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How to cite

AMOURA, Lamia et al. Interest of plasma microvesicles to monitor pancreatic islet graft dysfunction: beta cell- and leukocyte-derived microvesicles as specific features in a pilot longitudinal study. In: American Journal of Transplantation, 2020, vol. 20, n° 1, p. 40–51. doi: 10.1111/ajt.15534

This publication URL: https://archive-ouverte.unige.ch/unige:133073

Publication DOI: 10.1111/ajt.15534

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Article type : Original Article

Interest of plasma microvesicles to monitor pancreatic islet graft dysfunction: beta cell- and leukocyte-derived microvesicles as specific features in a pilot longitudinal study

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This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/ajt.15534

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Abbreviations

ASGPR: Asialoglycoprotein receptor, ß-cells: beta cells, DSA: Donor Specific Anti HLA antibody, F: Graft Failure, GAD: anti- glutamic acid decarboxylase, HLA-I, HLA-II: human leukocyte antigen class I or II, HV: Healthy volunteers, IA2: anti-tyrosine phosphatase, MVs: microvesicles, Phtdser: phosphatidylserine, PSA-NCAM: Poly Sialic Acid of Neural Cell Adhesion Molecule, PS: Partial Success, S: complete Success, TF: Tissue Factor.

Abstract

Markers of early pancreatic islet graft dysfunction and its causes are lacking. We monitored 19 T1D islet-transplanted patients for up to 36 months following last islet injection. Patients were categorized as Partial (PS) or complete (S) Success, or Graft Failure (F), using the β-score as an indicator of graft function. F was the subset reference of maximum worsened graft outcome. To identify the immune, pancreatic, and liver contribution to the graft dysfunction, the cell origin and concentration of circulating micro vesicles (MVs) were assessed, including MVs from insulin-secreting ß-cells typified by PSA-NCAM, and data were compared with values of the β-score. Similar ranges of PSA-NCAM$^+$-MVs were found in healthy volunteers and S patients, indicating minimal cell damage. In PS, a 2-fold elevation in PSA-NCAM$^+$-MVs preceded each β-score drop along with a concomitant rise in insulin needs, suggesting ß-cell damage or altered function. Significant elevation of liver ASGPR$^+$-MVs, endothelial CD105$^+$-MVs, neutrophil CD66b$^+$-MVs, monocyte CD 14$^+$-MVs,

and T4 lymphocyte $CD4^+$-MVs occurred before each β-score drop, $CD8^+$-MVs increased only in F, B lymphocyte $CD19^+$-MVs remained undetectable. In conclusion, PSA-$NCAM^+$-MVs are non-invasive early markers of transplant dysfunction, while $ASGPR^+$-MVs signal host tissue remodeling. Leukocyte MVs could identify the cause of graft dysfunction.

Introduction

DT1 patients with unstable diabetes have severe hypoglycemia and increased mortality risk (1, 2). Islet transplantation improves glycemic control (3), preventing severe hypoglycemia and microangiopathy (4). Despite tailored therapeutics, less than 20% of patients remain insulin-independent 5 years after transplantation, indicating progressive loss of graft function (5, 6).

Monitoring islet-transplanted patients remains challenging. A drop in C-peptide plasma concentration is a late indicator of ß-cell dysfunction, while random islet diffusion hinders informative liver biopsies (7). Furthermore, allo- and auto-immune antibodies, that are key in the diabetes pathogenesis remain poorly informative of the immune response. Their pre-transplantation prognosis value is debated (8, 9), whereas a de novo occurrence could not be associated with Cd3 specificity thereby precluding to a causative effect in graft failure (10).

Circulating granzyme and FasL characterizing activated cytotoxic lymphocytes, were reported indicative of ongoing islet transplant rejection (11). Strikingly, blood insulin mRNA (12, 13), miRNA-375 and miR-200c (14, 15), all early markers of ß-cell damage, do not identify the cause of islet dysfunction in allo- or auto-transplanted islet patients.

In a cellular model mimicking Instant Blood Mediated Immune Response (IBMIR), an early cause of islet damage, we demonstrated a redox and inflammatory driven ß-cell response with reduced insulin-secreting ability and up-regulation of procoagulant tissue factor (TF) (16), together with plasma membrane remodeling and ultimate shedding of ß-cell derived microvesicles (MVs) (17).

MVs are submicron plasma membrane vesicles shed from stimulated cells and circulating markers of cellular damage. They cargo characteristic features of the parental cell, correlate with disease severity (18) and are key cellular mediators of tissue remodelling and regeneration (19, 20). MV are often pathogenic as they convey procoagulant, proapoptotic, pro-inflammatory (21), and pro-senescent properties (22). Because MVs expose procoagulant phosphatidylserine(PhtdSer), and TF when shed from leukocytes or endothelial cells, their circulating concentrations they are associated with hypercoagulability and ischemia reperfusion. Procoagulant MVs of endothelial origin are elevated in the plasma of heart-transplanted patients during the first episode of acute rejection (23). Circulating MVs of endothelial origin or bearing TF are promising tools to monitor the immunosuppression-driven endothelial damage and graft dysfunction after renal transplantation (24, 25), and identify a worsening cardiovascular outcome (26). In recipients of stem cells, circulating TF^{+}-MVs are predictive of worsening outcome (27).

In a pilot study of three islet-transplanted patients, an early peak of procoagulant MVs of unknown cell origin was detected one month before increased insulin needs and decreased plasma C-peptide concentrations, suggesting an early MVs release before graft dysfunction. Furthermore, after new islet transplant, or corticosteroid treatment for suspected cellular rejection (28), MV levels returned to baseline. We hypothesized that circulating MVs of pancreatic origin could be early, non-invasive markers of graft loss and that other shedding

cells could characterize the cause of islet dysfunction (28, 29). PSA-NCAM^{+}-MVs were chosen to probe MVs from ß-cells in the plasma of transplanted patients. PSA-NCAM is a characteristic feature of adult pancreatic ß-cells (30, 31), also detected during organogenesis, its expression disappearing until adulthood (32).

