3-4c

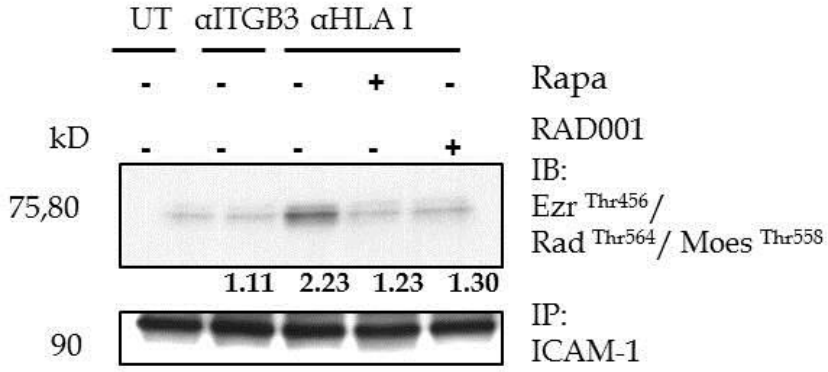

UT
αITGB3
αHLA I
- - - + -
Rapa
kD
- - - - +
RAD001
IB:
75,80
Ezr Thr456/
Rad Thr564/ Moes Thr558
1.11 2.23 1.23 1.30
IP:
90
ICAM-1

Figure 3-4d

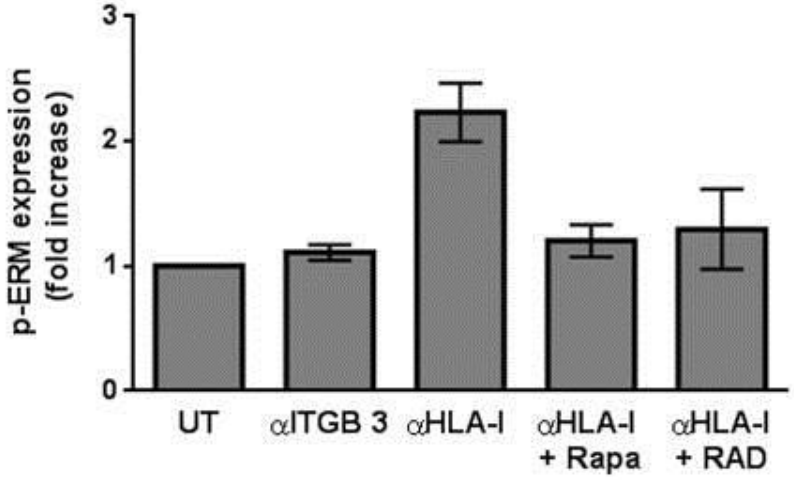

p-ERM expression
(fold increase)
3
2
1
0
UT
αITGB 3
αHLA-I
αHLA-I
+ Rapa
αHLA-I
+ RAD

Figure 3-4e

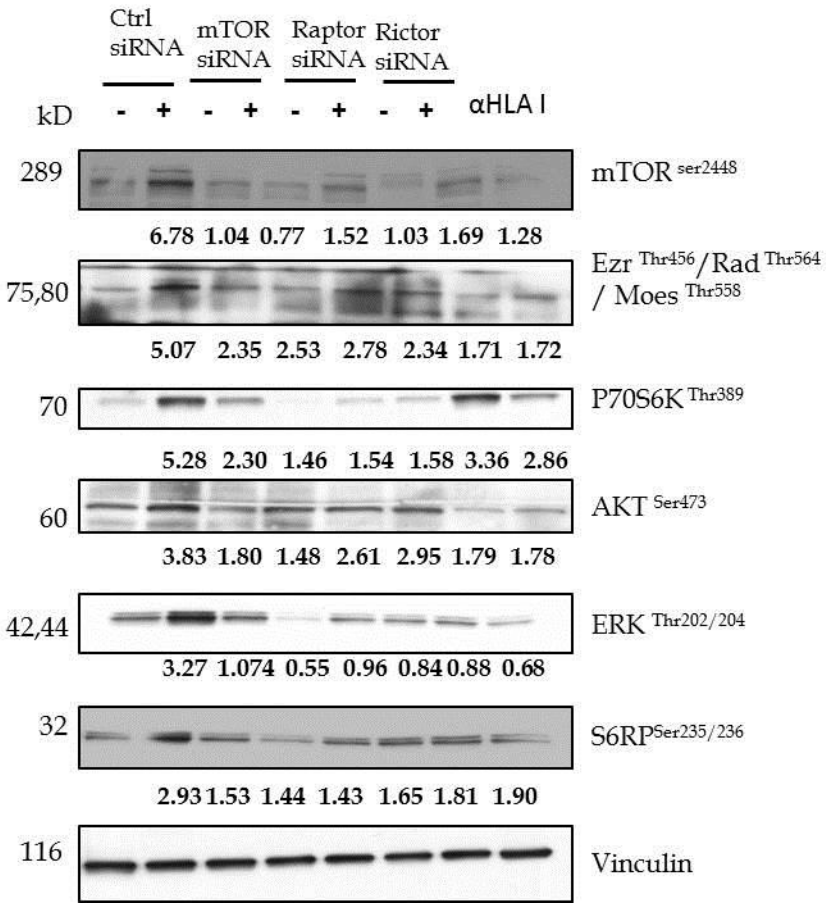

Ctrl siRNA
mTOR siRNA
Raptor siRNA
Rictor siRNA
kD
- + - + - + - +
αHLA I
289
mTOR ser2448
6.78 1.04 0.77 1.52 1.03 1.69 1.28
75,80
Ezr Thr456/Rad Thr564 / Moes Thr558
5.07 2.35 2.53 2.78 2.34 1.71 1.72
70
P70S6K Thr389
5.28 2.30 1.46 1.54 1.58 3.36 2.86
60
AKT Ser473
3.83 1.80 1.48 2.61 2.95 1.79 1.78
42,44
ERK Thr202/204
3.27 1.074 0.55 0.96 0.84 0.88 0.68
32
S6RP Ser235/236
2.93 1.53 1.44 1.43 1.65 1.81 1.90
116
Vinculin

Figure 3-4f

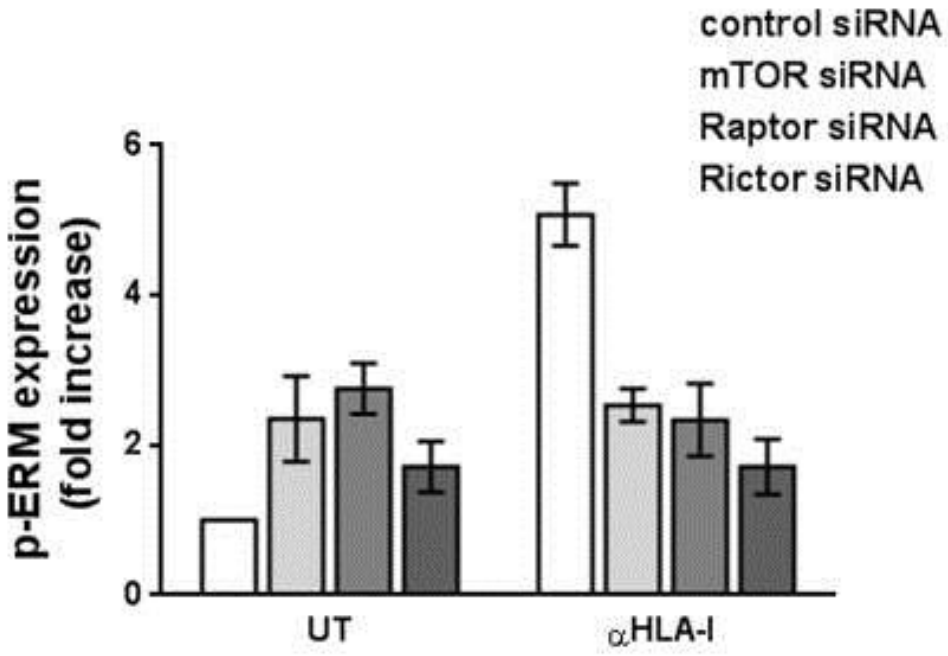


Figure 4. ERM phosphorylation is impaired by mTOR inhibition of EC.

(A) Quiescent HAECs were pretreated with Rapamycin at 30nM or RAD001 at 10nM for 24 h and stimulated with 0.1 mg/mL anti-HLA I mAb W6/32 for 10 min. Cells were lysed and proteins were separated by SDS–PAGE followed by immunoblotting with anti-phospho-mTOR Ser2448, anti-phospho-ERM, anti-phospho-p70S6K Thr389, anti-phospho-Akt Ser473, anti-phospho-ERK Thr202/Tyr204, or anti-phospho-S6RP Ser235/236 Abs. The membrane was probed with anti-vinculin Ab to confirm equal loading of proteins. **(B**) Phosphorylated protein band for p-ERM shown in (A) were quantified by densitometry scan analysis using ImageJ and results are expressed as the mean ± SEM fold increase in phosphorylation above untreated. Data are representative of three independent experiments. **(C)** HAECs were pre-treated as in (A); the lysates were immunoprecipitated with anti-ICAM-1 Ab followed by immunoblotting with anti-phospho-ERM. Vinculin was used to confirm equal loading. **(D)** Quantification corresponding to

p-ERM in (C) was determined as in (A). **(E)** HAECs were transfected with 100 nM mTOR, raptor, rictor control siRNA. After 48 h, EC were lysed and subjected to Western blot analysis using Abs to phospho-mTOR Ser2448, phospho-p70S6K Thr389, phospho-Akt Ser473, phospho ERK Thr202/204, phospho-S6RP Ser235/236 Abs, or Vinculin to confirm equal loading of proteins. **(F)** Phosphorylated protein band for p-ERM shown in (C) were quantified by densitometry scan analysis using ImageJ and results are expressed as the mean ± SEM fold increase in phosphorylation above untreated. Data are representative of three independent experiments. For all blots, the mean fold change over untreated is indicated as a number underneath each lane.

Figure 3-5

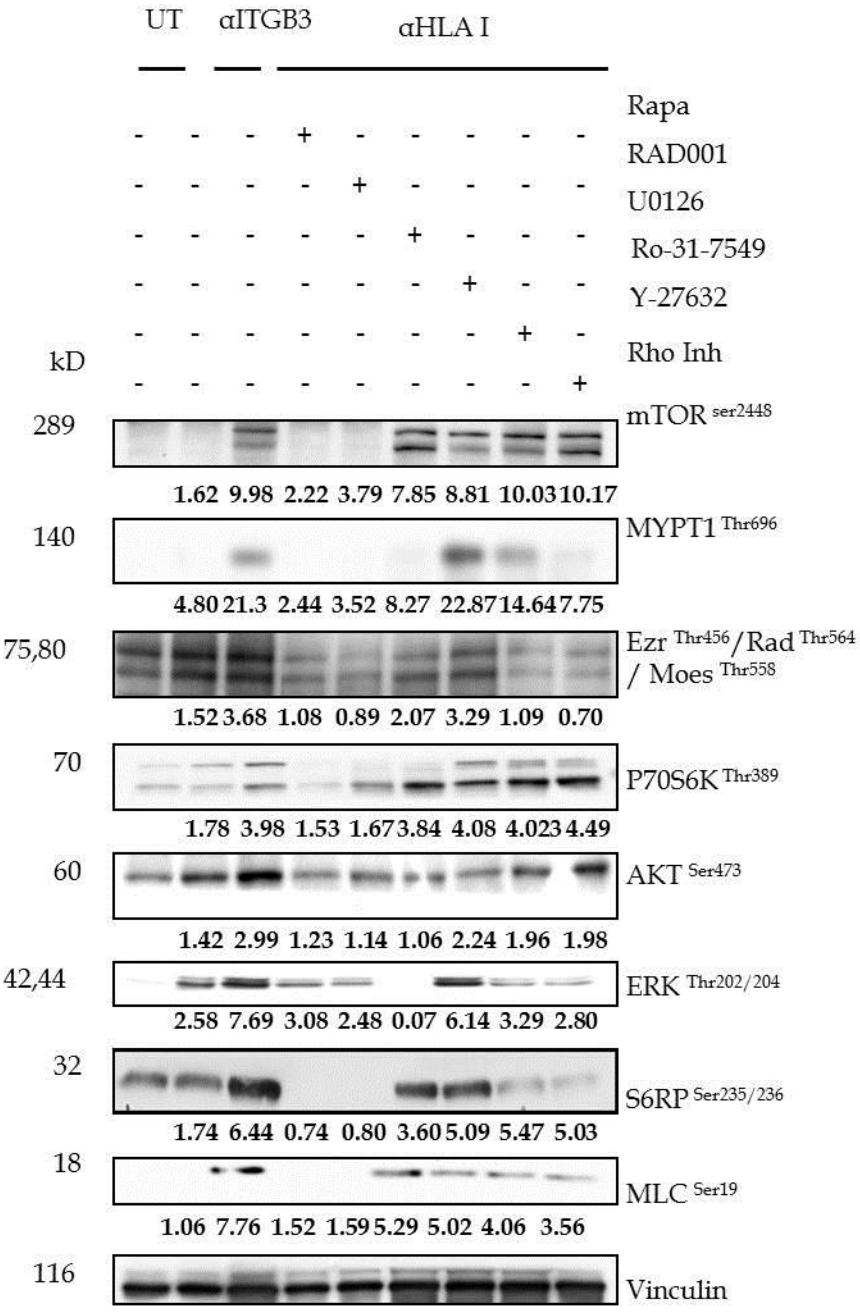


Figure 5. mTOR regulates HLA I-induced ERM phosphorylation through the RhoA and MAPK pathways.

