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Outside-in HLA class I signaling regulates ICAM-1 clustering and endothelial-monocyte interactions via mTOR in transplant antibody-mediated rejection

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Abstract

Antibody-mediated rejection (AMR) resulting in transplant allograft vasculopathy (TAV) is the major obstacle for long-term survival of solid organ transplants. AMR is caused by donor-specific antibodies to HLA which contribute to TAV by initiating outside-in signaling transduction pathways that elicit monocyte recruitment to activated endothelium. Mechanistic target of rapamycin (mTOR) inhibitors can attenuate TAV; therefore, we sought to understand the mechanistic underpinnings of mTOR signaling in HLA class I Ab-mediated endothelial cell activation and monocyte recruitment. We used an *in vitro* model to assess monocyte binding to HLA I Ab-activated endothelial cells and found mTOR inhibition reduced Ezrin/Radixin/Moesin (ERM) phosphorylation, ICAM-1 clustering and monocyte firm adhesion to HLA I Ab-activated endothelium. Further, in a mouse model of AMR, in which B6.RAG1$^{-/-}$ recipients of BALB/c cardiac allografts were passively transferred with donor-specific MHC I antibodies, mTOR inhibition significantly reduced vascular injury, ERM phosphorylation, and macrophage infiltration of the allograft. Taken together, these studies indicate mTOR inhibition suppresses ERM phosphorylation in endothelial cells which impedes ICAM-1 clustering in response to HLA class I Ab, and prevents macrophage infiltration into cardiac allografts. These findings indicate a

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AUTHOR CONTRIBUTIONS

SS and RAS contributed equally to this work. SS, RAS, MF, ER, JWKW and EFR designed research studies. SS, RAS, YJ, SK, and MF conducted experiments and/or acquired data. SS, RAS, YJ, EFR and MF analyzed data. SS, RAS and EFR wrote the manuscript. MF, ER, and JWKW provided critical review of the manuscript.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

novel therapeutic application for mTOR inhibitors to disrupt endothelial-monocyte interactions during AMR.

INTRODUCTION

The development of AMR persists as a major issue limiting long-term patient and allograft survival (1). AMR is mediated by the binding of HLA antibody (HLA Ab) to the mismatched donor HLA class I (HLA I) and class II (HLA II) antigens expressed on the graft endothelium, resulting in microvascular inflammation and intravascular activated mononuclear cells, with or without complement deposition (2, 3). Chronic exposure of heart, kidney and lung allografts to HLA Ab can lead to transplant allograft vasculopathy (TAV) resulting in graft dysfunction, loss and patient death (1, 4–7). Disruption of HLA Ab-mediated microvascular inflammation and leukocyte recruitment may prevent TAV. Recent experimental transplant studies depleting natural killer cells (8) or macrophages (9) support this concept.

Ligation of HLA I molecules expressed by endothelial cells (ECs) induces outside-in signaling pathways eliciting cytoskeletal remodeling, proliferation and migration (10). HLA I Ab signaling also triggers rapid mobilization of Weibel-Palade bodies (WPBs), induction of P-selectin, and recruitment of leukocytes to the activated endothelium (11–13). Mechanistic target of rapamycin (mTOR) can promote EC-leukocyte interactions, suggesting immunotherapy with rapalogs may be beneficial in preventing graft injury mediated by AMR (14–18). mTOR inhibitors are widely used for cancer and atherosclerosis (19, 20); however, they are also used as an immunosuppressive agent to prevent rejection of solid organ transplants (21). A number of studies have demonstrated efficacy of mTOR inhibitors for attenuating cardiac TAV (22, 23), yet the underlying mechanism remains unclear.

Here, we investigated the role of mTOR in monocyte recruitment to HLA I Ab-activated ECs. Our data demonstrate mTOR signaling induces ICAM-1 clustering on the EC surface which stabilizes monocyte firm adhesion, critical for transendothelial migration under conditions of physiological shear flow. Accordingly, mTOR inhibition in a murine model suppressed monocyte infiltration into allografts undergoing AMR with a concomitant decrease in capillary EC phosphorylation of downstream proteins. Taken together, these studies provide much needed mechanistic insight into the function of clinical mTOR inhibitors and their potential use in treating AMR.

MATERIALS AND METHODS

Reagents

Pan-HLA class I mAb recognizing distinct monomorphic epitopes were obtained from BioXCell (W6/32; mIgG2a; West Lebanon, NH) or Abcam (MEM-147; mIgG1; Cambridge, MA). Sheep pAb to ICAM-1/CD54, integrin αVβ3 (anti-ITGB3; mIgG1), and P-selectin were from R&D (Minneapolis, MN). Sirolimus (Rapamycin; Rapa) was purchased from LC Laboratories (Woburn, MA). Everolimus (RAD001; RAD) was from Novartis Pharma AG

(Switzerland). siRNAs were designed as previously described (15) and from Dharmacon/GE Healthcare Life Sciences (Lafayette, CO). Bapta-AM, UO126, Ro-31-7549, Y27632 and rabbit pAb to mTOR, Raptor, and Rictor were from Calbiochem/EMD Millipore (Billerica, MA). Rabbit pAb to phosphorylated mTOR^{Ser2448}, S6K^{Thr389}, S6RP$^{Ser235/236}$, Akt^{Ser473}, p44/42 MAPK (ERK1/2$^{Thr202/Tyr204}$), MYPT1^{Thr696}, or Ezrin^{Thr567}/Radixin^{Thr564}/Moesin^{Thr558} (ERM) were from Cell Signaling Technology (Danvers, MA) and used as we previously described (14, 15, 24–27). Goat-anti-rabbit and anti-mouse IgG were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit IgG isotype control, horseradish peroxidase (HRP) goat-anti-rabbit IgG, and 3,3'-diaminobenzidine (DAB) HRP Substrate Kit was purchased from Vector Labs (Burlingame, CA). Vybrant™ CFDA-SE (CFSE) Cell Tracer Kit, donkey-anti-sheep IgG, Phalloidin and DAPI were from Invitrogen (Rockville, MD). Rabbit-anti-mouse C4d Ab was a gift from Dr. William Baldwin (Cleveland Clinic). Rat anti-mouse Mac2 was from Cedarlane (Hornby, Ontario, Canada). Prior to use, all antibodies were titered to determine optimal working concentrations and their specificity confirmed using appropriate positive and negative controls.

Human Cells

Use of all human cells was approved by the UCLA Institutional Review Board (IRB#10-001689; IRB#00-01-023). Primary ECs were isolated from aortic rings of deceased donor hearts or from Clonetics (EC5555; San Diego, CA) and cultured as previously described (16, 28, 29). The monocytic cell line Mono Mac 6 (MM6; a gift from Dr. Loems Ziegler-Heitbrock; Germany) was cultured as described (11). Primary human monocytes were enriched from the peripheral blood of multiple healthy volunteers using Ficoll-Paque (GE Healthcare) density centrifugation, followed by MACS pan-monocyte negative selection (Miltenyi) as we previously described (12) at an average final purity of 89.8% based on positive CD14 staining as determined by flow cytometry using an LSRFortessa (BD Biosciences). Experiments were performed with early-passage ECs from 2–3 different donors. Monocyte Fc interactions with ECs were inhibited by incubation with human IgG (12).

Monocyte static and shear-based adhesion assays

Adherence of monocytes to ECs under static conditions was measured as described (11, 12, 30). Briefly, ECs were pre-treated with 30 μg/ml 10 nM Rapa or 30 nM RAD (Figure S1) or 100nM siRNA (siRNA efficiency and specificity are shown in Figure S2), then stimulated with 1 μg/ml anti-ITGB3 or anti-HLA I for 30 min, or 1 U/ml thrombin for 4–6 h. Monocytes were fluorescently labeled with 2 mM CFSE, then added 3:1 to ECs for 20 min. Non-adherent cells were removed by washing. Images of adherent monocytes were acquired from 8–10 4× objective fields on a Nikon Eclipse Ti and quantified using Imaris 7.6.2 (Bitplane; Concord, MA).

Adherence of monocytes to ECs under laminar flow conditions was measured using a closed perfusion system (ibidi) (31). ECs (10^5 cells/cm^2) were seeded in gelatin-coated flow chamber slides (ibidi) and cultured for 24 h. ECs were pre-treated with Rapa for 24 h or 20 μg/mL anti-ICAM-1 for 30 mins. ECs were then stimulated as above. 6.25×10^5/mL CFSE-labeled monocytes were perfused over ECs at 1 dynes/cm^2. Data was acquired on a Nikon

Eclipse Ti and analyzed using ImarisTrack 7.6.2 (BitPlane; Concord, MA). Exported videos show individual monocytes as objects, with each track representing an individual monocyte's movement over the indicated time.

Flow cytometry and ELISA Assays

Cell surface expression of P-selectin was measured on adherent ECs by cell-based ELISA (11, 30, 31). Cell surface expression of ICAM-1, E-selectin or VCAM-1 was measured by flow cytometry on an LSRFortessa (BD Biosciences; San Jose, CA). ECs were detached using Accutase (Innovative Cell; San Diego, CA) to preserve epitopes (32). von Willebrand Factor was measured in culture supernatants by ELISA (Helena Laboratories; Beaumont, TX) (11, 30).