We realized a 4-year program monitoring of the cell origin of circulating MVs in islet-transplanted patients. Longitudinal variations of PSA-NCAM^{+}-MVs were measured in the peripheral blood, before and after islet transplantation, together with circulating MVs of liver, immune, and vascular origin, to determine whether other identified cell sources of circulating MVs could characterize islet-graft loss of function.

Patients and methods

Study population

Twenty-seven T1D patients, who underwent intraportal transplantation via percutaneous transhepatic procedure at the Strasbourg University Hospital, were successively prospectively enrolled within the GRAGIL consortium between January 2010 and December 2017 (33). Eight patients were excluded because of pos-transplant clinical complications that precluded a complete harvest of the data (SuppTable 1). The 19 included patients underwent retrospective analysis after MV measurement in blood samples. Islet recipients received 11,000 IEQ/ kg of body weight in 1-3 infusions, depending on preparations. The original Edmonton steroid-free protocol was common to both the GRAGIL and TRIMECO immunosuppressive regimens used in the cohort (3, 4).

Monitoring of graft function and circulating MVs

Graft function was monitored by the β-score, scaling from 0 to 8 (34). Transplantation was considered a success (S, n=8) for β-score ≥6, partial success (PS, n=8) when β-score was 3-6, and a failure when ≤3 (F, n=3). Patients were categorized accordingly (Figure1). No modification of patients' allocation was found using either Igls (35) or β-2 scores (36), that both introduce intermediate clinical status (SuppTable 2), on-line with a recent report correlating β and β-2 scores (36). The F subset was only considered a reference of extreme worsened graft outcome.

Circulating MVs were measured during the whole follow-up period, starting from the second month following the last infusion. Three different types of data were generated: (i) mean MV values calculated by cumulating all MV measurements in each subset; (ii) mean baseline MV values, excluding values measured at the maximum elevation of circulating MVs that preceded β-score decrease; (iii) mean MV maximum elevation value (Ev), excluding baseline values. Wherever possible, the median time lapse between MV elevation and last islet infusion or the β-score decrease was calculated by referring to the MV peak median.

Blood sampling for MV quantification

Peripheral vein blood samples were harvested on 129mM sodium citrate (9:1 ratio), and processed within 60 min. Platelet Poor Plasma (PPP) containing MVs was obtained by double centrifugation at 1500g for 15 min followed by 13000g for 3 min at room temperature, then immediately frozen and stored at -80°C, according to recommendations of the International Society of Thrombosis and Haemostasis. Blood was collected prior to transplant, then monthly after each islet infusion for the first six months, and thereafter every

three months during the follow-up period (12-60 months). A total of 10-12 samples/patient were analyzed. PPP were also prepared from 13 healthy volunteers (HV).

MV quantification by prothrombinase enzyme assay and cell origin

Total MV concentration was determined by taking advantage of the MV ubiquitous Phtdser exposure(37). MVs from PPP were captured onto insolubilized biotinylated annexin-5, a protein with high affinity for Phtdser, using streptavidin-coated microtitration plates (Roche Diagnostics, Deutschland). After three washings, MVs were measured by prothrombinase assay in which blood clotting factors (FXa, FVa, FII) and calcium concentrations ensure that the MV PhtdSer is the rate-limiting parameter of the reaction (Supp methods)(37). To determine their cell origin, MVs from PPP were first washed by two centrifugation steps (60 min, 14.000g, 4°C), concentrated in 500µl before capture onto biotinylated antibodies against T-helper cells (anti-CD4), cytotoxic T-cells (anti-CD8), B-lymphocytes (anti-CD19), monocytes (anti-CD14) (all from Leinco, St Louis, Missouri, USA), activated neutrophils (anti-CD66b; BD Bioscience, New Jersey, USA) before prothrombinase assay. The MV concentration was obtained for each cell origin by subtracting the OD values measured using the isotype biotinylated control immunoglobulin (BD Bioscience).

Characterization of MV pancreas and liver origins by ultrasensitive prothrombinase assay

Washed concentrated MVs were insolubilized onto biotinylated antibodies against ß-cell (anti-PSA-NCAM IgM, eBioscience San Diego, USA) or hepatocyte cell (anti-ASGPR IgG, BD Bioscience) for 60 min at 37°C. After four washes, human coagulation factors were incubated for 20min at 37°C before prothrombinase assay.

Statistical analysis

Data are expressed as mean ± standard error mean (S.E.M.), and analyzed using GraphPad Prism6® (La Jolla,CA,USA). Statistical analysis of biological and clinical data variation between the 3 patients' subsets at baseline was performed using ANOVA followed by Kruskal Wallis test. Other comparisons between two subsets were realized using Mann–Whitney t-test. The threshold of significance was set at 5%. Intra-variations of the MV levels were established for each clinical subset. The minimum value of conventional clinical variables reflecting the loss of graft function and the maximum value for circulating MV level, were compared using an unilateral nonparametric Wilcoxon test. We also verified that the MV peak occurred before variation of each of the other relevant variables.

Results

Patients

No statistical difference in biological values or blood cell count was observed between the three subsets before transplantation by multi-comparison except for the body weight that was higher in PS *vs.* F (Table 1). Patients had a 56 years mean age, 38 years mean diabetes duration and a daily insulin requirement of 0.6 units/kg of body weight at baseline. Islet transplantation followed kidney grafting in 26% of patients in all subsets (5 patients, Table1). No patient had pre-formed DSA, six showed *de novo* HLA DSA, as a late event with respect to the drop of the β-score. (SuppTable 4). Nine Patients had pre-transplantation autoantibodies (anti-GAD, anti-IA2), 10 were positive post-transplantation, occurring as a late event compared to β-score drop. *De novo* anti-GAD or -IA2 autoantibodies were

detectable in in PS and F groups whereas several S patients did not escape conversion, in spite of stable islet function (SuppTable 5).