(A) Quiescent HAEC were pretreated with Rapamycin at 30nM or RAD001 at 10nM for 24 h, U0126, Ro-31-7549, Y-27632, all at 10 uM for 30 mins, Rho inhibitor at 1 ug/mL for 4 h, then stimulated with 0.1 mg/mL anti-HLA I mAb W6/32 for 10 min. Cells were lysed and proteins were separated by SDS–PAGE followed by immunoblotting with anti-phospho-mTOR Ser2448, anti-phospho-MYPT1 Thr696, anti-phospho-p70S6K Thr389, anti-phospho-Akt Ser473, anti-phospho ERK Thr202/204, anti-phospho-S6RP Ser235/236 Abs, anti-phospho MLC Ser19, or Vinculin to confirm equal loading of proteins. For all blots, the mean fold change over untreated is indicated as a number underneath each lane.

Figure 3-6a

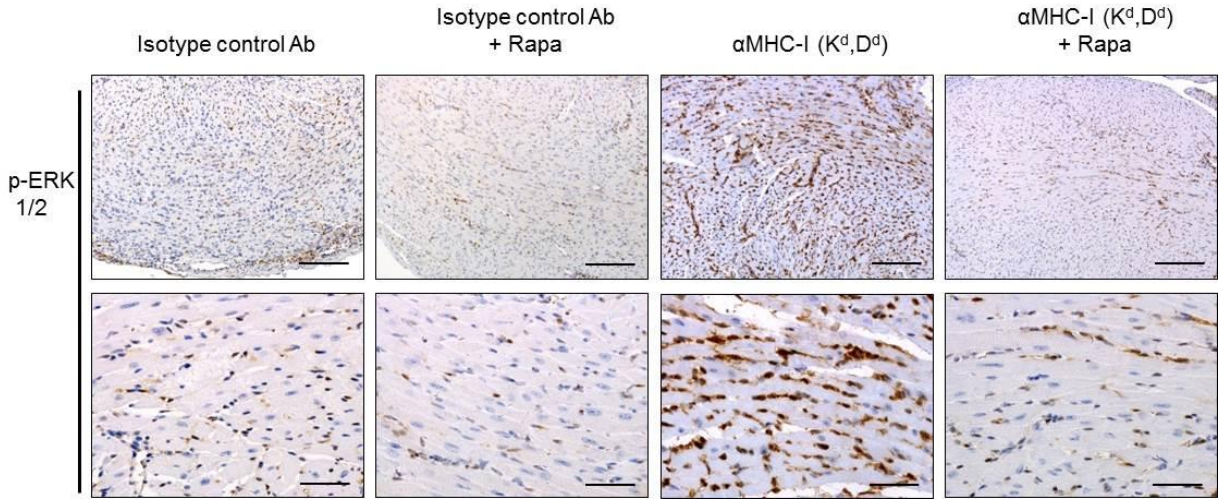

Isotype control Ab
Isotype control Ab
+ Rapa
αMHC-I (Kd,Dd)
αMHC-I (Kd,Dd)
+ Rapa
p-ERK
1/2

Figure 3-6b

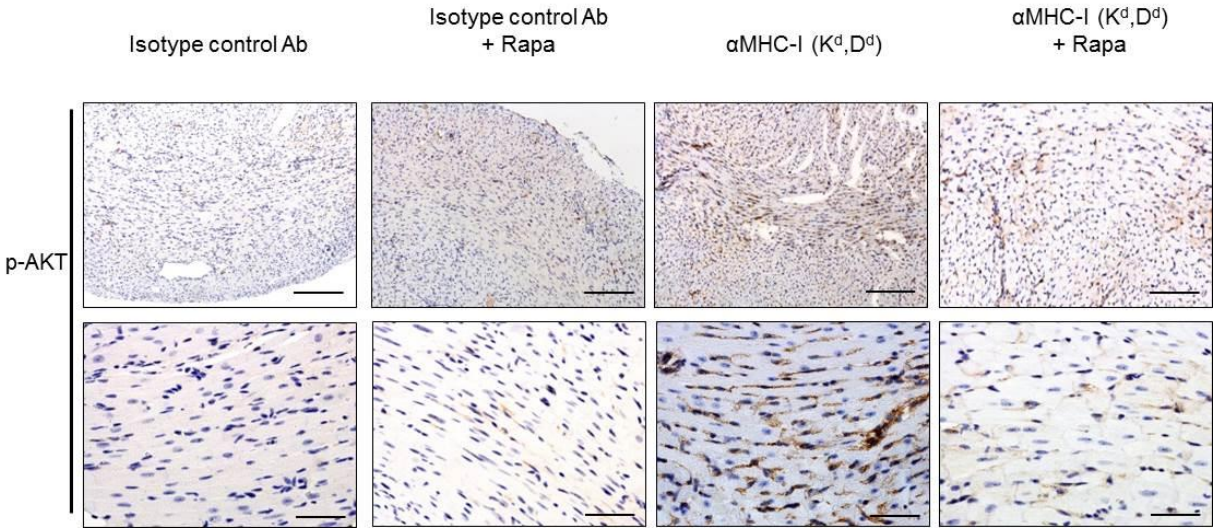

Isotype control Ab
Isotype control Ab + Rapa
αMHC-I (Kd,Dd)
αMHC-I (Kd,Dd) + Rapa
p-AKT

Figure 3-6c

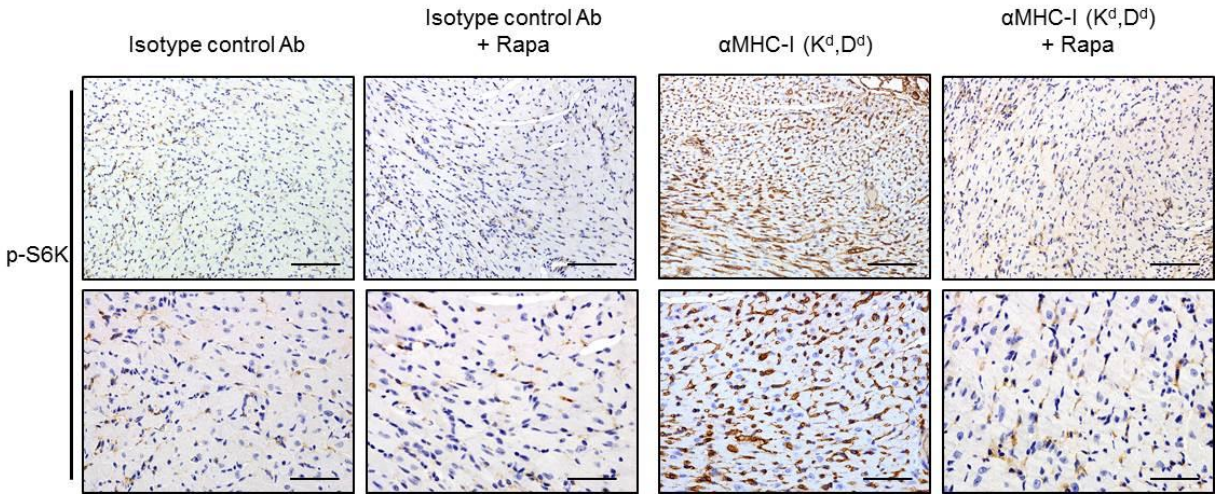

Isotype control Ab
Isotype control Ab
+ Rapa
αMHC-I (Kd,Dd)
αMHC-I (Kd,Dd)
+ Rapa
p-S6K

Figure 3-6d

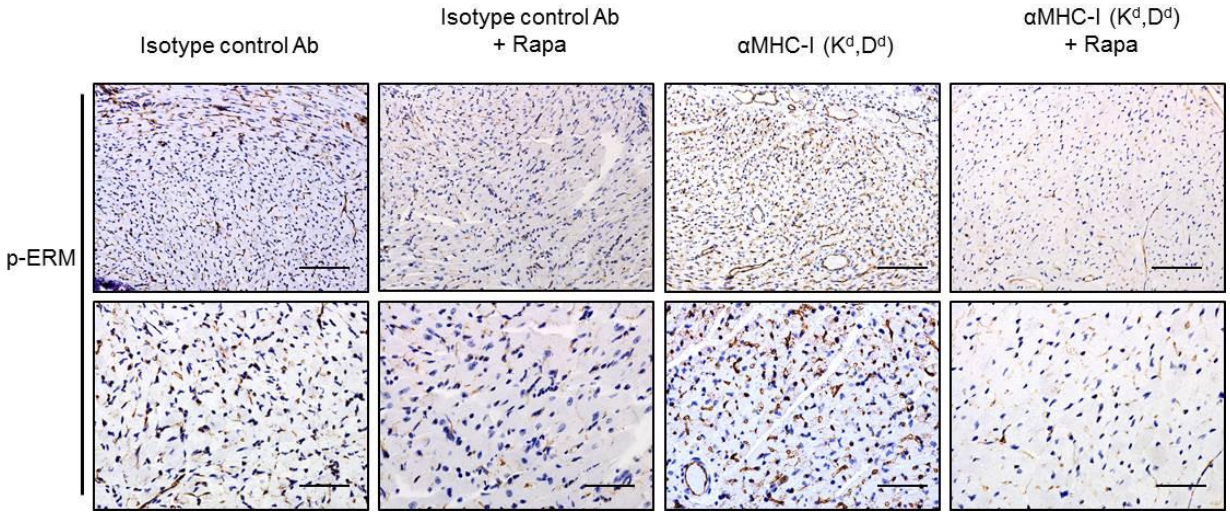

Isotype control Ab
Isotype control Ab + Rapa
αMHC-I (Kd,Dd)
αMHC-I (Kd,Dd) + Rapa
p-ERM

