Title: Circulating delta-like Notch ligand 1 is correlated with cardiac allograft vasculopathy and suppressed in heart transplant recipients on everolimus-based immunosuppression.

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Abbreviations

ANOVA	analysis of variance
BMI	body mass index
BPAR	biopsy-proven acute rejection
CAV	cardiac allograft vasculopathy
CNI	calcineurin inhibitor
CsA	cyclosporine A
DLK1	delta-like 1 homologue
DLL1	delta-like Notch ligand 1
ECM	extracellular matrix
EC	endothelial cell
EVR	everolimus
HF	heart failure
hsCRP	high sensitivity C-reactive protein
HTx	heart transplantation
IVUS	intravascular ultrasound
LVEDD	left ventricular end diastolic diameter
MCP-1	monocyte chemoattractant protein 1
MIT	maximal intimal thickness
MMF	mycophenolate mofetil
mTOR	mammalian target of rapamycin
mTORC	mTOR complex
NF	nuclear factor
NT-proBNP	N-terminal pro-B-type natriuretic peptide
PAV	percent atheroma volume
PBMC	peripheral blood mononuclear cell
POSTN	periostin
sCD25	soluble CD25
TNF	tumor necrosis factor
SIR	Sirolimus
VSMC	vascular smooth muscle cell

Abstract

Cardiac allograft vasculopathy (CAV) causes heart failure after heart transplantation (HTx), but its pathogenesis is incompletely understood. Notch signaling, possibly modulated by everolimus (EVR), is essential for processes involved in CAV. We hypothesized that circulating Notch ligands would be dysregulated after HTx.

We studied circulating delta-like Notch ligand 1 (DLL1) and periostin (POSTN) and CAV in *de novo* HTx recipients (n=70) randomized to standard or EVR-based, calcineurin inhibitor-free immunosuppression and in maintenance HTx recipients(n=41).

Compared to healthy controls, plasma DLL1 and POSTN were elevated in *de novo* (p<0.01; p<0.001) and maintenance HTx recipients (p<0.001; p<0.01). Use of EVR was associated with a treatment effect for DLL1. For *de novo* HTx recipients, change in DLL1 correlated with change in CAV at 1 (p=0.021) and 3 years (p=0.005). *In vitro,* activation of T cells increased DLL1 secretion, attenuated by EVR. *In vitro* data suggest that also endothelial cells and vascular smooth muscle cells (VSMC) could contribute to circulating DLL1.

Immunostaining of myocardial specimens showed colocalization of DLL1 with T cells, endothelial cells and VSMC.

Our findings suggest a role of DLL1 in CAV progression, and that the beneficial effect of EVR on CAV could reflect suppressive effect on DLL1.

Trial registration numbers

SCHEDULE trial: ClinicalTrials.gov NCT01266148

NOCTET trial: ClinicalTrials.gov NCT00377962

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Introduction

Heart transplantation (HTx) remains the definitive treatment for advanced heart failure (HF), offering a median survival after HTx of more than 13 years [1]. Within 5 years, however, ~30% of the patients develop cardiac allograft vasculopathy (CAV), an important contributor to adverse outcome after HTx [2]. Clinically, CAV resembles coronary heart disease, but is pathologically distinct from the usual coronary atherosclerosis and may affect any graft vessel, with concentric, progressive thickening along the length of the vessels that ultimately leads to ischemia, HF and death [3]. Multiple factors contribute to CAV development, including immunologic (e.g., chronic rejection process) and non-immune related responses (e.g., hyperlipidemia and viral infection), but the pathogenesis is incompletely understood.

Everolimus (EVR) is an alternative or supplement to calcineurin inhibitor (CNI) based immunosuppressive regimen after HTx and reduces the incidence and progression of CAV [4-6]. EVR, a mammalian target of rapamycin (mTOR) inhibitor, has antiproliferative effects on lymphocytes, vascular smooth muscle cells (VSMCs), endothelial cells (ECs) and fibroblasts [7] and attenuates the activation and inflammatory response of neutrophils *in vitro* [8]. However, the clinical benefit of EVR in HTx correlates poorly with changes in circulating inflammatory and vascular markers, and the mechanism by which EVR slows CAV progression remains unrevealed [5].