Mean values and longitudinal variations of total procoagulant MVs

No significant difference in mean annexin-5^{+}-MV values was observed between subsets. Longitudinal analysis indicated a trend to lower MV values post-transplantation at baseline (S, $p<0.05$; PS, $p<0.05$) and a significant difference between baseline values following transplantation, and those of the elevation peak (SuppFigure 2A,2B).

Detection of circulating tissue MVs post-transplantation

In PS, the median time lapse between the detection of circulating tissue-derived MVs and the β-score drop was shorter (PSA-NCAM^{+}-MVs:42; ASGPR^{+}-MVs:55.8 days, Table2) compared to vascular cell-derived MVs (CD105^{+}-MVs:78 days; leukocytes >83.1 days), such MV release sequence being also observed in patients with graft failure (SuppTable 7). To better characterize the impact of vascular and tissue stress on circulating MV profiles, MV cell origins were characterized.

Circulating PSA-NCAM^{+}-MVs and islet-graft function.

MVs shed by ß-cells measured by ultrasensitive prothrombinase assay detected low levels of circulating PSA-NCAM^{+}-MVs. Concentrations were significantly different between S and PS subsets, F patients showing highest values (0.56±0.08nM), altogether suggesting that PSA-NCAM^{+}-MVs is a reliable sensor of the severity of pancreatic damage or dysfunction (PS: 0.51±0.05nM *vs.* S:0.29±0.02nM, $p<0.0001$; Figure 2A). Accordingly, PSA-NCAM^{+}-MVs circulated at lower concentrations in S and HV subsets (S:0.29±0.02nM *vs.* HV: 0.34±0.01nM).

Longitudinal analysis of circulating PSA-NCAM$^+$-MVs revealed a 2-fold elevation in the PS subset occurring 30-60 days before the β-score drop (Ev:0.92±0.16 *vs.* baseline:0.44±0.02nM, p<0.0001), and a 3-fold increase in F patients (Ev:1.5± 0.8 *vs.* baseline:0.41± 0.04nM, p<0.01) (Figure 2B, Table2). Of note, insulin treatment was never discontinued in either PS or F patients.

Conversely, S patients exhibited low PSA-NCAM$^+$-MVs concentrations, circulating levels remaining stable and similar to values measured in HV (S:0.31±0.03nM *vs.* HV:0.34±0.01nM, Figure2).

Variations in hepatocyte-derived MV plasma concentrations

An early release of hepatocyte-derived ASGPR$^+$-MVs, was concomitant with islet infusion in all subsets. Thereafter, the ASGPR$^+$-MVs baseline values significantly decreased below pre-transplantation values. However, in PS the β-score drop was preceded by an up to 10-fold rise in circulating ASGPR$^+$-MVs (PS:0.43±0.08 *vs.* baseline:0.03±0.01nM, Figure 3A), a similar raise being observed in the F reference subset (F:0.48±0.05 *vs.* baseline:0.03±0.01nM, p<0.0001). Conversely, in the S subset, ASGPR$^+$-MVs concentrations remained unchanged.

CD105- bearing endothelial MV plasma concentrations

As reported in other transplantations (26, 27, 38), baseline post-transplantation endothelial CD105$^+$-MVs were reduced in all subsets (p<0.05). In PS patients a 2-fold elevation occurred 78 days before the β-score drop (0.56±0.08 *vs.* baseline: 0.23±0.04nM, p<0.01) and 79.8 days before in F (0.54±0.05 *vs.* baseline: 0.17±0.05nM, p<0.01) (Figure 3B, Table2, SuppTable7).

Leukocyte MV release in the plasma of islet-transplanted patients

In contrast to PSA-NCAM$^+$-MVs, tissue ASGPR$^+$-MVs-MVs, endothelial CD105$^+$-MVs, and leukocyte-derived MVs levels increased in PS or F subsets within the 4 months prior to the β-score drop (Table2, SuppTable7).

Kinetics of the CD4$^+$ T- derived MV release

Concentrations of plasma CD4$^+$-MVs shed from T-helper cells were analyzed as a ratio to the circulating lymphocyte count. CD4$^+$-MVs circulated at lower levels post-transplantation in S and PS subsets (S:PreTx:0.19±0.02 *vs.* baseline: 0.10±0.02nmoles/10^9lymphocytes, $p<0.05$; PS:PreTx:0.57±0.29 *vs.* baseline:0.24±0.03nmoles/10^9lymphocytes, $p<0.05$, Figure 4A) as well as in F (PreTx:0.41±0.11 *vs.* baseline: 0.18±0.03nmoles/10^9lymphocytes, $p<0.05$). In the PS or F subsets, a 2-fold CD4$^+$-MVs elevation occurred within a 147 days before the β-score drop (Ev:0.46±0.04 *vs.* baseline:0.24±0.03nmoles/10^9lymphocytes, $p<0.0001$; Ev:0.37±0.04 *vs.* baseline: 0.18±0.03nmoles/10^9lymphocytes, $p<0.01$, respectively, Figure 4A). CD4$^+$-MV concentrations remained independent from total lymphocyte counts, and elevations lasted longer than those of other leukocyte-derived MVs (SuppTable 6).

Kinetics of the CD8$^+$ T cell-derived MV release

CD8$^+$-MVs shed from cytotoxic CD8 T-cells increased by 3 folds within the 126 days prior the β-score drop (PS:0.10±0.03 *vs.* baseline:0.01±0.01nM, $p<0.0001$; F:0.38±0.04 *vs.* baseline:0.13±0.04nM, $p<0.01$, respectively, SuppTables 7,6). Interestingly, when reported as a ratio of the lymphocyte count, the CD8$^+$-MV elevation was no longer observed in PS, while still evidenced in the F subset (0.15±0.09 *vs.* baseline: 0.04±0.04nmoles/10^9lymphocytes, $p<0.05$) (Figure 4B).

