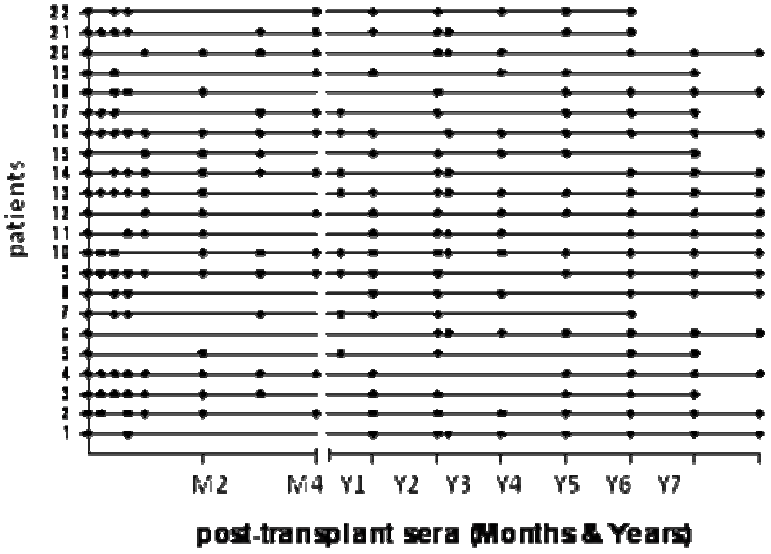
SUPPLEMENTAL DIGITAL CONTENT 1 (SDC 1- COMBINED FILE)

SDC 2 – Figure 1. A schematic representation of the sera (n=256) collected post-transplantation from kidney allograft recipients (n=22) and tested in a donor-specific ECXM.

Canet, E. *et al.*

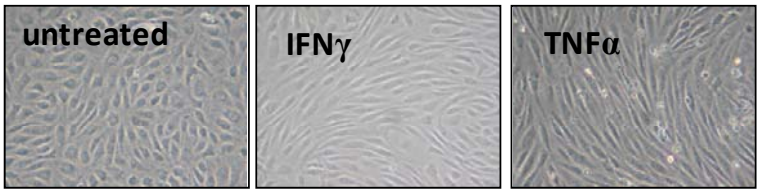


SUPPLEMENTAL DIGITAL CONTENT 1 (SDC 1- COMBINED FILE)

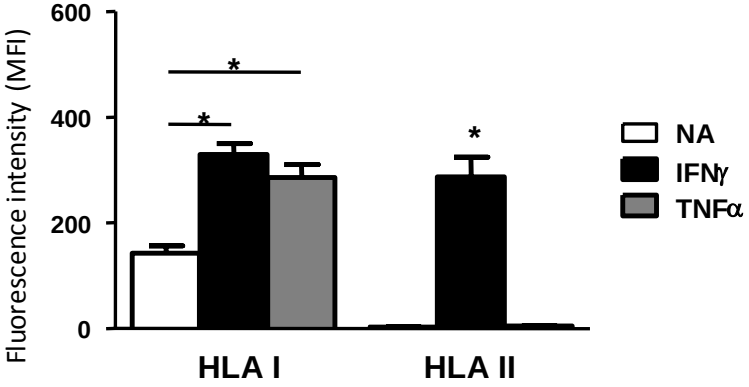
SDC 3 – Figure 2. Endothelial cell cultures (A) Morphology of resting and cytokine-activated HAEC investigated by phase microscopy (B) HLA class I and HLA class II relative expression measured by flow cytometry on resting non activated (NA) *versus* cytokines-activated vascular EC. Results shown are means of median of fluorescence intensity (MFI) ± SEM from 3 independent experiment *p<0.05.

Supplemental Figure 2 Canet, E. *et al.*

A



B



SDC 4 – Fig 3. Regulatory effect of circulating EC-reacting alloantibodies (DSA versus NonDSA) on endothelial gene transcripts. A schematic representation of transcripts repressed (green) or induced (red) on EC cultures by patient's sera containing either DSA or Non DSA determined by a dedicated PCR array. Means are shown as fold changes compared to untreated EC Controls are EC treated with a pool of AB sera for non DSA and EC prestimulated with IFNγ (100U/ml) and subsequently treated with a pool of AB sera. (**B, C**) Validation of transcript regulation by qRT-PCR in ECs. Results are representative of 3 independent experiments. *$p<0.05$ versus controls.