Western blots

EC lysates for Western blot analysis, with/without anti-ICAM-1 immunoprecipitation, were prepared as described (29) and quantified with Image J software (NIH; Bethesda, MD).

ICAM-1 localization and clustering

Following HLA I Ab stimulation and/or mTOR inhibition, ECs were incubated for 1 h with sheep-pAb to ICAM-1 then 30 min with donkey-anti-sheep IgG conjugated with AF594 before being fixed with 4% PFA. Fixed cells were counterstained with AF488-conjugated Phalloidin and DAPI. Cellular localization and clustering of ICAM-1 expression on ECs was quantified using three-dimensional analysis of confocal laser scanning microscopy (CLSM) images (33, 34). Images were acquired on a TCS-SP5 inverted Acousto Optical Beam Splitter (AOBS) confocal microscope (Leica) using LAS X 1.9 (Leica Biosystems, Vista, CA). Three-dimensional z-stacks were analyzed for ICAM-1 expression using Imaris 8.1 by creating a discrete quantitative "spot" objects layer from ICAM-1 labeling data in the red channel. ICAM-1 clustering was evaluated by counting the number of spots per cell, and measuring their three-dimensional sizes in voxels (volumetric pixels).

Murine Heterotopic heart transplantation

Male B6.129S7-Rag1^{tm1Mom} (B6.RAG1$^{-/-}$, H-2^{b}) and BALB/c (H-2^{d}) mice aged 7–10 weeks old were obtained from The Jackson Laboratory (Bar Harbor, ME) and housed under pathogen-free conditions in the Division of Laboratory Animals at UCLA. BALB/c hearts were heterotopically transplanted into B6.RAG1$^{-/-}$ recipients as we previously described (11). Beginning day 3 post-transplant, 30 μg/kg of donor-specific anti-MHC I (anti-H-2Kd, SF1-1.1; or anti-H-2Dd, 34-2-12) or isotype control (mIgG2a, MOPC-173) from Biolegend (San Diego, CA) were injected biweekly by tail vein. Indicated mice received intraperitoneal injection of 1 mg/kg/day Rapa. Graft function was monitored by abdominal palpation daily.

Immunohistochemistry and histopathological grading

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 at 5 μm before H&E and IHC staining was performed as we previously described for Mac-2 and C, or p-ERK, p-AKT and p-S6K (14, 15, 24–27). IHC staining for p-ERM was conducted as

follows. Sections were rehydrated and antigen was recovered in a steamer with citrate buffer (pH 6.0) before endogenous peroxide activity was quenched with 3% hydrogen peroxide. Sections were blocked with normal goat serum in PBS, incubated with rabbit pAb to ERM, or isotype control overnight at 4°C then HRP goat anti-rabbit IgG for 40 min at room temperature before being developed with DAB and counterstained with hematoxylin. Staining was scored by a single blinded pathologist (35). The presence of Mac2-positive intravascular activated mononuclear cells, or positive EC staining in capillaries and intramyocardial arteries/veins for C4d or phosphorylated proteins were scored on a scale of 0–3 (0, no staining; 1, focal staining; 2, multifocal staining; 3, diffuse staining).

Statistical Analysis

Differences between groups were determined using one or two-way ANOVA using Prism 5 (GraphPad). P-values of <0.05 were considered statistically significant.

RESULTS

mTOR inhibition in ECs significantly reduces HLA I Ab-induced monocyte recruitment

We characterized the capacity of two different FDA-approved pharmacologic mTOR inhibitors, sirolimus (Rapamycin; Rapa), and everolimus (RAD001; RAD), to halt recruitment of monocytes to HLA I Ab-activated human aortic ECs. Stimulation of ECs with HLA I Ab increased recruitment of the human monocytic cell line MonoMac6 (MM6 cells) to a level comparable to that induced by thrombin (Figure 1A, **left panel**). In contrast, treating ECs with control IgG (not shown) or Ab against ITGβ3 failed to evoke monocyte adhesion. Similar effects were observed using PBMC-derived monocytes freshly isolated from third-party healthy donors (Figure 1A, **right panel**). Pre-treatment of ECs with Rapa completely abolished enhanced monocyte recruitment to HLA I Ab-activated EC (Figure 1B, 1C), comparable to ECs pre-treated with the calcium-chelator (11) BAPTA-AM. Again, we observed similar results between propagated and PBMC-derived (Figure 1B, **left** vs **right panel**) monocytes. Collectively, these data indicate mTOR is necessary for HLA I Ab-induced monocyte binding to activated ECs.

mTOR can complex with regulatory-associated protein of mTOR (Raptor) to form mTOR complex 1 (mTORC1) or rapamycin-insensitive companion of mTOR (Rictor) to form mTOR complex 2 (mTORC2), with differing downstream effects. We used two complementary approaches to investigate mTOR complex involvement in HLA I Ab-mediated monocyte recruitment to ECs (Figure 1D, E). First, we treated ECs with short-term Rapa or RAD for 2 h prior to stimulation with HLA I Ab to determine the contribution of mTORC1 protein complexes to monocyte recruitment, as rapalogs require >6 h to inhibit mTORC2 formation, but inhibit mTORC1 formation rapidly (15, 16). Adherence of MM6 cells was significantly attenuated with 2 h Rapa or RAD pre-treatment of ECs (Figure 1D), although not as completely as 24 h pre-treatment to block formation of both complexes (Figure 1B). Second, we used siRNA to knockdown Raptor, Rictor or mTOR expression in ECs prior to their stimulation with HLA I Ab (Figure 1E). High efficiency and specificity of mTOR (>99%), Raptor (>85%) and Rictor (>80%) protein depletion was achieved by siRNA-mediated knockdown (Figure S2). Although silencing of either mTORC1 or

mTORC2 individually suppressed adherence of MM6 cells to near baseline levels, simultaneous depletion of mTORC1 and mTORC2 via mTOR siRNA resulted in its complete suppression (Figure 1E), suggesting a synergistic interaction between mTORC1 and mTORC2 in this process. These results indicate monocyte recruitment to HLA I Ab-activated ECs is regulated via both mTORC1- and mTORC2-dependent pathways.

Additionally, results obtained using HLA I Ab directed against a different monomorphic epitope on HLA I (clone MEM-147) were comparable to those obtained with clone W6/32 (Figure S3).

In vivo blockade of mTOR ameliorates MHC class I Ab-mediated AMR

We then tested the requirement for mTOR in monocyte recruitment to HLA I Ab-activated endothelium *in vivo* using a clinically relevant murine model (11). B6.RAG1$^{-/-}$ mice (H-2^{b}) received a complete mismatch Balb/c (H-2^{d}) heterotopic heart transplant and were passively transferred with anti-donor MHC class I Ab biweekly for 30 days (Figure 2A). H&E and Mac2 staining confirmed MHC I Ab transfer resulted in EC swelling with associated intravascular activated mononuclear cells in the allograft that were significantly reduced in Rapa-treated animals to levels similar to isotype control-treated mice (Figure 2B, 2C). As expected, Rapa did not inhibit MHC I Ab-mediated activation of the complement cascade. These data are consistent with the *in vitro* experimental findings, and indicate administration of mTOR inhibitors *in vivo* causes a reduction in HLA I Ab-induced monocyte recruitment to the allograft.

mTOR regulates ICAM-1-induced firm adhesion of monocytes to HLA I Ab stimulated ECs

We next sought to characterize the mechanism by which mTOR regulates HLA I Ab-mediated monocyte adherence. To investigate the potential impact of mTOR inhibition on HLA I Ab-induced WPb exocytosis, we measured endothelial P-selectin surface expression and von Willebrand Factor (vWF) release (Figure 3). Stimulation of ECs with thrombin or HLA I mAb induced rapid WPb exocytosis as demonstrated by P-selectin expression and vWF release (Figure 3A), while exposure to anti-ITGβ3 did not. Pre-treatment of ECs with Rapa or RAD for 24 h (Figure 3B) or 2 h (Figure S4A) had no effect on HLA I Ab-induced WPb mobilization, as neither P-selectin expression nor vWF secretion were affected (Figures 3B, S4A) like they were upon pre-treatment with BAPTA-AM.

Rapa is known to directly inhibit protein synthesis (36); however, we did not find any significant changes in the basal surface expression levels of key molecules in the leukocyte adhesion cascade (37), including ICAM-1 (Figure 3C), E-Selectin or VCAM-1 (Figure S4B) upon activating ECs with HLA I Ab, with or without Rapa pre-treatment.