Kinetics of neutrophil-derived MV release

Post-transplantation $CD66b^+$-MV baseline was low in all patients (S:0.3±0.06; PS:0.1±0.02; F:0.07±0.03nmoles/10^9neutrophils). A 2-fold elevation occurred 83.1 days prior the β-score decrease in PS (0.5±0.1 *vs.* baseline: 0.1±0.02nmoles/10^9 neutrophils, $p<0.001$, Figure 5A, Table2) and 94.2 days in F (SuppTables 7).

Kinetics of monocyte-derived MV release

In PS, $CD14^+$-MV levels were 8 folds elevated before the β-score decrease (0.5±0.07 *vs.* baseline: 0.06±0.02nmoles/10^9monocytes, $p<0.0001$, 87.6 days, Figure 5A,Table2), and 5-6 folds elevated in F patients at similar median time lapse (0.23±0.12 *vs.* baseline: 0.04±0.01nmoles/10^9monocytes, respectively, $p<0.05$, (SuppTables 7).

B lymphocyte-derived MVs

In all subsets, $CD19^+$-B cell-derived MVs exhibited no significant variation at any time, in either pre- or post-transplantation mean values, suggesting that no B-lymphocyte damage or major cell activation could be detected (Figure 4C).

Longitudinal overview of MV variations

PSA-$NCAM^+$ and $ASGPR^+$-MVs elevation indicating graft and tissue damage in PS patients characterized by moderate graft dysfunction, occurred at shortest delay prior to the β-score decrease (42, 55.8 days, respectively), and was preceded by the endothelial $CD105^+$-MV peak (78 days), while MVs identifying the innate immune response were detected earlier, within 83.1-102 days, when the β-score was still above the threshold of graft dysfunction

(Table2, Figure6). The first $CD8^+$-MVs detected post-islet transplantation was solely associated with complete graft failure.

Discussion

This study relies on the well-established observation that circulating MVs are the signature of a pathophysiological state and of the severity of graft damage (18, 21). Using the β-score as the international reference evaluating islet graft function and taking advantage of the longitudinal follow-up, we compared Success and Partial subsets MV phenotypes, the Failure subset constituting a reference of worsened outcome with graft dysfunction. We herein demonstrate that (i) circulating PSA-$NCAM^+$-MVs are early indicators of pancreatic islet-graft dysfunction; (ii) variations in liver- and vascular-cell-derived MV levels characterize host and graft tissue remodeling; (iii) leukocyte MVs sense immune responses, altogether holding potential value in the identification of the causes of graft dysfunction.

Plasma PSA-$NCAM^+$-MVs as early indicators of islet dysfunction

In contrast to PS, PSA-$NCAM^+$-MVs concentrations were low in both S and HV subsets, indicating minimal islet damage and complete ß-cell function recovery after full engraftment. As proof, PSA-$NCAM^+$-MV concentrations in HV patients were low and similar to values measured in S patients (HV:0.34±0.01nM *vs.* S:0.29±0.02nM). Given the 3-folds increase before β-score drop in F, circulating PSA-$NCAM^+$-MVs appear biomarkers of ß-cell damage and graft dysfunction with potential prognosis value in PS patients.

Circulating MVs are surrogate markers of host tissue remodeling post-islet transplantation

IBMIR prompts the early loss of a proportion of islets(39), while allo- and autoimmune responses endanger the function of engrafted ones. We reasoned that identifying the variations in MVs from vascular cells, liver, pancreas tissues would help in deciphering the cellular causes of graft dysfunction. A similar approach using hematopoietic stem-cell $CD34^+$-MVs and hepatocyte $ASGPR^+$-MVs probed the severity of alcoholic hepatitis and predicted steroid therapy resistance(40). Furthermore, $CD105^+$-MVs are the sole characteristic and predictive endothelial markers of acute cardiac transplant rejection(38). Because annexin-5 positive MVs as well as platelet and endothelial-derived microvesicles were reported correlated with HbA1c values in DT1 patients(41), we studied pre-transplantation biological and clinical parameters of potential incidence on MV generation. Strengthening the proposed prognosis value of MVs, we could not evidence statistical differences at baseline in MV phenotypes between the 3 subsets of the cohort despite a trend for higher HbA1C in PS that was not statistically relevant (7.1% *vs.* 8.1%; Table1)

In our study, low baseline post-transplantation liver $ASGPR^+$-MVs and endothelial $CD105^+$-MVs levels were rapidly reached in all subsets, suggesting minimal remodeling in the peri-transplantation period, as reported within the month after kidney transplantation(24), or the next week of uncomplicated liver transplantation(42).

In the S subset, the stable baseline plateau suggests a restored graft function. In PS or F subsets, remote elevation of both $ASGPR^+$-MVs and $CD105^+$-MVs occurred at distance from islet injection and prior to the β-score fall and graft failure. Compared to the PSA-$NCAM^+$-MVs elevation, the time lapse between peak and onset of the β-score drop was longer, suggesting that upstream events caused endothelial damage and liver tissue remodeling,

before ß-cell damage became detectable. Transient and immediate elevation of endothelial VE-cadherin^{+}-MVs and hepatocyte-derived MVs, characterize enhanced vessel permeability and tissue remodeling post liver transplantation (42). Strikingly, before islet transplantation all patients exhibited higher circulating concentrations of liver- and endothelial-derived MVs, suggesting that vessel and liver damage are common features in brittle T1D (43). Our data bring new evidence that at distance from the islet transplantation, endothelial and hepatocyte cells are prime actors in tissue remodeling, possibly related to the progression of graft dysfunction. Because the endothelium of hepatic sinusoïd vessels is highly susceptible to oxidation, prolonged ischemia at sites of islet embolization may also have contribute to the shedding of noxious endothelial MVs possibly impairing engraftment (44, 45). If so, graft success could be improved by pre-treatments designed to ensure endothelial cyto-protection.

Leukocyte-derived MVs characterize the severity of graft damage or dysfunction

At sites of ischemia-induced damage, interactions between endothelial MVs, platelets, and leukocytes constitute a driving force for the recruitment of neutrophils and monocytes at the surface of the inflamed endothelium (46), resulting in the sustained release of pro-inflammatory MVs and endothelial permeability associated with primary graft dysfunction (47, 48).

