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Genetic and functional profiling of CD16-dependent natural killer activation identifies patients at higher risk of Cardiac Allograft Vasculopathy

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Abstract

Background Cardiac transplantation is an effective therapy for end-stage heart failure. As cardiac allograft vasculopathy (CAV) is the major cause of late mortality after Heart transplant (HT), there is an urge to identify markers that reflect inflammatory or cytotoxic immune mechanisms contributing to its onset. Non-invasive and early stratification of patients at risk remains a challenge to adapt individualized therapy. While the CD16 (FCGR3A) receptor was recently identified as a major determinant of antibody-mediated natural killer (NK) cell activation in heart transplant biopsies, little is known about its role in promoting allograft vasculopathy. This study aimed to investigate whether markers that reflect CD16-dependent circulating NK cell activation may identify patients at higher risk of developing CAV after HT.

Methods Blood samples were collected in 103 patients undergoing routine coronarography angiography for CAV diagnosis (median 5 years since HT). Genomic and phenotypic analyses of FCGR3A/CD16 Fc receptor (FcR) profiles were compared in CAV-positive (n=52) and CAV-free patients (n=51). The level of CD16 expression and rituximab-dependent cell cytotoxic activity of peripheral NK cells in heart transplant recipients (HTRs) was evaluated by using a non-invasive NK-cellular humoral activation test (NK-CHAT).

Results The enhanced levels of CD16 expression and antibody-dependent NK cell cytotoxic function of HTR were associated with the FCGR3A-VV genotype. The frequency of the FCGR3A-VV genotype was significantly higher in the CAV+ group (OR: 3.9, p=0.0317). The FCRGR3A-VV genotype was identified as an independent marker that correlates with the presence of CAV at time of coronary angiography using multivariate logistic regression models. The FCRGR3A-VV genotype was also identified as a baseline independent predictor of CAV risk (OR: 4.7, p=0.023).

Conclusions This study unravels a prominent role for the CD16-dependent NK cell activation pathway in the complex array of factors that favor progression of transplant arteriosclerosis. It highlights the clinical potential of a non-invasive evaluation of the FCGR3A/CD16 in the early stratification of CAV risk. Recognizing CD16 as a major checkpoint controlling immune surveillance may promote the design of individualized NK cell-targeted therapies to limit vascular damage in highly responsive sensitized patients.

Clinical Perspective

What Is New?

- We used a non invasive NK-Cellular humoral activation test (NK-CHAT) to evaluate the association between genetic and phenotypic markers of the CD16-dependent NK cell activation pathway and development of Cardiac Allograft Vasculopathy (CAV).
- We report that the FCGR3A-VV polymorphic variant, encoding the highly responsive CD16 Fc-receptor, can be identified as an independent baseline predictor of CAV and can be useful for stratifying patients at higher risk of rejection.
- This study identifies a potential immune mechanism underlying the inter-individual variability of the recipient's complement-independent humoral response that relies on its FCGR3A genotype and CD16-dependent NK cell activation profile.

What Are the Clinical Implications?

- The non-invasive profiling of FCGR3A polymorphism and CD16-dependent NK cell responses may warrant personalized monitoring of heart transplant outcomes.
- NK-CHAT appraisal of risk could potentially limit the recommendation of invasive biopsies to sensitized patients with high humoral responsiveness.
- Indexing of patient-to-patient variability regarding NK cell responses may be of value in defining potency markers that anticipate the response to rituximab or IV-Ig treatment of AMR.
- Recognizing CD16 as a major checkpoint controlling immune surveillance may promote the possibility of using NK cell-targeted therapies to limit vascular damage in patients identified being at high humoral risk.

Keywords: Cardiac allograft vasculopathy, Natural killer cell cytotoxicity, Fc-gamma receptor polymorphism, Heart transplantation, Donor-specific anti-HLA antibodies

Abbreviations and acronyms

ATG: anti-thymocyte globulin

BMI: body mass index

CAV: cardiac allograft vasculopathy

CKD: chronic kidney disease

CMV: cytomegalovirus

CNI: calcineurin inhibitor

DSA: donor-specific antibodies

eGFR: estimated glomerular filtration rate

FCGR: Fc gamma receptor

HLA: human leucocyte antigen

HT: heart transplantation

HTR: heart transplant recipient

MFI: mean fluorescence intensity

MMF: mycophenolate mofetil

NK: natural killer

NK-CHAT: NK cell- Cellular Humoral Activation Test

PBMC: peripheral blood mononucleated cells

SNP: single-nucleotide polymorphism

Introduction

Heart transplantation (HT) is a life-saving treatment for patients with advanced heart failure. Predicting the risk of a given patient developing coronary artery disease in the transplanted heart, also known as cardiac allograft vasculopathy (CAV), remains a major issue in the prevention of allograft loss and long-term mortality after heart transplantation¹⁻³. In 2016, the International Society for Heart and Lung Transplantation (ISHLT) updated the guidelines and risk factors for development of CAV⁴.

Diagnosis of CAV often relies on asymptomatic patients receiving a coronary angiography on a regular basis, which is an invasive procedure that is often performed at stages when lesions have already progressed and remain poorly accessible to treatment. The current challenge is to identify non-invasive biomarkers that allow for the early stratification of patients at risk^{2, 5-8} to tailor individualized therapeutic approaches preventing heart transplant failure^{6, 9}.

CAV is the result of cumulative endothelial aggression resulting from activation of alloreactive cells. The humoral component is a major determinant of allograft injury after organ transplantation¹⁰⁻¹³. The inflammatory signals resulting from stimulation of activating Fc- receptors (Fc-R) for C-reactive protein (CRP) serum amyloid P component (SAP) and IgG expressed on endothelial cells, vascular smooth muscle cells, monocytes/macrophages are identified as contributors and biomarkers of coronary heart disease pathogenesis¹⁴. Fc-R polymorphisms have been reported as individual predisposition factors contributing to vascular disease¹⁴, notably when development of cardiovascular disease associate with auto or alloimmune disorders and cannot be explained by traditional cardiovascular risk factors. Donor-specific anti-human leukocyte antigen (HLA) circulating antibodies (DSA) with C1q binding activity have been associated with worsened prognosis after HT, but these markers lack the specificity to predict graft outcome in a given patient¹⁵. In addition to complement-dependent mechanisms, the Fc segment of IgG can stimulate the CD16-dependent

cytotoxicity and inflammatory function of immune natural killer (NK) cells^{16, 17}, whose critical role has been identified in antibody-driven allograft vasculopathy using animal models^{18, 19}. Recent reports have highlighted the clinical value of tissue-based gene profiling of NK-cell transcripts within the heart allograft in the classification of antibody-mediated rejection (AMR)²⁰. These approaches have identified a role for the CD16/FcR activation pathway in promoting antibody-mediated vascular damage of heart allograft²¹. A single-nucleotide substitution (rs396991) in the FCGR3A gene leads to an allelic variation of amino acid 158 (phenylalanine-F or valine-V) in the IgG binding domain of the CD16 receptor that impacts the antibody-dependent cell cytotoxic (ADCC) function of NK cells. The FCGR3A-VV polymorphic variant binds with higher affinity to human IgG1 and IgG3 than does the FCGR3A-FF variant. The host FCGR3A genotype has been identified as a major determinant of the clinical response to therapeutic IgG such as rituximab²². In addition, the FCGR3A polymorphisms have been reported to contribute to infection susceptibility²³⁻²⁵ and coronary artery diseases^{26, 27} and to predispose patients to severe complications after organ transplantation²⁸⁻³². In the current study, we investigated whether CD16-dependent NK cell activation profiles and FCGR3A polymorphism may be associated with the development of CAV.

Methods

Patient population and CAV grading

We performed a prospective single-centre observational study including 103 HT recipients (HTRs) undergoing a coronary angiography in the framework of their routine follow-up (performed on the first year after HT, every two years or in case of unexplained left ventricular dysfunction) in the cardiac surgical unit of Assistance Publique-Hôpitaux de Marseille (AP-HM) from 2011 to 2014. The study was approved by the Agence Française de

Sécurité Sanitaire des Produits de Santé (June 2011, reference B101345-70) and the Comité de Protection des Personnes Sud-Méditerranée I (January 2011, reference 10-61) and supervised by AP-HM under ID numbers NCT01569334/2010-A01145-34). Written informed consent was obtained from all participants. The healthy donors' blood samples were anonymized according to the French Blood Centre regulation procedures. CAV diagnosis was defined by coronary angiography and graded according to the ISHLT recommendations. All patients received perioperative induction therapy with rabbit anti-thymocyte globulin (ATG) or basiliximab. Statin therapy was given postoperatively. Maintenance immunosuppressant therapy included calcineurin inhibitors (CNIs), such as cyclosporine or tacrolimus, mycophenolate mofetil (MMF) or everolimus and steroids. Blood samples were collected in a fasting state 12 hours after CNI administration.