Figure 3-6e

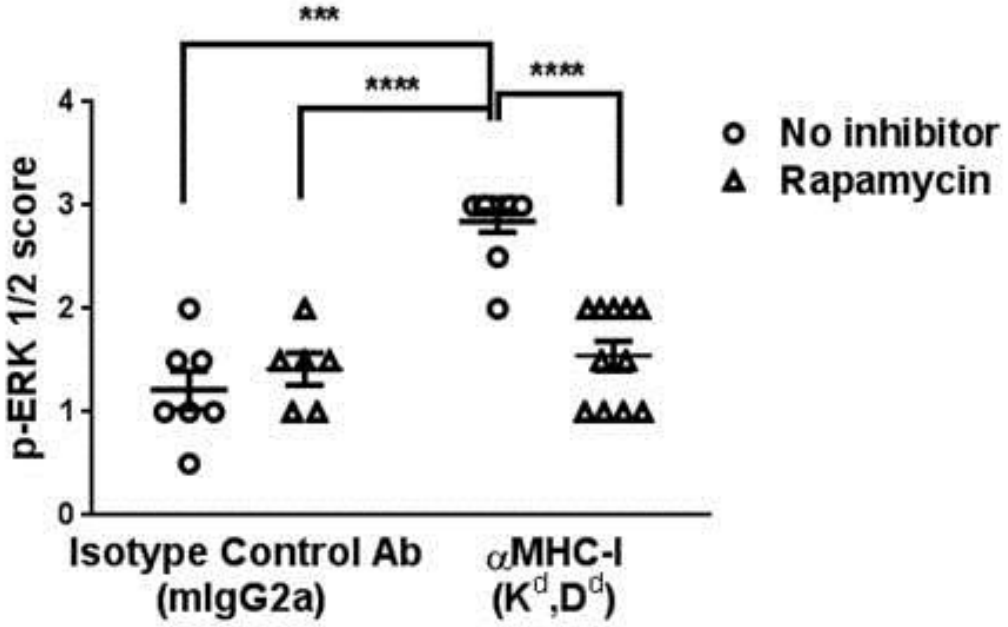

***
****
****
p-ERK 1/2 score
4
3
2
1
0
No inhibitor
Rapamycin
Isotype Control Ab
(mIgG2a)
αMHC-I
(K^d,D^d)

Figure 3-6f

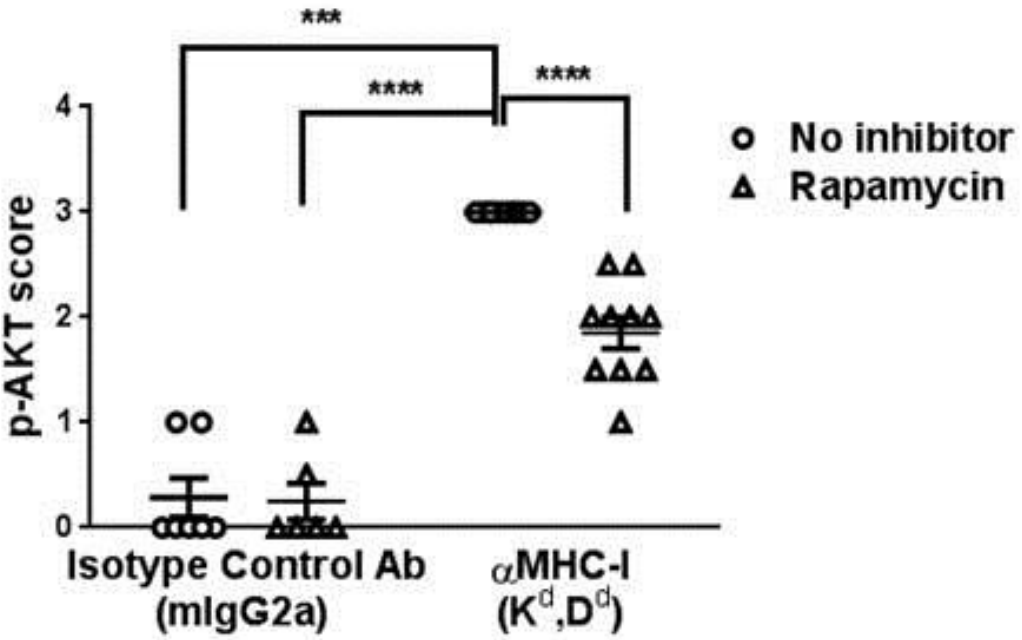

***
****
****
p-AKT score
4
3
2
1
0
No inhibitor
Rapamycin
Isotype Control Ab
(mIgG2a)
αMHC-I
(K^d,D^d)

Figure 3-6g

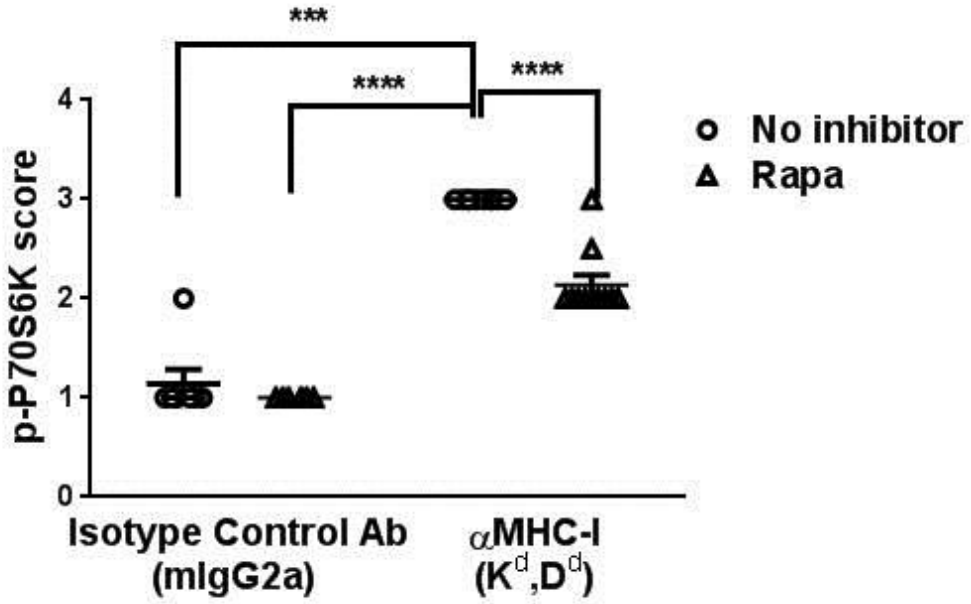

***
****
****
p-P70S6K score
0
1
2
3
4
No inhibitor
Rapa
Isotype Control Ab
(mIgG2a)
αMHC-I
(K^d,D^d)

Figure 3-6h

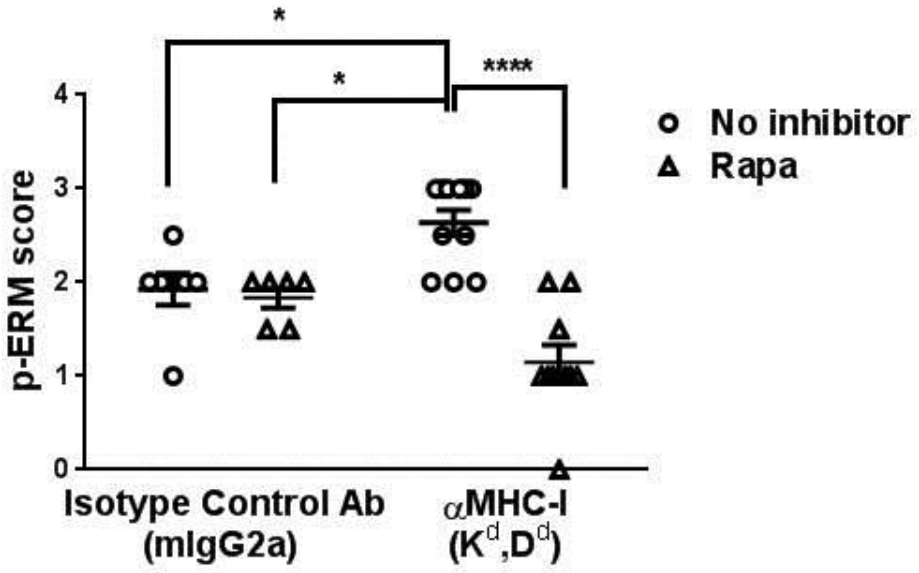


Figure 6: Rapamycin treatment inhibits activation of HLA I Ab-induced mTOR proteins *in vivo*. Immunohistochemical analysis of proteins involved in MHC class I-induced cell survival or proliferation pathway in all four treatment groups, as scored by a blinded pathologist. Representative histology of phosphorylated cell survival and proliferation proteins in cardiac allografts following indicated treatments. Analysis was determined by immunoperoxidase single staining for (A, E) p-ERK1/2 Thr202/Tyr204, (B,F) p-AKT Ser473, (C,G) p-P70S6K Thr421/Ser424, or (D, H) p-Ezrin (Thr567)/Radixin (Thr564)/Moesin (Thr558) in allografts treated with 30 ug MHC I (anti-H-2K^d + anti-H-2D^d) Ab or mIgG isotype controls, with or without concurrent rapamycin treatment. (A-D) Images in the top row are presented at 100X magnification, and the bottom row at 200X magnification. Images were viewed with an Olympus BX50 microscope and recorded with an Olympus DP21 camera. All phosphorylated proteins visible (dark brown stain, arrows) in cardiac vessels and endocardium. (mean ± SEM; n ≥6). *P

< 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. scale bars, top rows=100 ɥm, bottom rows= 50 ɥm.

Figure 3-7a

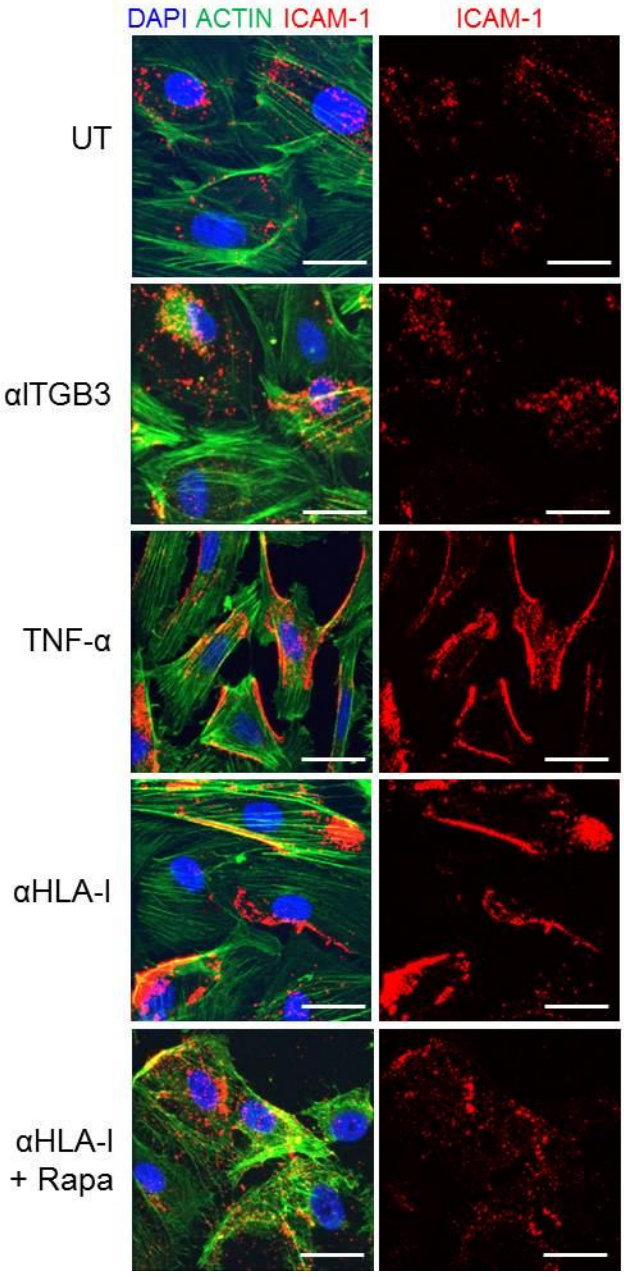


Figure 3-7b- c

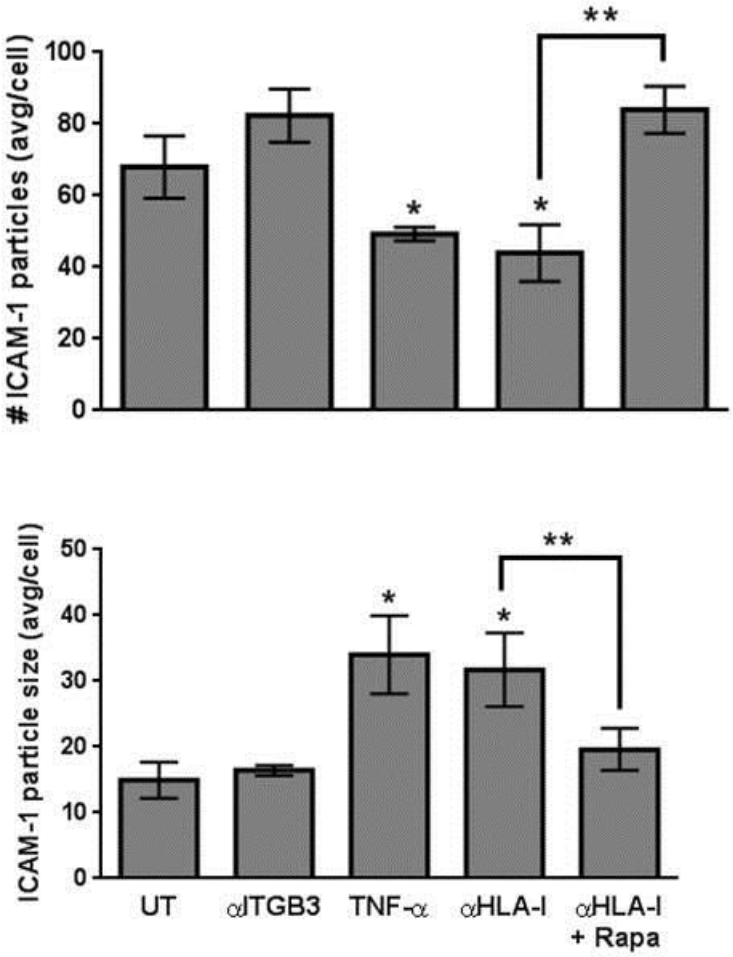
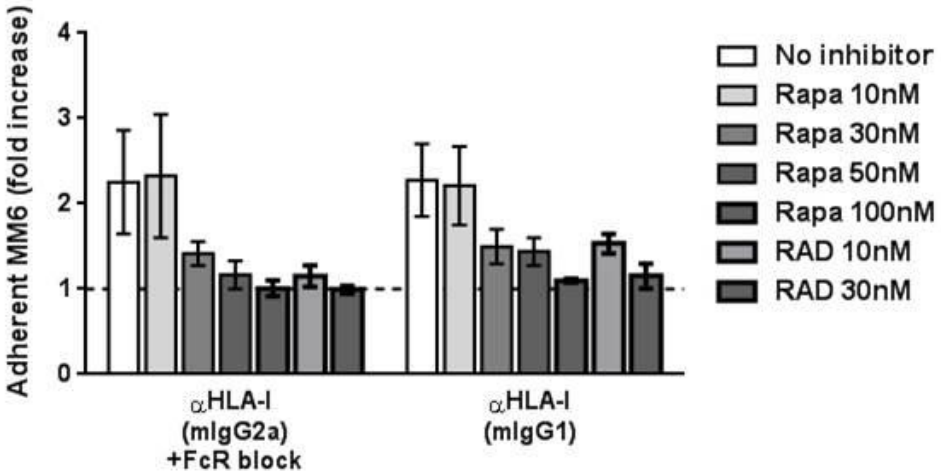


Figure 7: mTOR is required for ICAM-1 clustering in HLA-I activated HAEC.

(A) HAECs were either left untreated or pre-treated with rapamycin at 30nM for 24hours. EC were then stimulated with 1 mg/mL anti-HLA I mAb W6/32 for 30 min, or 10ng/ml TNF-α for 4 h. EC were incubated for 1 h with sheep anti-ICAM-1 antibody, followed by 30 min incubation with secondary FITC-labeled donkey-anti-sheep secondary antibody to cross-link ICAM-1 and then fixed. F-actin fibers were stained with Phalloidin. Cells were viewed and imaged by a Zeiss confocal microscope. Clustering of ICAM-1 at the cell margins is shown in EC treated with TNF-α and HLA I Ab, and diffuse distribution of ICAM-1 is shown in UT EC, as well as those treated with ITGB3 Ab and where cells were pre-treated with rapamycin. scale bars=10 ųm .**(B,C)** ICAM-1 clustering was quantified by ImageJ and results are expressed as the mean ± SEM over untreated. Data are representative of three or more images per treatment.