Since expression levels of adhesion molecules were not altered, we chose to investigate the effects of mTOR inhibition on the adhesive function of the endothelium under physiological shear flow conditions (Figure 3D, Videos S1–3). When we perfused MM6 cells over HLA I Ab-activated endothelial monolayers, we observed increased adhesion compared to untreated control ECs (Figure 3D, **left panel** and Videos S1–2). However, the percent of firmly adherent MM6 cells was significantly decreased when ECs were pre-treated with

Rapa compared to no inhibitor treatment (Figure 3D, **left panel** and Videos S2–3). The capacity of Rapa to inhibit firm adhesion of MM6 cells was comparable to treatment of ECs with ICAM-1 neutralizing Ab. Further stratification of MM6 cells by their rolling speed confirmed no difference in monocytes not directly interacting with the ECs (Figure 3D, **right panel**). We observed a significant decrease in the percentage of firmly adherent MM6 cells (speed 0–2 μm/s) between ECs with no inhibitor treatment (18%) and Rapa-treated ECs (5.3%) with a corresponding increase in the percentage of slow rolling MM6 cells (speed 2–10 μm/s) for these groups (9.1% vs 25.1% respectively). These data indicate the proportion of monocytes that would normally firmly adhere to HLA I Ab-stimulated ECs are no longer able to do so in the presence of mTOR inhibition, and instead continue to slowly roll along the endothelium, implicating a role for mTOR in regulating monocyte firm adhesion to ICAM-1 on ECs following HLA I Ab stimulation.

mTOR inhibition in HLA I Ab-stimulated EC impairs ICAM-1 clustering

ICAM-1 clusters at the apical membrane of ECs and promotes leukocyte firm adhesion by forming a “ring” or “cup” around the adhered leukocyte (37–39). As this clustering event is essential for firm adherence, we questioned if mTOR inhibition disables ICAM-1 clustering. We first examined the capacity of HLA I Ab-stimulation to mediate ICAM-1 clustering by imaging ECs using super-resolution confocal immunofluorescence microscopy. Surface expression levels of ICAM-1 were not altered after HLA I Ab ligation (Figure 3C). Therefore, we measured ICAM-1 aggregation with the Imaris spot detection algorithm. In ECs that were untreated, or treated with negative control ITGβ3 Ab, ICAM-1 was distributed diffusely, with a large number of small sized spots (Figure 4A, 4B). Treatment with HLA I Ab triggered dramatic ICAM-1 clustering into a significantly smaller number of spots with a significantly larger size at the margins of ECs, similar to ECs treated with positive control thrombin (Figure 4A, 4B). Pre-treatment of ECs with Rapa resulted in a diffuse scattering of small-sized ICAM-1 spots distributed throughout the cell surface similar to untreated control ECs (Figure 4). These findings indicate HLA I Ab-mediated mTOR activation regulates ICAM-1 clustering, promoting monocyte firm adhesion to the endothelium.

HLA I Ab-induced ERM phosphorylation is impaired by mTOR inhibition in ECs

ERM complex activation is required for ICAM-1 clustering and association with the actin cytoskeleton (40); therefore, we investigated the effect of mTOR inhibition on ERM signaling. Stimulation of ECs with HLA I Ab induced a 3-fold increase in the levels of phosphorylated ERM compared to control ECs (Figure 5A). HLA I Ab-ligation on ECs pre-treated with rapalogs for 24 h decreased phosphorylation of ERM (Figure 5A), suggesting ERM phosphorylation depends on mTOR. As a control, we verified pre-treatment of ECs with Rapa or RAD inhibited HLA I Ab-induced phosphorylation of mTOR, p70S6K, Akt, ERK, and S6RP (Figure S5A), known downstream targets of the HLA class I outside-in signaling pathway (16).

Using siRNA to investigate the independent contribution of mTOR complexes in HLA I Ab-mediated ERM phosphorylation (Figure 5B), we found HLA I Ab-induced ERM phosphorylation in ECs was reduced 3-fold following Raptor (mTORC1) and 2.8-fold after

Rictor (mTORC2) knockdown. Complete inhibition was achieved upon mTOR knockdown. We have verified that pre-treatment of ECs with Rapa or RAD inhibited HLA I-induced phosphorylation of known downstream targets of the HLA class I outside-in signaling pathway (15, 26). These experiments substantiate the synergistic role for both mTORC1 and mTORC2 in the phosphorylation of ERM.

Co-immunoprecipitation with anti-human ICAM-1 showed stimulation of ECs with HLA I Ab increased complex formation between ICAM-1 and phosphorylated ERM by 2.2-fold compared to control (Figure 5C). Pre-treatment of ECs with either mTOR inhibitor significantly reduced complex formation between ICAM-1 and phosphorylated ERM, supporting our hypothesis that mTOR signaling downstream of HLA class I engagement with Ab regulates ERM phosphorylation and physical association with ICAM-1.

mTOR regulates HLA I Ab-induced ERM phosphorylation through the Rho pathway

EC activation leads to ERM phosphorylation through the GTP-binding protein RhoA (39, 41). Rho signals through the ROCK and PKC pathways resulting in an accumulation of phosphorylated ERM (42, 43). To examine the signaling relationships between Rho and mTOR following HLA class I ligation, we used pharmacological inhibitors targeting PKC (Ro-31-7549), ROCK (Y-27632), and Rho (C3 transferase) (Figure 6). Rho activation was determined by quantitating levels of phosphorylated myosin light chain (MLC) and myosin phosphatase target subunit 1 (MYPT1), as myosin phosphatase has been shown to regulate actin:myosin interactions (44, 45) and they are both downstream of Rho signaling. HLA I Ab-stimulation of ECs caused a significant increase in levels of phosphorylated MYPT1 and MLC compared to ECs stimulated with isotype control Ab (not shown) or untreated (Figure 6A, 6B) that was reduced to baseline levels by mTOR inhibition with Rapa or RAD, situating the mTOR pathway upstream of Rho signaling. As previously reported, rapalogs blocked HLA I Ab-mediated phosphorylation of downstream mediators of the mTOR pathway including mTOR, p70S6K, Akt, ERK and S6RP (Figure S6) (15). Inhibition of the Rho pathway via indirect ROCK inhibition or direct Rho inhibition blocked HLA I Ab-induced phosphorylation of MYPT1, MLC and ERM (Figure 6A, 6B); however, we saw no effect on inhibition of the mTOR pathway, with no changes in Akt^{473} or $p70S6K^{389}$ (Figure S6). Pre-treatment of ECs with pharmacological inhibitors targeting Rho or ROCK, significantly reduced adherence of monocytes to HLA I Ab-activated endothelium (Figure S7). However, PKC inhibition had no effect on levels of phosphorylated target proteins or monocyte adherence (Figure 6A, 6B, S6 and S7). Collectively, these data indicate that following HLA class I engagement by Ab, mTOR signals through the Rho pathway via ROCK to mediate ERM phosphorylation and subsequent monocyte adhesion to ECs.

Blockade of mTOR prevents MHC class I Ab-activation of endothelial ERM phosphorylation in murine cardiac allografts during AMR

Our *in vitro* findings indicated that inhibiting mTOR signaling prevents HLA class I Ab-mediated ICAM-1 clustering in ECs by inhibiting ERM phosphorylation. Therefore we evaluated the effect of Rapa on MHC I Ab-induced phosphorylation of proteins in the ERM pathway in our murine model of cardiac AMR. Mice treated with anti-donor MHC class I Ab demonstrated diffuse capillary staining of phosphorylated ERK, AKT, S6K and ERM

(Figure 7) compared to control mAb-treated groups. In the Rapa + MHC I Ab-treated group, expression of all four phosphorylated proteins was significantly diminished. These results provide evidence that mTOR regulates ERM protein activation *in vivo* similar to the regulation we observed in our *in vitro* studies.

DISCUSSION

Our results provide the first evidence that ligation of HLA I Ab on ECs leads to ICAM-1 clustering and monocyte firm adhesion through an mTOR/ERM-dependent signaling pathway. Both mTORC1 and mTORC2 were involved in mTOR-mediated monocyte adherence to HLA I Ab-activated ECs. Inhibiting mTOR diminished monocyte adherence *in vitro* as well as monocyte recruitment and allograft injury in an *in vivo* model of cardiac AMR, supporting the clinical relevance of these findings.

Leukocyte adhesion to the activated endothelium is tightly regulated by the selectin family of molecules. E- and P-selectin mediate the initial steps of monocyte tethering and adhesion to ECs, whereas ICAM-1 and VCAM-1 are needed for firm adhesion (37). Since previous studies showed crosslinking HLA I on ECs by Ab induces P-selectin expression and monocyte capture (13, 30), we reasoned that mTOR inhibition might affect this process. However, while Rapa inhibits VCAM-1 expression by TNFα-activated ECs (46), neither Rapa nor RAD altered protein expression of VCAM-1, E-selectin, P-selectin, or ICAM-1 in HLA I Ab-activated ECs. Instead, mTOR inhibition abrogated ICAM-1 translocation to the site of leukocytes, which is consistent with previous studies (47).

Phosphorylation of ERM causes a conformational change allowing it to bind ICAM-1's cytoplasmic tail at its N-terminus and the actin cytoskeleton at its C-terminus, forming a physical bridge which mediates clustering of ICAM-1 on the surface of the cell (38, 47, 48). Here, we provide several lines of evidence that HLA I Ab-mediated ICAM-1 clustering is due to upstream mTOR regulation of ERM phosphorylation. First, mTOR inhibition or protein depletion of Raptor or Rictor in HLA I Ab-stimulated ECs disrupts physical complex formation between phosphorylated ERM and ICAM-1. Second, mTOR inhibition led to a significant reduction in the expression of phosphorylated ERM. Finally, in our model of antibody-mediated cardiac transplant injury, we show a dramatic reduction in phosphorylated ERM accompanied by a significant decrease in intravascular activated mononuclear cells within the capillaries of heart allografts from mice treated with Rapa, consistent with *in vitro* studies.