Cells, plasma and sera

Cell and sera samples of HTRs and healthy donors were collected from AP-HM and from EFS respectively. Peripheral blood mononuclear cells (PBMCs) were isolated from HTR and healthy donor blood samples using Ficoll gradients.

Anti-HLA antibody detection

Sera from all patients were tested for anti-HLA antibodies using multianalyte bead assays performed on the Luminex-based LABScan™ 200 flow analyzer. The antibody specificity and binding levels (mean fluorescence intensity: MFI), were determined by the LABScreen® Single Antigen bead assay (One Lambda, Thermo Fisher Scientific, California, USA) and analyzed with the HLA Fusion software. The binding reactivity was considered saturated with an MFI >15000, positive with a MFI >500 to 10000 or negative with an MFI <500. The

HLA antibody specificities were analyzed in reference to the HLA typing of donors to identify DSA.

C1q detection

The C1qScreen™ assay was used to test the capacity of DSA to bind C1q (One Lambda, Part of ThermoFisher Scientific). The C1q complement component bound by the antigen-antibody complex was detected with an R-PE-labeled anti-C1q antibody. Fluorescence intensity was measured using a Luminex-based LABScan™ 200 flow analyzer. MFI>1000 for C1qScreen™ assay was considered positive.

FCGR3A genotyping

The detection of FCGR3A (F158V/rs396991) polymorphisms was performed by using the SNAPSHOT technique. Data were analyzed using the GeneMapper 4.0 and GeneRate computer tool package (<http://geneva.unige.ch/generate>). For each of the different alleles obtained, both strands of the PCR products were sequenced using the BigDye Terminator v1.1 Sequencing Kit (Applied Biosystems) and analyzed on an automated ABI PRISM 3130 XL genetic analyzer.

Phenotypic analysis of peripheral lymphocyte cell subset

Multiparameter flow cytometry analysis of NK cells was conducted on PBMCs isolated with Ficoll-Paque (Eurobio, Les Ullis, France). Antibodies were obtained from Beckman Coulter: CD3-ECD (IgG1, UCHT1); CD56-PC7 (IgG1, N901); CD16-AA750 (IgG1, Clone 3G8). The cells were labeled for 15 min at room temperature, and the NK cells were defined as the percentage of CD3–CD56+ NK cells expressing the CD16 receptor. The MFI of CD16 staining was further evaluated within the CD16+CD3–CD56+ NK cell subset (Supplemental

figure 2). At least 3000 events were analyzed in the NK lymphocyte gate using the Navios cytometer (Beckman Coulter).

Phenotypic analysis of antibody-dependent NK cell activation

The NK-CHAT analysis was performed as previously detailed³³. Briefly, effector PBMCs and 500,000 target cells (CD20⁺ B-EBV-transformed or K562 cell lines) were incubated with 10% SVF for 3 h at 37°C using a 1:1 effector/target ratio in the presence of Golgi Stop and CD107a-PC5 antibodies (Becton Dickinson). Cells were washed and labeled with CD3-ECD, CD16-PE and CD56-PC7 antibodies (Beckman Coulter) for 15 min at room temperature. Data acquisition was performed on a Navios cytometer. CD107a/Lamp1 expression patterns were analyzed in the CD3⁻CD56⁺ NK subset gated within the PBMCs. Analysis of the K562-induced NK cell expression of CD107a/Lamp1 cytotoxic granule exocytosis allowed for the evaluation of the natural cytotoxic activity of NK cells. NK-ADCC activity was evaluated after addition of 10 μ g/ml of rituximab to the PBMC-B cell target co-culture for 3h by measuring the % of CD107a/Lamp1-positive CD3⁻CD56⁺ NK cells. The level of CD16 engagement was quantified as the ratio between the MFI of NK cell CD16 expression (CD16MFI) observed when PBMCs were incubated with CD20⁺ B cell targets in the presence or absence of rituximab. The level of rituximab-induced NK cell CD16 engagement was defined as the CD16 down-regulation index (CD16DRI).

Statistical analysis

Statistical analysis was performed using Graph Pad Prism 5 software (GraphPad Software, La Jolla, CA) and IBM SPSS Statistics for Windows, Version 20.0 (Armonk, NY: IBM Corp). Quantitative variables were summarized by means and standard deviation (\pm SD) or medians and interquartile ranges. Continuous variables were compared using Student's t-test or non-

parametric Mann-Whitney test. One-way analysis of variance (ANOVA) was used to compare more than two groups. The association between continuous variables was analyzed using Spearman's or Pearson's rank correlation analysis. Categorical variables were reported as counts or percentages and compared using a chi-square or Fisher's exact test. A p value <0.05 was considered to represent a statistically significant difference. Time of first CAV diagnosis was compared across the FCGR3A genotypes with the use of Kaplan-Meier estimation combined with the log-rank test. Association between CAV and clinical, demographic and immunological variables were assessed by using a univariate analysis. Variables identified as significantly associated with CAV (p value <0.05 , Table 1) allowed for the construction of logistic regression multivariate models enabling the calculation of adjusted tests characterizing the risk factors associated with CAV.

Results

Clinical characteristics of HTRs

The characteristics of the study cohort are shown in Table 1. Coronary angiograms were used to define a CAV- group of 51 HTRs without detectable angiographic lesions and a CAV+ group of 52 patients exhibiting mild (grade 1, $n=35$), moderate (grade 2, $n=6$) or severe (grade 3, $n=11$) CAV. Time post-transplant was highly associated with the occurrence of CAV and the median time at first CAV diagnosis was 3 years (25-75 percentile: 1-7.2). CAV was diagnosed in 8 of 29 HTRs (28%) examined 1-year post-transplant, 12 of 29 (41%) HTRs evaluated between 1 and 5 years post-transplant, 14 of 35 HTRs (40%) evaluated between 6 and 9 years post-transplant, and 32 of 45 HTRs (71%) evaluated after 10 years post-transplant. Donor age and recipient body mass index (BMI) were significantly higher in the CAV⁺ group. The rates of CAV tended to be higher when a female recipient received a heart

from a male donor (n=13 CAV⁺ out of 20 patients, p=0.161) or when the patient had signs of hypertension before transplant (p=0.071). Renal dysfunction at the time of enrolment (eGFR <60 ml/min/1.73 m²) was significantly associated with CAV (Table 1).

Maintenance immunosuppressant mainly consisted of a tritherapy regimen combining calcineurin inhibitors (CNIs), MMF and steroids (group A, n=68) that associated with lower CAV incidence (Table 1), or everolimus, CNI and steroids (group B, n=15), or bi- or monotherapy (group C, steroid or MMF withdrawal n=18; CNI monotherapy n=2). Post-transplant infections (viral, fungal, bacterial or parasitic) were observed in 48 patients (30 of 52 CAV⁺ HTR). Post-transplant viral (15 CAV⁺ out of 21 HTR) and bacterial infections (24 CAV⁺ out of 36 HTRs) were associated with CAV (Table 1). Post-transplant CMV infectious rates were higher in CAV⁺ HTR (14 CAV⁺ out of 18 CMV⁺ HTR). The occurrence of CAV was also significantly higher when the recipient was not immunized against Epstein-Barr virus (EBV) prior to the graft (16 CAV⁺ out of 21 EBV⁻ patients at transplant). Circulating anti HLA-antibodies were detected in 30 HTRs (29%). Circulating DSAs were detectable in 22% of HTRs. Among the DSA, 14 were further identified as C1q-binding DSA, mostly associating DSA with MFI>10000 and HLA Class II recognition (online Supplemental Table 1). No significant association was noted between the presence of circulating DSA or C1q-binding DSA or a history of cellular rejection and the occurrence of CAV.

Quantitative analysis of lymphocyte cell subset counts

The distribution (% and cell numbers) of peripheral blood CD3⁻CD56⁺ NK, CD19⁺ and CD4⁺/CD8⁺ T lymphocyte subsets was analyzed in HTRs. CD3⁺CD8⁺ and NK peripheral blood cell counts were preserved in HTRs and exhibited values that were comparable to the reference values observed in a cohort of healthy controls. In contrast, B and CD4⁺ T lymphocyte cell counts were significantly lower in HTRs than in healthy controls. No

significant difference in the distribution of lymphocyte cell subsets was observed between the CAV⁺ or CAV⁻ groups (online Supplemental Figure 1).