The Notch signaling pathway is essential for communication between neighboring cells [9] and processes known to contribute to CAV [10], like EC activation [11], accumulation and activation of immune cells [12], proliferation of VSMC and extracellular matrix (ECM) remodeling [13,14] and T cell activation [15]. Activation or inhibition of Notch signaling depends on the configuration and interactions of canonical Notch ligands [16]. Proteolysis induces secretion of extracellular domains which together with a large group of non-canonical ligands may modulate Notch signaling [17]. The canonical delta-like Notch ligand 1 (DLL1) regulates cell fate of monocytes [18], macrophages [19], T cells [20] and EC [21], and in HF, circulating DLL1 is elevated and associated with impaired cardiac function and adverse outcome [22,23]. To our knowledge, DLL1 is the only detectable canonical Notch ligand in human plasma/serum. For

non-canonical Notch ligands, delta-like 1 homologue (DLK1) is the most studied and has inhibitory effects on angiogenesis [24]. Periostin (POSTN), a matricellular protein functioning as a non-canonical Notch ligand [25], is regulated in clinical myocardial injury [26], and is associated with cardiac dysfunction in end-stage HF [23]. POSTN may modify Notch signaling through inhibition of DLK1 [27] or maintenance of Notch1 receptor expression [28].

Based on the involvement of Notch signaling in processes central to development and progression of HF and CAV, and potential modulation of Notch signaling by mTOR inhibition [29], we hypothesized that i) circulating Notch ligands would be dysregulated in HTx recipients, ii) EVR would affect levels of circulating Notch ligands in HTx recipients; and iii) circulating levels of Notch ligands would be associated with CAV progression. We tested our hypothesis by measuring the Notch ligands DLL1, POSTN and DLK1 in plasma from two cohorts of HTx recipients with EVR-based and standard immunosuppression, at different timepoints and accompanied by assessment of CAV.

Materials and Methods

Patient population

Two adult HTx cohorts were included: i) *de novo* HTx recipients from the Scandinavian heart transplant everolimus *de novo* study with early CNI avoidance (SCHEDULE) [4], and ii) maintenance HTx recipients from the NOrdic Certican Trial in HEart and Lung Transplantation (NOCTET) [30].

The *SCHEDULE trial* (ClinicalTrials.gov NCT01266148) was a 1 year, controlled and randomized multicenter trial and has been reported in detail [4]. In brief; the trial was conducted in five Scandinavian HTx centers to evaluate if early initiation of EVR and early cessation of the CNI cyclosporine A (CsA) could improve renal function and hamper the progression of CAV. After antithymocyte globulin induction therapy, *de novo* HTx recipients were randomized to an immunosuppressive regimen consisting of either i) low-dose EVR, CsA, mycophenolate mofetil (MMF) and corticosteroids with CsA withdrawal and stepwise increments in EVR dose up to full dose after 7-11 weeks (hereafter: Everolimus group, EVR)

or ii) standard regimen with CsA, MMF, and corticosteroids (hereafter: CNI group, CNI). The two regimens were initiated no later than the fifth postoperative day. After the 1 year trial, immunosuppression was according to the investigator's preference, and the patients were reassessed at 3 years post HTx [31]. A total of 70 patients (CNI, n=34; EVR, n=36) with plasma collected at ≥3 timepoints: 7-11 weeks (i.e., baseline), 6 months, 1 year and 3 years post HTx, were included in the present follow-up substudy.

The *NOCTET trial* (ClinicalTrials.gov: NCT00377962) was a 1 year, controlled and randomized multicenter study conducted in five Scandinavian transplant centers and reported in detail earlier [30]. In brief; maintenance HTx recipients with renal impairment were randomized to either i) EVR + reduced CNI (hereafter: Everolimus group, EVR) or ii) standard CNI-based regimen for maintenance immunosuppression ≥ 1 year post HTx (hereafter: CNI group, CNI). The aim of the study was to evaluate whether introduction of EVR with a simultaneous protocol-specified reduction in CNI exposure would improve renal function. After the 1 year core trial, the study was extended for 1 year and patients reassessed at their annual visit 2 years after enrollment [32]. A total of 41 patients (CNI, n=22; EVR, n=19) (mean 4.7 years, range 0.9-16.9 years post HTx) with plasma collected at ≥ 3 timepoints: inclusion (i.e., baseline), 6 weeks and 1 and 2 years after inclusion, were included in the present follow-up substudy.

For comparison, 20 age and sex matched healthy subjects were included.

For both studies written informed consent was obtained after institutional review board approval. The studies were carried out in accordance with the ICH Harmonized Tripartite Guidelines for Good Clinical Practice, applicable local regulations and the Declaration of Helsinki.

T cell isolation and activation

Peripheral blood mononuclear cells (PBMCs) were obtained from heparin-anticoagulated blood from healthy donors by Isopaque-Ficoll (Lymphoprep; Axis-Shield, Oslo, Norway) gradient centrifugation [33]. Isolation of T cells from PBMCs was performed by negative selection using Pan T cell isolation kit and MACS Separator (Miltenyi Biotec, Bergisch

Gladbach, Germany). Isolated T cells were resuspended in AimV medium (Gibco, ThermoFisherScientific, Waltham, MA), seeded into a 48 well plate (Costra, Cambridge, MA; 250.000 cells/well), and incubated for 40 minutes in a humidified CO_2 incubator at 37ºC before adding EVR (Sigma-Aldrich, St. Louis, MO), sirolimus (SIR) (Rapamycin, InvivoGen, San Diego, CA), or CsA (Sigma-Aldrich) in different concentrations. After 90 minutes of incubation the cells were activated by beads coated with antibodies to CD2, CD3 and CD28 (T cell activation/expansion kit, Miltenyi Biotec). Cell-free supernatants and cell pellets were harvested at 24, 48 and 72 hours and stored at -80ºC.

Results

Patient population

Baseline demographics and characteristics were comparable between the two treatment groups of *de novo* HTx recipients (*SCHEDULE population*), except for measured glomerular filtration rate, hsCRP and total cholesterol that were higher and uric acid and NT-proBNP that were lower in the EVR group (Table 1). For maintenance HTx recipients (*NOCTET population)*, no differences in baseline demographics and characteristics were found (Table 1). For comparison , 20 healthy control subjects were recruited. Controls (mean age±SD: 59.4±8.8 years) were of similar age as the NOCTET population (58.9±9.3), but non-significantly older than the SCHEDULE population (50.5±13.7, p=0.11). Further, creatinine in the controls was lower (76±11 μmol/l) compared to both SCHEDULE (111±41 μmol/l, p<0.001) and NOCTET (121±26 μmol/l, p<0.001). Women represented 35% of controls, which was not statistically different from SCHEDULE (24%) or NOCTET (12%) (Chi square p=0.11).

Notch ligands in plasma at baseline

Compared to healthy controls and adjusting for differences in age and creatinine, baseline plasma DLL1 levels were higher in *de novo* (SCHEDULE) (p<0.01) and in maintenance (NOCTET) (p<0.001) HTx recipients. In SCHEDULE, DLL1 levels correlated with

creatinine (r=-0.45, p<0.001), hsCRP (r=0.36, p=0.004), NT-proBNP (r=0.38, p=0.004) and uric acid (r=0.53, p<0.001). Similar, but more modest associations were observed for baseline DLL1 in NOCTET (creatinine r=-0.27, p=0.088; hsCRP r=0.28, p=0.078; uric acid r=0.33, p=0.042, and hemoglobin r=0.32, p=0.044). In addition, DLL1 levels at baseline in NOCTET were correlated with time since HTx (r=0.35, p=0.026) (Figure 1).

Baseline POSTN levels were also higher in both groups of HTx recipients compared to controls, but in contrast to DLL1, the highest levels of POSTN were seen in *de novo* HTx recipients (SCHEDULE). No significant correlations between POSTN and baseline biochemical variables were observed in SCHEDULE. For maintenance HTx recipients (NOCTET), baseline POSTN levels correlated with uric acid (r=0.39, p=0.014) but not with time since HTx (r=0.28, p=0.085) (Figure 1).

For DLK1, no significant differences were found (Figure 1).

Effect of EVR on Notch ligands in plasma

. A significant overall treatment effect was observed in the temporal course of circulating DLL1 for each study population (Figure 2). In *de novo* HTx recipients (SCHEDULE) plasma levels of DLL1 decreased after 6 months of EVR treatment and remained low compared to baseline levels, but above those of healthy controls, throughout the study. Comparing the change in DLL1 after adjusting for baseline DLL1 levels and change in kidney function (i.e., creatinine) in the same period revealed a significantly larger decrease in the EVR group at all timepoints. Among maintenance HTx recipients (NOCTET) a similar, but less distinct pattern was observed with a significant treatment effect at 1year follow-up.

No treatment effects were observed for POSTN or DLK1 (Figure 2). POSTN levels declined after commencing on either of the immunosuppressive regimens in *de novo* HTx (SCHEDULE) recipients and remained low, but above those of healthy controls, for both treatment groups compared to baseline (Figure 2). In contrast, POSTN increased compared to baseline at all subsequent timepoints in both treatment groups in maintenance HTx recipients (NOCTET) (Figure 2). Plasma DLK1 remained stable and comparable to levels in healthy controls in both treatment arms and study populations (Figure 2).

While DLL1 displayed similar profiles in SCHEDULE and NOCTET, POSTN exhibited a marked early decline in SCHEDULE, independent of study arm. The level of POSTN at 1 and 3 years in SCHEDULE was quite similar to NOCTET levels (i.e., median 4.7 years post HTx) and it seems that at 6 months, “steady state” levels of POSTN are achieved. The reason for this pattern is unclear, but as POSTN is induced during tissue injury and during ECM remodeling [34], we suggest that the initial high POSTN levels after HTx reflect ongoing ECM remodeling.

Notch ligands and acute rejections

Similar to the original report [31], there was a trend association between biopsy-proven acute rejection (BPAR) and the use of EVR (32% vs. 13%, p=0.056), EVR and CNI, respectively). As depicted in Supplemental Table 1, we observed, however, no difference in change in DLL1 between patients with and without BPAR, although there was a trend toward lower DLL1 levels in patients who experienced BPAR within the first year (p=0.090). No differences in change- or 1-year levels of DLL1 in relation to BPAR were observed within the treatment arms. For the other ligands, the decline in POSTN during 1 year follow-up was larger in those who experienced BPAR.

DLL1 and CAV development

Recently, we reported attenuated CAV development in the EVR group of *de novo* HTx recipients (SCHEDULE) [5]. Herein, we observed a treatment effect for DLL1. We therefore evaluated the relationship between change in DLL1 (ΔDLL1) and CAV development in the SCHEDULE population. A positive correlation between ΔDLL1 and change in maximal intimal thickness (ΔMIT) from baseline to 1 (r=0.31, p=0.016) and 3 years (r=0.39, p=0.006) was noted, primarily reflecting changes in the CNI group (Figures 3A and 3B). However, we found no correlation between MIT and DLL1 levels at the individual time points. ΔDLL1 could potentially reflect improved allograft function, but correlations with changes in allograft function [i.e., left ventricular ejection fraction (LVEF), left ventricular end diastolic diameter (LVEDD), peak early diastolic mitral inflow velocity/ peak late diastolic mitral inflow velocity (E/A ratio) and NT-proBNP] were poor for both ΔMIT and ΔDLL1

(Supplemental Table 2). However, ΔDLL1 correlated with change in CRP (r=0.30, p=0.018), mainly driven by a correlation in the CNI group (r=0.58, p=0.001), and creatinine (r=0.49, p<0.001) where the correlation was present in both treatment groups (Supplemental Table 2). For the whole study population, the association between ΔDLL1 and ΔMIT was sustained when treatment, ΔNT-proBNP and Δcreatinine were included in a linear regression model (Figure 3C). These data support that the association between changes in circulating DLL1 and MIT are independent of changes in allograft or kidney function. Moreover, changes in percent atheroma volume (ΔPAV), an additional marker of CAV development also downregulated by EVR in the SCHEDULE population [5], were also significantly correlated with ΔDLL1 at 1 and 3 years, primarily reflecting changes in the CNI group, and again, independently of allograft or kidney function (Figure 3C).

Effect of EVR on Notch ligand DLL1 in myocardial tissue

To assess whether the observed effects of EVR on DLL1 could be observed in the myocardium, we determined expression of mRNA for DLL1 and Notch receptors 1 and 2 in myocardial tissue from both study arms (EVR, n=14; CNI, n=18) in the SCHEDULE population. Whereas *DLL1* was downregulated in the EVR group, *NOTCH1/2* were similar between the groups (Figure 4C). Importantly, *DLL1* correlated with MIT 1 year post-HTx for both study groups (n=28, r=0.40, p=0.003) (Figures 4D and 4E), and a similar finding was also found between *DLL1* and PAV (r=0.36, p=0.057). *DLL1* also correlated with LVEDD and *NOTCH1* was correlated with NT-proBNP, suggesting some association with allograft function (Figure 4D).

EVR suppresses DLL1 in activated T cells

Activated T cells are involved in the pathogenesis of CAV [35], and Notch and mTOR signaling are crucial contributors in T cell-mediated immune responses. We therefore evaluated the effect of different concentrations of EVR (4, 12 and 40 ng/mL) on DLL1 release. Activation of T cells was associated with a time dependent increase in DLL1 and the T cell activation marker soluble (s)CD25 as compared to unstimulated cells (Figure 5A). EVR attenuated DLL1 and sCD25 release in a time and dose dependent manner, but with only modest effects

in further decreasing DLL1 and sCD25 release at the highest doses (Figure 5 B/C). Using even higher doses (100 and 500 ng/mL) had limited effects (Supplemental Figure 1A). A similar dose-response pattern has previously been reported of rapamycin on activated T cell production of Th1/Th2 cytokines [36], and importantly, the lower doses are relevant to targeted concentrations of EVR in the HTx patients (6-10 ng/mL in the SCHEDULE and 3-8 ng/mL in the NOCTET study). Stimulation of activated T cells with the mTOR inhibitor sirolimus, but not the CNI CsA, dampened the DLL1 and sCD25 release in a pattern similar to EVR, whereas CNI reduced sCD25 release dose dependently at 72 hours (Supplemental Figure 2). These results suggest that the observed effects on circulating DLL1 in the EVR group may be mediated by T cells, attributable to EVR exposure rather than freedom from CNI. Moreover, in activated T cells, EVR attenuated mRNA expression of the mTOR-responsive gene UTP15 (Supplemental Figure 1B) in a similar pattern to DLL1 and sCD25, supporting a link between mTOR complex (mTORC) signaling and DLL1 release. Contrary to the effects on DLL1, the effect of EVR on mRNA levels of Notch receptors 1 and 2 was more complex with a reduction of *NOTCH1* in un-activated T cells, and a dose dependent increased *NOTCH1* in activated T cells at 72 hours (Supplemental Figure 3).

ECs and VSMCs may be sources of circulating DLL1

DLL may be released from other cells than T cells, potentially contributing to circulating DLL1 in HTx recipients. To elucidate this, we examined the release DLL1 from VSMCs, human aortic and umbilical vein ECs and cardiac fibroblasts. These experiments revealed (Supplemental Figures 4/5/6): i) VSMC and ECs, but not cardiac fibroblasts, secrete DLL1 in the conditioned medium and could be potential sources for circulating DLL1 in addition to T cells, ii) Activation of VSMC with the prototypical upstream inflammatory cytokine interleukin 1β enhanced DLL1 secretion, while the release of DLL1 upon tumor necrosis factor (TNF)-mediated endothelial cell activation, another prototypical upstream inflammatory cytokine, was more modest, iii) EVR attenuated DLL1 release.

Expression of DLL1 in myocardial tissue

Our findings so far show that DLL1 mRNA are expressed in myocardial tissue following HTx. Our in vitro studies suggest that T cells, ECs and VSMCs could be important cellular sources of DLL1. To further elucidate these findings, we performed immunohistochemistry analysis of myocardial specimens from two HTx recipients with high MIT (0.90 and 0.81mm), indicative of presence of CAV. The biopsies were obtained at the first annual post-HTx visit for participants in the SCHEDULE trial (Figure 6). DLL1 protein was expressed in myocardial tissue and exhibited a distribution pattern corresponding to presence of T cells, ECs and VSMCs. Although we were unable to firmly establish the proximity to CAV in our specimens, the strong staining of the EC marker CD31, in the upper right corner of Figure 6C could reflect endothelial activation, a common feature of CAV [37].

Discussion

We studied circulating Notch ligands DLL1, POSTN and DLK1 and CAV in HTx recipients receiving standard or EVR-based, CNI-free immunosuppression and in maintenance HTx recipients. Our main findings were: i) In HTx recipients, plasma DLL1 and POSTN were elevated compared to healthy controls, ii) use of EVR-based, CNI-free immunosuppression was associated with decreased plasma DLL1 levels, iii) during long-term follow-up, changes in plasma DLL1 in *de novo* HTx recipients correlated with changes in markers of CAV (MIT and PAV), iv) in myocardial tissue from *de novo* HTx recipients, the DLL1 protein was expressed in a distribution pattern similar to T cells, ECs and VSMCs, and DLL1 mRNA levels were attenuated in the EVR group and correlated with CAV (MIT and PAV), v) *in vitro*, activation of T cells increased DLL1 secretion, and EVR and sirolimus, but not CsA, attenuated this release, vi) ECs and VSMCs released DLL1, with attenuating effects of EVR in EC. These findings suggest a link between CAV development and DLL1 involving a complex interaction between T cells, VSMCs and ECs, also within the heart allograft.

As in chronic HF, circulating DLL1 levels at baseline in *de novo* HTx recipients correlated with kidney function [38]. More importantly, enhanced DLL1 levels compared to healthy controls persisted after adjustment for creatinine. DLL1 was positively correlated to

systemic inflammation as reflected by hsCRP and in particular uric acid, which is markedly elevated early after HTx, potentially reflecting increased metabolic and oxidative stress [39]. The persistently elevated DLL1 levels, suggesting enhanced Notch pathway activity, could mean that DLL1 is a marker of these processes, but also indicate that Notch signaling and DLL1 contribute to inflammation, endothelial activation and metabolic and oxidative stress in HTx recipients. Furthermore, DLL1 is central in T cell-mediated responses [40], and differentiation and activation of T cells require DLL1-mediated Notch signaling [41 40]. While T cell activation may be important for maintaining adaptive immune responses, dysregulated T cell activation may cause maladaptive immune response characterized by extensive production of inflammatory cytokines. The release of DLL1 from activated T cells in our *in vitro* study may propose DLL1 to be a part in a pathogenic loop leading to enhanced T cell activation in HTx recipients and potentially maladaptive T cell responses.

A major finding was that EVR downregulated DLL1 *in vivo*. Our *in vitro* findings show a similar effect of sirolimus, but not the CNI CsA, suggestive of mTORC involvement, but the underlying mechanisms are not clear. mTORC regulates several pathways relevant for T cell activation such as Nuclear Factor (NF)-κB, and is activated by the Akt pathway, which links T cell activation to mTORC [42]. Since the pattern of DLL1 release mimics that of sCD25, it is tempting to hypothesize that DLL1 could be directly regulated through mTORC. TNF, the prototypical activator of NF-κB, has been shown to increase DLL1 expression in fibroblast like cells [43]. Furthermore, another mTORC inhibitor, Torin-1, decreased the expression of DLL1 *in vivo* in colorectal cancer xenografts [44]. Moreover, the release of DSL ligands including DLL1 is achieved by shedding induced by ADAM9/10/12 [45-47], and interestingly rapamycin (i.e., sirolimus) has been shown to inhibit ADAM10 [48] and ADAM12 [49,50]. Thus, both direct mTOR mediated effects as well as protease activity that is also influenced by the mTOR pathway could contribute to DLL1 release.

Neointimal proliferation and infiltration by macrophages are principal to CAV development and likely driven by several processes including upstream cytokine production from activated T cells. Indeed, T cells dominate among of the immune cells accumulating in

the thickened intima [51], and the beneficial effect of EVR on CAV development as reported in the SCHEDULE population [5] may mirror attenuation of T cell activation. However, the molecular mechanism for the beneficial effect of EVR on CAV is not clear. Our previous attempts to identify associations between the effect of EVR on CAV and effects on a wide range of inflammatory markers, including CRP, have failed. Our present findings that EVR downregulates DLL1 both in plasma and the myocardium, and that DLL1 correlates with MIT and PAV, reflective of CAV development, may suggest that the beneficial effects of EVR on CAV could involve downregulation of DLL1 and Notch signaling. Interestingly, in experimental HTx, blockade of transcriptional activation downstream of all Notch receptors in T cells eliminated the effects of canonical Notch signaling and improved graft survival [52]. EC injury from immunologic and non-immunologic factors is central to CAV development [3], and Notch signaling is involved in EC dysfunction [11]. Herein we show that ECs release DLL1, attenuated by EVR. Thus, it is tempting to hypothesize that the beneficial effect of EVR in CAV could involve attenuated Notch signaling with inhibitory effects on DLL1 release from T cells and ECs. Although we lack data on the effects of EVR on DLL1 protein levels in the myocardium, the immunostaining showing colocalization of DLL1 to markers of T cells and ECs within the myocardium in CAV patients, may suggest that such mechanisms also could be operating *in vivo* within the myocardium in HTx recipients with CAV.

In vivo, EVR decreased DLL1 in plasma and myocardium from HTx recipients. In contrast to circulating DLL1 where the temporal change in DLL1 and not the level at a specific time point correlated with change in MIT and PAV, myocardial DLL1 correlated with MIT and PAV in biopsies taken 1 year after post-HTx. This “disassociation” between plasma and myocardial DLL1 may suggest that circulating DLL1 at a single timepoint may reflect the contribution of multiple cell types in the body, some of which may not be involved in CAV progression, while the change in circulating DLL1 over time to a larger extent may mirror concerted long-term changes during CAV progression. On the other hand, myocardial mRNA levels of DLL1 presumably mainly reflects DLL1 in ECs, VSMCs and immune cells in close proximity to CAV.

Limitations to the present study include the relatively small sample sizes and the homogenous Scandinavian population. Thus, the SCHEDULE and NOCTET study populations may differ from the global HTx recipient population, which may constrain the extrapolation of our results. Moreover, lack of blood samples obtained before HTx limits the ability to interpret the pattern of DLL1 levels after HTx. Further, associations do not necessarily imply any causal relationship, and we lack data on Notch signaling within the CAV lesions and relevant cells (i.e., T cells and ECs), and analysis of their DLL1 mRNA and protein expression could illuminate our findings. Mechanistic data on effects of mTOR inhibition on ligand-receptor interactions would have strengthen our findings. For our experiment on the effect of EVR on NOTCH1/2 mRNA expression in activated T cells it might have been informative to in parallel quantify the corresponding receptor proteins and DLL1, as others have reported an upregulation of NOTCH1 proteins after anti-CD3/28 stimulation of T cells [53]. Whereas plasma DLL1 performs technically well, and displays good coefficients of variation and no diurnal or postprandial variation [54], less is known for POSTN. To illuminate the robustness of our findings, the stability of DLL1 in samples thawed more than once, could have been measured. Unfortunately, we have no data on freeze/ thaw stability for DLL1 for the present study. Finally, other Notch ligands, such as DLL3/4, Jagged 1/2 could also be involved in CAV development. Future studies should address the limiting issues of the present trial.

We conclude that in HTx recipients, DLL1 is increased and downregulated by EVR-based immunosuppression, and change in DLL1 is correlated with CAV progression. Our findings may suggest a role for Notch signaling and DLL1 in CAV progression.

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Disclosures

The authors of this manuscript have conflicts of interest to disclose as described by the American Journal of Transplantation. Dag Solbu is an employee of Novartis Scandinavia. The SCHEDULE and NOCTET studies were supported by Novartis Scandinavia.

Figure Legends

Figure 1. Circulating Notch ligands at baseline in the SCHEDULE (n = 70, 7-11 weeks post heart transplant) and NOCTET (n = 41, mean time since heart transplant: 4.7 years) trials and healthy controls (CTR, n = 20). Data are given as median and interquartile range. **p<0.01, ***p<0.001P-values adjusted for age and creatinine using ANCOVA with Bonferroni adjusted post-hoc tests.

Figure 2. Circulating Notch ligands during long-term follow-up in the SCHEDULE and NOCTET trials. Data are given as estimated marginal means and 95% CI. The p-value indicates the group effect of treatment from repeated measures ANOVA using baseline levels as a covariate. The grey shaded areas represent the geometric mean (grey line) and 95% CI for healthy controls, n = 20. *p<0.05, **p<0.01, ***p<0.001 vs. baseline (BL). †p<0.05 comparing change at the indicated timepoint between standard treatment (CNI) *vs.* everolimus adjusting for baseline levels and change in creatinine in the same period.

Figure 3. Association between cardiac allograft vasculopathy progression and change in DLL1. The plots show the correlation between change in maximal intimal thickness (MIT) and percent atheroma volume (PAV) and DLL1 at **A)** 1 and **B)** 3 years. The numbers show the Spearman correlation coefficient and p-value for the total population (black) and in the CNI (blue) and everolimus (red) treatment arms. **C)** linear regression analysis of change in MIT and PAV at 1 and 3 years with treatment and change in DLL1, creatinine and N-terminal pro-B-type natriuretic peptide (NT-proBNP) for the same period as predictors. Beta: standardized regression coefficient; *t*: t statistic, *p*: probability.

Figure 4. Effect of everolimus on the expression of Notch in myocardial tissue. **A)** integrity of mRNA from endomyocardial biopsies (n=27) obtained at the 1 year control from the SCHEDULE population. Left lane is the ladder while the two right lanes are mRNA from biopsies. **B)** melting curve from real-time polymerase chain reaction showing specific primers

(DLL1, NOTCH1/2, GAPDH cardiac tissue; NOTCH3/4). **C)** mRNA expression of NOTCH, NOTCH2 and DLL1 in endomyocardial biopsies (CNI group n=15; Everolimus group n=12) obtained 1 year after heart transplantation. *p<0.05(Mann-Whitney U test). **D)** correlation between mRNA of DLL1, NOTCH1, NOTCH2 and circulating (c) biomarkers and indices of allograft function. The numbers represent Spearman correlation coefficients (blue p<0.05, red p<0.01). **E)** correlation between maximal intimal thickness (MIT) and DLL1 mRNA 1 year after heart transplantation (showed as ln transformed values). Experiments were run in duplicates.

Figure 5. Modifying effect of everolimus (EVR) on DLL1 secretion from activated T cells. **A.** DLL1 and sCD25 levels in conditioned media from T cells activated with beads coated with antibodies to CD2, CD3 and CD28 for 24, 48 and 72 hours. **B.** Effect of 4, 12 and 40 ng/mL EVR on DLL1 and sCD25 secretion from activated T cells (anti-CD2/3/28) after 24 hours and **C.** 72 hours. *p<0.05, **p<0.01, ***p<0.001 vs. activated T cells (anti-CD2/3/28). †p<0.05, ††p<0.01, †††p<0.001 vs. cells activated T cells treated with 4 ng/mL EVR (Student T-test). T cells from three healthy donors, experiments were run in triplicates. US: unstimulated T cells. White bars: unstimulated T cells; grey bars: activated T cells; black bars: activated T cells treated with EVR.

Figure 6. Expression of DLL1 in endomyocardial biopsies obtained at the first annual post-HTx visit. Localization of A) $CD3^+$ cells as a marker of T cells, and B) expression in consecutive tissue slide of DLL1 as identified by immunostaining with 3,3′-Diaminobenzidine (DAB); C) $CD31^+$ cells as a marker of endothelial cells, and D) expression in consecutive tissue slide of DLL1; E) Alfa smooth muscle actin $(\alpha SMA)^+$ cells as a marker of vascular smooth muscle cells, and F) expression in consecutive tissue slide of DLL1. Immunofluorescence visualization was performed by the Tyramide signal amplification (TSA) method. Arrowheads indicate T cells (panel A), endothelial cells (panel C) and

vascular smooth muscle cells (panel E) and corresponding DLL1 expression (panels B,D and F). Scale bar 100 μm in 10x and 10 μm in 40x.

Supporting information

Additional Supporting Information may be found online in the supporting information tab for this article. The available information includes

Supplemental Materials and Methods

In this section, blood collection protocol and methods for

biochemical analyses, studying ECs, cardiac fibroblasts and VSMCs, obtaining myocardial biopsies, performing RNA isolation and real-time quantitative polymerase chain reaction, immunohistochemistry, echocardiography, IVUS and statistical analysis are provided.

Supplemental Table 1. Associations between DLL1, POSTN and DLK1 levels and acute rejection.

Supplemental Table 2. Associations between changes in MIT (ΔMIT), DLL1 (ΔDLL1) and POSTN (ΔPOSTN) and changes in indices of allograft function (i.e., LVEF, LVEDD and E/A ratio), biochemical cardiac and inflammatory markers (i.e., NT-proBNP and CRP) and kidney function (i.e., creatinine) in the total population and the two treatment arms separately at 1 year post-HTx.

Supplemental Figure 1. Modifying effect of everolimus (EVR) in extended doses on DLL1 secretion and on UTP15 mRNA expression from activated T cells.

Supplemental Figure 2. Modifying effect of sirolimus (SIR) and cyclosporine A (CsA) on DLL1 secretion from activated T cells.

Supplemental Figure 3. Modifying effect of EVR expression of NOTCH1 mRNA and NOTCH2 mRNA in activated T cells.

Supplemental Figure 4. Modifying effect of everolimus (EVR) on DLL1 secretion from activated vascular smooth muscle cells (VSMCs).

Supplemental Figure 5. Modifying effect of everolimus (EVR) on DLL1 secretion from activated human aortic endothelial cells (HAoECs).

Supplemental Figure 6. Modifying effect of everolimus (EVR) on DLL1 secretion from activated human umbilical vein endothelial cells (HUVECs).

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