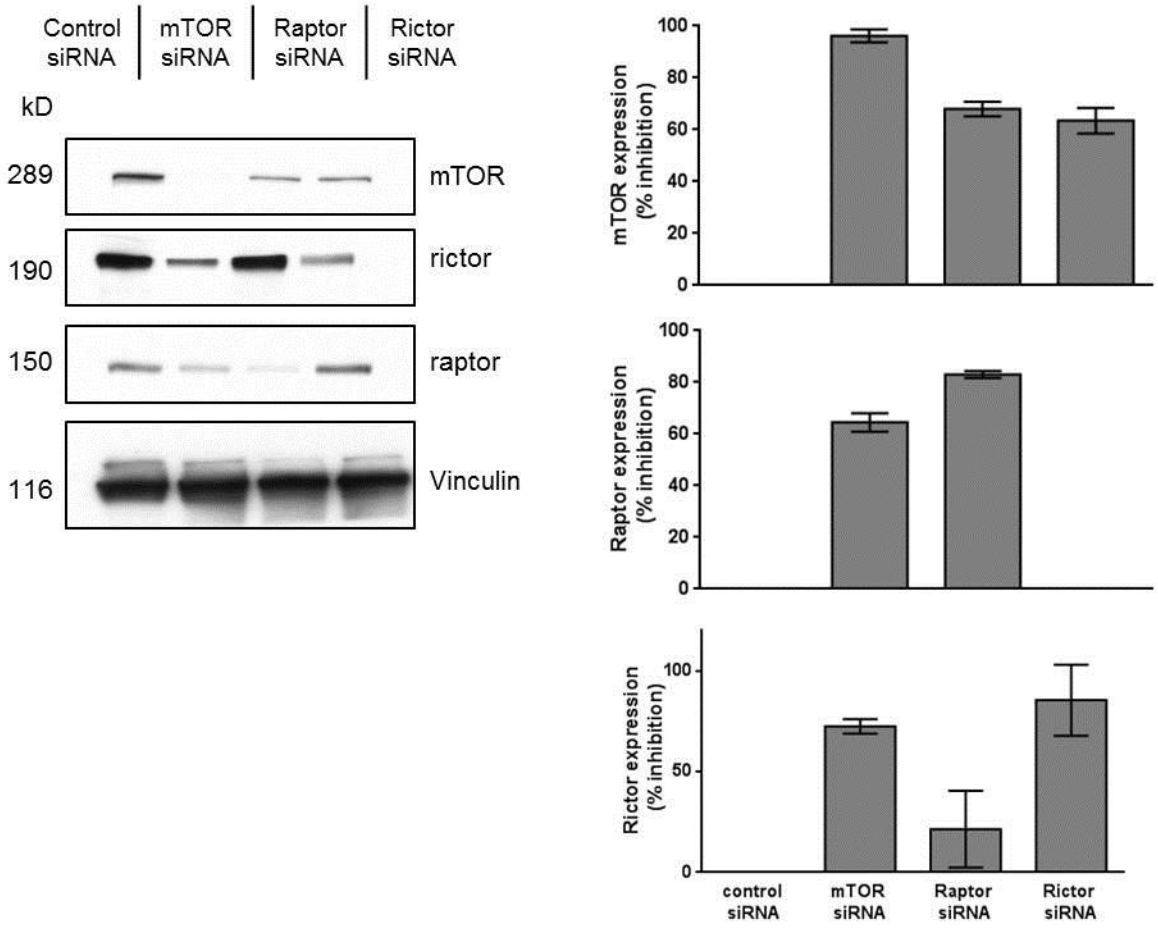
Supplementary Figure 3-1



Supplemental Fig 1. Determination of ideal mTOR inhibitor dosage.

HAECs were pre-treated with the indicated mTOR inhibitors at the listed dosages for 24h. Experiment was carried out as described in Figure 1.

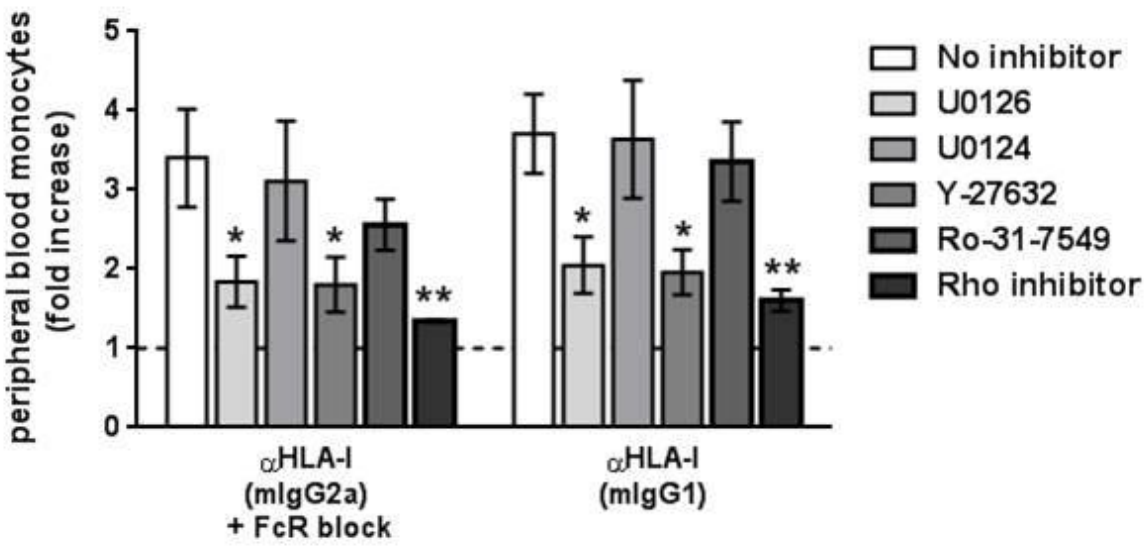
Supplementary Figure 3-2



Supplemental Fig 2. siRNA knock-down efficiency in the mTOR pathway.

HAECs were transfected with 100 nM mTOR, raptor, rictor control siRNA. After 48 h, EC were lysed and subjected to Western blot analysis using Abs to mTOR, raptor, rictor, and vinculin. Results are representative of two independent experiments.

Supplementary Figure 3-3



Supplemental Fig 3. Monocyte adherence to HLA-class I Ab-stimulated endothelium is blocked when the mTOR pathway is inhibited in EC

HAECs were pre-treated with the indicated inhibitors at the dosage and for the amount time, as listed in Figure 6. Experiment was carried out as described in Figure 1.

3.6 References

Amico, P., G. Honger, M. Mayr, J. Steiger, H. Hopfer, and S. Schaub. 2009. Clinical Relevance of Pretransplant Donor-Specific HLA Antibodies Detected by Single-Antigen Flow-Beads. *Transplantation* 87:1681-1688.

Andreassen, A.K., B. Andersson, F. Gustafsson, H. Eiskjaer, G. Radegran, E. Gude, K. Jansson, D. Solbu, K. Karason, S. Arora, G. Dellgren, and I. Gullestad. 2015. Three Year Follow Up of the Randomized SCHEDULE Trial With Everolimus Initiation and Early Withdrawal of Calcineurin Inhibitor Therapy in De Novo Heart Transplant Recipients - A Multicenter, Randomized Scandinavian Trial. *Journal of Heart and Lung Transplantation* 34:S129-S130.

Barilli, A., R. Visigalli, R. Sala, G.C. Gazzola, A. Parolari, E. Tremoli, S. Bonomini, A. Simon, E.I. Closs, V. Dall'Asta, and O. Bussolati. 2008. In human endothelial cells rapamycin causes mTORC2 inhibition and impairs cell viability and function. *Cardiovascular Research* 78:563-571.

Barreiro, O., M. Yanez-Mo, J.M. Serrador, M.C. Montoya, M. Vicente-Manzanares, R. Tejedor, H. Furthmayr, and F. Sanchez-Madrid. 2002. Dynamic interaction of VCAM-1 and ICAM-1 with moesin and ezrin in a novel endothelial docking structure for adherent leukocytes. *Journal of Cell Biology* 157:1233-1245.

Colvin, R.B., and R.N. Smith. 2005. Antibody-mediated organ-allograft rejection. *Nature Reviews Immunology* 5:807-817.

Duhart, B.T., Jr., W.A. Ally, A.G. Krauss, J.Q. Hudson, J.D. Eason, V. Rao, and J.M. Vanatta. 2015. The benefit of sirolimus maintenance immunosuppression and rabbit antithymocyte

globulin induction in liver transplant recipients that develop acute kidney injury in the early postoperative period. *Journal of transplantation* 2015:926168.

Eisen, H.J., J. Kobashigawa, R.C. Starling, D.F. Pauly, A. Kfoury, H. Ross, S.S. Wang, B. Cantin, A. Van Bakel, G. Ewald, S. Hirt, H. Lehmkuhl, A. Keogh, M. Rinaldi, L. Potena, A. Zuckermann, G. Dong, C. Cornu-Artis, and P. Lopez. 2013. Everolimus Versus Mycophenolate Mofetil in Heart Transplantation: A Randomized, Multicenter Trial. *American Journal of Transplantation* 13:1203-1216.

Eisen, H.J., E.M. Tuzcu, R. Dorent, J. Kobashigawa, D. Mancini, H.A. Valantine-von Kaeppler, R.C. Starling, K. Sorensen, M. Hummel, J.M. Lind, K.H. Abeywickrama, P. Bernhardt, and R.B.S. Grp. 2003. Everolimus for the prevention of allograft rejection and vasculopathy in cardiac-transplant recipients. *New England Journal of Medicine* 349:847-858.

Farkas, S., M. Hornung, C. Sattler, M. Guba, M. Steinbauer, M. Anthuber, H. Herfarth, H.J. Schlitt, and E.K. Geissler. 2006. Rapamycin decreases leukocyte migration in vivo and effectively reduces experimentally induced chronic colitis. *International Journal of Colorectal Disease* 21:747-753.

Fishbein, G.A., and M.C. Fishbein. 2012. Morphologic and immunohistochemical findings in antibody-mediated rejection of the cardiac allograft. *Human Immunology* 73:1213-1217.

Fox, R., T.Q. Nhan, G.L. Law, D.R. Morris, W.C. Liles, and S.M. Schwartz. 2007. PSGL-1 and mTOR regulate translation of ROCK-1 and physiological functions of macrophages. *Embo Journal* 26:505-515.

Geissler, E.K., A.A. Schnitzbauer, C. Zulke, P.E. Lamby, A. Proneth, C. Duvoux, P. Burra, K.W. Jauch, M. Rentsch, T.M. Ganten, J. Schmidt, U. Settmacher, M. Heise, G. Rossi, U.

Cillo, N. Kneteman, R. Adam, B. van Hoek, P. Bachellier, P. Wolf, L. Rostaing, W.O. Bechstein, M. Rizell, J. Powell, E. Hidalgo, J. Gugenheim, H. Wolters, J. Brockmann, A. Roy, I. Mutzbauer, A. Schlitt, S. Beckebaum, C. Graeb, S. Nadalin, U. Valente, V.S. Turrion, N. Jamieson, T. Scholz, M. Colledan, F. Fandrich, T. Becker, G. Soderdahl, O. Chazouilleres, H. Makisalo, G.P. Pageaux, R. Steininger, T. Soliman, K.P. de Jong, J. Pirenne, R. Margreiter, J. Pratschke, A.D. Pinna, J. Hauss, S. Schreiber, S. Strasser, J. Klempnauer, R.I. Troisi, S. Bhoori, J. Lerut, I. Bilbao, C.G. Klein, A. Konigsrainer, D.F. Mirza, G. Otto, V. Mazzaferro, P. Neuhaus, and H.J. Schlitt. 2016. Sirolimus Use in Liver Transplant Recipients With Hepatocellular Carcinoma: A Randomized, Multicenter, Open-Label Phase 3 Trial. *Transplantation* 100:116-125.

Gerhardt, T., and K. Ley. 2015. Monocyte trafficking across the vessel wall. *Cardiovascular Research* 107:321-330.

Heiska, L., K. Alfthan, M. Gronholm, P. Vilja, A. Vaheri, and O. Carpen. 1998. Association of ezrin with intercellular adhesion molecule-1 and -2 (ICAM-1 and ICAM-2) - Regulation by phosphatidylinositol 4,5-bisphosphate. *Journal of Biological Chemistry* 273:21893-21900.

Ho, E.K., G. Vlad, E.R. Vasilescu, L. de la Torre, A.I. Colovai, E. Burke, M. Deng, J. Schwartz, C. Marboe, D. Mancini, G. Opelz, and N. Suciu-Foca. 2011. Pre- and posttransplantation allosensitization in heart allograft recipients: Major impact of de novo alloantibody production on allograft survival. *Human Immunology* 72:5-10.

Huo, Y.L., V. Iadevaia, and C.G. Proud. 2011. Differing effects of rapamycin and mTOR kinase inhibitors on protein synthesis. *Biochemical Society Transactions* 39:446-450.

Ishibashi, T., T. Sakamoto, H. Ohkawara, K. Nagata, K. Sugimoto, S. Sakurada, N. Sugimoto, A. Watanabe, K. Yokoyama, N. Sakamoto, M. Kurabayashi, Y. Takuwa, and Y. Maruyama. 2003. Integral role of RhoA activation in monocyte adhesion-triggered tissue factor expression in endothelial cells. *Arteriosclerosis Thrombosis and Vascular Biology* 23:681-687.