NK cell cytotoxic activity is preserved in HTRs

Flow-cytometry analysis of the membrane expression of the cytotoxic granule CD107a/Lamp1 NK cell marker revealed that constitutive NK cell activation (% CD107a/Lamp1-positive NK cells in the absence of target stimulation) and K562-driven natural cytotoxicity of NK cells were comparable in the HTR and controls groups (Figure 1A). Quantification of the antibody-dependent cytotoxic activity of peripheral NK cells towards autologous B-lymphocytes challenged with rituximab (NK-ADCC) revealed a high inter-individual heterogeneity among HTRs (Figure 1A). The NK-ADCC intensity was correlated with the number of autologous circulating B-lymphocytes expressing the CD20 target antigen (Figure 1B). Flow-cytometry analysis of CD16 expression on CD3-CD56⁺ NK cells gated within PBMCs in a standardized NK-CHAT assay³³ further allowed for the measurement of the level of Fc-dependent activation of HTR NK cells towards rituximab-coated B cell targets (online supplemental figure 2A). To normalize the variability of baseline CD16 expression in HTRs, the CD16DRI was calculated as the ratio between the NK cell CD16 staining (MFI) measured in response to B cells coated with control serum (baseline) and the NK cell CD16MFI measured in response to rituximab-coated cells (illustrated in online supplemental figure 2B). The CD16DRI exhibited high inter-individual variability among HTRs (median CD16DRI: 8.6-fold rituximab-induced down-regulation, 25-75 percentile: 5.5-13.7) and was correlated with the cytotoxic function of HTR NK cells, as measured by CD107a/Lamp1 granule exocytosis in response to rituximab (Figure 1C).

The FCGR3A polymorphism is associated with the level of CD16-dependent NK cell activation

The CD16DRI was also shown to be significantly associated with the constitutive CD16 expression on circulating NK cells (CD16MFI, Figure 2A).

We then investigated whether such inter-individual NK-ADCC variation may relate to polymorphic variations in the FCGR3A gene encoding CD16. We showed that the CD16DRI was associated with the FCGR3A genotype of HTRs and was higher in homozygous FCGR3A-VV patients than in HTRs bearing the FCGR3A-VF or FCGR3A-FF genotype (Figure 2B). Similarly, we confirmed that the basal level of CD16 expression (CD16MFI) in circulating NK cells was significantly higher in recipients bearing the homozygous FCGR3A-VV genotype (Figure 2C).

The FCGR3A-VV genotype is an independent predictor of CAV

The frequency of the FCGR3A-VV genotype was significantly higher in the CAV⁺ group (Odds ratio: 3.9, 95%, p=0.0317, CI: 1.18 to 12.99, positive predictive value=0.92, negative predictive value=0.25) than in the CAV-free or healthy control groups (p=0.03, Figure 3A). A Kaplan-Meier analysis adjusting for the time post-transplant at first CAV diagnosis confirmed that the median CAV-free survival was 5 years in FCGR3A-VV HTRs, while individuals who bear the FF/VF genotype had a median CAV-free survival of 9 years post-HT (Figure 3B). The FCGR3A-VV genotype was also associated with a higher incidence of post-transplant viral infections (p=0.005, Odds ratio: 4.92 CI: 1.6-15.1), whereas the FCGR3A-FF genotype (35 patients) was associated with an enhanced rate of skin cancers occurring between transplant and enrolment in the study (6 out of 9 patients, p=0.021), CKD (p=0.028, 28 out of 35 FCGR3A-FF HTRs with eGFR < 60 ml/min/1.73 m²), and an enhanced frequency of circulating DSA with MFI >10000 or C1q binding activity (p=0.037) (Online Supplemental Table 2). A majority of patients who developed CMV infection also developed CAV. Indeed 14 out of the 18 patients who developed CMV infection after heart transplant had a diagnosis

of CAV (3 grade 3, 1 grade 2 and 10 grade 1). It is of note that 50% of these patients who developed CMV and CAV were FCGR3A-VV, while none of the patients that developed CMV infection in absence of CAV exhibited this FCGR3A-VV susceptibility genotype (Online Supplemental Table 3).

We further challenged the independent value of these markers of the CD16 NK cell activation pathway as susceptibility markers for CAV using regression multivariate analysis models introducing confounding factors identified in the univariate analysis (Table 1). We identified an elevated risk of developing CAV in FCGR3A-VV (Table 2), independent of other CAV risk factors such as time since transplantation, donor age, recipient age and BMI, occurrence of post-transplant viral and bacterial infections, CKD and immunosuppressive regimen. We further characterized the risk associated with the FCGR3A genotype at the time of transplant by constructing a predicting multivariate model that restricts the adjusting variables to pre-transplant features. The FCGR3A-VV genotype remained a strong predictor of CAV development independent of other risk factors such as donor age, hypertension, and male donor to female recipient status (Table 3). High constitutive NK cell surface expression of CD16 (online supplemental Table 4) and HTR NK-ADCC responsiveness, as evaluated by the CD16DRI (online supplemental table 5) were also correlated with the angiographic CAV diagnosis at the time of enrolment.

Discussion

As CAV is a major cause of late mortality after HT, there is an urge to identify early non-invasive markers that reflect immune mechanisms contributing to its onset. Non-invasive

early stratification of patients at risk is also a major challenge that favors the personalized indication of treatment. The potential contribution of genetic variation conditioning inflammatory processes resulting from CRP ligation to Fc-R has been evoked as a mechanism promoting vascular injury and predisposition to cardiovascular disease. Targeted anti-inflammatory therapies have been proposed as a novel approach to reduce cardiovascular risk³⁴. Our study is the first establish a link between the genetic variation of the FCGR3A/CD16 activating Fc-R and the predisposition for heart vasculopathy. Recent reports have identified the Fc-Receptor CD16 as a major checkpoint controlling inflammatory and vascular cytotoxicity resulting from immune alloreactivity. A recent report²¹ illustrated the clinical potential of biopsy measurement of CD16 and NK cell transcripts to refine classification and diagnosis of antibody mediated heart transplant rejection. In this context, our study provides evidence that CD16 profiling identifies non-invasive biomarkers that reflect the intrinsic potential of a given recipient to mount deleterious cytotoxic immune responses towards heart vasculature. The strength of this study is to provide evidence that the FCGR3A-VV genetic variant encoding the CD16 receptor with high affinity for the Fc portion of antibodies is associated with an enhanced CD16-driven NK cell activation potential and behaves as a susceptibility factor identifying patients at higher risk to develop coronary artery disease of the transplanted heart. At the individual level, such a highly responsive NK cell profile associated with the VV genotype and CD16 expression levels is expected to shape the intensity of NK cell antibody-dependent cellular cytotoxic and inflammatory paracrine function through the release of CD107a/Lamp1 cytotoxic granules and inflammatory cytokines such as interferon (IFN)- γ .

We show that despite immunosuppression, the number and cytotoxic function of circulating immune NK cells are maintained in HTRs and exhibit high patient-to-patient variability. Although preservation of this immune potential may be beneficial for thwarting the neoplastic

and infectious complications that often arise in immunosuppressed HTRs, it may cause adverse effects of auto or alloantibodies towards graft endothelium in immunized patients bearing the high-affinity FCGR3A-VV allele. The phenotypic NK-CHAT evaluation of peripheral blood NK cell activation in a relevant cohort of HTRs showed that patients developing post-transplant CAV are predominantly those that exhibit the highest CD16 expression and CD16-dependent NK cell activation potential in response to antibodies. We show that the intensity of the NK cell response to rituximab is dependent not only on the number of HTR peripheral B cells expressing the CD20 antigen but also on the intrinsic level of CD16 expression of the recipient, which is in part conditioned by the FCGR3A-VV genotype. These data provide a mechanistic link between the CD16 signaling pathway and the highly variable capacity of a given recipient to trigger immune activation in response to a viral or humoral challenge, both identified as risk factors of CAV.

As previously reported, history or persistent chronic kidney disease (CKD, eGFR <60), evaluated in transplant recipients before heart transplant and at time of enrolment in the study, was identified as an independent risk factor for CAV. The pathological crosstalk between heart and kidney organ dysfunction, also referred to as the cardio-renal syndrome is now widely recognized. It is admitted that various factors participate to alteration of renal function in HTR, both before and after heart transplant. While multivariable analysis retained a kidney dysfunction before graft, recipient age and % LVEF as independent risk factors associating with CKD (eGFR <60), no significant association could be observed between renal dysfunction and calcineurin inhibitors dosage or CD16-dependent NK cell activation.