SUPPLEMENTAL DIGITAL CONTENT 1 (SDC 1- COMBINED FILE)

SDC 4 – Figure 3

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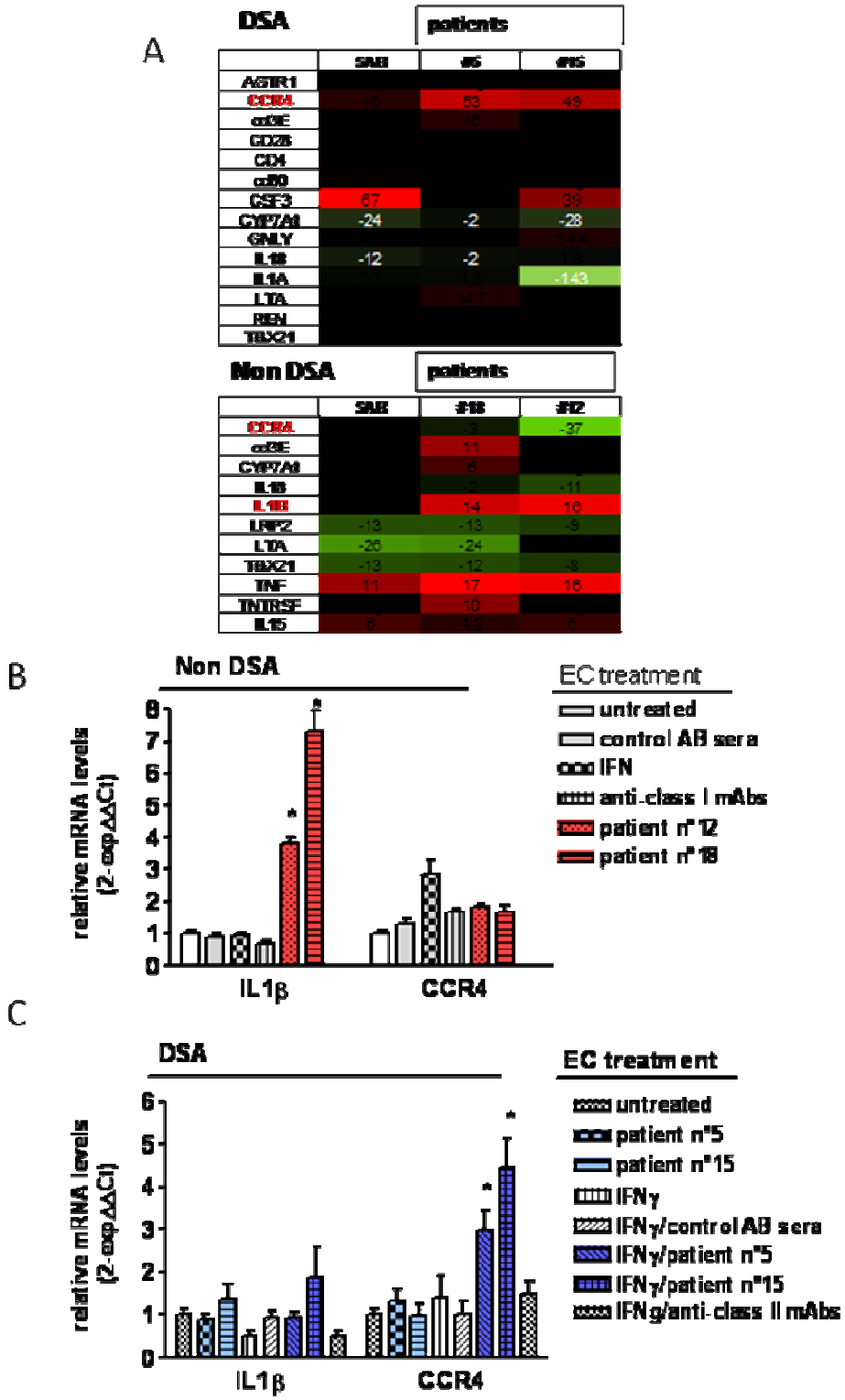
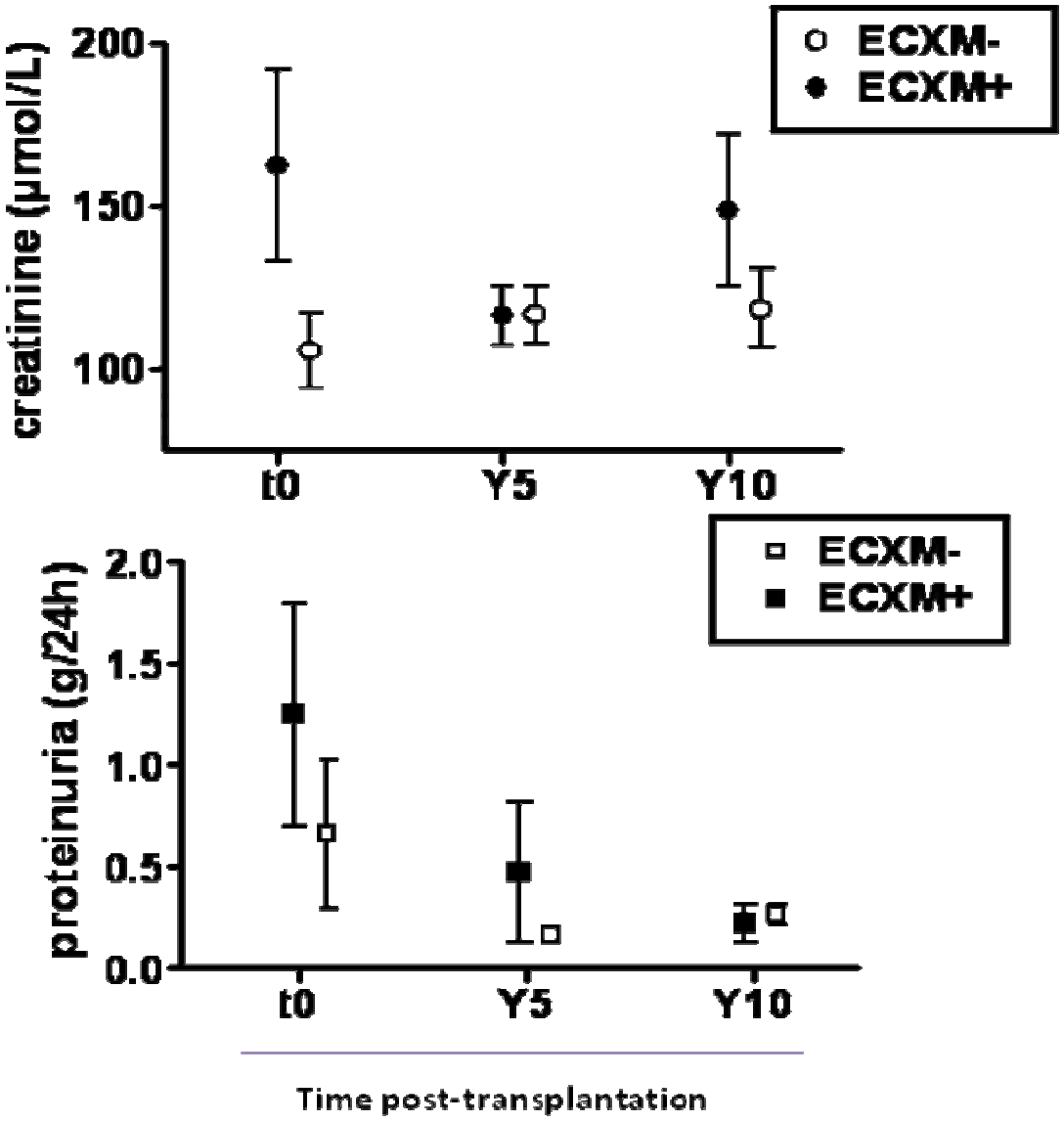


SDC 5 – Fig 4. Clinical impact of an ECXM+ evaluated by comparing graft function of the 6 ECXM+ recipients with their respective paired mate kidney recipients which were ECXM-. Graft function was assessed at the time of detection (t0) of a positive ECXM and 5 years post-t0 by comparing serum creatinine and proteinuria.

Canet, E. *et al.*



Canet E. *et al.*

SDC 6 – Materials and Methods

Patients and sera

A total of 256 sera taken before and after transplantation from 22 transplanted patients receiving cadaveric kidney allografts at the CHU de Nantes from 1999 to 2001 (patient's follow-up: 6-8 years) were tested. All recipients were followed at our institution. This cohort was selected to fit the following criteria: (1) availability of EC cultures isolated from their own transplant donor, (2) homogeneity of immunosuppressive regimen and recipient's follow-up, follow-up > 5 years and (3) use of paired mate kidney recipients that allows comparative studies. Five out of the 22 recipients (22.7%) have experienced an episode of acute rejection. Biopsies were performed when clinically indicated and analysed by microscopy. Clinical rejection was determined according to Banff 97 criteria (1). Sera from healthy blood donors (n=9 individuals and a pool of 20 male AB donors) were provided by EFS (Nantes, France) and used as controls. The study was performed according to the guidelines of the local ethics committee (CCPRB, CHU de Nantes, France).

HLA typing and Pretransplant cross-match

Recipient's HLA A, B, DR, and DQ typing were performed by genomic DNA typing, using PCR–sequence specific primer (SSP) for the determination of HLA class I (Kit Olerup-SSP; Genovision, Oslo, Norway), or HLA-DR and DQ subtypes (MicroSSP class II; One Lambda. Donors HLA class I and II typing were performed using routine lymphocytotoxicity assay. Pretransplant cross-match was performed by CDC on separated T and B lymphocytes, according to National Institutes of Health recommendations.

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Clinical data

Clinical data including patient's demographics, number of transplants, HLA PRA sensitization, histocompatibility matching (number of HLA mismatches), rejection episodes, graft loss, post-transplant serum creatinine and proteinuria levels were collected from DIVAT (Données Informatisées Validées en Transplantation, a national registry for kidney transplantation. Graft loss was defined as a loss of graft function (return in dialysis or retransplant) or death of patient. Patients were followed up until graft loss or 30 september 2007.

EC isolation, culture and activation

Human arterial endothelial cells (HAEC) were isolated and cultured as we previously described {Le Bas-Bernardet, 2003 #43}. Briefly,HAEC were isolated from unused aortic pieces collected at the time of kidney transplantation and harvested according to good medical practice and stored in the DIVAT Biocollection (French Health Minister Project number 02G55). Unused aortic pieces were harvested in culture medium and kept at 4°C until cell isolation that take place in the few hours following retrieval. For HAEC isolation, arteries were incubated with collagenase A (Roche) for 30 min at 37°C. Cells were harvested and EC were selected using CD31-Dynabeads (Dynal,, France). HAEC were characterized as we previously described {Le Bas-Bernardet, 2003 #43} before storage. ECs were grown in Endothelial Cell Basal Medium (ECBM) supplemented with 10% fetal calf serum (FCS), 0.004 mL/mL ECGS/Heparin, 0.1 ng/mL hEGF, 1 ng/mL hbFGF, 1 µg/mL hydrocortisone, 50 µg/mL gentamicin and 50 ng/mL amphotericin B (C-22010, PromoCell, Heidelberg, Germany). For activation, confluent EC monolayers were starved overnight in ECBM supplemented with 2% FCS without growth factors and incubated with recombinant human TNFα (100 U/mL, kindly provided by Prof. P. Neuman, BASF, Ludwigshafen, Germany) or IFNγ (100 U/mL, Imukin, Boehringer Ingelheim, Germany) for the indicated period of time. ECs were used between passage 2 and 5.

Flow cytometry and donor-specific EC cross-match assays

For EC phenotype analysis, immunostaining was performed as we previously reported {Le Bas-Bernardet, 2003 #43}. Briefly, cells (1–2 $\times$ 10^5 cells/sample) were suspended with Trypsin-EDTA (Gibco BRL), washed twice with PBS containing 1% BSA and 0.1% NaN_3, and then incubated on ice for 30 min with a saturating concentration of first antibody. After three washes, cells were incubated

with a PE- or FITC-labeled goat anti-mouse F(ab')2 IgG (Jackson Lab. West Grove, PE) at 4 °C for 30 min. Cells were washed twice and fixed in 1% paraformaldehyde. Antibodies used in this study are the following: anti-pan HLA class I (clone W6/32 from American Tissue Culture Collection, Manassas, VA), anti-HLA-DR (Clone L243, ATCC).

For ECXM, EC (1–2 $\times$ 10^5 cells/sample) were suspended with Trypsin-EDTA (Gibco BRL), washed twice with PBS containing 1% BSA and 0.1% NaN_3, and then incubated on ice for 30 min with 25µL of patient's sera (dilution ¼ in PBS/BSA/NaN3). After three washes, cells were incubated with a PE- or FITC-labeled goat anti-human F(ab')2 IgG or IgM (Jackson Lab.) at 4 °C for 30 min. Cells were fixed in 1% paraformaldehyde. Negative controls were performed using a pool of normal human AB sera (NHS) from 20 healthy males donors (EFS, Nantes, France), 9 individual AB sera from healthy blood donors (EFS) or istotype-matched IgG control(Jackson Lab.). Fluorescence was measured on 10,000 cells/sample using a FACScalibur® (Becton Dickinson, Mountain View, CA) and analyzed using CellQuestPro® software (Becton Dickinson). We currently used a serum from sensitized patient containing defined anti- HLA class I antibodies to assess our ability to detect known alloantibodies in our EC crossmatch assay (data not shown). Data are depicted in histograms plotting median of fluorescence intensity (MFI) on a four-decade logarithmic scale (x-axis) *versus* cell number (y-axis). Experiments were repeated at least three times. A positive ECXM was defined by antibody binding leading to a median of fluorescence intensity at least equal of twice the median obtained with controls.

Antibody titer and IgG subclass determination

Determination of specific antibody titer was performed by facs analysis. Briefly, serially diluted sera (1/2 – 1/1024) were incubated with recipient EC and titration was achieved by performing ECXM as described above. IgG subclass of EC reactive antibodies was assess using specific mouse anti-human IgG1, IgG2, IgG3 and IgG4 as secondary antibodies in ECXM experiments (Sigma).

TaqMan Low Density Arrays (TLDA) and qPCR validation

Profiling of gene expression on EC was performed using the TaqMan® Array Human Immune and Human apoptosis Gene Signature arrays (Applied Biosystems, CA, USA), microfluidic cards designed to performed series of qRT-PCR simultaneously as we previously described {Quillard, 2009 #119}. The TLDA contains 96 TaqMan® RNA Assays enabling the simultaneous quantitation of 90 human

mRNA and 6 endogenous controls. RNA was isolated using Trizol reagent (Invitrogen) and treated with Turbo DNase® (Ambion, Invitrogen) before reverse transcription (RT). Total RNA was reverse-transcribed with the Multiplex RT pool set, then quantitated using an Applied Biosystems 7900 HT Real-Time PCR system (PE Applied Biosystems, Foster City, CA, USA) according to manufacturer's recommendations. A total of 16 experimental conditions have been performed on 2 EC cultures including treatment with 4 patient's sera (2 with DSA [#5 and #15] and 2 with NonDSA [#12 and #18]) and monoclonal antibodies directed against HLA class I (clone W6/32) or class II (HLA-DR, clone L243). Controls also included EC treated with IFNg (for HLA Class II induction on EC culture), treatment with pooled male AB sera from healthy controls and treatment with patient's sera containing DSA without pretreatment with IFN. The samples have been used for TLDA experiments as well as for subsequent QPCR validation. Concerning data analysis, criteria for the selection of candidates from TLDA were as follows:

<u>For DSA:</u>

- No regulation in the absence of IFN stimulation (i.e. class II expression)
- No regulation by a pool of normal human sera
- A similar regulation observed for at least 2 patient's sera as compared with EC treated with IFNγ alone

<u>For Non DSA:</u>

- A similar regulation observed for the 2 patient's sera (patients #12 and #18) compared with EC treated with culture medium alone.
- A differential regulation compared to EC treated with DSA.

To exclude a non specific regulatory effect mediated by cytokines in the sera, cells were treated with TNF or IFNγ alone in TLDA experiments to induce the typical gene expression profiles. A criterion for the selection of candidate genes was to be differentially regulated as compared to the controls including IFNγ or TNF alone.

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Single-well qPCR analysis was conducted to validate the regulation of the selected transcripts with the following primers and probe from Applied Biosytems: IL1beta (Hs_00174097_m1), CCR4 (Hs_99999919_m1) and HPRT (H99999909_m1). Each experiment was conducted in triplicate. For quantification, replicates were normalized by the concomitant quantification of hypoxanthine-guanine phosphoribosyl transferase (HPRT). The normalized expression level was then calculated as $\log_2|2^{-\Delta Ct}|$.

Statistical analysis

The statistical analysis was performed using GraphPad Prism Version 5.00 software. Comparison of median of fluorescence intensity (MFI) obtained with sera on TNF- or IFN-activated EC was performed using a Wilcoxon test. Correlation between a positive ECXM and HLA immunization was investigated using a Fisher test. HLA class I and class II at cell surface according to culture condition was compared by means of non parametric Kruskal-Wallis test. For clinical data, a Wilcoxon test was also used to compare creatinine and proteinuria between paired-mate kidney transplant recipients. Results with *p*-values of less than 0.05 were considered statistically significant.

(1): Racun LC, Solez K, colvin RB, et al. The Banff 97 working classification of renal allograft pathology. *Kidney Int* 1999; 55:713.

SUPPLEMENTAL DIGITAL CONTENT 1 (SDC 1- COMBINED FILE)

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