The GTP-binding protein Rho regulates actin cytoskeletal remodeling and the formation of cell-cell and cell-substratum adhesion (49). Previous observations that ICAM-1 associates with components of the actin cytoskeleton, together with our studies showing HLA I Ab induces MLC phosphorylation to promote stress fiber formation via Rho kinase (50), suggested likely involvement of Rho family proteins in HLA I Ab-induced ERM phosphorylation. Furthermore, HLA I Ab-ligation promotes hydrolysis of phosphatidylinositol bisphosphate (PIP_2) to inositol (1,4,5)-triphosphate (IP_3) (51), a requirement for activation of the ERM proteins (52). Notably, RhoA plays an important role

in monocyte adhesion to TNFα-activated human ECs (39) and EC activation can lead to Rho-induced ERM phosphorylation (38, 39).

Here, we show that Rho is required for HLA I Ab-mediated phosphorylation of ERM. While Rho or Rho kinase inhibition prevented HLA I-induced ERM phosphorylation, it had no effect on HLA I Ab-induced activation of the mTOR signaling, indicating mTOR is upstream of Rho. RhoA activity has been reported to be dependent on both mTORC1 and mTORC2, which mediate its expression and GTPase activity, respectively (39, 53). HLA I Ab-ligation prompted Rho activation, but also stimulated MLC/MYPT1 phosphorylation and subsequent stress fiber formation in an mTOR-dependent manner. Given its dual role in regulating ERM phosphorylation and actin stress fiber formation, it was unsurprising Rho inhibition dramatically reduced monocyte binding to HLA I Ab-activated endothelium.

Macrophages profoundly influence various aspects of transplantation, representing important effector cells in the process of antibody-mediated rejection (4, 54). Our findings are consistent with a model in which HLA class I Ab/mTOR-dependent outside-in signaling mechanisms trigger clustering of endothelial ICAM-1 into a membrane structure that provides a platform for stable monocyte firm arrest (Figure 8). Class I mediated activation of mTOR signals through the Rho pathway to mediate ERM phosphorylation. mTOR inhibition in HLA class I antibody-stimulated ECs prevents HLA I antibody-mediated p-ERM and disrupts the association of p-ERM with ICAM-1. Although rapalogs are commonly used as a component of maintenance immunosuppressive therapy, they are primarily credited with directly suppressing T cell activation; however, our findings demonstrate a novel mechanism of suppressing monocyte recruitment to the HLA Ab-activated endothelium during AMR. mTOR inhibitors have therapeutic benefit in reducing the incidence and progression of TAV in cardiac recipients (21–23); therefore, targeting the mTOR pathway may alleviate vascular injury in the setting of AMR by reducing microvascular inflammation. We did not characterize the effect of rapamycin on the thrombin inflammatory pathway that can also mediate monocyte recruitment during AMR via PKC signaling (55). Given the observation of thrombotic microangiopathy in early kidney AMR (56), and the role of thrombin in amplifying the production of monocyte chemoattractant protein-1 (57) it would be important to determine whether or not rapamycin therapy attenuates PKC signaling. It is known that mTORC2 phosphorylates PKCs, as it phosphorylates AKT. Based on previously reported data (58–60), we would argue that long-term treatment with rapamycin is likely to affect PKCs. However, it remains to be tested in primary human endothelial cells. In addition, mTOR's regulation of monocyte:EC adhesive interactions are likely important in other immune and inflammatory disorders including atherosclerosis and cancer. As such, the signaling pathways we identified here may be broadly applicable to other inflammatory diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

Ab	Antibody
AMR	Antibody-mediated rejection
ECs	Endothelial cells
ERM	Ezrin/Radixin/Moesin
HLA class I antigens	HLA-A, B, C antigens
ICAM-1	Intercellular adhesion molecule 1
MLC	Myosin light chain
mTOR	Mechanistic Target of Rapamycin
mTORC1	mTOR complex I
mTORC2	mTOR complex II
MYPT1	myosin phosphatase target subunit 1
Rapa	Rapamycin
TAV	Transplant allograft vasculopathy
VCAM-1	vascular cell adhesion protein 1
vWF	von Willebrand Factor
WPBs	Weibel Palade Bodies

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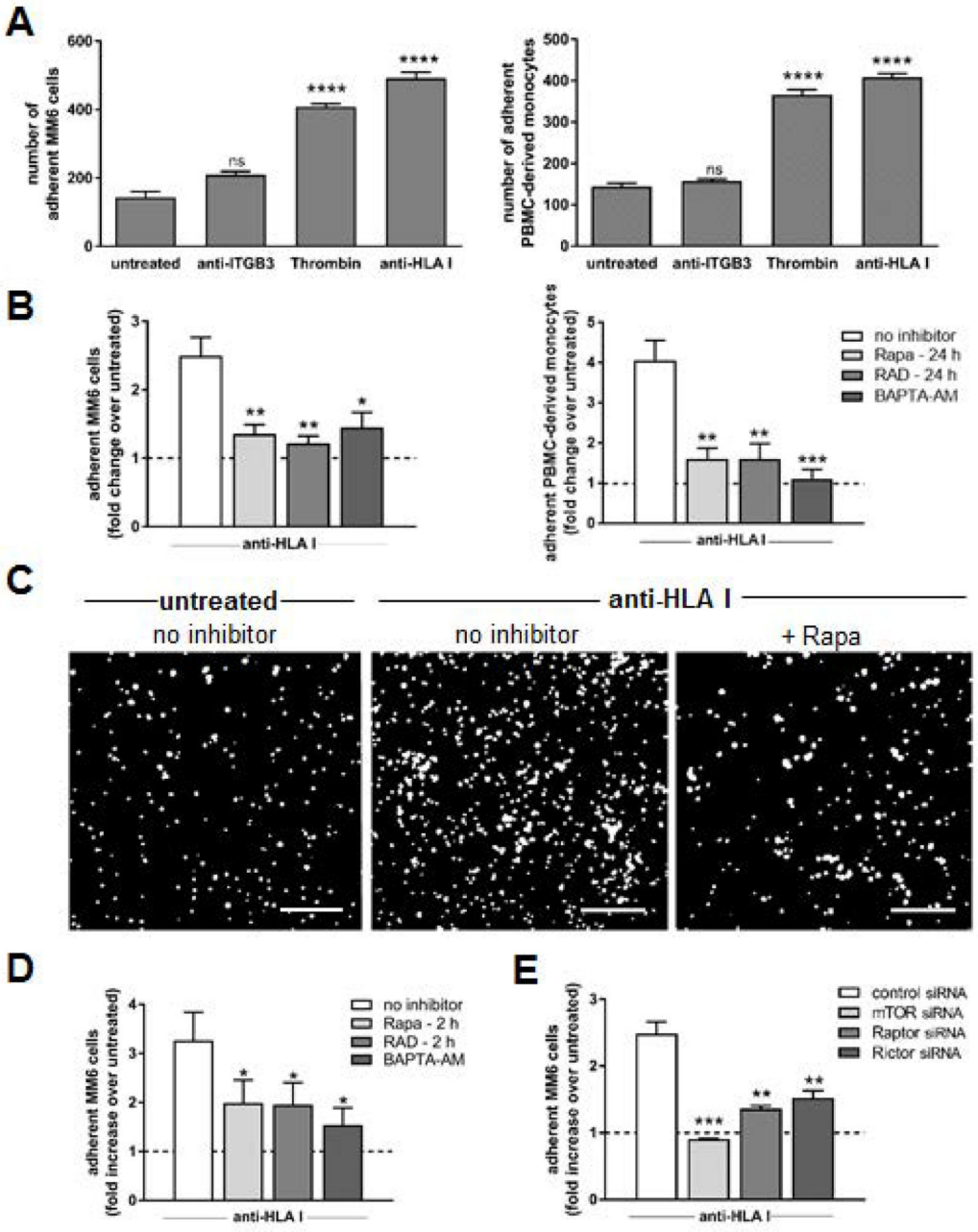

Figure 1. Monocyte adherence to HLA class I Ab-stimulated endothelium is suppressed when the mTOR pathway is inhibited in ECs