Strength of the study is to provide evidence that the identification of CD16 profile of the recipient as a risk factor associated to development of CAV is in part independent of other risk factors that associate to the cardio renal syndrome such as donor/recipient age, uremic or infectious status and immunosuppressive regimen.

Strength of the study is to provide evidence that the identification of CD16 profile of the recipient as a risk factor associated to development of CAV is in part independent of other risk factors that associate to the cardio renal syndrome such as donor/recipient age, uremic or infectious status and immunosuppressive regimen.

Genotyping of the FCGR3A polymorphic variant would be easy to implement in the pre-transplant setting; therefore, we challenge the view that this susceptibility marker may represent an independent predictor with potential value in the identification of patients at higher risk of developing CAV. Although previous studies have reported associations between FCGR polymorphisms and adverse infectious events or accelerated atherosclerosis after organ transplant^{28, 30, 31}, the link between the FCGR3A SNP and infection has yet to be elucidated in the context of HT. A recent study reported that patients with a combined FCGR2A-131H/H and FCGR3A-FF (low IgG binding affinity) genotype were more susceptible to blood-borne infections and exhibited increased mortality after liver transplant. Previous studies have identified CMV infection as a risk factor associated with the development of allograft vascular lesions^{35, 36}. In the present cohort, the FCGR3A-VV genotype was identified as a risk factor associated with viral infections. We provide evidence that the FCGR3A-VV genotype is associated with an increased susceptibility to CAV independent of the pre-transplant recipient and donor CMV combination, post-transplant infectious status, or immunosuppressive regimen. This suggests that, upon CMV infectious challenge, FCGR3A-VV HTRs may develop more potent humoral inflammatory responses that favor the development of allograft vasculopathy.

Interestingly, the association of CAV with the genetic and functional biomarkers of the CD16 activation pathway was independent of the DSA C1q binding activity. Our study thus reveals a potential role for the FCGR3A-VV SNP as a susceptibility factor that controls the chronic cytotoxicity of antibodies independent of the complement pathway. Although this association remains unclear, enhanced MFI and C1q binding activity of DSA and skin cancer rates were associated with the FCGR3A-FF genotype, thus suggesting that the threshold of innate immune NK cell immunosurveillance may be lowered in these HTRs. Interestingly, among the 8 patients who exhibited the highest levels of circulating DSA (MFI>15000), all 4 patients who remained CAV-free at the time of enrollment had the FCGR3A-FF low-responsive genotype. As shown in the kidney transplant setting (28), we suggest that such non-invasive NK-CHAT evaluation of CD16-mediated reactivity may reveal features of underlying humoral mechanisms involving NK cell chronic activation towards the donor heart that may not be fully appreciated from a conventional Luminex assessment of HLA circulating antibodies. We expect that the non-invasive CD16DRI evaluation of recipient NK cell reactivity towards donor cells could reveal the pathogenic activity of anti-endothelial antibodies that remains undetectable with conventional assays but exhibits a high potential to promote vasculopathy in high responders with the FCGR3A-VV genotype.

Our work has several limitations. First, the FCGR3A profiles were only analyzed in 103 HTRs, and the ability of the NK-CHAT to predict CAV needs to be further demonstrated in larger prospective and multicentric cohorts of HTRs with defined pre- and post-transplant time points. Nevertheless, given the multiple risk factors evaluated, none of the other Fc-R polymorphisms evaluated proved to be significantly associated to CAV in the present cohort. Considering this primary analysis, we also need to challenge the predictive value of these CD16-dependent NK cell activation indexes evaluated at time of transplant with more

stringent end-points, such as graft failure, cancer and viral immune surveillance, AMR and patient mortality. FcγRs are also expressed on neutrophils and monocyte/ macrophage subsets; another limitation of our study is that we only focused on the implication of CD16 in NK cell immune cytotoxic activation.

Our study provides evidence of a role for NK immune cells as effectors of cardiac allograft dysfunction. As CD16 is a major component that controls complex IgG-mediated effector functions of immune cells in response to alloimmune, neoplastic and infectious challenge, we expect that such a genetic and phenotypic assessment of CD16/FCGR3A may provide biomarkers that reflect the complexity of immune mechanisms associated with heart allograft loss.

In addition, as shown in the kidney transplant setting ³³, the non invasive assay NK-CHAT monitoring of Fc-R and antibody dependent NK cell alloreactivity may be a valuable tool to identify patients at higher risk to develop CAV after heart transplantation. Such indexing of patient-to-patient variability regarding NK cell responses also suggests that NK-CHAT may also be of value in defining potency markers that anticipate response to therapy, notably in the rituximab or IV-Ig treatment of AMR.

Conclusion Our study unravels the relevance of the CD16 axis in controlling the intensity of IgG-mediated NK cell cytotoxic activation in HTR. Our work suggests that individualized scoring of the recipient's intrinsic capacity to elicit CD16-dependent NK cell activation may provide an integrated view on the interplay between susceptibility SNPs, humoral innate cell alloreactivity and CMV in the promotion of coronary artery disease in the transplanted heart. Such indexing of patient-to-patient variability regarding NK cell responses may be of value in defining non-invasive biomarkers that anticipate CAV risk and response to therapy.

Translated to a simplified test, such analysis of the FCGR3A polymorphism and NK-ADCC activation potential may warrant informative non-invasive monitoring of heart transplant outcomes both before transplant and after transplant. Such an appraisal of risk could potentially limit the recommendation of invasive biopsies to a subset of immunized patients with high ADCC responsiveness, whereas less stringent monitoring may be considered in patients with a lower ADCC reactive potential. This method may also provide novel tools to quantify the individual benefit/risk profile of the patient to adapt immunosuppressive therapies. Recognizing CD16 as a major checkpoint controlling immune surveillance in the setting of HT may promote the possibility of designing and testing novel individualized NK cell-targeted therapies to limit vascular damage in sensitized patients identified being at high humoral risk.

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Figure Legends

Figure 1 (A) Phenotypic analysis of NK cell degranulation: % of CD3-CD56⁺ NK cells expressing the CD107/Lamp1 in the absence of targets, the presence of anti-CD20 rituximab (ADCC), or the presence of K562 targets (natural cytotoxicity). (B) The level of rituximab-induced NK cell activation (% of CD3-CD56⁺ peripheral NK cells expressing the CD107/Lamp1 degranulation marker in response to rituximab) correlated with the number of autologous B-lymphocytes. (C) The level of rituximab-induced NK cell activation correlated with the level of rituximab-induced down-regulation of CD16 on NK cells (CD16DRI).

Figure 2 (A) The level of rituximab-induced down-regulation of CD16 on NK cells (CD16DRI) correlated with the level of NK cell CD16 constitutive expression (CD16MFI) (B) Levels of CD16DRI across CAV⁺ and CAV⁻ HTR groups with the FCGR3A-FF, FCGR3A-VF or FCGR3A-VV genotypes. (C) Levels of constitutive CD16 expression (MFI) in HTR NK cells across the FCGR3A-FF, FCGR3A-VF or FCGR3A-VV genotypes and CAV⁺ and CAV⁻ patients.

Figure 3 (A) Distribution of the FCGR3A genotype in the CAV⁺ (n=52) and CAV⁻ (n=51) HTR and healthy control groups (n=85). (B) Kaplan-Meier analysis of the impact of the FCGR3A-VV risk genotype in the transplant recipient CAV free survival period at time of first post-transplant CAV diagnosis.