Ivetic, A., and A.J. Ridley. 2004. Ezrin/radixin/moesin proteins and Rho GTPase signalling in leucocytes. *Immunology* 112:165-176.

Jin, Y.P., Y. Korin, X.H. Zhang, P.T. Jindra, E. Rozengurt, and E.F. Reed. 2007. RNA interference elucidates the role of focal adhesion kinase in HLA class I-mediated focal adhesion complex formation and proliferation in human endothelial cells. *Journal of Immunology* 178:7911-7922.

Jin, Y.P., R.P. Singh, Z.Y. Du, A.K. Rajasekaran, E. Rozengurt, and E.F. Reed. 2002. Ligation of HLA class I molecules on endothelial cells induces phosphorylation of Src, paxillin, and focal adhesion kinase in an actin-dependent manner. *Journal of Immunology* 168:5415-5423.

Jin, Y.P., N.M. Valenzuela, M.E. Ziegler, E. Rozengurt, and E.F. Reed. 2014. Everolimus Inhibits Anti-HLA I Antibody-Mediated Endothelial Cell Signaling, Migration and Proliferation More Potently Than Sirolimus. *American Journal of Transplantation* 14:806-819.

Jindra, P.T., A. Hsueh, L. Hong, D. Gjertson, X.D. Shen, F. Gao, J. Dang, P.S. Mischel, W.M. Baldwin, M.C. Fishbein, J.W. Kupiec-Weglinski, and E.F. Reed. 2008a. Anti-MHC class I antibody activation of proliferation and survival signaling in murine cardiac allografts. *Journal of Immunology* 180:2214-2224.

Jindra, P.T., Y.P. Jin, R. Jacamo, E. Rozengurt, and E.F. Reed. 2008b. MHC class I and integrin ligation induce ERK activation via an mTORC2-dependent pathway. *Biochemical and Biophysical Research Communications* 369:781-787.

Jindra, P.T., Y.P. Jin, E. Rozengurt, and E.F. Reed. 2008c. HLA class I antibody-mediated endothelial cell proliferation via the mTOR pathwayle. *Journal of Immunology* 180:2357-2366.

Kluger, M.S. 2004. Vascular endothelial cell adhesion and signaling during leukocyte recruitment. *Advances in dermatology* 20:163-201.

Kobashigawa, J., L. Miller, D. Renlund, R. Mentzer, E. Alderman, R. Bourge, M. Costanzo, H. Eisen, G. Dureau, R. Ratkovec, M. Hummel, D. Ipe, J. Johnson, A. Keogh, R. Mamelok, D. Mancini, F. Smart, H. Valantine, and I. Mycophenolate Mofetil. 1998. A randomized active-controlled trial of mycophenolate mofetil in heart transplant recipients. *Transplantation* 66:507-515.

Ley, K., C. Laudanna, M.I. Cybulsky, and S. Nourshargh. 2007. Getting to the site of inflammation: the leukocyte adhesion cascade updated. *Nature Reviews Immunology* 7:678-689.

Li, F., X.H. Zhang, Y.P. Jin, A. Mulder, and E.F. Reed. 2011. Antibody ligation of human leukocyte antigen class I molecules stimulates migration and proliferation of smooth muscle cells in a focal adhesion kinase-dependent manner. *Human Immunology* 72:1150-1159.

Lien, Y.-H.H. 2015. Top 10 things primary care physicians should know about maintenance immunosuppression for transplant recipients. *Am. J. Medicine*

Lones, M.A., L.S.C. Czer, A. Trento, D. Harasty, J.M. Miller, and M.C. Fishbein. 1995. CLINICAL-PATHOLOGICAL FEATURES OF HUMORAL REJECTION IN CARDIAC ALLOGRAFTS - A STUDY IN 81 CONSECUTIVE PATIENTS. *Journal of Heart and Lung Transplantation* 14:151-162.

Mahoney, T.S., A.S. Weyrich, D.A. Dixon, T. McIntyre, S.M. Prescott, and G.A. Zimmerman. 2001. Cell adhesion regulates gene expression at translational checkpoints in human myeloid leukocytes. *Proceedings of the National Academy of Sciences of the United States of America* 98:10284-10289.

Meng, L.H., and X.F.S. Zheng. 2015. Toward rapamycin analog (rapalog)-based precision cancer therapy. *Acta Pharmacologica Sinica* 36:1163-1169.

Millan, J., and A.J. Ridley. 2005. Rho GTPases and leucocyte-induced endothelial remodelling. *Biochemical Journal* 385:329-337.

Mutin, M., F. George, G. Lesaule, and J. Sampol. 1996. Reevaluation of Trypsin-EDTA for Endothelial Cell Detachment before Flow Cytometry Analysis. Endothelium.

Narayanan, K., M.D. Jendrisak, D.L. Phelan, and T. Mohanakumar. 2006. HLA class I antibody mediated accommodation of endothelial cells via the activation of PI3K/cAMP dependent PKA pathway. *Transplant Immunology* 15:187-197.

Neff, G.W., M. Montalbano, and A.G. Tzakis. 2003. Ten years of sirolimus therapy in orthotopic liver transplant recipients. *Transplantation Proceedings* 35:209S-216S.

Petit, V., and J.P. Thiery. 2000. Focal adhesions: structure and dynamics. *Biology of the Cell* 92:477-494.

Qiu, Y., X. Wang, J. Fan, Z. Rao, Y. Lu, and T. Lin. 2015. Conversion From Calcineurin Inhibitors to Mammalian Target-of-Rapamycin Inhibitors in Heart Transplant Recipients:

A Meta-Analysis of Randomized Controlled Trials. *Transplantation Proceedings* 47:2952-2956.

Raught, B., A.C. Gingras, and N. Sonenberg. 2001. The target of rapamycin (TOR) proteins. *Proceedings of the National Academy of Sciences of the United States of America* 98:7037-7044.

Salehi, S., and E.F. Reed. 2015. The divergent roles of macrophages in solid organ transplantation. *Current Opinion in Organ Transplantation* 20:446-453.

Schindelin, J., I. Arganda-Carreras, E. Frise, V. Kaynig, M. Longair, T. Pietzsch, S. Preibisch, C. Rueden, S. Saalfeld, B. Schmid, J.Y. Tinevez, D.J. White, V. Hartenstein, K. Eliceiri, P. Tomancak, and A. Cardona. 2012. Fiji: an open-source platform for biological-image analysis. *Nature methods* 9:676-682.

Schindelin, J., C.T. Rueden, M.C. Hiner, and K.W. Eliceiri. 2015. The ImageJ ecosystem: An open platform for biomedical image analysis. *Molecular reproduction and development* 82:518-529.

Sosa, R.A., C. Murphey, N. Ji, A.E. Cardona, and T.G. Forsthuber. 2013. The Kinetics of Myelin Antigen Uptake by Myeloid Cells in the Central Nervous System during Experimental Autoimmune Encephalomyelitis. *Journal of Immunology* 191:5848-5857.

Sosa, R.A., C. Murphey, R.R. Robinson, and T.G. Forsthuber. 2015. IFN-gamma ameliorates autoimmune encephalomyelitis by limiting myelin lipid peroxidation. *Proc Natl Acad Sci U S A*

Thomas, K.A., N.M. Valenzuela, D. Gjertson, A. Mulder, M.C. Fishbein, G.C. Parry, S. Panicker, and E.F. Reed. 2015a. An Anti-C1s Monoclonal, TNT003, Inhibits

Complement Activation Induced by Antibodies Against HLA. *American Journal of Transplantation* 15:2037-2049.

Thomas, K.A., N.M. Valenzuela, and E.F. Reed. 2015b. The perfect storm: HLA antibodies, complement, Fc gamma Rs, and endothelium in transplant rejection. *Trends in Molecular Medicine* 21:319-329.

Tran, T.M., P. Ivanyi, I. Hilgert, T. Brdicka, M. Pla, B. Breur, M. Flieger, E. Ivaskova, and V. Horejsi. 2001. The epitope recognized by pan-HLA class I-reactive monoclonal antibody W6/32 and its relationship to unusual stability of the HLA-B27/beta 2-microglobulin complex. *Immunogenetics* 53:440-446.

Valenzuela, N.M., L. Hong, X.D. Shen, F. Gao, S.H. Young, E. Rozengurt, J.W. Kupiec-Weglinski, M.C. Fishbein, and E.F. Reed. 2013a. Blockade of P-Selectin Is Sufficient to Reduce MHC I Antibody-Elicited Monocyte Recruitment In Vitro and In Vivo. *American Journal of Transplantation* 13:299-311.

Valenzuela, N.M., L. Hong, X.D. Shen, F. Gao, S.H. Young, E. Rozengurt, J.W. Kupiec-Weglinski, M.C. Fishbein, and E.F. Reed. 2013b. Blockade of p-selectin is sufficient to reduce MHC I antibody-elicited monocyte recruitment in vitro and in vivo. *American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons* 13:299-311.

Valenzuela, N.M., A. Mulder, and E.F. Reed. 2013c. HLA Class I Antibodies Trigger Increased Adherence of Monocytes to Endothelial Cells by Eliciting an Increase in Endothelial P-Selectin and, Depending on Subclass, by Engaging Fc gamma Rs. *Journal of Immunology* 190:6635-6650.

Valenzuela, N.M., A. Mulder, and E.F. Reed. 2013d. HLA class I antibodies trigger increased adherence of monocytes to endothelial cells by eliciting an increase in endothelial P-selectin and, depending on subclass, by engaging FcgammaRs. *Journal of immunology* 190:6635-6650.

Valenzuela, N.M., and E.F. Reed. 2011. The link between major histocompatibility complex antibodies and cell proliferation. *Transplantation Reviews* 25:154-166.

Valenzuela, N.M., K.R. Trinh, A. Mulder, S.L. Morrison, and E.F. Reed. 2015. Monocyte Recruitment by HLA IgG-Activated Endothelium: The Relationship Between IgG Subclass and FcgammaRIIa Polymorphisms. In U.o.C.a.L. Angeles, editor American Journal of Transplantation. 17.

Wang, C., L.F. Qin, T.D. Manes, N.C. Kirkiles-Smith, G. Tellides, and J.S. Pober. 2014. Rapamycin antagonizes TNF induction of VCAM-1 on endothelial cells by inhibiting mTORC2. *Journal of Experimental Medicine* 211:395-404.

Wang, C., T. Yi, L.F. Qin, R.A. Maldonado, U.H. von Andrian, S. Kulkarni, G. Tellides, and J.S. Pober. 2013. Rapamycin-treated human endothelial cells preferentially activate allogeneic regulatory T cells. *Journal of Clinical Investigation* 123:1677-1693.

Willicombe, M., P. Brookes, E. Santos-Nunez, J. Galliford, A. Ballow, A. McLean, C. Roufosse, H.T. Cook, A. Dorling, A.N. Warrens, T. Cairns, and D. Taube. 2011. Outcome of Patients with Preformed Donor-Specific Antibodies Following Alemtuzumab Induction and Tacrolimus Monotherapy. *American Journal of Transplantation* 11:470-477.

Wojciak-Stothard, B., L. Williams, and A.J. Ridley. 1999. Monocyte adhesion and spreading on human endothelial cells is dependent on Rho-regulated receptor clustering. *Journal of Cell Biology* 145:1293-1307.

Yamakuchi, M., N.C. Kirkiles-Smith, M. Ferlito, S.J. Cameron, C. Bao, K. Fox-Talbot, B.A. Wasowska, W.M. Baldwin, J.S. Pober, and C.J. Lowenstein. 2007. Antibody to human leukocyte antigen triggers endothelial exocytosis. *Proceedings of the National Academy of Sciences of the United States of America* 104:1301-1306.

Chapter 4:
The role of mTOR in monocyte activation and recruitment in response to HLA-I Ab activated EC.

4.1 Abstract

Macrophages are powerful contributors to graft injury in antibody-mediated allograft rejection. Though the mTOR pathway has been implicated as a mediator of activity in innate immune cells, additional mechanistic studies are needed. The purpose of this study was to determine if mTOR inhibition in monocytes can modulate their capacity to adhere to HLA I antibody-activated endothelium. We used an in vitro model to examine binding of mTOR-inhibited monocytes to endothelial cells stimulated with HLA I antibodies and found binding to be blocked. Furthermore, we determined by solid phase assay that blockade of monocytic binding may be due to dampening of PSGL-1 signaling in monocytes.

4.2 Introduction

AMR persists as a major issue confounding solid organ transplants. As the main feature of AMR, donor specific HLA antibodies contribute to decreased graft function and poor outcome[1]. The presence of intragraft macrophages is another key feature of AMR and is associated with increased incidence of rejection[2, 3] . The many functions of macrophages, including release of pro-inflammatory, proliferative and fibrotic factors are injurious to the allograft depletion of recipient macrophages or inhibition of monocyte activation has been shown to attenuate graft injury and prolong survival[4-7]. Though currently used immunosuppressive drugs are through to exert effects on innate immune cells[8, 9], the full impact of thes drugs on the function of monocytes is still unknown.

Of these drugs, mTOR inhibitors such as rapamycin (sirolimus) and its derivative everolimus are potent drugs used to treat a variety of inflammatory diseases and graft dysfunctions, in which monocyte recruitment and activity underlies the mechanisms of cell injury.