In islet transplantation, it remains unclear whether the initial inflammation and innate immune responses further promote acute episodes or are the first step of chronic graft dysfunction. Despite additional islet injections, early and significant islet mass loss can persist, due to recurrent inflammatory episodes also involving the adaptive immune response. In this study, we demonstrate that leukocyte MVs mark islet ischemia reperfusion injury and graft dysfunction. Their prognosis value is strongly suggested by the fact that MV elevations always occurred prior to the β-score decrease and that concentrations in healthy subjects are

lower than those of transplanted patients. Furthermore, peaks of neutrophil CD66b$^+$-MVs and CD14$^+$-MVs characterizing PS and F had occurred at a longer median time lapse to the β-score drop than those of endothelial, liver, and ß-cell origins, suggesting an earlier contribution to graft dysfunction. In addition, CD14$^+$-MVs discriminated partial from complete graft failure. Most likely, MVs from monocytes and neutrophils contribute to early endothelial inflammation, which in turn accelerates the endothelial dysfunction (49).

MV profile and monitoring of humoral or cellular immune responses

Given the exclusive HLA-I or II distribution observed among the S (HLA-II) and F (HLA-I) subsets, it is tempting to speculate that innate immunity remains pivotal in islet-graft dysfunction. Indeed, while CD8 T-cells may contribute to late allo-specific immune islet damage (50), pancreatic islets do not stimulate primarily CD4-independent and CD8-dependent immune responses. Therefore, high levels of CD8$^+$-MVs only detected in F patients would sense ß-cell destruction, their release possibly primed by acute CD4-independent CD8$^+$T cytotoxic injury (51), and the later loss of islet allografts being potentially accelerated by a secondary alloreactive response (50).

Our data support previous hypotheses that cross-reactivity jeopardizes long-term islet allograft survival. Because autoreactive HLA-I-A2 CD8 antibodies destroy ß-cells that highly express insB-18 in T1D patients and although our study was not designed to identify antibodies to insB-18, a worsened outcome perspective to the activation of CD8 associated with the occurrence of HLA-I DSA was evidenced (52).

Strikingly, CD4$^+$-MVs and CD8$^+$-MVs elevations were often concomitant and always preceded the β-score decrease, with stable T lymphocyte count. Since no plasma CD4$^+$-MV elevation or variation in T-cell count was found in the Success subset, it is likely that CD4$^+$-

MVs reflect major T-cell activation in partial or complete graft failure, rather than a systemic response. Although CD4-dependent pathways in the islets are controlled by conventional immunotherapies, they eventually remain active in the host liver (53), thereby explaining hepatic MV shedding and tissue remodeling in the F subset (Figure 4A, Table2).

In islet-transplanted patients, cellular rejection may be misdiagnosed as humoral response. While confirmation will require detailed leukocyte subset cell counts, no significant variation in $CD19^+$-B cell-derived MVs could be detected in the PS or F subsets, suggesting that B lymphocytes are not prime actors of rejection, on-line with the absence of accelerated graft attrition following anti-HLA DSA conversion in 27 islet graft recipients (10).

Altogether, our findings indicate a specific sequence of cellular activation characterizing cells and tissues involved in the graft loss of function, initiated by leukocyte activation until detectable massive ß-cell damage, the latest event preceding the β-score drop (Table2, Figure 6). Following the first $CD8^+$-MVs and $CD4^+$-MVs rise characterizing acquired immune response to the graft while questioning immunosuppressive therapy escape. At distance from the islet injection, MVs typifying neutrophils and monocyte activation are detected before endothelial and liver MVs elevation, indicating abnormal host liver tissue remodeling, potentially leading to islet loss and suggesting a MV-driven graft damage (Table2, Figure 6).

In conclusion, circulating MVs of tissue origin appear biomarkers of graft damage, while the leukocyte MVs pattern is a signature of the immune response, likely to promote islet loss. PSA-$NCAM^+$-MVs could prove valuable for the early and non-invasive detection of islet dysfunction before the assessment of ß-cell damage by conventional tools. Combining circulating MVs from the host tissue and immune cells might offer enhanced sensitivity and help to decipher the causes of graft dysfunction and monitor immunotherapy. Whether the

individual profile of circulating MVs could prove useful for patients' stratification and monitoring however needs confirmation in larger multicenter longitudinal studies.

Study limitations

1-In this pilot monocentric longitudinal study, 12 separate measurements per patient were performed to overcome the limited patient number. Advantageously, baseline and peak MV values followed small distribution ranges within each clinical subset, indicating that these biomarkers were nonetheless robust. To strengthen the MV prognosis value, data analysis focused on the sixteen S and PS patients, the three F patients being considered as a reference of worsened irreversible outcome.

2-The study was not designed to identify $CD4^+$-MVs originating from Treg cells, and no conclusion can be drawn about the proportion of T4 and Treg cells that eventually shed MVs.

3-The β-2 score that better stratifies islet function or the Igls Score that takes into account severe hypoglycemia events were not used for stratification. Nevertheless, no modification of patients' allocation was obtained by either scoring, individual β-2 or β scores variations being closely related (SuppFigure1, SuppTable2), transition from S to PS and PS to S occurred at similar time lapse by either β-2 or β scores. Owing to scarce severe hypoglycemia, patients from the S subset were equally distributed in the "optimal" or "good" Igls sub-categories, with no significant MV variation.

Acknowledgments

Authors wish to thank Blandine Yver for expert technical assistance in sampling and measuring vascular MV. This work was partly supported by a grant from the French Society of Transplantation and the french National Research Agency ANR Grant COCERP n° ANR-16-CE29-0009-03 and ASDIA France.