Primary endothelial cells (ECs) were stimulated with Ab against Integrin β3 (anti-ITGB3), HLA class I (anti-HLA I; clone W6/32) or thrombin for 5 min. CFSE-labeled MonoMac6 (MM6 cells) or human monocytes enriched from third-party peripheral blood (PBMC-derived monocytes) were pre-treated with polyclonal human IgG to block FcγR interactions, then allowed to adhere to ECs for 20 min, then un-adhered monocytes were washed off and adherent monocytes in 8–10 fields were imaged and quantified. (A) Shown are mean numbers of MM6 cells (left panel) or PBMC-derived monocytes (right panel) adherent to treated ECs over untreated ECs ± SEM from five independent experiments. ****P<0.0001,

ns=not significant when comparing treated ECs to untreated ECs by two-way ANOVA with Tukey's multiple comparisons test (B) ECs were pre-treated with Rapa, RAD or no inhibitor for 24 h, or BAPTA-AM for 30 min before monocytes were allowed to adhere. Shown are fold changes in mean number of MM6 cells (left panel) or PBMC-derived monocytes (right panel) adherent to treated ECs over untreated control ECs (dotted line) ± SEM from five independent experiments. *P<0.05, **P<0.01, ***P<0.001 for ECs given the indicated inhibitor to ECs given no inhibitor by two-way ANOVA with Tukey's multiple comparisons test (C) Representative images showing CFSE-labeled MM6 cells adherent to the endothelial monolayer following indicated treatment. scale bars = 100 μm (D) ECs were pre-treated with no inhibitor, Rapa or RAD for 2 h (targets mTORC1 or 2 respectively), or BAPTA-AM for 30 min before MM6 cells were allowed to adhere. Shown are fold changes in mean numbers of MM6 cells adherent to treated ECs over untreated control ECs (dotted line) ± SEM from five independent experiments. *P<0.05 for ECs given the indicated inhibitor to ECs given no inhibitor by two-way ANOVA with Tukey's multiple comparisons test (E) ECs were pre-treated with control siRNA or siRNA directed against mTOR, Raptor (mTORC1), or Rictor (mTORC2) before MM6 cells were allowed to adhere. Shown are fold changes in numbers of MM6 cells adherent to treated ECs over untreated control ECs (dotted line) ± SEM from five independent experiments. **P<0.01, ***P<0.001 for ECs given the indicated siRNA to ECs given no siRNA by two-way ANOVA with Tukey's multiple comparisons test

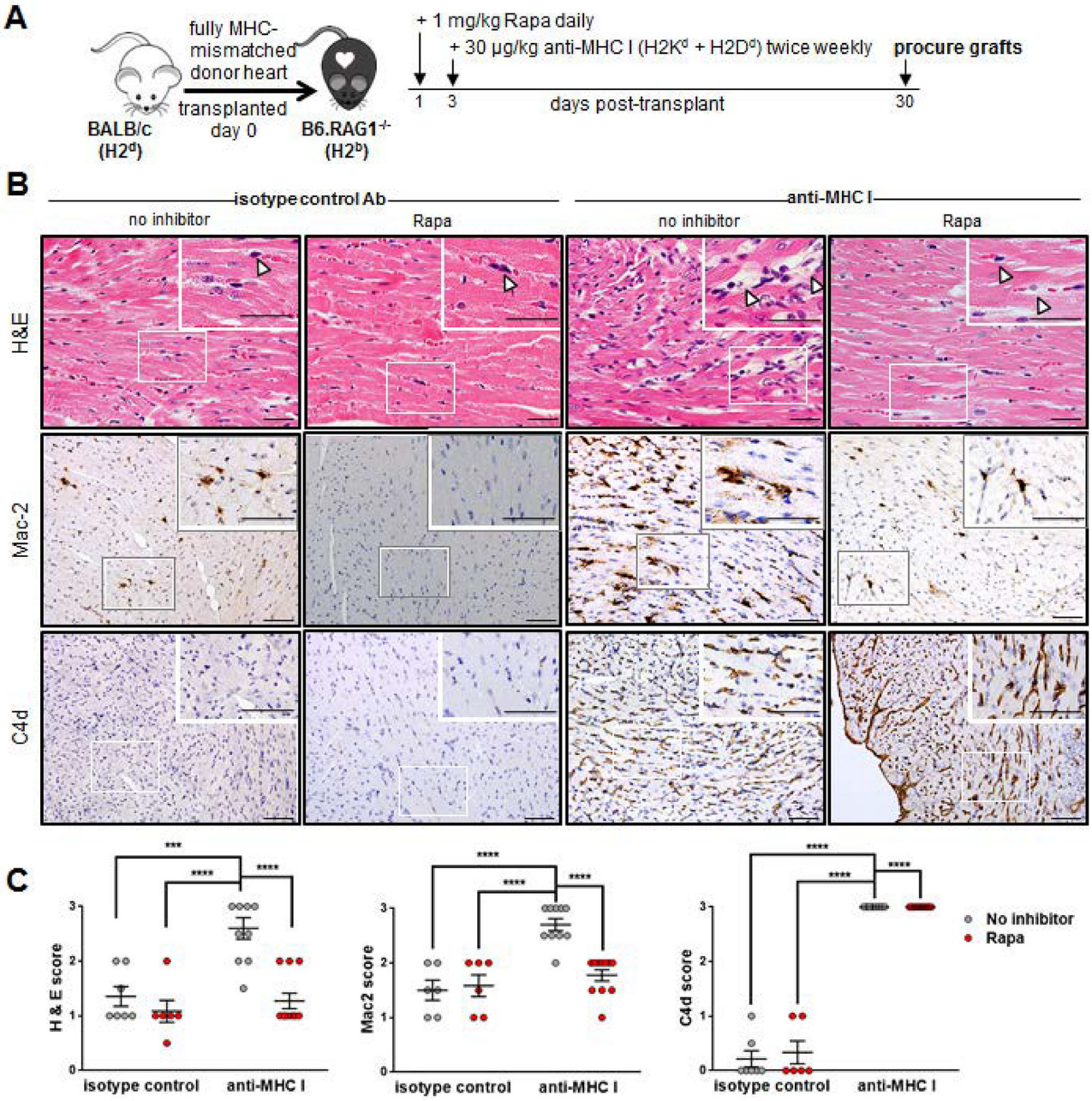

Figure 2. Rapamycin treatment ameliorates acute injury by blocking monocyte recruitment in a murine model of cardiac antibody mediated rejection

(A) BALB/c cardiac allografts were transplanted into B6.RAG1$^{-/-}$ recipients and passively transfused with 30 µg/kg anti-MHC I (anti-H2K^{d} + anti-H2D^{d}) mAb or isotype control mAb biweekly beginning 3 days post-transplant. Rapa treatment at 1mg/kg/daily was initiated 1 day post-transplant. (B) Grafts were procured at day 30 post-transplant and evaluated for microvascular abnormalities by H&E, as well as intravascular activated mononuclear cells and/or complement deposition by immunohistochemical staining for MAC2 or C4d respectively. Arrow heads in top row indicate prominent nuclei of cells in distended capillaries of anti-MHC I-treated animals that was not seen in the anti-MHC I group treated

with Rapa, or either group that received control mIgG mAb where arrow heads indicate flat, thin nuclei in collapsed capillaries. scale bars = 50 μm (C) Shown are mean scores ± SEM for n≥6 per group with each dot representing one animal. *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001 by two-way ANOVA with Tukey's multiple comparisons test.

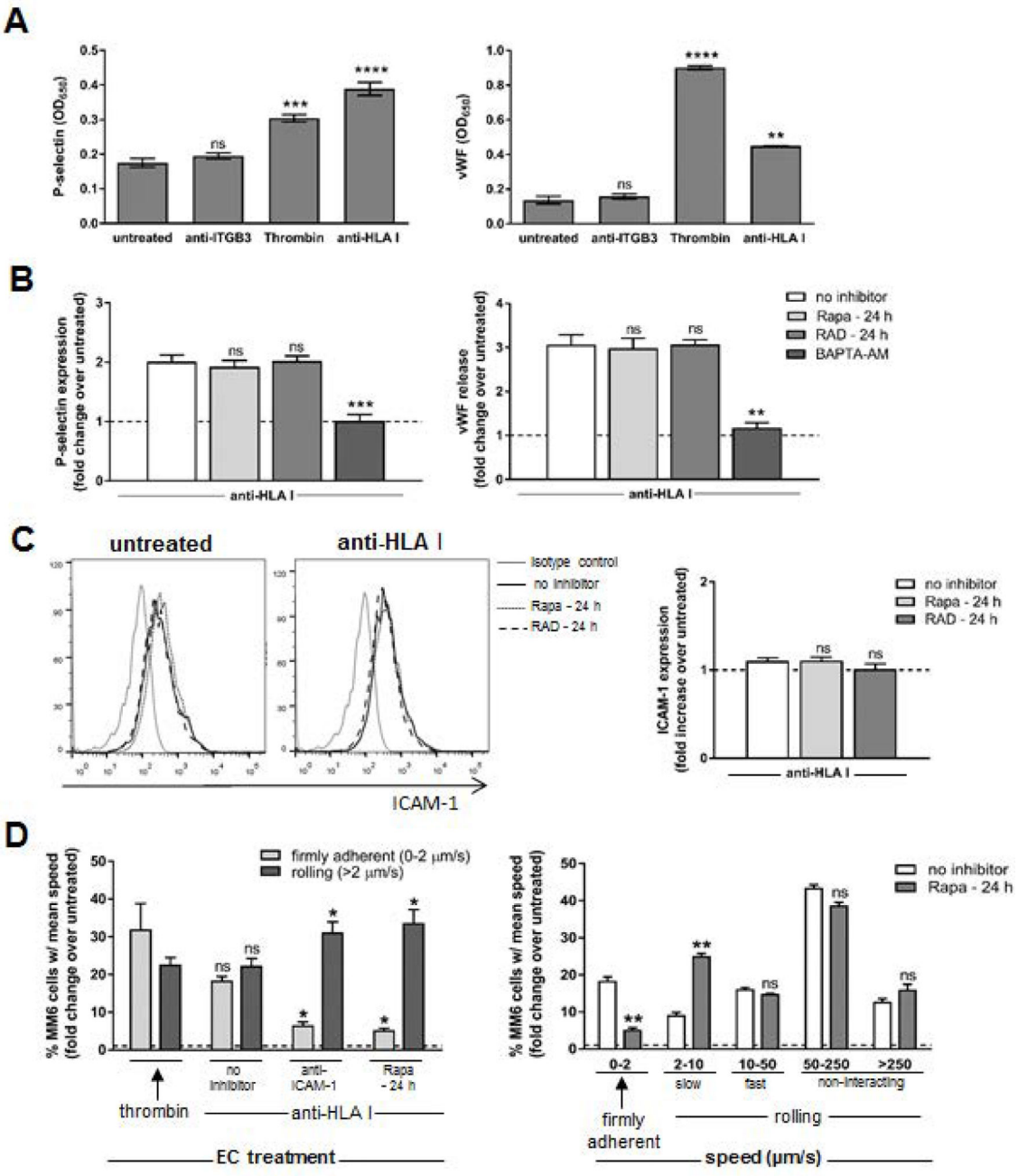

Figure 3. mTOR regulates ICAM-1-induced firm adhesion of monocytes to HLA class I Ab-stimulated ECs

Primary endothelial cells (ECs) were pre-treated with Rapa, RAD or no inhibitor for 24 h, or BAPTA-AM for 30 min before they were stimulated with Ab against Integrin β3 (anti-ITGB3), HLA I (anti-HLA I; clone W6/32) or thrombin for 5 min. **(A)** EC P-selectin expression was detected by cell-based ELISA, and EC-release of von Willebrand Factor (vWF) was assessed by ELISA of supernatant. Shown are the average optical density values (OD_{650}) ± SEM from 5 independent experiments. ***P<0.001, ****P<0.0001, ns=not significant when comparing treated ECs to untreated ECs by two-way ANOVA with Tukey's multiple comparisons test **(B)** ECs were pre-treated with no inhibitor, Rapa or RAD for 24 h,

or BAPTA-AM for 30 min. EC P-selectin expression was detected by cell-based ELISA, and EC-release of vWF was assessed by ELISA of supernatant. Shown are the mean fold change of protein expression from treated ECs over untreated ECs (dotted line) ± SEM from 5 independent experiments. ***P<0.001, ns=not significant when comparing ECs given the indicated inhibitor to ECs given no inhibitor by two-way ANOVA with Tukey's multiple comparisons test **(C)** ICAM-1 expression was detected by flow cytometry. Shown is representative histograms and mean fold increase in ICAM-1 positive cells in each condition over untreated cells (dotted line on graph) from 2 independent experiments. ns=not significant when comparing ECs given the indicated inhibitor to ECs given no inhibitor by one-way ANOVA with Tukey's multiple comparisons test **(D)** MM6 cells were pre-treated with polyclonal human IgG to block FcγR interactions, perfused at 1 dynes/cm^2 over an endothelial monolayer pre-treated with Rapa for 24 h, then anti-ICAM-1 neutralizing Ab for 30 min then stimulated with anti-HLA I for 30 min. Three 5–10s videos (Videos S1–3) were collected in real-time for each condition, and mean velocity (mm/sec) of each MM6 cell over video duration was determined using ImarisTrack. Shown are average percentages of total MM6 cells observed with each indicated speed ± SEM from 5 independent experiments *P<0.05, ns=not significant by two-way ANOVA with Tukey's multiple comparisons test (left panel) or **P<0.01; ***P<0.001, when comparing ECs treated with Rapa to ECs left untreated by Student's T test (right panel).

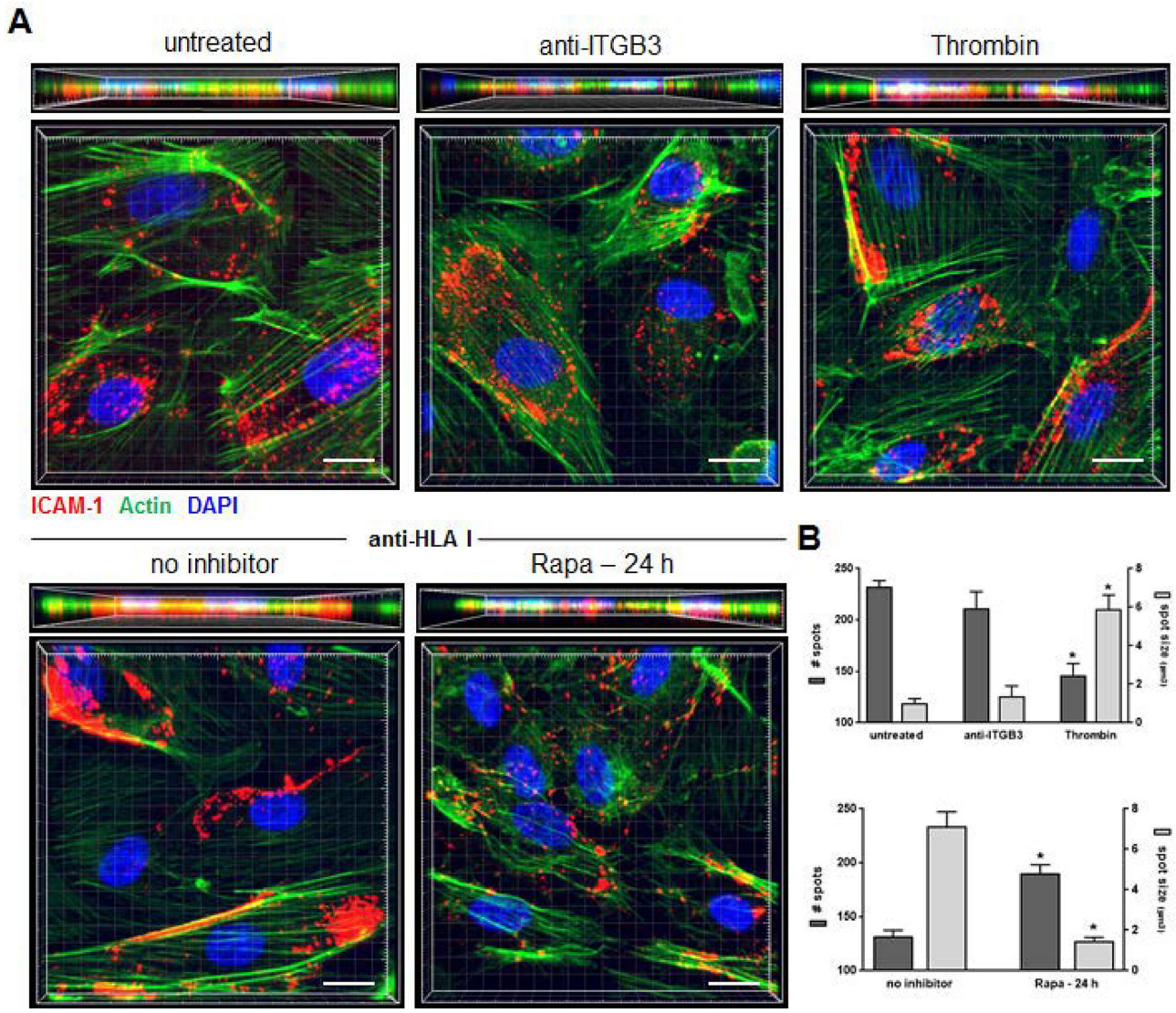

Figure 4. mTOR is required for ICAM-1 clustering in HLA class I Ab-activated ECs

Primary endothelial cells (ECs) were pre-treated with Rapa or no inhibitor for 24 h, before they were stimulated with Ab against Integrin β3 (anti-ITGB3), HLA I (anti-HLA I; clone W6/32) or thrombin for 5 min, then CFSE-labeled MM6 cells were allowed to adhere for 20 min. ECs were stained by immunofluorescence for ICAM-1 (red), as well as Phalloidin for actin filaments (green) and DAPI to detect cell nuclei (blue). (A) Shown are representative three-dimensional volumetric confocal images of ECs following indicated treatments. scale bars=10 μm. (B) Imaris 3D analysis software was used to quantify ICAM-1 clustering. Shown are the number of individual spots per cell detected in the red channel as well as their size, and results are expressed as the mean number of spots or mean three-dimensional size (μm^3) ± SEM of each treated group over untreated ECs (n≥30 cells per group). *P<0.05 by unpaired two-tailed Student's t test.

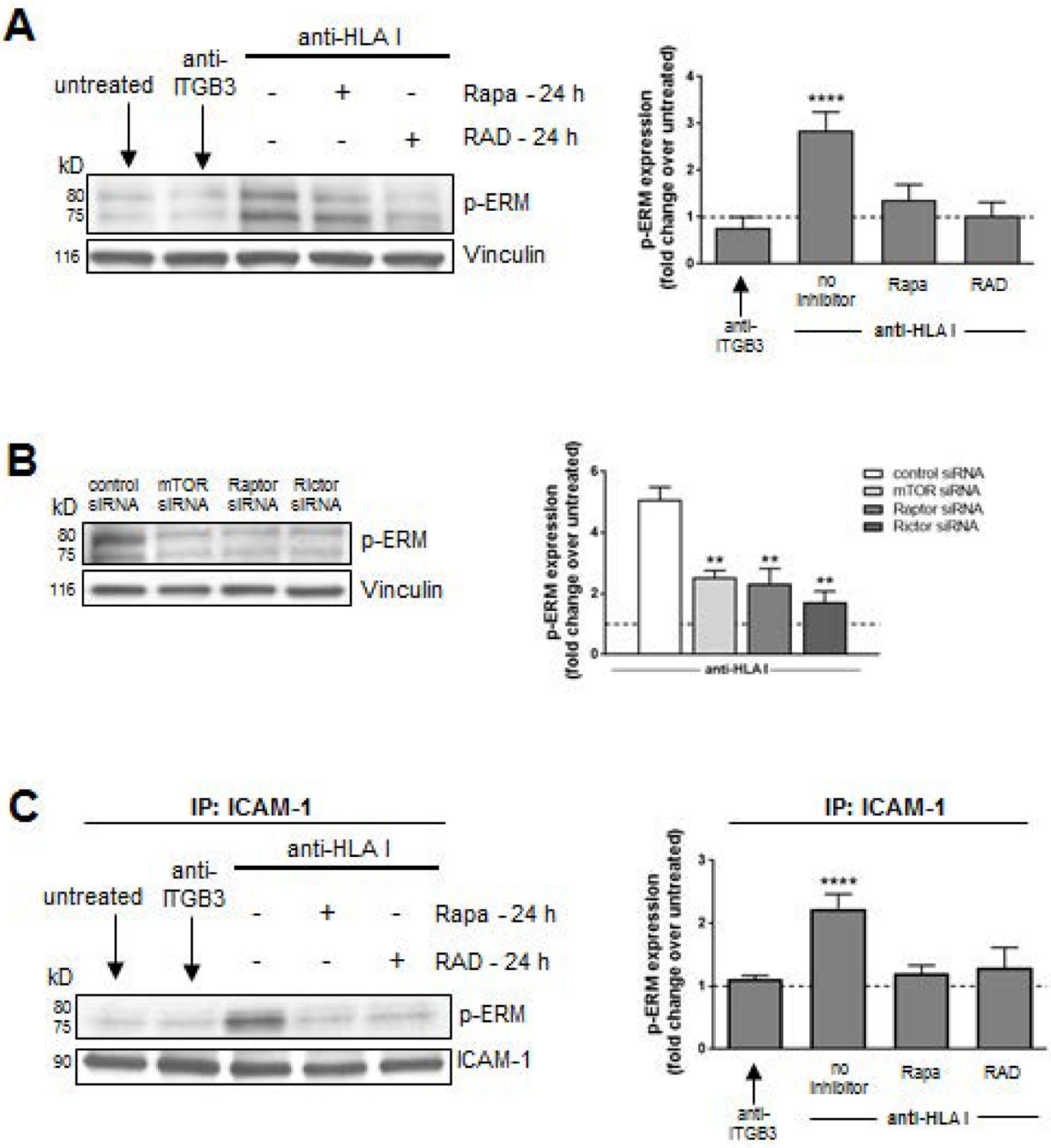

Figure 5. ERM phosphorylation is impaired by mTOR inhibition in HLA class I Ab-activated ECs

ECs were pre-treated with Rapa or RAD for 24 h and stimulated with HLA I Ab (anti-HLA I; clone W6/32) for 10 min. **(A)** Cells were lysed and proteins were separated by SDS–PAGE followed by immunoblotting to detect phosphorylated Ezr^{Thr456}/Rad^{Thr564}/ $Moes^{Thr558}$ (p-ERM) as well as Vinculin to confirm equal loading of proteins and were quantified by densitometry scan analysis using ImageJ. Results are expressed as the mean fold increase in p-ERM expression over untreated ECs ± SEM over 3 independent experiments. **(B)** ECs were transfected with either control siRNA, or siRNA directed against mTOR, Raptor (MTORC1), or Rictor (MTORC2) before treatment with anti-HLA I for 10

min. Cells were lysed after 48 h and proteins were separated by SDS–PAGE followed by immunoblotting to detect p-ERM as well as anti-Vinculin mAb to confirm equal loading of proteins and were quantified by densitometry scan analysis using ImageJ. Results are expressed as the mean fold increase in p-ERM expression over untreated ECs ± SEM over 3 independent experiments. **(C)** ECs were pretreated with Rapa or RAD for 24 h and stimulated with anti-HLA I for 10 min. Cells lysates were immunoprecipitated (IP) with anti-ICAM-1 followed by immunoblotting to detect p-ERM as above. Results are expressed as the mean fold increase in p-ERM expression over untreated ECs ± SEM over 3 independent experiments. **P<0.01; ****P<0.0001 by two-way ANOVA with Tukey's multiple comparisons test.

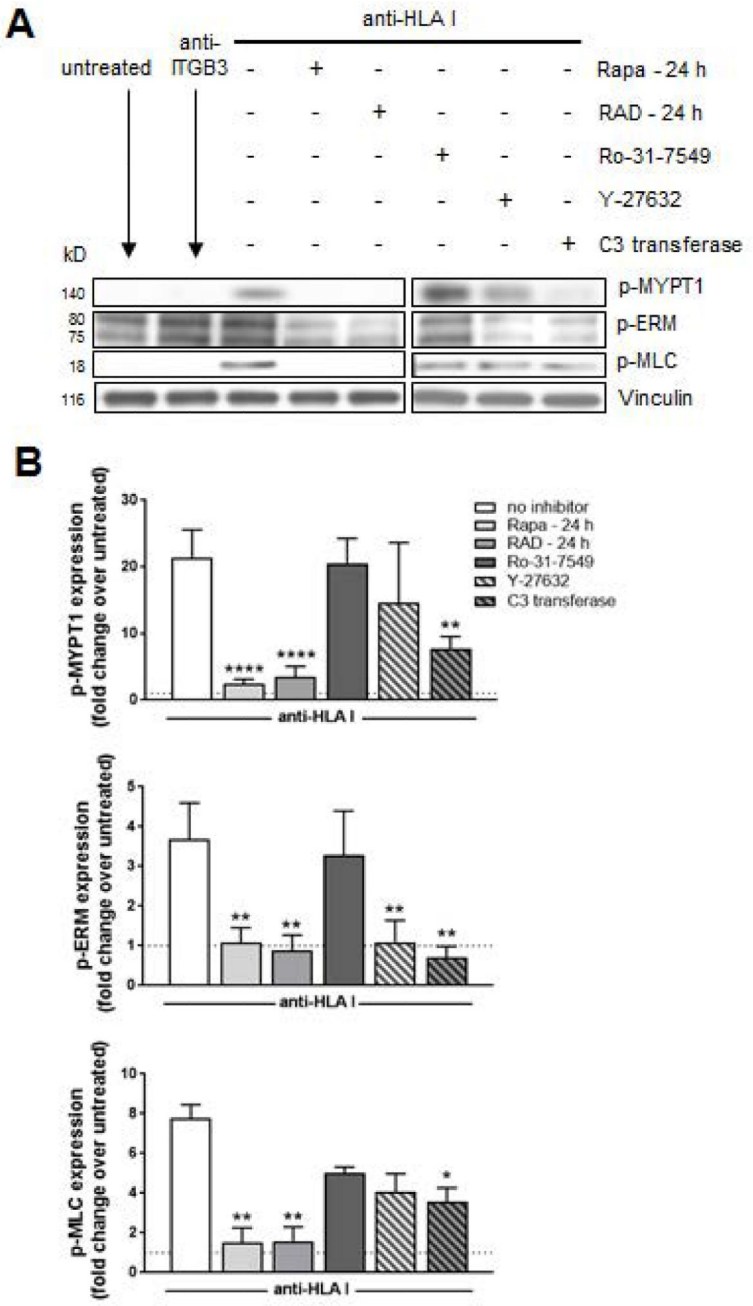

Figure 6. mTOR regulates HLA class I Ab-induced ERM phosphorylation through the Rho pathway

ECs were pretreated with Rapa or RAD for 24 h, or Ro-31-7549 (PKC inhibitor), Y-27632 (ROCK inhibitor), or U0126 (C3 transferase; Rho inhibitor) for 4 h, then stimulated with HLA I Ab (anti-HLA I; clone W6/32) for 10 min. **(A)** Cells were lysed and proteins were separated by SDS–PAGE followed by immunoblotting to detect phosphorylated MYPT1^{Thr696} (p-MYPT1), p-ERM, and phosho-MLC^{Ser19} (p-MLC), as well as Vinculin to confirm equal loading of proteins. Images from different parts of the same gel are separated by boxes. **(B)** Protein bands were quantified by densitometry scan analysis using ImageJ and results are expressed as the mean fold increase in phosphorylation above untreated with and

without the indicated inhibitor ± SEM over 3 independent experiments. *P<0.05; **P<0.01; ****P<0.0001 by two-way ANOVA with Tukey's multiple comparisons test.

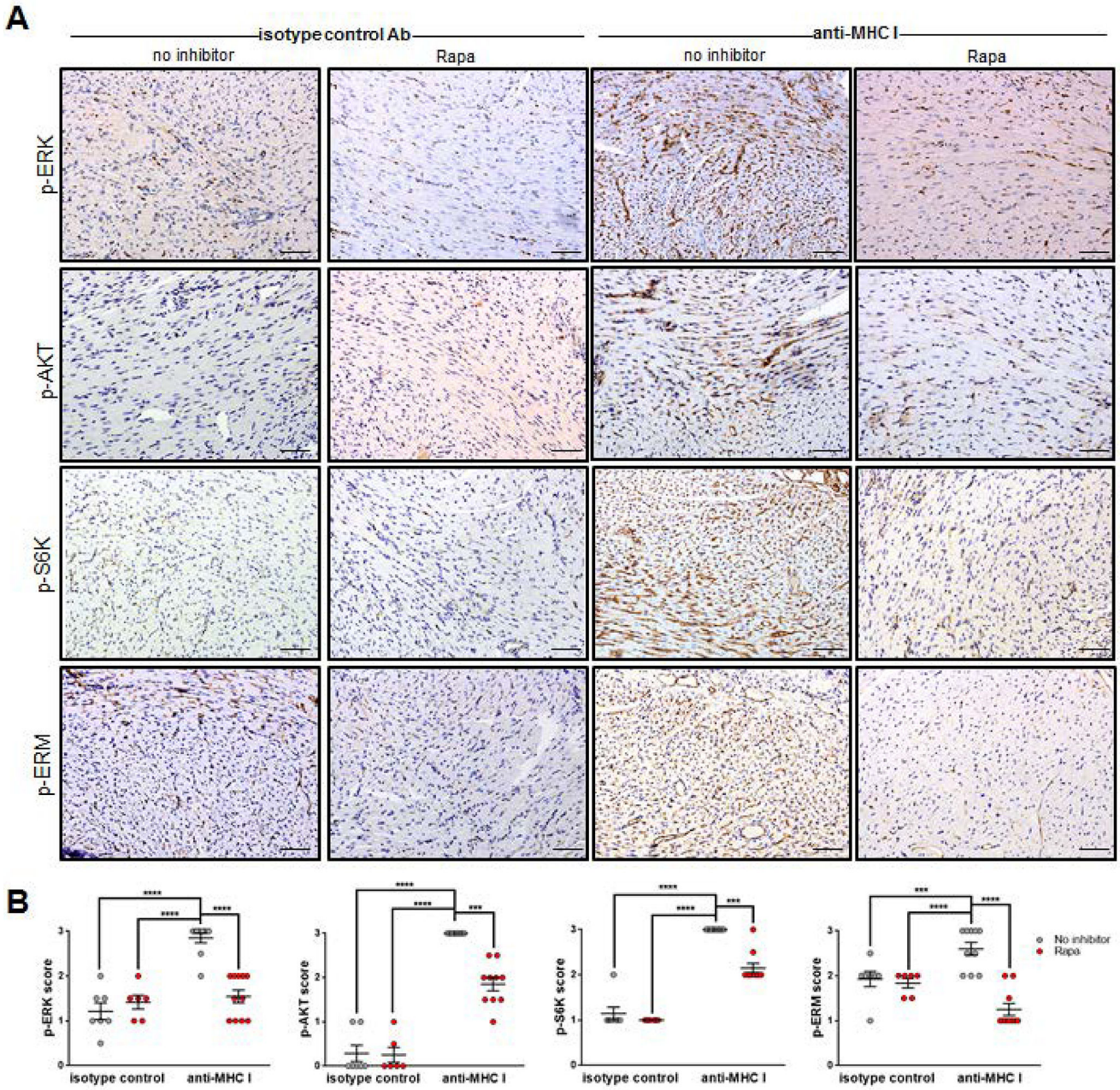

Figure 7. Rapamycin treatment inhibits activation of anti-HLA class I mAb-induced mTOR signaling proteins *in vivo*

BALB/c cardiac allografts were transplanted into B6.RAG1$^{-/-}$ recipients, passively transfused with anti-MHC I or isotype control and treated with daily Rapa before immunohistochemical analysis of proteins involved in MHC I-induced cell survival/ proliferation pathways. (A) Grafts were procured at day 30 post-transplant and evaluated for p-ERK, p-AKT, p-S6K and/or p-ERM expression by immunohistochemical staining. All phosphorylated proteins are visible in cardiac vessels and endocardium (dark brown) in cardiac vessels and endocardium. scale bars = 50 μm (C) Shown are mean scores ± SEM for

n≥6 per group with each dot representing one animal. *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001 by two-way ANOVA with Tukey's multiple comparisons test.

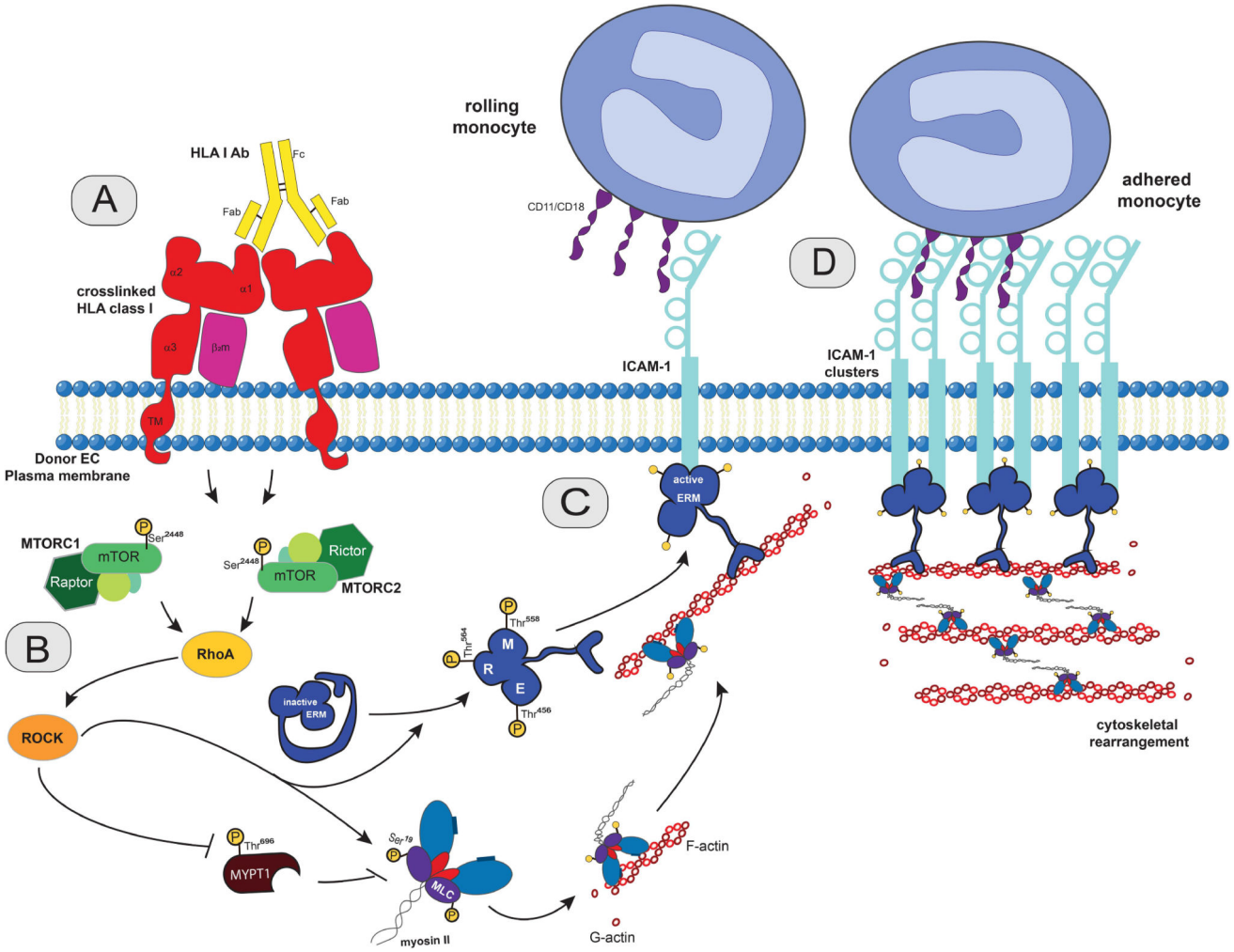

Figure 8. Proposed model of outside-in HLA class I-mediated regulation of ICAM-1 clustering and EC-monocyte interactions via mTOR in transplant antibody-mediated rejection

(A) Antibody-ligation and cross-linking of HLA class I molecules on the surface of ECs initiates outside-in signaling. (B) HLA class I cross-linking triggers mTOR complex 1 (MTORC1) and complex 2 (MTORC2) signaling and downstream activation of Rho to phosphorylate ERM (p-ERM) (C) p-ERM physically associates with both the cytoskeleton (via its F-actin binding site) and ICAM-1 (via its N-terminal FERM domain). (D) This physical association results in ICAM-1 clustering at the surface of ECs and monocyte firm adhesion to ECs.