Experimental evidence suggests that mTOR inhibition can modulate the leukocyte recruitment system at the level of the monocytes recruited to the activated endothelium of allografts[10-12]. mTOR inhibition by sirolimus of THP-1 cells and primary monocytes suppressed the in vitro production of the monocyte chemoattractant protein-1 (MCP-1), IL-8, regulated on activation, normal T cell expressed and secreted protein (RANTES), the macrophage inflammatory protein MIP-1α, and MIP-1β following stimulation by LPS[13]. Additionally, mTOR has been shown to be required for production of pro-inflammatory cytokines IL-6 and TNF-α by monocytes in patients with coronary artery disease[14]. Given these findings, we investigated the role of the mTOR signaling network in monocytes recruited to HLA I-activated ECs.

Following PSGL-1-mediated leukocyte rolling or FcγRs engagement, intracellular signaling pathways are activated in monocytes, allowing for firm adhesion to the endothelium[15]. The ability of monocytes to firmly adhere to activated endothelium is regulated by conformational changes in the β2 integrins MAC-1 and LFA-1, through which monocytes firmly adhere to ICAM-1[15]. Recent studies suggest that mTOR signaling may be required downstream of PSGL-1[10, 11] or Fc γ receptor-mediated[16] activation in monocytes. As such, we tested if mTOR is involved in monocyte adherence to ICAM-1 in response to PSGL-1 signaling.

4.3 Methods

Antibodies and Chemicals

Pan-HLA antibodies were used as they are well-characterized and recognize monomorphic epitopes on all HLA class I antigens [17]. Pan-HLA antibody MEM-147 (mIgG1) was purchased from Abcam (Cambridge, MA). Human anti-integrin αVβ3 mIgG1 (R&D; Minneapolis, MN) was used as negative control as indicated. PMA (Phorbol 12-myristate 13-acetate) was purchased from Abcam (Cambridge, MA).

Anti-human ICAM-1 (CD54) antibody for solid phase assay was obtained from R&D (Minneapolis, MN). Recombinant human P-selectin (CD62P) dimer was purchased from R&D (Minneapolis, MN). Rapamycin (sirolimus) was purchased from LC Laboratories (Woburn, MA).

Cell Culture

Primary human aortic endothelial cells (HAECs) were isolated from the aortic rings of deceased donor hearts (H126 or F1153) as previously described, or obtained from Clonetics (lot number: EC5555) and cultured as described previously[18-20]. Briefly, HAECs were seeded at confluence on 0.1 % gelatin-coated tissue culture plates in complete medium containing 20% heat-inactivated fetal bovine serum (FBS), antibiotics (P/S) (both from Invitrogen Life Technologies), heparin (Sigma-Alrich) and endothelial cell growth serum (ECGS) (Fisher Scientific. All experiments were repeated with early-passage endothelial cells from 2-3 different donors to rule out donor-specific responses.

Static monocyte adhesion assay

Adherence of monocytic cells to endothelium under static conditions was measured as previously described[3, 21]. ECs were stimulated with control antibody anti-human integrin αVβ3 or antibody against HLA class I. Polyclonal human IgG (20ug/mL) was used to block FcγR interactions.

Solid Phase Assay

A 96 well high protein affinity nunc plate was coated with recombinant human ICAM-1 and P-selectin dimer (or anti-CD18) (R&D Cat #ADP4-200). Monocytes were pre-treated with mTOR inhibitor and added to the plate. Adherent cells were counted by fluorescence microscopy on a live cell fluorescence microscope (Nikon Eclipse Ti). Data was analyzed by Cell Profiler.

Statistical Analysis

Differences between groups were determined using one or two-way ANOVA using Prism v5 software (GraphPad Software Inc., La Jolla, CA). p values of <0.05 were considered statistically significant.

4.4 Results

4.4.1. Inhibition of mTOR in monocytes significantly reduces adhesion to HLA I antibody-activated endothelium.

We previously found that crosslinking FcγRs or PSGL-1 activates monocyte β2 integrins and promotes adherence to ICAM-1 (data not shown). Furthermore, our group determined that PI3K and Src are upstream of mTOR activation in endothelial HLA I signaling[22, 23]. Moreover, it is well-established that the cytoskeleton is regulated by mTORC2 through PKCα and Rho GTPases[24] . Therefore, we hypothesized that mTOR signaling networks are involved in monocyte adhesion downstream of P-selectin/PSGL-1 and HLA class I antibody/FcγR interactions.

To validate this theory, we measured adhesion of mTOR-deficient monocytes to HLA I antibody-activated endothelial cells. ECs were treated with HLA-I Abs to stimulate WPb exocytosis and upregulate P-selectin expression. MM6 were pre-treated with varying doses and times (2 and 24 hours) of the pharmacological mTOR inhibitor rapamycin and overlayed on stimulated EC in static adherence assays and changes in adherence were determined by fluorescent microscopy and quantitated by Cell Profiler.

mTOR inhibition in monocytes significantly prevented binding to endothelium in response to HLA I antibodies (**Figure 4-1**). These results point to a role for mTOR in regulation of monocyte adhesiveness, and suggest that rapamycin may also reduce the capacity of monocytes to adhere to graft vessels during AMR.

4.4.2 Inhibition of mTOR in monocytes significantly reduces adhesion to ICAM1 following PSGL-1 cross-linking.

Monocytes firmly adhere to ICAM-1 through surface β2 integrins, including LFA-1 (αLβ2) and Mac-1 (αMβ2). Though resting monocytes express high levels of LFA-1 or Mac-1 on their cell surface, they only bind to ICAM-1 following exposure to activation signals such as, chemokines and rolling on P-selectin, which crosslinks PSGL-1 on the monocyte surface. These signals induce conformational changes of β2 integrins, which then allow it to bind ICAM-1 or the extracellular matrix[25]. Recent data suggests that the mTOR pathway is activated in monocytes following PSGL-1 engagement[11, 26] . To explore this theory, we next tested the effect of mTOR inhibition on inducible monocyte adherence to ICAM-1 following PSGL-1 interactions.

In order to assess the effect of mTOR inhibition on inducible monocyte adherence to ICAM-1 following PSGL-1 interactions, we developed a solid phase assay in which purified human ICAM-1 protein is immobilized on Nunc Maxisorp 96 well plates with or without recombinant P-selectin, which serves to activate monocytes by crosslinking PSGL-1 on their surface. The monocytic cell line Mono Mac 6 (MM6) was pre-treated with the mTOR inhibitor rapamycin for 24 hours and monocytic binding was measured. Positive control PMA is used as a strong stimulus of monocyte activation. We found that mTOR inhibition followed by PSGL-1 engagement significantly impairs monocytic adherence (**Figure 4-2**).

This mechanistic study further implicates the mTOR pathway in monocyte activation in response to PSGL-1 engagement, and suggests that PSGL-1 signaling in monocytes is hampered by mTOR inhibition.

4.5 Future Directions

As crosslinking FcγRs increases monocyte binding to ICAM-1[15], we will use a murine IgG2a antibody to crosslink FcγRs following monocytic treatment with mTOR inhibitors. The ability of these monocytes to adhere to immobilized ICAM-1 will then be determined by a solid phase assay.

Additionally, intracellular signaling events will be studied by Western blot following FcγR and PSGL-1 engagement. MM6 will be treated with mIgG2a Ab and crosslinking 2nd antibody, and the recombinant soluble P-selectin chimera. Cells will be lysed and immunoblotted with phosphospecific Abs against p70 S6 Kinase, S6 ribosomal protein, Akt, and ERK. The role of mTOR inhibition on FcγR/PSGL-1 induced protein phosphorylation will be established.

siRNA of mTOR, rictor and raptor will be used to confirm results and to examine the role of mTORC2 and mTORC1 in this process.

We will use siRNA against mTOR, raptor and rictor to confirm our findings and to explore the role of mTORC1/mTORC2 in contributing to the capacity of monocytes to adhere to HLA-activated endothelium.

Figure 4-1a

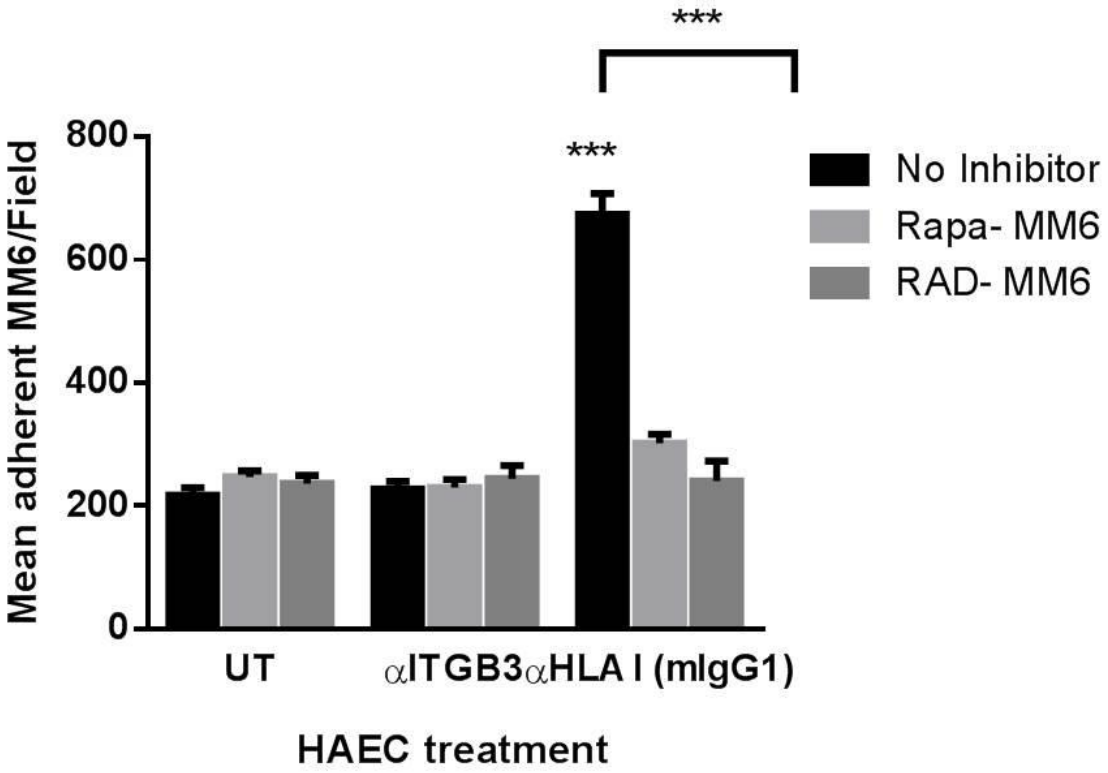

***
***
800
600
400
200
0
Mean adherent MM6/Field
No Inhibitor
Rapa- MM6
RAD- MM6
UT
αITGB3αHLA I (mIgG1)
HAEC treatment

Figure 4-1b

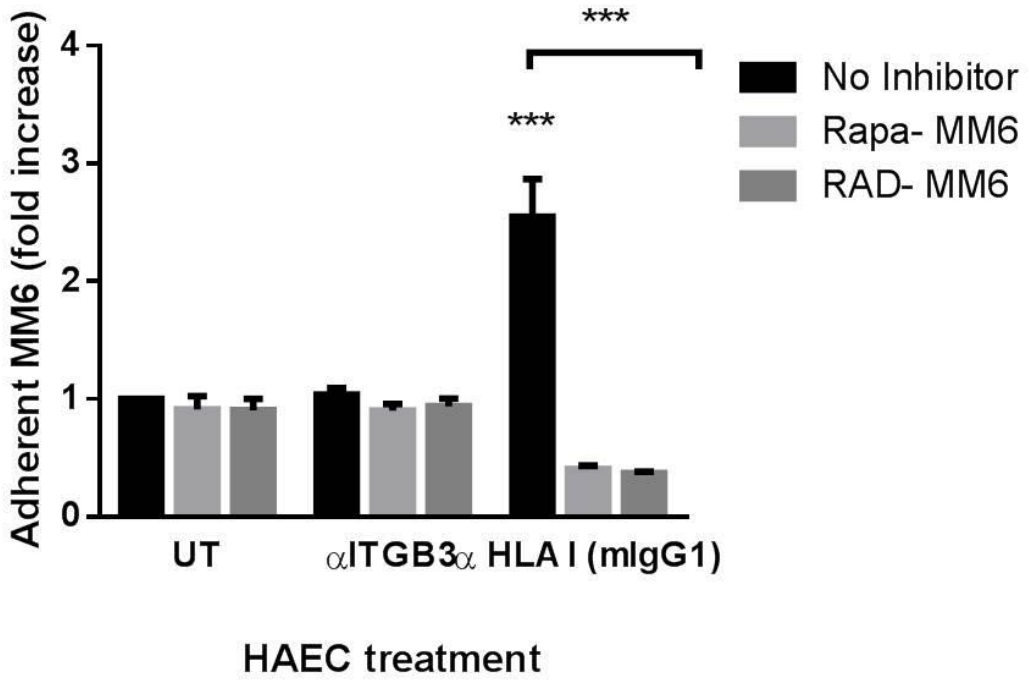

***
***
No Inhibitor
Rapa- MM6
RAD- MM6
Adherent MM6 (fold increase)
0
1
2
3
4
UT
αITGB3α HLA I (mIgG1)
HAEC treatment

Figure 4-1c

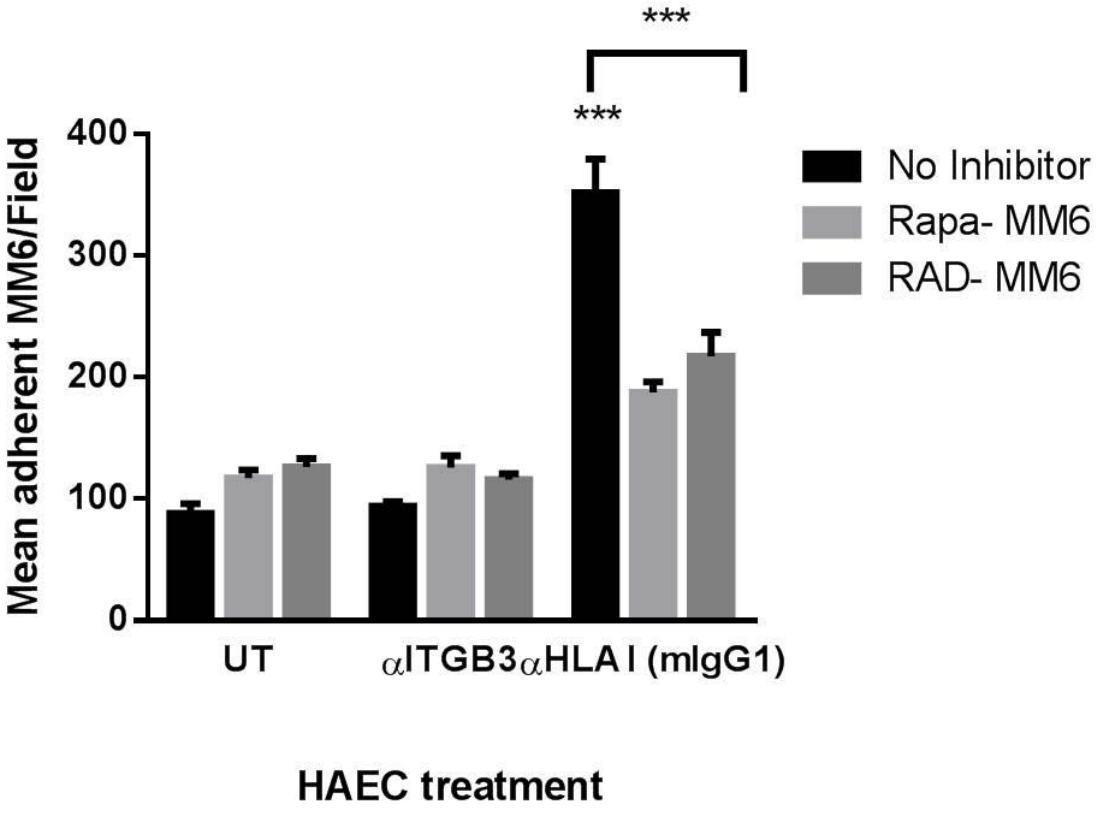

***
***
400
300
200
100
0
Mean adherent MM6/Field
No Inhibitor
Rapa- MM6
RAD- MM6
UT
αITGB3αHLA I (mIgG1)
HAEC treatment

Figure 4-1d

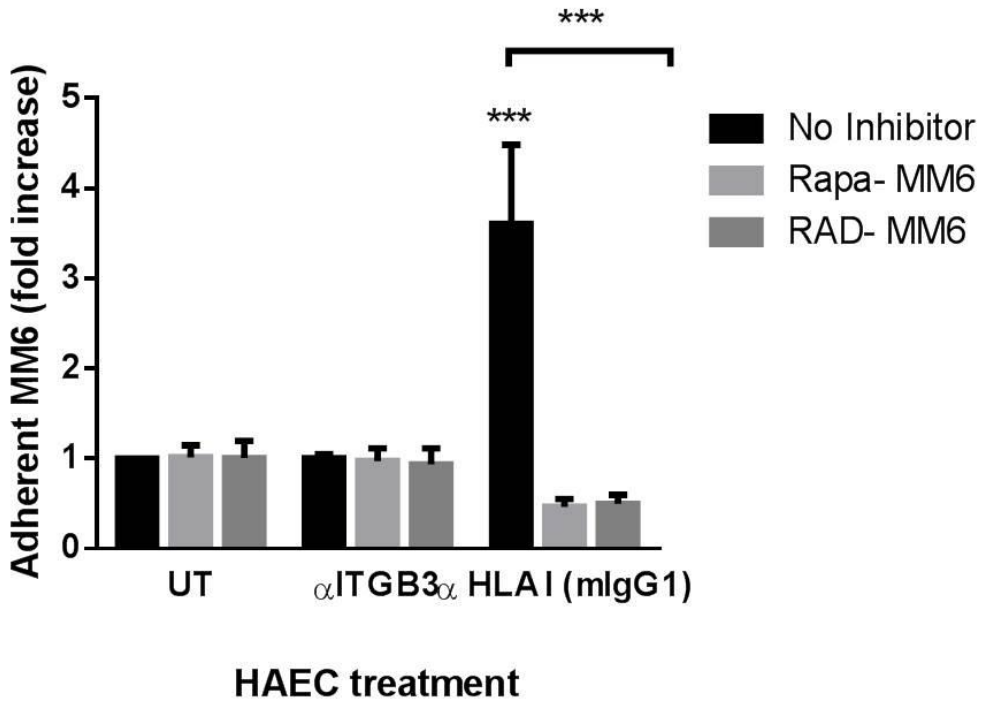


Figure 4-1. Monocyte adherence to HLA-class I Ab-stimulated endothelium is blocked when the mTOR pathway is inhibited in monocytes. Primary human aortic endothelium (EC, donor H126, 3F1153, or X127) were either left un-stimulated or stimulated with control antibody against Integrin B3 or HLA I intact antibody (MEM-147 mIgG1) at lg/mL diluted in M199 supplemented with 5% FBS for 5 min. Mono Mac6 were either untreated, pretreated with Rapamycin at 30nM or RAD001 at 10nM for 24 **(A,B)** or 2 **(C,D)** hours. Monocytes were fluorescently labeled with CFSE, resuspended in RPMI supplemented with 5% FBS, and were added at a ratio of 2–3 monocytes to 1 endothelial cell and allowed to adhere for 20 min. After removal of non-adherent monocytes by washing, adherent monocytes were counted by fluorescence microscopy and automated software (CellProfiler) in 8-10 fields per condition as

previously described (Chapter 3). Results from one representative experiment are shown **(A, C).** Bar graph represents mean fold increase in the number of adherent Mono Mac 6 **(B,D)** from multiple ($n \geq 3$) independent experiments ± SEM. *, $p<0.05$; ** $p<0.01$ versus untreated. *$p<0.05$, **$p<0.01$ versus comparing no inhibitor to inhibitor.

Figure 4-2

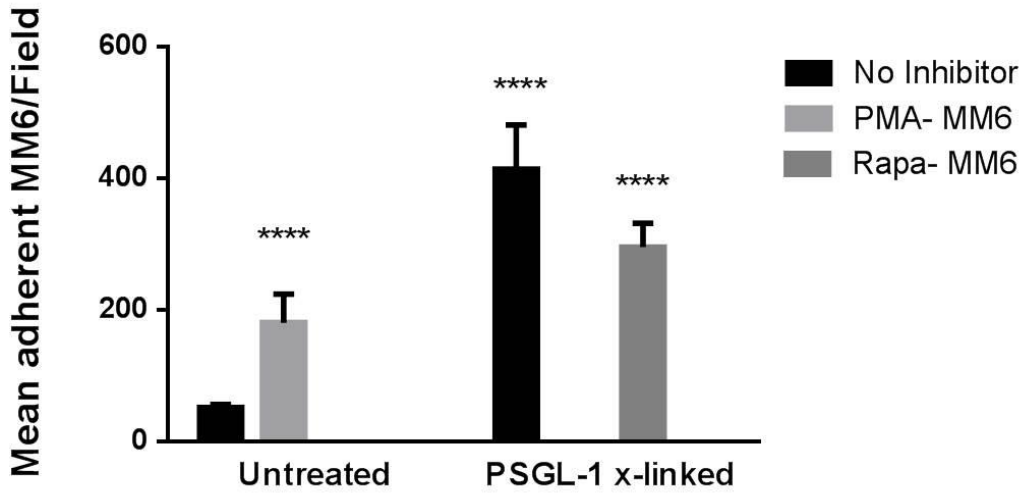


Figure 4-2. Inhibition of mTOR in monocytes significantly reduces adhesion to ICAM-1 following PSGL-1 cross-linking A 96 well high protein affinity nunc plate was coated with 1ug/well of recombinant human ICAM-1 and 5ug/well P-selectin dimer (or anti-CD18) diluted in PBS and incubated overnight at 4˚ C. Mono Mac6 were either untreated o pre-treated with Rapamycin at 30 nM for 24 hours or stimulated with PMA for 15 minutes, the labeled with CFSE for 10 mins. Monocytes were then added to the immobilized ICAM-1 and P-selectin dimer. Adherent cells were counted by fluorescence microscopy on a live cell fluorescence microscope (Nikon Eclipse Ti). Data was analyzed by Cell Profiler.

4.6 References

1. Amico P, Honger G, Mayr M, Steiger J, Hopfer H, Schaub S. Clinical relevance of pretransplant donor-specific hla antibodies detected by single-antigen flow-beads. *Transplantation*. 2009;87:1681-1688
2. Fishbein MC, Kobashigawa J. Biopsy-negative cardiac transplant rejection: Etiology, diagnosis, and therapy. *Current Opinion in Cardiology*. 2004;19:166-169
3. Valenzuela NM, Mulder A, Reed EF. Hla class i antibodies trigger increased adherence of monocytes to endothelial cells by eliciting an increase in endothelial p-selectin and, depending on subclass, by engaging fc gamma rs. *Journal of Immunology*. 2013;190:6635-6650
4. Kakuta Y, Okumi M, Miyagawa S, Tsutahara K, Abe T, Yazawa K, Matsunami K, Otsuka H, Takahara S, Nonomura N. Blocking of ccr5 and cxcr3 suppresses the infiltration of macrophages in acute renal allograft rejection. *Transplantation*. 2012;93:24-31
5. Kitchens WH, Chase CM, Uehara S, Cornell LD, Colvin RB, Russell PS, Madsen JC. Macrophage depletion suppresses cardiac allograft vasculopathy in mice. *American Journal of Transplantation*. 2007;7:2675-2682
6. Akashi S, Sho M, Kashizuka H, Hamada K, Ikeda N, Kuzumoto Y, Tsurui Y, Nomi T, Mizuno T, Kanehiro H, Hisanaga M, Ko S, Nakajima Y. A novel small-molecule compound targeting ccr5 and cxcr3 prevents acute and chronic allograft rejection. *Transplantation*. 2005;80:378-384
7. Salehi S, Reed EF. The divergent roles of macrophages in solid organ transplantation. *Current Opinion in Organ Transplantation*. 2015;20:446-453

8. Weimer R, Mytilineos J, Feustel A, Preiss A, Daniel V, Grimm H, Wiesel M, Opelz G. Mycophenolate mofetil-based immunosuppression and cytokine genotypes: Effects on monokine secretion and antigen presentation in long-term renal transplant recipients. *Transplantation*. 2003;75:2090-2099

9. Ma W, Mishra S, Gee K, Mishra JP, Nandan D, Reiner NE, Angel JB, Kumar A. Cyclosporin a and fk506 inhibit il-12p40 production through the calmodulin/calmodulin-dependent protein kinase-activated phosphoinositide 3-kinase in lipopolysaccharide-stimulated human monocytic cells (vol 282, pg 13351, 2007). *Journal of Biological Chemistry*. 2012;287:804-804

10. Mahoney TS, Weyrich AS, Dixon DA, McIntyre T, Prescott SM, Zimmerman GA. Cell adhesion regulates gene expression at translational checkpoints in human myeloid leukocytes. *Proceedings of the National Academy of Sciences of the United States of America*. 2001;98:10284-10289

11. Fox R, Nhan TQ, Law GL, Morris DR, Liles WC, Schwartz SM. Psgl-1 and mtor regulate translation of rock-1 and physiological functions of macrophages. *Embo Journal*. 2007;26:505-515

12. Farkas S, Hornung M, Sattler C, Guba M, Steinbauer M, Anthuber M, Herfarth H, Schlitt HJ, Geissler EK. Rapamycin decreases leukocyte migration in vivo and effectively reduces experimentally induced chronic colitis. *International Journal of Colorectal Disease*. 2006;21:747-753

13. Lin HYH, Chang KT, Hung CC, Kuo CH, Hwang SJ, Chen HC, Hung CH, Lin SF. Effects of the mtor inhibitor rapamycin on monocyte-secreted chemokines. *Bmc Immunology*. 2014;15:9

14. Gao SS, Liu WM, Zhuo XD, Wang LJ, Wang G, Sun T, Zhao Z, Liu JH, Tian YL, Zhou J, Yuan ZY, Wu Y. The activation of mtor is required for monocyte pro-inflammatory response in patients with coronary artery disease. *Clinical Science*. 2015;128:517-526

15. Ley K, Laudanna C, Cybulsky MI, Nourshargh S. Getting to the site of inflammation: The leukocyte adhesion cascade updated. *Nature Reviews Immunology*. 2007;7:678-689