Authors' contributions

Study design: LA, FT, LK

Patient recruitment and data collection: LA, FZ, LK, MK, AE, FT

Statistical design, analysis, and interpretation of data: LA

Writing up of the first draft of the paper and rewriting: LA; LK, FT

Editing of the manuscript: LA, FT

Review of the manuscript: LA; LK, FT, DB, NJ, TB, PYB, GK

Approval of the final manuscript: LA, LK, FT

Contributions: FZ, SS, MK

Disclosure

The authors of this manuscript have no conflicts of interest to disclose as described by the *American Journal of Transplantation*.

Figure Legends

Figure 1: **Flow chart of patients enrolled in the study**

Figure 2: Variation ranges and cumulative values of circulating PSA-NCAM$^+$-MVs in transplanted patients with graft success, partial success or failure

(A) Mean of cumulative MV longitudinal values measured on 10-12 separate occasions for each patient in each subset. Data presented as mean of all individual values including elevation and baseline values during the follow-up period (48 months)

(B) MVs were measured within one month prior to transplantation (PreTx), at maximum elevation (Ev) and baseline (Bsl) following last islet infusion (10-12 samples/ each patient)

S: Success; PS: Partial Graft Success; F: Graft Failure; HV: healthy volunteers

# p <0.0001(PS *vs.* S); *** p <0.0001 (F *vs.* S); p=ns (HV *vs.* S)

*$ p <0.0001 (Ev vs. Bsl; PS); **p <0.01(Ev vs. Bsl; F)*

Figure 3: Circulating hepatic and endothelial cell-derived MVs in transplanted patients with graft success, partial success or failure. MVs were measured within one month prior to transplantation (PreTx) at maximum elevation (Ev) and baseline (Bsl) following last islet infusion (10-12 samples/patient)

S*: Success; PS: partial graft success; F: graft failure*

(A) ASGPR$^+$-MVs: *# p <0.0001 (Ev vs. Bsl; PS); $ p <0.0001(Ev vs. Bsl; F)*

(B) CD105$^+$-MVs: ** *p <0.01 (Ev vs. Bsl; PS); **p <0.01(Ev vs. Bsl; F)*

Figure 4: Circulating T-helper, Cytotoxic-T, and ß-cell derived MVs in transplanted patients with graft success, partial success or failure. MVs were measured within one month prior to transplantation (PreTx) at maximum elevation (Ev) and baseline (Bsl) following last islet infusion (10-12 samples/patient). MV values were reported for the total count of circulating lymphocytes for each sample

S*: Success; PS: partial graft success; F: graft failure*

(A) $CD4^+$-MVs: *# p <0.0001 (Ev vs. Bsl; PS); **p <0.01(Ev, vs. Bsl; F)*
(B) $CD8^+$-MVs: *p=ns (Ev vs. Bsl; PS); ***p <0.001(Ev vs. Bsl; F)*
(C) $CD19^+$-MVs *p=ns (Ev vs. Bsl; PS); p=ns (Ev vs. Bsl; F)*

Figure 5: Circulating neutrophil- and monocyte -derived MVs in transplanted patients with graft success, partial success or failure. MVs were measured within one month prior to transplantation (PreTx) at maximum elevation (Ev) and baseline (Bsl) following last islet infusion (10-12 samples/patient).

S: Success; PS: partial graft success; F: graft failure

(A) $CD66b^+$ -MVs *values were reported for the number of circulating neutrophils for each sample. *** p <0.001 (Ev vs. Bsl; PS); **p <0.01(Ev vs. Bsl; F).*
(B) $CD14^+$*-MV values were reported for the number of circulating monocytes for each sample. # p <0.0001 (Ev vs. Bsl; PS); *p <0.05 (Ev vs. Bsl; F).*

Figure 6: Sequence of circulating MVs release during islet transplantation, with respect to their cell origin.

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Tables and Figures

Table 1: Pre-transplantation demographic values and biological characteristics of islet transplanted patients and healthy volunteers.

Variables	Transplanted T1D	Success	Partial Success	Failure	HV
N	19	8	8	3	13
M/F(n)	10/9	6/2	5/3	2/1	3/10
Age (years)	56 (29-74)	58 (44-74)	61 (29-68)	57(43-63)	40 (29-61)
Weight (kg)	62 (49-77)	60 (55-74)	71 (63-77)	55 (49-60) *	62 (50-95)
Diabetes duration (years)	38 (14-44)	25 (14-36)	34 (15-44)	26 (20-32)	-
HbA1c (%)	7.5 (5.2-8.7)	7.1 (5.2-8.1)	8.1(7.2 -8.7)	7.5 (8.1-7.1)	-
Glucose fasting level (mg/dl)	160 (57-241)	110 (57-234)	119 (62-195)	180 (138-241)	-
Creatinin (µmol/l)	65.2 (48-119.8)	88.5 (56-110)	84 (58-119.8)	52 (48-56)	-
Insulin needs (IU/kg/d)	0.6 (0.3-1.05)	0.5 (0.42-1.05)	0.48 (0.3-0.87)	0.76 (0.56-0.8)	-
Islet Tx alone (n)	14 (74)	6 (75)	6 (75)	2 (67)	-
Islet after kidney Tx (n, %)	5 (26)	2 (25)	2 (25)	1 (33)	-
Graft follow-up (months)	48 (12-60)	59 (45-60)	60 (30-60)	60 (12-60)	-
Graft ummunogenicity					
HLA mismatches	Negative	Negative	Negative	Negative	-
Autoantibodies (n patients)					
Anti GAD	7	2	3	2	-
Anti IA2	2	1	1	0	-
Immunosuppression					
Depleting induction (n, %)					
Rituximab/ Daclizumab	5 (26)	2 (25)	2 (25)	1 (33)	-
Tymoglobulin	14 (74)	6 (75)	6 (75)	2 (67)	-
Maintenance regimen (n, %)					
CNI + Sirolimus	5 (26)	6 (75)	6 (75)	2 (67)	-
CNI + MMF	14 (74)	2 (25)	2 (25)	1 (33)	-

Values are expressed as median and interquartile range (IQR). Routine blood tests, as well as pancreatic and kidney function tests, were performed using standard clinical methods.