Table 1 Patient characteristics

	Patients Without CAV, n=51	Patients With CAV, n=52	p Value
At Transplant			
Recipient age at transplant (yrs)	46 ± 1.8	47.6 ± 1.9	0.544
Recipient gender (male,%)	68.6%	65.4%	0.726
Donor age (yrs)	32 (22-45)	41 (36-47)	0,016
Donor gender (male, %)	81.6 %	88.2%	0.355
Sex Mismatch	18.36%	27.45%	0.281
Recipient EBV+	90.2%	68.6%	0.007
Recipient CMV+	70.6%	63.5%	0.442
Donor cause of death			
Trauma	66.7%	56.8 %	0.331
Stroke, Cerebrovascular	20.8 %	38.63%	0.061
Recipient Preoperative Primary Heart Disease			
Ischemic cardiomyopathy	44%	42.3 %	0.863
Idiopathic dilated cardiomyopathy	30%	40.4%	0.273
Other	26%	17.3%	0.286
HTA at Transplant	5.9%	17.3%	0.071
Previous transplant n,%	0	3, (5.8%)	0.079
post transplant infections: bacterial (%)	23.5%	46.2%	0.016
post transplant infections:CMV (%)	7.8%	26.9%	0.011
Parameters at enrolment in the Study			
Time after heart transplantation (yrs)	3 (1-6)	7 (3-13)	< 0.0001
Recipient age (yrs)	51.3 (44-62)	57.8 (47-65)	0,070
Body mass index (kg/m2)	23.6 ± 0.7	26.3 ± 0.7	0,010
Current smoker (%)	19.6%	23.5%	0.630
Diabetes (%)	14%	13%	0.891
Dyslipidemia (%)	46%	52%	0.545
Platelet count (109/L)	264 (216-316)	229 (200-279)	0.0358
echocardiographic ejection fraction %	73 (68-80)	71 (61-77)	0.141
eGFR (CKDEPI, ml/min/1.73m²)	63.9 ± 3.6	51.8 ± 3.1	0,021
CKD (CKDEPI<60)	47.1%	66.7%	0,046
history of Acute cellular rejection	53%	52%	0.918
Therapies			
Induction therapy (% Basiluximab vs ATG)	51%	25%	0.007
Group A: Tritherapy MMF, steroids, CNI	76.5%	55.8%	0.027
Group B: Everolimus , steroids ,CNI	11.8%	17.3%	0.425
Group C : Bitherapy/monotherapy	11.8%	26.9%	0.052
Immunisation status			
DSA positive (n)	9 (18%)	14 (28%)	0.235
DSA MFI >10000	4 (8%)	7 (14%)	0.303
DSA C1q binding (n)	5 (10%)	9 (18%)	0.249

Data are presented as the median (25-75 percentiles) or mean ± SD or values n (% within group).

Table 2 Multivariate predictors of cardiac allograft vasculopathy

Risk factor for CAV	Odds Ratio	<i>p</i>	95% CI
CD16VV	7.45	0.034	1,163 – 47.784
CKD at time of enrolment (eGFR<60ml/min/1.73m2)	7.25	0.030	1.146 – 15.729
Donor Age at Transplant	1.08	0.004	1.026 – 1.144
Years since transplantation	1.34	0.009	1.077 – 1.665
post transplant bacterial infection	3.2	0.037	1.070 – 9.031
Recipient BMI at time of enrolment	1.13	0.049	1.000 – 1.287
post transplant CMV infection	2.76	0.182	0.625 – 12.262
Immunosuppressive regimen (ABC)	0.83	0.736	0.294 – 2.373
Recipient Age at time of enrolment	0.96	0.166	0.907 – 1.017

Table 3: Multivariate analysis for the prediction of CAV risk at time of transplant

Risk factor for CAV at transplant	Odds Ratio	<i>p</i>	95% CI
CD16VV	4.7	0.023	1.244 – 17.778
Recipient EBV+ at Transplant	0.24	0.059	0.055 – 1.057
Donor Age at Transplant	5.93	0.006	1.677 – 20.995
Donor Stroke	1.06	0.012	1.013 – 1.110
Sex Mismatch MaleD/FemaleR	3.9	0.049	1.005 – 15.116
Recipient Hypertension	1.56	0.476	0.457 – 5.358

Graphical abstract: The genotypic and phenotypic NK-cellular humoral activation test (NK-CHAT) allows early and non invasive individualized appraisal of the CD16-dependent immune responsiveness of HTR to chronic infectious and humoral factors associated to CAV.