16. Ganesan LP, Wei G, Pengal RA, Moldovan L, Moldovan N, Ostrowski MC, Tridandapani S. The serine/threonine kinase akt promotes fc gamma receptor-mediated phagocytosis in murine macrophages through the activation of p70s6 kinase. *Journal of Biological Chemistry*. 2004;279:54416-54425

17. Tran TM, Ivanyi P, Hilgert I, Brdicka T, Pla M, Breur B, Flieger M, Ivaskova E, Horejsi V. The epitope recognized by pan-hla class i-reactive monoclonal antibody w6/32 and its relationship to unusual stability of the hla-b27/beta 2-microglobulin complex. *Immunogenetics*. 2001;53:440-446

18. Jin YP, Valenzuela NM, Ziegler ME, Rozengurt E, Reed EF. Everolimus inhibits anti-hla i antibody-mediated endothelial cell signaling, migration and proliferation more potently than sirolimus. *American Journal of Transplantation*. 2014;14:806-819

19. Jin YP, Singh RP, Du ZY, Rajasekaran AK, Rozengurt E, Reed EF. Ligation of hla class i molecules on endothelial cells induces phosphorylation of src, paxillin, and focal adhesion kinase in an actin-dependent manner. *Journal of Immunology*. 2002;168:5415-5423

20. Jin YP, Korin Y, Zhang XH, Jindra PT, Rozengurt E, Reed EF. Rna interference elucidates the role of focal adhesion kinase in hla class i-mediated focal adhesion

complex formation and proliferation in human endothelial cells. *Journal of Immunology*. 2007;178:7911-7922

21. Valenzuela NM, Hong L, Shen XD, Gao F, Young SH, Rozengurt E, Kupiec-Weglinski JW, Fishbein MC, Reed EF. Blockade of p-selectin is sufficient to reduce mhc i antibody-elicited monocyte recruitment in vitro and in vivo. *American Journal of Transplantation*. 2013;13:299-311
22. Jindra PT, Hsueh A, Hong L, Gjertson D, Shen XD, Gao F, Dang J, Mischel PS, Baldwin WM, Fishbein MC, Kupiec-Weglinski JW, Reed EF. Anti-mhc class i antibody activation of proliferation and survival signaling in murine cardiac allografts. *Journal of Immunology*. 2008;180:2214-2224
23. Jindra PT, Jin YP, Rozengurt E, Reed EF. Hla class i antibody-mediated endothelial cell proliferation via the mtor pathwayle. *Journal of Immunology*. 2008;180:2357-2366
24. Thomson AW, Turnquist HR, Raimondi G. Immunoregulatory functions of mtor inhibition. *Nature Reviews Immunology*. 2009;9:324-337
25. Montresor A, Toffali L, Constantin G, Laudanna C. Chemokines and the signaling modules regulating integrin affinity. *Frontiers in Immunology*. 2012;3:10
26. Zarbock A, Abram CL, Hundt M, Altman A, Lowell CA, Ley K. Psgl-1 engagement by e-selectin signals through src kinase fgr and itam adapters dap12 and fcr gamma to induce slow leukocyte rolling. *Journal of Experimental Medicine*. 2008;205:2339-2347

Chapter 5:
Discussion and Future Directions

5.1 Key Findings

Accumulating evidence argues for a central role of anti-donor HLA antibodies in a substantial proportion of graft injury and rejection. For this reason, HLA antibodies and their role in AMR have become a point of major focus in transplantation research. Antibodies target the HLA molecules on the endothelium of the donor graft, inducing vascular damage and subsequent leukocyte recruitment.

As mTOR has been shown to be required for endothelial activation following HLA class I ligation by antibody, we postulated that mTOR inhibition can mitigate features of HLA class I-induced AMR by abrogating monocyte recruitment to endothelial cells. Our findings indicate a novel application for rapamycin in the prevention of antibody-mediated rejection and graft injury, as no current therapies target endothelial-leukocyte interactions or endothelial activation.

The goal of this dissertation was to investigate the effects of mTOR inhibition on ECs in the context of HLA I antibody-mediated cellular recruitment in an *in vitro* and *in vivo* model of acute AMR, and to elucidate the mechanisms and signaling pathways involved. Briefly, we investigated if monocyte-specific mTOR inhibition will modulate monocyte recruitment to HLA I antibody-activated ECs. There are three key findings.

First, we demonstrated that mTOR inhibition of HLA I-stimulated ECs *in vitro* reduces monocyte recruitment to the endothelium, identifying a novel mechanism by which mTOR inhibitors exert their effect. While monocyte recruitment and infiltration into areas of vascular inflammation have been studied in several disease models[1-3], the impact of mTOR inhibition on monocyte recruitment in a model of AMR was unknown until now. Notably, we validated this finding *in vivo*. Administration of the mTOR inhibitor rapamycin, which is commonly used in the clinical setting, abrogated anti-donor MHC I antibody-induced graft pathology and intragraft macrophage infiltrates. We also determined that MHC I antibody-elicited mTOR activation was abolished in the endothelium; however, future studies should examine the effect of rapamycin treatment on the innate immune cells in this model. These results indicate that mTOR inhibitors may be effective in the prevention of macrophage infiltration during AMR by dampening endothelial cell activation and innate effector adhesivity triggered by donor specific HLA I antibodies. It should be noted, however, that our model is limited in that it only address acute AMR. It is necessary to develop a murine model of chronic AMR to fully understand the impact of mTOR inhibition in AMR and to be able to translate these findings to the clinic.

Next, we made use of a flow-based assay to study monocyte-EC interactions under physiological conditions. We found that mTOR regulates endothelial ICAM-1 function following HLA I engagement. ICAM-1 is the main endothelial adhesion molecule involved in firm arrest of leukocytes and disrupting its function impairs the ability of ECs to support firm adhesion. Future studies should investigate the impact of mTOR inhibition on the functionality of VCAM-1 and adhesion molecules involved in transmigration of leukocytes.

Finally, we found that following ligation of HLA I on ECs, mTOR signals through RhoA and MAPK to phosphorylate ERM which associates with ICAM-1, allowing for adhesion molecule clustering on the surface of the endothelium, which in turn is required for firm adhesion of leukocytes. While we elucidated an intricate pathway by which mTOR-mediated class I signaling in ECs regulates monocyte adherence, it should be noted that additional pathways are likely involved and should be investigated in the future.

The novel findings from this project pave the way for better understanding the molecular pathways and signal transduction networks that trigger tissue injury and rejection, thereby facilitating identification of targets for new therapies. The results presented herein raise several questions, such as the role of mTOR in HLA II-antibody-induced endothelial activation and potential modulation of macrophage subsets by mTOR signaling. These are addressed below.

5.2 The Role of mTOR in HLA II-Mediated Monocyte Recruitment

Donor-reactive HLA class II antibodies are generally considered to be more pathogenic and deleterious than HLA class I antibodies, causing more extensive injury and greater pathology in patients with AMR. As such, they have garnered recent attention and novel endeavors are surfacing to address this problem[4-6].

Our group has been conducting studies to understand the effects of HLA II antibodies on ECs and the subsequent signaling cascades involved. We reported that HLA II-mediated signaling activates endothelial cell migration and is dependent upon mTOR[7]. Future studies should determine if mTOR regulates the recruitment of immune effector cells into the allograft in response to HLA II antibodies, and if so, by what mechanisms. Furthermore, it would be beneficial to examine the role of mTOR in monocyte recruitment to HLA II-activated endothelium *in vivo*, though an appropriate animal model based upon HLA II antibody-driven AMR does not yet exist.

5.3 The Impact of mTOR Inhibition on Monocyte Subsets

The changing platform of macrophage subsets and polarization are of key interest in transplantation. It is well known that macrophages can polarize and acquire distinctive phenotypes and physiological functions in response to environmental factors. Traditionally, macrophages were classified as either pro-inflammatory M1 or alternatively-activated M2 macrophages. Accumulating data has revealed there to be a multitude of macrophage subsets, including an unpolarized naïve M0 state, an mDC state, and several distinct M2 subsets.[8] Despite these advances, the mechanisms that regulate macrophage polarization are poorly defined.

Research conducted by Zhu et al. found a broad defect in M2 polariztion when regular mTOR signaling was disrupted. Constitutive mTORC1 activity in a myeloid specific manner demonstrated enhanced M1 function. In response to IL-4 and IL-13, macrophages were unable to

polarize to M2, though M1 polarization was not affected.[9] An unrelated study found that mTOR inhibition in the same murine model rescued the defective M2 polarization[10], though the M1 phenotype was unaffected. In a rat model of induced ischemic strokes, rapamycin treatment was shown to prevent brain macrophage polarization toward an M1 phenotype. There was a corresponding decrease in the levels of pro-inflammatory cytokine and chemokine production by macrophages[11]. Collectively, these data point to a key role for the mTOR pathway in regulating macrophage polarization, though they fail to clearly elucidate the impact of mTOR signaling in macrophage differentiation.

A potential implication of mTOR regulation of endothelial-leukocytic interactions may be the modulation of macrophage subsets. We conducted preliminary studies to investigate the underlying mechanisms of macrophage polarization as relates to mTOR signaling (preliminary data, not shown). Initial findings suggest that HLA I Ab-stimulated ECs moderate skewing of primary monocytes to an mDC phenotype. mTOR inhibition prevented this and maintained macrophages in an unpolarized M0 state. In order to confidently make a conclusion regarding the role of mTOR in macrophage polarization in AMR, we must first fully define the impact of HLA antibodies on macrophage differentiation and polarization, and then explore the contribution of the mTOR pathway.

Elucidating the impact of HLA antibodies on macrophage phenotype and activity could be used to modulate patients' immunosuppressive regiments accordingly.

5.4 The Future of mTOR Inhibitors in The Clinic

Our data highlight the importance of macrophages in the pathogenesis of AMR and the progression of rejection. We found that mTOR inhibitors may potentially moderate the presence of intragraft macrophages, reducing the extent of HLA I antibody-triggered injury. More investigation is required in the form of clinical trials to determine the impact of mTOR inhibitors on recipient monocytes and macrophages.

5.5 Conclusion

The importance of AMR in graft survival has become apparent in recent years as it is usually unresponsive to conventional therapies and remains a therapeutic challenge. The process of AMR is multifactorial, resulting from the integration of multiple mechanisms which are currently incompletely understood.

This project demonstrates the multifaceted network of signaling pathways that are activated following endothelial HLA class I ligation by antibody, and how mTOR signaling modulates monocyte recruitment in the setting of AMR. mTOR inhibitors used in the clinical setting potentially moderate the risk of rejection. Our results give a glimpse into the interconnected mechanisms of antibody-mediated injury that involve the mTOR pathway.

5.6 References

1. Farkas S, Hornung M, Sattler C, Guba M, Steinbauer M, Anthuber M, Herfarth H, Schlitt HJ, Geissler EK. Rapamycin decreases leukocyte migration in vivo and effectively reduces experimentally induced chronic colitis. *International Journal of Colorectal Disease*. 2006;21:747-753
2. Baetta R, Granata A, Canavesi M, Ferri N, Arnaboldi L, Bellosta S, Pfister P, Corsini A. Everolimus inhibits monocyte/macrophage migration in vitro and their accumulation in carotid lesions of cholesterol-fed rabbits. *Journal of Pharmacology and Experimental Therapeutics*. 2009;328:419-425
3. Martinet W, Verheye S, De Meyer GRY. Everolimus-induced rntor inhibition selectively depletes macrophages in atherosclerotic plaques by autophagy. *Autophagy*. 2007;3:241-244
4. Barocci S, Valente U, Nocera A. Detection and analysis of hla class i and class ii specific alloantibodies in the sera of dialysis recipients waiting for a renal retransplantation. *Clinical Transplantation*. 2007;21:47-56
5. Colvin RB. Antibody-mediated renal allograft rejection: Diagnosis and pathogenesis. *Journal of the American Society of Nephrology*. 2007;18:1046-1056
6. Iniotaki-Theodoraki A. The role of hla class i and class ii antibodies in renal transplantation. *Nephrology Dialysis Transplantation*. 2001;16:150-152
7. Jin YP, Reed EF. Signaling networks regulating hla class ii antibody-mediated activation and migration of endothelial cells. *Human Immunology*. 2013;74:46-46
8. Salehi S, Reed EF. The divergent roles of macrophages in solid organ transplantation. *Current Opinion in Organ Transplantation*. 2015;20:446-453

9. Byles V, Covarrubias AJ, Ben-Sahra I, Lamming DW, Sabatini DM, Manning BD, Horng T. The tsc-mtor pathway regulates macrophage polarization. *Nature Communications*. 2013;4:11

10. Zhu LN, Yang T, Li LJ, Sun LN, Hou YZ, Hu XL, Zhang LJ, Tian HL, Zhao QJ, Peng JX, Zhang HB, Wang RY, Yang ZZ, Zhang LF, Zhao Y. Tsc1 controls macrophage polarization to prevent inflammatory disease. *Nature Communications*. 2014;5:13

11. Xie LK, Sun F, Wang JX, Mao XO, Xie L, Yang SH, Su DM, Simpkins JW, Greenberg DA, Jin KL. Mtor signaling inhibition modulates macrophage/microglia-mediated neuroinflammation and secondary injury via regulatory t cells after focal ischemia. *Journal of Immunology*. 2014;192:6009-6019