Statistical significance by multivariate analysis, using ANOVA and Kruskal Wallis test **: p <0.05 (PS vs. F).*

CNI: calcineurin inhibitor: HV: healthy volunteers; MMF: mycophenolate mofetil; HLA: human leukocyte antigen; HbA1c: glycated hemoglobin; M/F: male/female; Tx: transplantation; GAD: anti- glutamic acid decarboxylase; IA2: anti-tyrosine phosphatase.

Table 2: Time delay between microvesicle (MV) elevation and the subsequent β-score decrease in partial graft success.

MVs	Days to drop of the β-score median (range) in PS
Insulin secreting ß-cells PSA-NCAM^{+}-MVs	42 (29 - 63)
liver cells ASGPR^{+}-MVs	55.8 (31.8 - 118.8)
Endothelial cells CD105^{+}-MVs	78 (60 - 216)
Neutrophils CD66b^{+}-MVs	83.1 (66 - 102)
Monocyte/macrophages CD14^{+}-MVs	87.6 (70.2 - 102)
T 8 lymphocytes CD8^{+}-MVs	-
T 4 lymphocytes CD4^{+}-MVs	147 (37.8 - 196.2)
Procoagulant MVs Annexin V^{+}-MVs	85.2 (30 - 129)

Values are expressed as days (median and interquartile range, IQR).
Annexin V^{+}: Procoagulant MVs; PSA-NCAM^{+}-MVs: beta cell-derived MVs; CD66b^{+}-MVs: neutrophil; CD14^{+}-MVs: monocyte cell-derived MVs; CD4^{+}-MVs: T helper and CD8^{+}-MVs cytotoxic T: cell-derived MVs; ASGPR^{+}-MVs: hepatic MVs; CD105^{+} -MVs: endothelial cell-derived MVs.

Figure 1 :

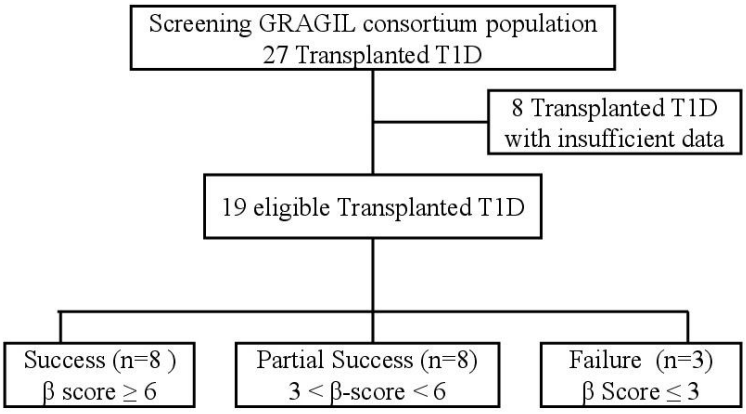
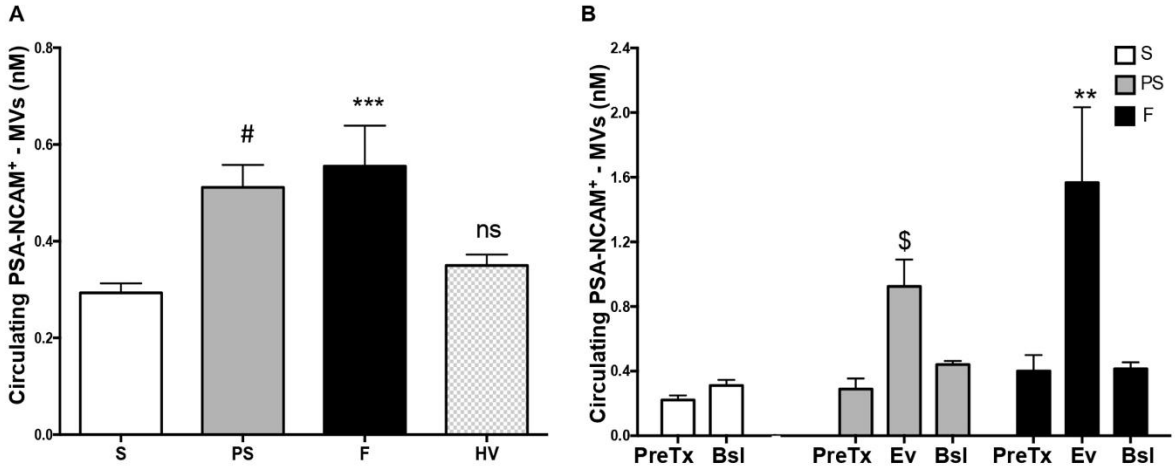


Figure 2 :



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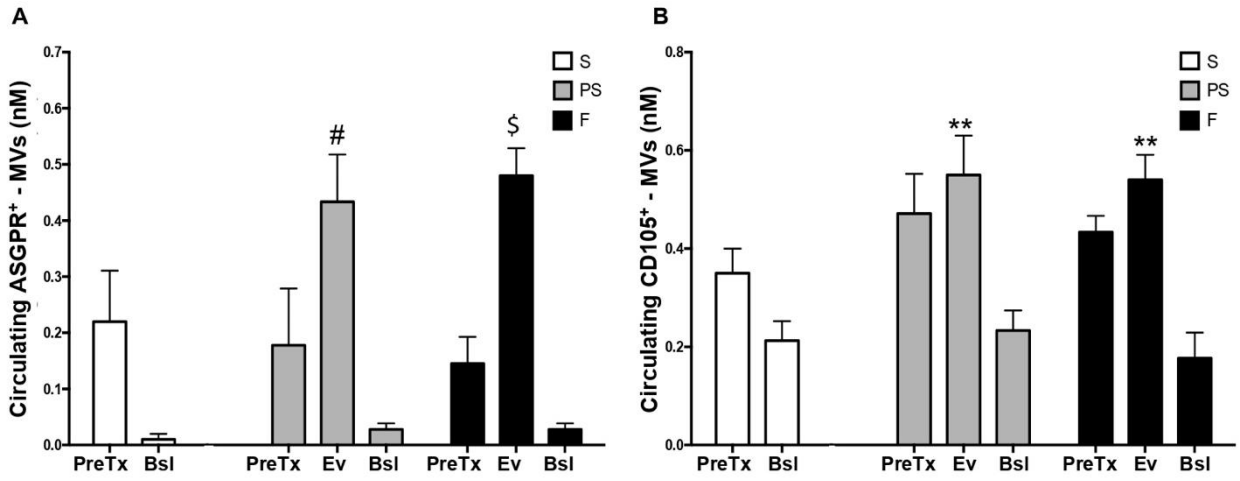