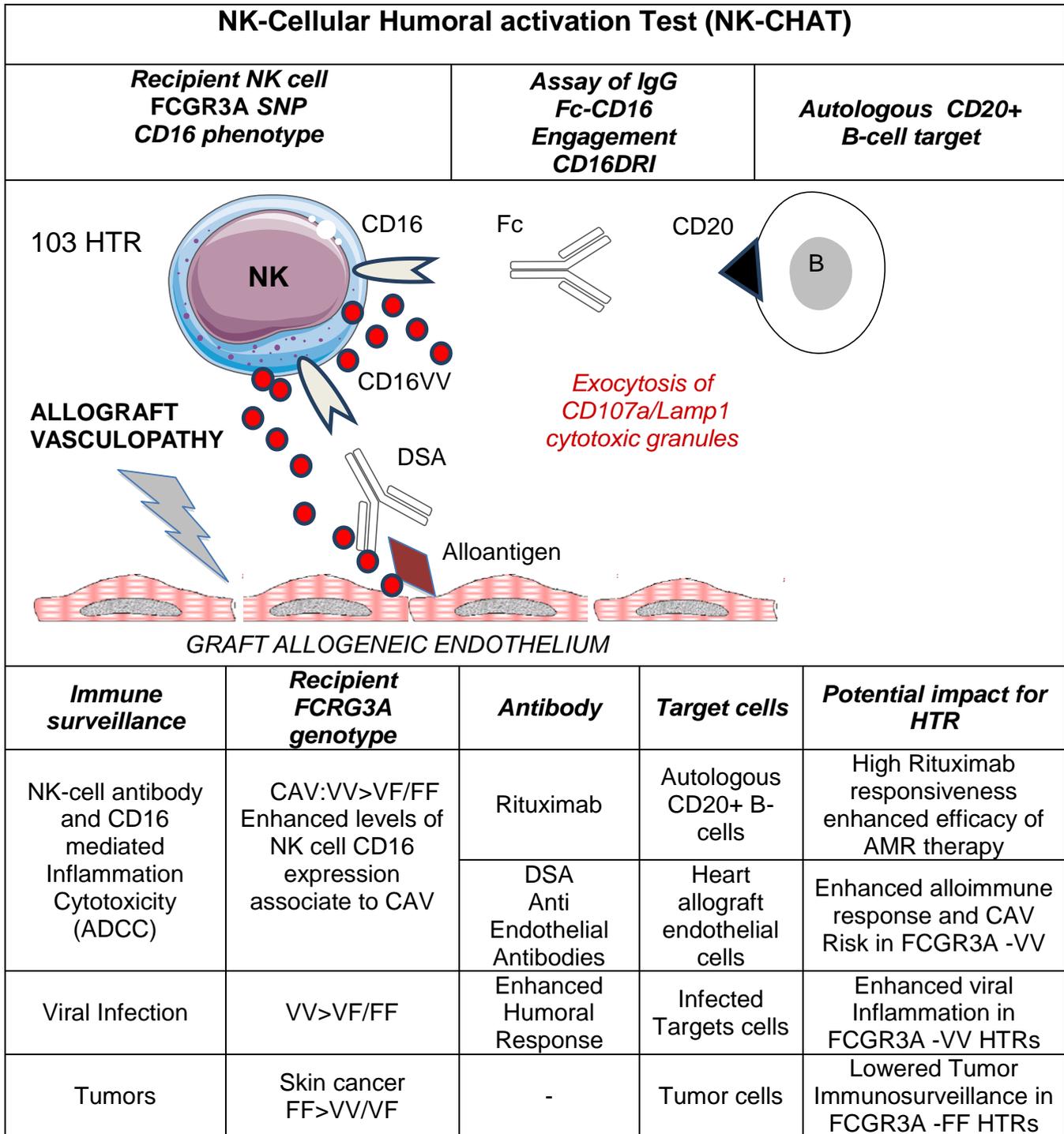


Figure 1

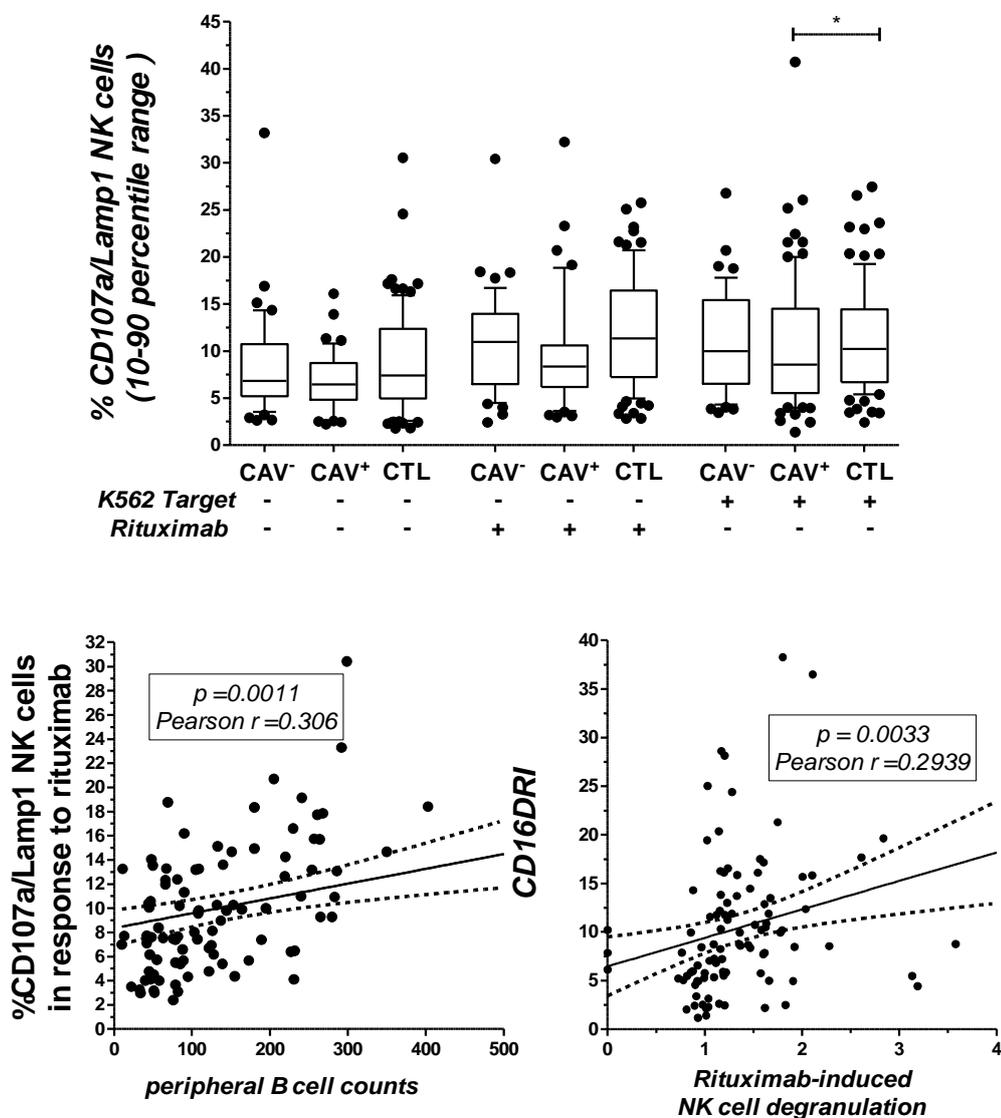


Figure 1 (A) Phenotypic analysis of NK cell degranulation: % of CD3-CD56⁺ NK cells expressing the CD107/Lamp1 in the absence of targets, the presence of anti-CD20 rituximab (ADCC), or the presence of K562 targets (natural cytotoxicity). **(B)** The level of rituximab-induced NK cell activation (% of CD3-CD56⁺ peripheral NK cells expressing the CD107/Lamp1 degranulation marker in response to rituximab) correlated with the number of autologous B-lymphocytes. **(C)** The level of rituximab-induced NK cell activation correlated with the level of rituximab-induced down-regulation of CD16 on NK cells (CD16DRI).

Figure 2

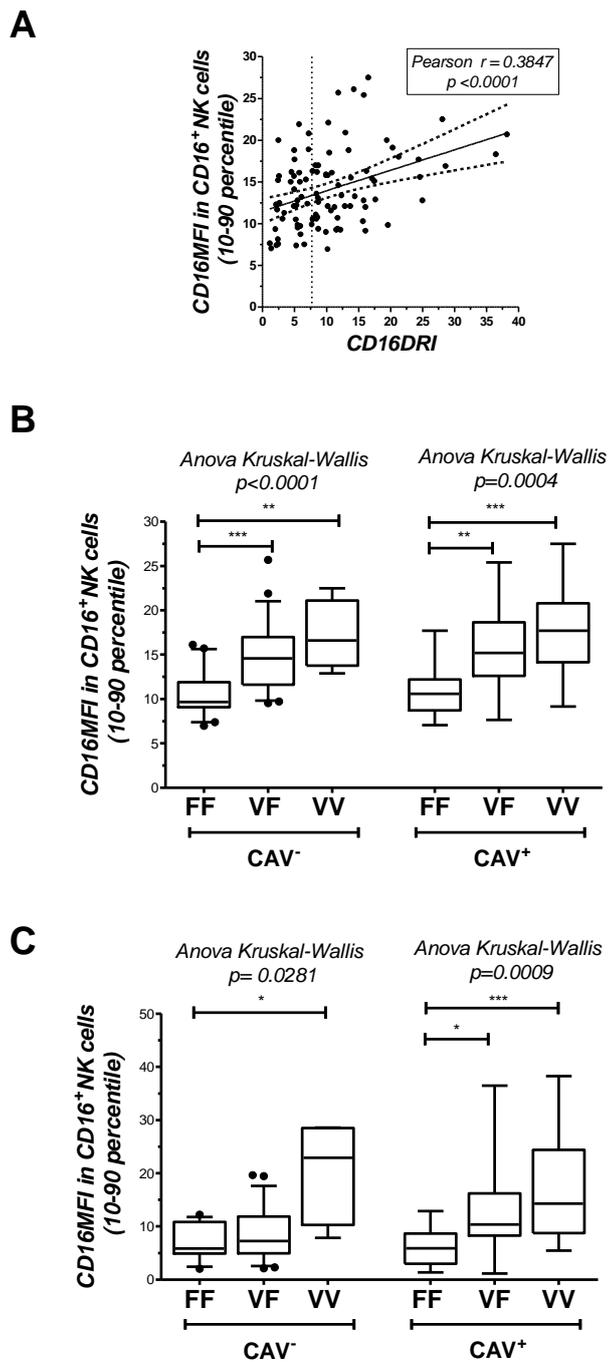


Figure 2 (A) The level of rituximab-induced down-regulation of CD16 on NK cells (CD16DRI) correlated with the level of NK cell CD16 constitutive expression (CD16MFI) (B) Levels of CD16DRI across CAV⁺ and CAV⁻ HTR groups with the FCGR3A-FF, FCGR3A-VF or FCGR3A-VV genotypes. (C) Levels of constitutive CD16 expression (MFI) in HTR NK cells across the FCGR3A-FF, FCGR3A-VF or FCGR3A-VV genotypes and CAV⁺ and CAV⁻ patients.

Figure 3

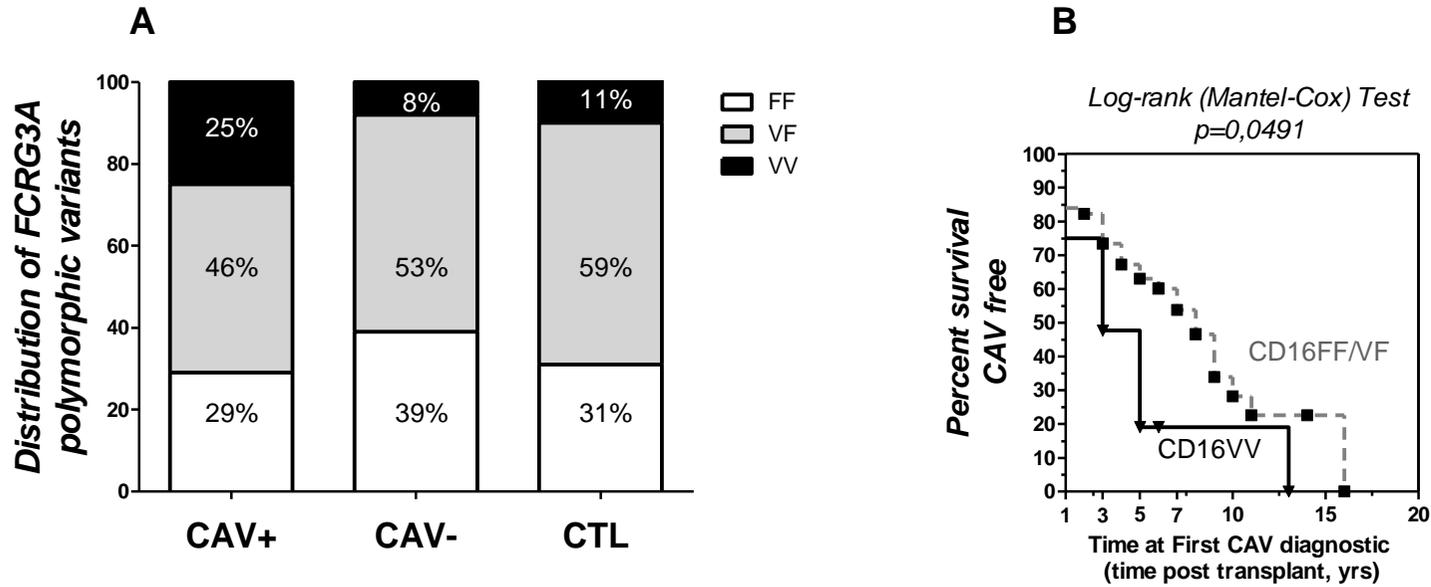


Figure 3 (A) Distribution of the FCGR3A genotype in the CAV+ (n=52) and CAV- (n=51) HTR and healthy control groups (n=85). **(B)** Kaplan-Meier analysis of the impact of the FCGR3A-VV risk genotype in the transplant recipient CAV free survival period at time of first post-transplant CAV diagnosis.

Supplementary data

Supplemental Table 1: Panel reactive antibodies (PRA), DSA and C1q binding activity of circulating anti-HLA antibodies

idPatient	Id HTR	PRA/DSA	Years postTX at 1st CAV diagnosis	CAV diagnosis	MFI DSA HLA Class I	MFI DSA HLA Class II	C1q Binding	C1q+ Class I (MFI)	C1q+ Class II (MFI)	FCRG3A Genotype
114	1	Class I and II	5	CAV-	>15000	>15000	Class I and II	>15000	>15000	FF
99	2	Class I and II	5	CAV-	12000	>15000	Class I and II	7000	>15000	FF
122	3	Class I and II	14	CAV-	7000	>15000	Class II	0	>15000	FF
17	4	Class I and II	1	CAV grade 1	4000	2500	negative	0	0	FF
24	5	Class I and II	1	CAV grade 3	3000	8000	negative	0	0	VV
32	6	Class I	14	CAV-	2000	0	negative	0	0	FF
41	7	Class I	1	CAV-	3000	0	negative	0	0	VF
92	8	Class I	3	CAV grade 1	3000	0	Class I	600	0	VV
10	9	Class I	4	CAV grade 2	1400	0	Class I	7000	0	FF
78	10	Class I	3	CAV grade 2	1000	0	negative	0	0	FF
60	11	Class II	7	CAV-	0	>15000	Class II	0	>15000	FF
35	12	Class II	9	CAV-	0	10000	Class II	0	5000	VF
15	13	Class II	1	CAV-	0	10000	negative	0	0	VF
113	14	Class II	5	CAV-	0	2000	negative	0	0	VF
108	15	Class II	8	CAV grade 1	0	>15000	Class II	0	>15000	FF
77	16	Class II	3	CAV grade 1	0	>15000	Class II	0	>15000	FF
21	17	Class II	1	CAV grade 1	0	>15000	Class II	0	>15000	FF
5	18	Class II	1	CAV grade 1	0	>15000	Class II	0	>15000	VF
98	19	Class II	3	CAV grade 1	0	12000	Class II	0	8000	VV
20	20	Class II	3	CAV grade 1	0	14000	Class II	0	3000	VF
9	21	Class II	1	CAV grade 3	0	5000	Class II	0	2000	VF
26	22	Class II	8	CAV grade 1	0	1200	negative	0	0	VF
25	23	Class II	3	CAV grade 3	0	1000	negative	0	0	VF

Supplemental Table 2: Baseline characteristics of the patients according to the FCGR3A genotype

	HTR Genotyping n=103			
	FCRG3A FF n= 35	FCRG3A VF n= 51	FCRG3A VV n= 17	FCRG3A SNP p Value
CAV Status				
CAV+ HTR (n=52)	15 (43%)	24 (47%)	13 (76%)	0.0629
CAV- HTR (n=51)	20 (57%)	27 (53%)	4 (24%)	0.0629
Infectious and Neoplastic status				
Post transplant viral infection (n=21)	5 (14%)	8 (38%)	8 (47%)	0.0196
post transplant CMV infections (n=18)	4 (11%)	7 (39%)	7 (41%)	0.0320
post transplant Bacterial Infection (n=36)	12 (34%)	16 (4%)	8 (47%)	0.509
Post Transplant Pneumocystis (n=5)	1 (3%)	1 (2%)	3 (18%)	0.055
Post transplant cutaneous Cancer (n=9)	6 (17%)	2 (4%)	1 (6%)	0.082
Immunisation status				
DSA positive (n=23)	11 (31%)	9 (18%)	3 (18%)	0.223
DSA MFI >10000 (n=11)	7(20%)	2 (4%)	2 (12%)	0.040
DSA C1q binding (n=14)	8(23%)	4 (8%)	2 (12%)	0.090
Kidney and heart function				
CDKEPI eGFR (ml/min/1.73m2)	39	50.5	58	0.132
Echocardiographic ejection fraction %	73	71.5	72	0.884
NK cells				
% NK peripheral cells	10	11.9	7.4	0.015
NK cell counts	154	174	144	0.143
CD16 expression (CD16 MFI)	10.5	15.2	16.9	< 0.0001
NK cell function (%CD107a/Lamp1)				
no target	7.09	6.47	6.35	0.246
K562 target	10.04	9.57	9.47	0.849
in response to Rituximab	8.655	9.73	12.11	0.400
Rituximab- NK CD16DRI (Fold CD16 Down regulation index)	5.825	9.917	16.09	< 0.0001

Data are presented as median values or n (% within the FCGR3AFF, VF or VV genotype group of HTRs). P values across the FCGR3A genotype were tested by one-way analysis of variance or the Kruskal-Wallis test for continuous variables and the Fisher exact test for categorical variables.

Supplemental Table 3: Analysis of the distribution of the FCGR3A-VV genotype of HTR patients across CMV Infection and development of CAV

	FCGR3A-VV	FCGR3A-FFVF	<i>n</i>
CMV+CAV+	7	7	14
CMV+CAV-	0	4	4
CMV-CAV+	7	32	39
CMV-CAV-	3	43	46
<i>n</i>	17	86	103

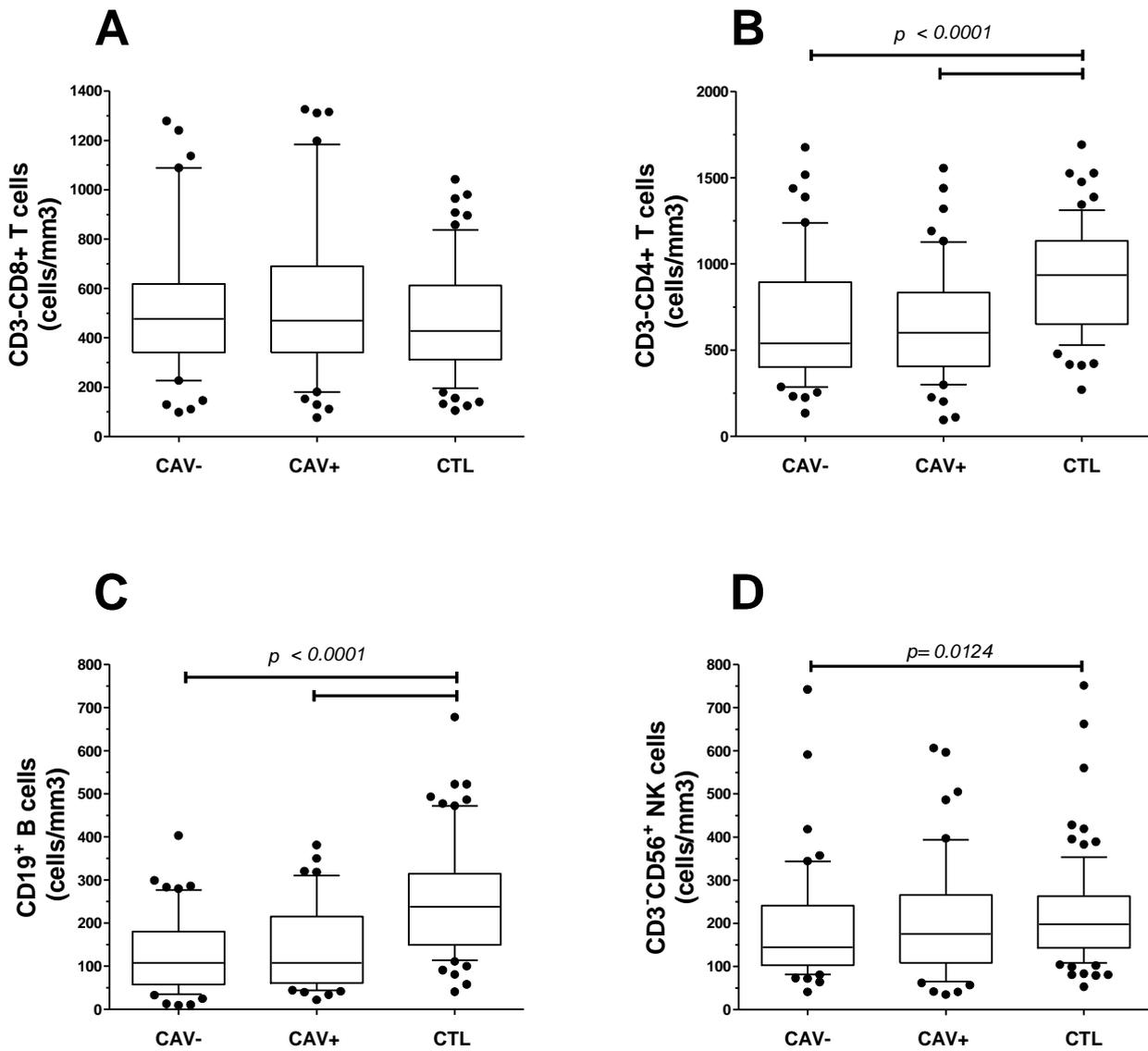
Supplemental Table 4: Multivariate Logistic Regression models: correlation of CD16 expression and presence of cardiac allograft vasculopathy at time of coronary angiographic evaluation

<i>CAV at enrolment</i>	<i>Odds Ratio</i>	<i>p</i>	<i>95% CI</i>	
MFI CD16 within CD16+CD3-CD56+ NK cells	1.15	0.016	1.026	– 1.288
Time since transplant at Inclusion	1.39	0.007	1.095	– 1.759
Donor Age at Transplant	1.08	0.001	1.032	– 1.134
CKD at time of Inclusion (eGFR <60)	5.44	0.016	1.379	– 21.439
post transplant CMV infection	3.835	0.046	1.027	– 14.324
Immunosuppressive Regimen (group A/B/C)	.869	0.693	.428	– 1.757
Recipient Age a time of Inclusion	.954	0.102	.902	– 1.009

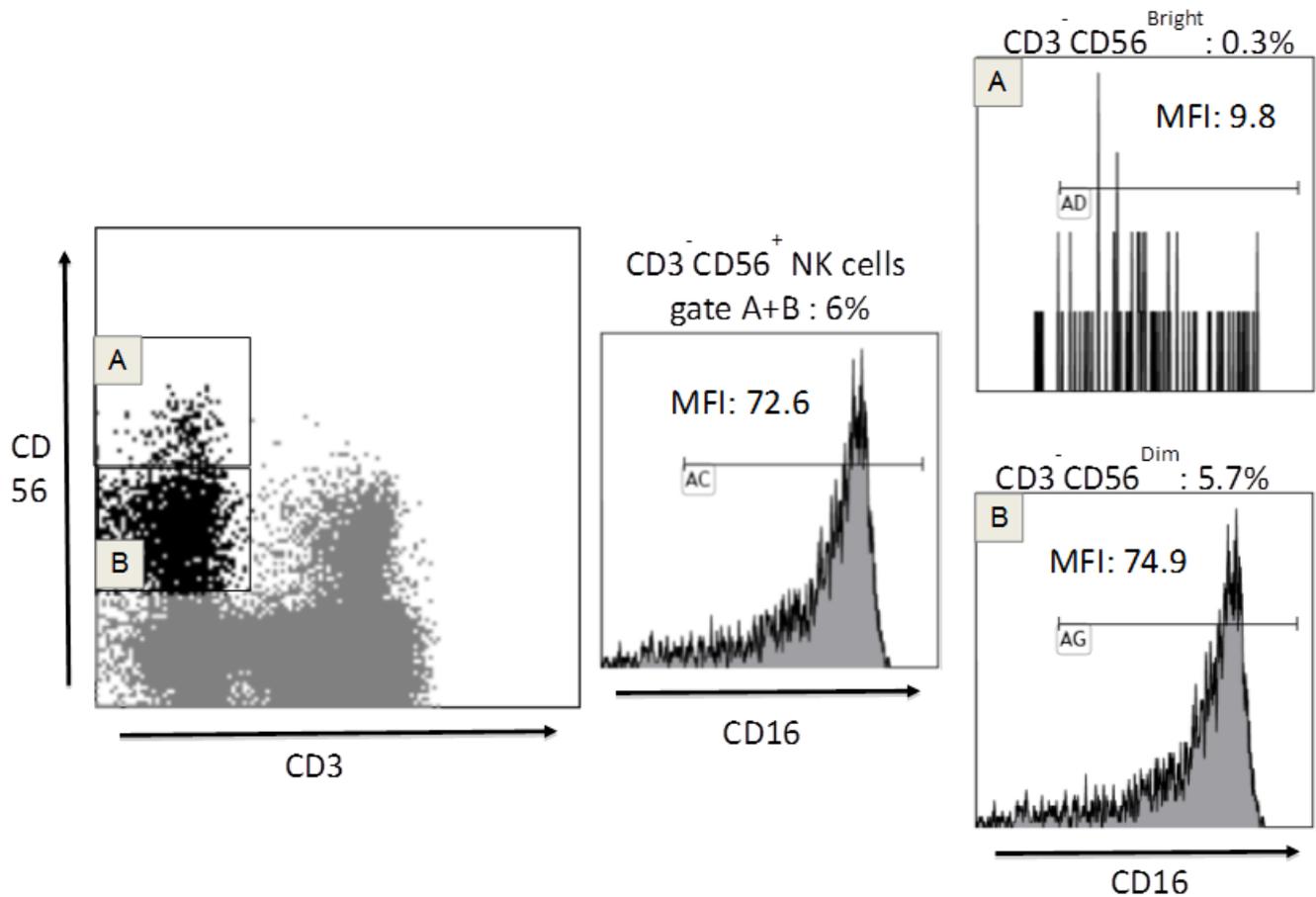
Supplemental Table 5: Multivariate Logistic Regression models: correlation of CD16DRI and presence of cardiac allograft vasculopathy at time of coronary angiographic evaluation

<i>CAV at enrolment</i>	<i>Odds Ratio</i>	<i>p</i>	<i>95% CI</i>		
CD16DRI (fold Rituximab-induced CD16 downregulation)	1.12	0.012	1.030	–	1.220
Donor Age at Transplant	1.08	0.001	1.031	–	1.140
Time since transplant at Inclusion	1.35	0.006	1.089	–	1.660
CKD at time of Inclusion (eGFR <60)	7.44	0.01	0.000	–	34.920
post transplant CMV infection	4.5	0.025	1.210	–	16.780
Recipient Age a time of Inclusion	0.96	0.15	0.910	–	1.010
Immunosuppressive regimen (group A/B/C)	1.10	0.805	0.500	–	2.420

Supplemental Figure 1. Phenotypic analysis of CD3+, NK and B cell lymphocyte counts in heart transplant recipients (HTRs) and healthy individuals (CTL)



Supplemental Figure 2 A. Illustration of the Flow Cytometry gating strategy. NK cells were defined within peripheral blood mononucleated cells (PBMCs) as a CD3⁻ CD56⁺ cell subset. CD3⁻CD56^{dim} (gate B) NK cells were identified as the major subset expressing CD16 while the CD56^{bright} NK cell subset was CD16 negative. Variation of CD16 expression (MFI) resulting from antibody recognition was therefore further evaluated within HTR PBMC on the whole CD3⁻CD56⁺ NK cell subset without further discrimination of the CD56^{bright} and CD56^{dim} subsets.



Supplemental Figure 2B: Down-regulation of CD16 surface expression upon NK cell recognition of Rituximab-coated CD20⁺ B-EBV cell targets Representative fluorescence-activated cell sorter plots of NK cell CD16 expression was measured after activation of HTR PBMC with B-EBV cell targets or rituximab-Coated cell targets for 3 hours. CD16 expression (% and MFI) was evaluated within CD3-CD56⁺ NK cells cell sorter plot of CD16 expression before and after activation. The Rituximab-induced CD16 down regulation index (CD16DRI) was evaluated as a fold reduction in NK CD16 expression (MFI) induced by Rituximab. Upper panel illustrates evaluation of CD16 DRI in a DSA⁺ patient with a FCGR3A-VV genotype that developed CAV 1 year post heart transplant (Supplemental Table 1 Patient 24). Lower panel illustrates evaluation of CD16 DRI in a DSA⁺ patient with a FCGR3A-FF genotype that exhibits no sign of CAV 5 years post Transplant (Supplemental Table 1 Patient 24).