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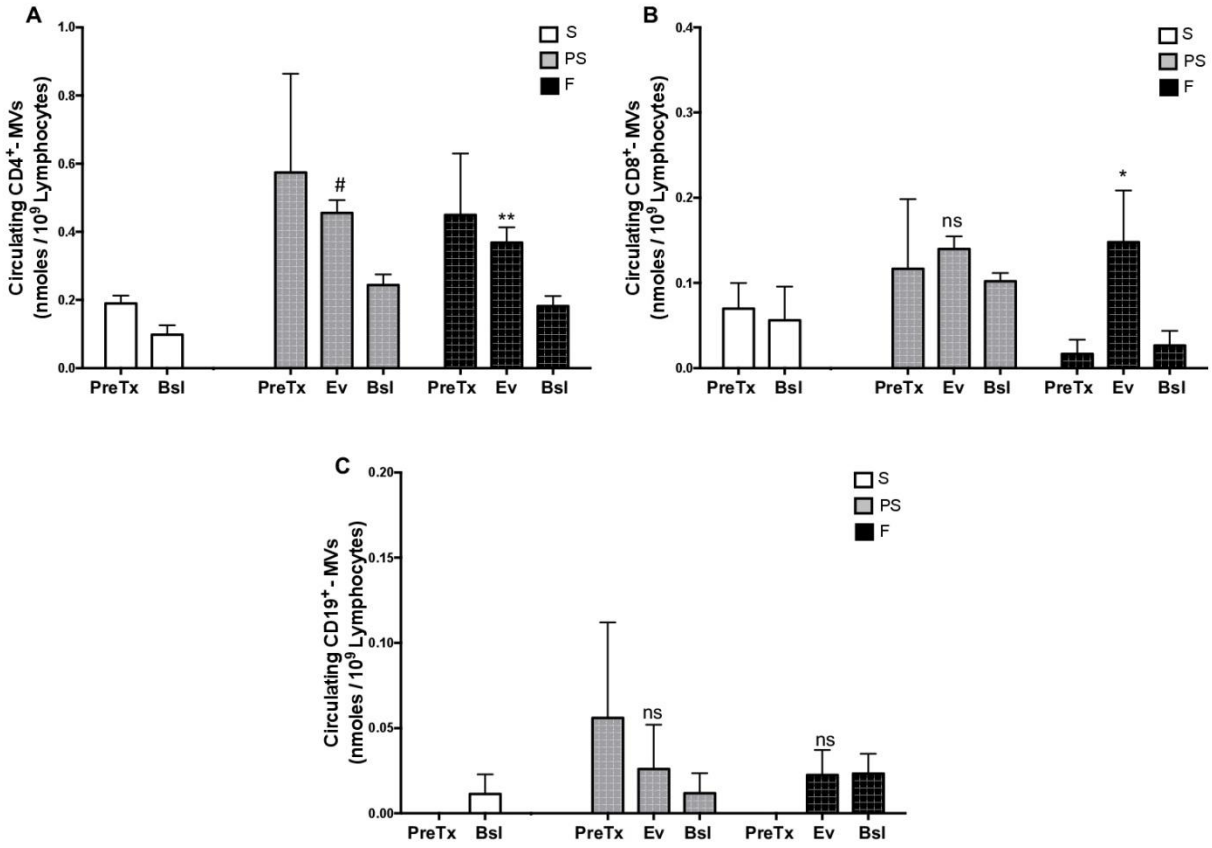

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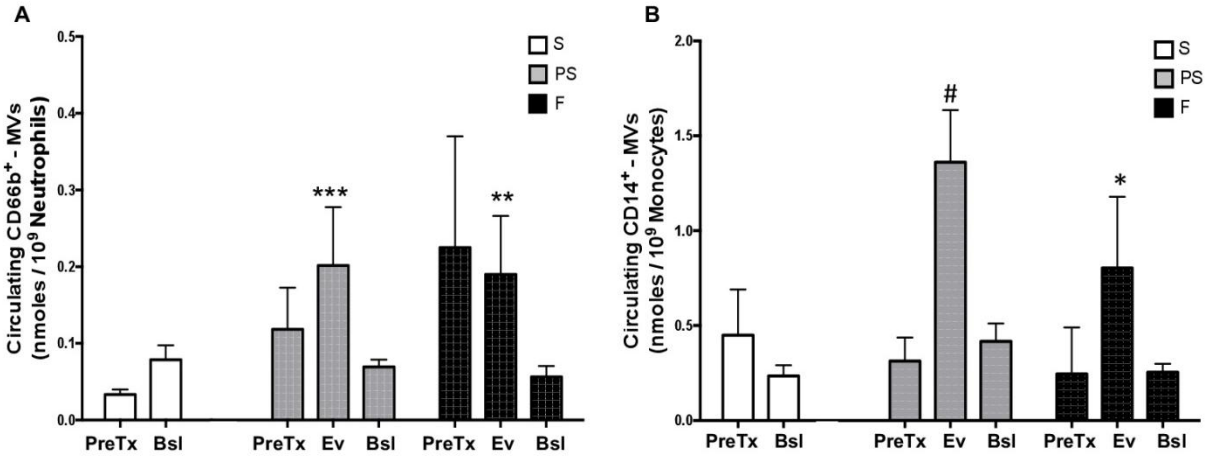

Figure 6: