TITLE PAGE

Title: Influence of the immunogenetic KIR and HLA systems on long-term renal transplant outcome

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ABSTRACT

Natural killer (NK) cells are implicated in transplant tolerance induction. NK cells express surface killer-cell immunoglobulin-like receptors (KIRs). By recognizing major histocompatibility complex class I antigens, KIRs prevent autologous cell killing and promote lysis of non-self antigen presenting cells. This study assessed whether incompatibility between recipient KIR and donor HLA class I ligands is a useful predictor of long-term graft survival.

We genotyped 113 kidney transplant patients and their donors for HLA A, B, C, DR, and KIR genes. Patients underwent immunosuppressive protocols and were followed-up for 5 years.

Our results show that KIR2DS3 gene expression in recipients was associated with better performance over time, but this effect was not evident using multivariate analysis. Conversely, presence of the donor HLA ligand for KIR2DS3 had a negative long-term impact on serum creatinine and MDRD trends, which was maintained in multivariate analysis (p=0.0308). In the univariate analysis, recipient KIR2DL1 gene absence was associated with elevated creatinine levels after 5 years. Lastly, transplantation of HLA-A3/A11-negative donor kidneys into KIR3DL2-positive patients had a statistically significant protective effect (p=0.0389) versus transplantation of HLA-A3/A11-positive kidneys into these patients.

Finally the study shows how KIR-HLA histocompatibility could be involved in the long-term immune response to kidney transplantation.

Keywords: outcome; NK cells and receptors; HLA-structure/function; rejection.
**Abbreviations:** CAD, chronic allograft dysfunction; DGF, delayed graft function; GFR, glomerular filtration rate; GVHD, graft-versus-host disease; HLA, human leukocyte antigen; ITIMs, immune tyrosine-based inhibitory motifs; KIR, killer-cell immunoglobulin-like receptor; MDRD, modification of diet in renal disease; MHC, major histocompatibility complex; MMF, mycophenolate mofetil; MPA, mycophenolate acid; NK, natural killer cell; PRA, panel-reactive antibody; SRF, stable renal function; WRF, worse renal function
INTRODUCTION

Immunosuppressive therapy has modified the early outcome of kidney transplantation, reducing the incidence of acute rejection from 30% to less than 10%. Despite this advance, long-term renal allograft survival has not mirrored the short-term improvements. Unfortunately, new approaches based on drug research, induction therapies, and immunosuppressive treatment, integrated with low-toxicity regimens, do not have a significant impact on the long-term prognosis after kidney transplantation. In the long term, renal transplantation is commonly characterized by chronic allograft dysfunction (CAD), a multifactorial pathology resulting in graft failure. A series of inflammatory events involving both cell-mediated and humoral immunity leads to progressive kidney allograft loss. CAD represents an endpoint precipitated by several biological processes that generate intolerance towards the transplanted organ, but is only partly due to involvement of major histocompatibility complex (MHC) class I antigens.

The innate immune system represents a new frontier in transplantation medicine. Innate immunity is the body's first line of defense, involving diverse mechanisms to respond to attack by foreign agents. These mechanisms include the complement system, cytokines and other soluble proteins, neutrophils, macrophages, and natural killer (NK) cells. NK cells play a special role as a bridge between innate and adaptive immunity, and thereby, critically mediate transplantation success or failure. NK cells promote both acute and chronic damage. However, recent studies have also implicated NK cells in transplant tolerance induction [1-3].

Killer cell immunoglobulin-like receptors (KIRs) are glycoproteins expressed on the surface of NK cells and subsets of T cells. KIRs interact with specific motifs on HLA class I molecules, thus modulating NK cytotoxicity. KIRs have been divided into distinct groups, depending on the number of external immunoglobulin domains (2D or
A long cytoplasmic tail containing two immune tyrosine-based inhibitory motifs (ITIMs) allows the transduction of inhibitory signals and characterizes the inhibitory KIRs (2DL, 3DL). Short cytoplasmic tails correspond to activating KIRs (2DS, 3DS).

Many studies have associated KIR genes with susceptibility to several diseases, immune responsiveness, and events following allogenic transplantation, namely haematopoietic stem cell transplantation [4-6]. A large body of evidence indicates that the risk of acute rejection in solid organ transplantation is influenced by recipient KIR and donor HLA antigen interactions. However, few studies have elucidated potential KIR system involvement in long-term transplant outcome [1, 7-12].

Our study evaluated the role of KIR and KIR-HLA compatibility on long-term renal function. From 2003 to 2005, we monitored kidney transplant recipients using standardized center regimens and well-established and structured transplant procedures to determine whether the KIR system per se, or KIR-HLA compatibility between recipient and donor, impacts renal function in a 5-year follow-up.

PATIENTS AND METHODS

Patients

We enrolled 113 patients who received kidney transplantation between 2003 and 2005 at our center. Exclusion criteria were cancer or other diseases with a fatal prognosis, hyperimmunization or previous transplant, renal transplantation combined with other organs (heart, liver), major urological complications, primary non function, presence of neoplasia, or acute vascular rejection.
Study design

We evaluated patient serum creatinine levels and estimated GFR (Glomerular filtration rate) at discharge, at 1 and 3 months, and at 1, 2, 3, 4, and 5 years after transplantation. Patients whose serum creatinine levels were above the population median at 5-years post transplantation were deemed to have a deteriorated or worsened outcome. Based on these diagnostic criteria, the enrolled patient population was divided into two groups: patients with stable renal function (SRF) and patients who experienced a worsening of renal function (WRF) at the 5-year follow-up. Patients in the two groups were comparable for sex distribution, donor and recipient age, deceased or living donor, time on dialysis, cold ischemia time, HLA mismatches, and immunosuppressive therapy schemes.

Immunosuppressive therapy

Patients received a standard immunosuppressive therapy protocol based on induction therapy with intravenous Basiliximab (20 mg on days 0 and 4), followed by steroids at decreasing dosages, and calcineurin inhibitors (cyclosporine or tacrolimus), with or without mycophenolate mofetil (MMF) or mycophenolat acid (MPA). The study protocol was approved by the Institutional Ethics Committee, and written informed consent was obtained from all subjects.

Biochemical analysis

Patient serum creatinine levels were measured at discharge, at 1 and 3 months, and at 1, 2, 3, 4, and 5 years after transplantation. Glomerular filtration rate was calculated using
the 4-variable MDRD CKD EPI equation (using SI Units), a formula that takes age, sex, race, and serum creatinine level into account. Patients whose serum creatinine levels were above the population median at 5-years post transplantation were deemed to have a deteriorated or worsened outcome.

**Genotyping**

Donor and recipient KIR and HLA genotyping was performed by serological and molecular biology techniques that are detailed in the “Genotyping assay” section of the Supplemental Digital Content 1. KIR haplotypes were assigned according to the presence of only one stimulator gene 2DS4, whereas group-B haplotypes contain various combinations of 2DS1, 2DS2, 2DS3, 2DS5, 3DS1, and 2DS4. HLA-C alleles were divided into two groups: HLA-C1 alleles with serine at position 77 and asparagine at position 80, and HLA-C2 alleles with asparagine at position 77 and lysine at position 80. HLA-A3 and -A11, and HLA-Bw4 assignment was based on the typing specificity.

**Statistical analysis**

We evaluated the effect of transplant recipient KIR genes and of various KIR-HLA combinations in recipient/donor pairs on serum creatinine and MDRD GFR over time, using analysis of variance (ANOVA) nonparametric repeated measures. In the presence of a significant main effect of time, a post-hoc analysis was subsequently performed using Tukey's test for evaluating differences between all possible pairs of mean creatinine values at each follow-up. Multivariate analysis of variance (MANOVA) was implemented to control for possible confounding effects of sex, hypertension, dgf, donor and recipient age, and immunosuppressive regimen. The frequencies of HLA-
KIR matches were evaluated using contingency tables, and the observed values were compared with expected values using a chi-squared test or by Fisher’s exact test as appropriate. Row and adjusted relative risk (RR) with 95% confidence intervals (95% CI) was also calculated using a modified Poisson regression model with robust variance estimation.

RESULTS

Specific KIR gene frequencies in our patients were consistent with those reported for the general Italian population [13] and are detailed in Table A of the “Supplemental Digital Content 2.” Table 1 reports the KIR gene effects on serum creatinine and MDRD GFR in the enrolled 113 transplant patients at the 5-year follow-up. KIR2DS3 carriers had lower overall serum creatinine concentrations than KIR2DS3-negative patients (Figure 1A), and this difference approached statistical significance according to ANOVA (F=3.41; p=0.0681). KIR2DS3-positive transplant recipients also had significantly improved MDRD GFRs than did KIR2DS3-negative patients after 5 years (F=4.90; p=0.0294).

Specific combinations of recipient KIRs and donor HLA antigens altered long-term renal function outcome as shown in table 2. KIR2DS3-positive transplant recipients who received an HLA-C1-positive kidney had higher serum creatinine levels than KIR2DS3-positive patients who received HLA-C1-negative kidneys (Figure 1B; F=3.95; p=0.0227). Univariate creatinine analysis over time also suggested that KIR2DL1 gene absence in recipients was associated with higher creatinine levels at 5 years post-transplant (Table 1; F=3.88; p=0.0520). We subsequently performed multivariate analysis of variance on the KIR gene identified by ANOVA. The multivariate approach did not confirm the statistical significance of the results obtained
regarding renal function in KIR2DS3-positive and KIR2DL1-negative recipients. However, renal function outcome in the coupled KIR2DS3-recipient/HLA-C1 donor scenario remained statistically significant (Table 2; F=3.95; p=0.0227).

To confirm these data, we evaluated these associations after dividing our patient population according to the median creatinine values at 5 years following transplantation. Sixty patients with a serum creatinine at 5 years post-transplant below the median (1.5 mg/dL at 5 years) were included in the stable renal function group (SRF), whereas 53 patients with creatinine levels above the median value were grouped as worsened renal function (WRF). Demographic, clinical, and transplant-related parameters of the patient populations are shown in Table B of the “Supplemental Digital Content 3”. Table 3 compares KIR gene frequencies in SRF and WRF groups. KIR2DS3-negative recipients had an increased relative risk of 5-year creatinine levels being elevated above the median level (RR=1.453, 95% CI: 0.938-2.253), but this was not statistically significant. Additionally, compared to the KIR2DL1-negative patients, those who did not carry the KIR2DL1 gene, which is in linkage disequilibrium with KIR2DP1, showed a 2-fold higher relative risk of having a serum creatinine above the median value (RR 2.176, 95% CI: 1.779-2.663).

Table 4 compares frequencies of different combinations of recipient KIR genes donor HLA ligands in the SRF and WRF groups. The combination of recipient KIR3DL2 gene and its HLA-A3/A11 ligand in the donor appeared significantly more frequently in the WRF group. An approximately 33% decreased risk of 5-year creatinine values above the median level was associated with the absence of HLA-A3, HLA-A11, or both (RR 0.661, 95% CI 0.453-0.964). After adjusting for potential confounders, the estimated relative risk of worsened long-term renal function due to the combinations of recipient KIR2DL1 or KIR3DL2, and the donor HLA-A3/A11 ligand, were confirmed (respectively: RR=2.186, 95% CI: 1.497-3.193; RR=0.680, 95% CI: 0.465-0.994).
DISCUSSION

This study evaluated NK alloreactivity modulation by the immunogenic KIR system in renal transplantation. Our goal was to test whether certain combinations of KIR genes in recipients and reciprocal HLA Class I ligands on donors altered long-term kidney graft function.

Although a number of studies have investigated the role of KIR in acute rejection after kidney transplantation, the potential role of KIR in the long term has only been analysed in a single study. Cirocco et al. reported an association between the absence of KIR2DL2 and KIR2DS2 and poor renal function in HLA-identical sibling pairs [14]. Van Bergen et al. indicated that alloreactive NK cells may thwart the success of HLA-compatible kidney transplantation, and suggested that the suppression of NK-cell activity can improve the survival of kidney graft [12]. The results of our study show a correlation between the presence of KIR2DS3-HLA-C1 and renal function as regards serum creatinine and MDRD GFR in the enrolled transplant patients at the 5-year follow-up. The KIR2DS3 gene codes for an activator receptor that promotes NK cell cytotoxic activity. In our population, the carriers of KIR2DS3 activator, through univariate analysis, showed a trend of better renal function over time, as determined by improved serum creatinine levels and MDRD GFR values. However, this result was not confirmed by multivariate analysis. Nonetheless, the presence of the KIR ligand, HLA-C1, in the donor reverses the effect of recipient KIR2DS3, an effect confirmed by multivariate analysis.

Some studies on hematopoietic stem cell transplantation demonstrated a correlation between the presence of KIR2DS3 in the donor DNA and the development of chronic graft-versus-host disease (GVHD) in the recipient [15, 16]. This suggests that the KIR2DS3 activating receptor on donor tissues, together with its absence in the recipient,
increase the alloreactivity of donor-derived NK and T cells. However, in line with our results, previous studies reported an association between recipient KIR2DS3-positivity and a protective effect against chronic GVHD and acute myeloid leukemia, resulting in greater long-term survival [17, 18]. These associations are difficult to interpret as KIR2DS3 gene function is not completely understood. However, KIRs are clonally expressed on NK cells in a stochastic way, so that each NK cell clone expresses only a portion of its KIR genome. Thus, a substantial fraction of patient NK cells may not express KIR2DS3 even if the corresponding gene is present [19].

We investigated whether distinct HLA class I ligands on donor tissues can influence long-term renal function after transplantation. In our study, KIR2DS3-positive patients who received HLA-C1-positive kidneys displayed a worse long-term creatinine profile than KIR2DS3-negative patients coupled with HLA-C1-negative donors, or KIR2DS3-positive patients who received an HLA-C1-negative kidney. Despite our small population, these results suggest that the presence of the KIR2DS3 ligand in the donor can activate the recipient KIR and function. Specifically, the presence of recipient KIR2DS3 in association with donor kidney HLA-C1 ligand promotes cytotoxic and cytolytic NK cell activity in the recipient, thereby inducing the destruction of allogeneic cells.

In comparing SRF and WRF patients over the long term, we found that absence of the KIR2DL1 gene in recipients is strongly associated with elevated creatinine levels at 5 years following transplantation. This finding was further confirmed by relative risk analysis, which showed that absence of recipient KIR2DL1 results in >2-fold increased risk of elevated creatinine levels at 5 years post-transplantation. The KIR2DL1 gene has an immune-inhibitory function. The observed association between KIR2DL1-absence and a renal function outcome supports the hypothesis that NK cytotoxicity is less inhibited in patients without this gene. A study by Kunert et al. performed in kidney
transplant recipients, reported that recipient KIR2DL1-positivity was associated with decreased acute rejection risk, especially in the concurrent presence of the appropriate ligand HLA-C2 [8]. This confirmed the influence of KIR2DL1 on the short-term outcome of renal transplantation.

One noteworthy finding of our study concerns the concurrent presence of the HLA-A3/A11 ligand in the donor and KIR3DL2 in the recipient, a so-called "gene structure" present in all KIR haplotypes, with a primarily inhibitory action on NK cytotoxicity. We observed that the absence of donor HLA-A3/A11 in the presence of recipient KIR3DL2 was more common in the group with better renal function. Moreover, transplantation of an HLA-A3/A11-negative kidney, versus a positive kidney, was associated with a graft-protective effect in the long term (RR=0.661), demonstrated by improved renal function at 5 years post-transplantation. We also confirmed this last finding by multivariate analysis.

The specificity of these HLA ligands for the KIR3DL2 gene is well-substantiated [20-22]. A recent study showed that HLA-A11 (HLA-A*1102) also binds with high affinity to KIR2DS4. Both KIR2DS4 and KIR3DL2 share a valine-proline motif in position 71-72 [23]. Because of this structural similarity, we cannot exclude an inducing effect of HLA-A11 on NK cell cytotoxicity, by binding to the activating receptor KIR2DS4.

The role of KIR3DL2-HLA-A3/A11 interactions in vivo is still debated [20-22, 24]. Several studies have demonstrated that during maturation, NK cells go through a process of "education" by self MHC class I molecules to become functionally competent [25-29]. In the absence of inhibitory KIRs, or in the presence of inhibitory KIRs but in the absence of its ligands, the cells are hyporesponsive. Fauriat et al. studied NK cell alloreactivity not only in terms of genotype, but also phenotype. Their results showed that KIR3DL2-positive NK cells were hyporesponsive even in the presence of the respective ligand HLA-A3/A11. This observation suggests that probably
no peptide in the donor was present at sufficient levels to "educate" NK cells and make them functionally competent. However, these authors consider the KIR3DL2-HLA-A3/A11 interaction inhibitory at the level of the target cell [30]. The results of the current study suggest that NK cells in patients who received a transplant from an HLA-A3/A11-negative donor were at a less functional maturation stage, and therefore, hyporesponsive to non-self antigens. We believe that such NK cell hyporesponsiveness was translated into the better serum creatinine concentration observed over the long term in these recipients.

Finally, we noted that KIR-ligand incompatibility with regard to the KIR3DL2-HLA-A3/A11 combination favors improved long-term serum creatinine levels. Prior studies have shown that the absence of some KIR ligands (including KIR3DL2) is associated with an anti-leukemic affect that results in a lower risk of disease recurrence and increased survival rates, after hematopoietic stem cell transplantation [5, 31, 32].

In conclusion, this study demonstrates a likely correlation between the innate immune system and long-term kidney transplant outcome. Formerly, allogenic graft survival or failure has been almost exclusively attributed involvement of the acquired cell-mediated immune system and HLA recognition mechanisms. Here, we have shown the potential for KIR/HLA interactions to modulate renal graft survival. The immunogenic KIR system remains a fascinating field of investigation and deserves further exploration into its role in mediating kidney transplantation outcomes.

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**LEGENDS TO FIGURES**

**Figure 1.** A) Mean creatinine values ± standard deviation measured over time in patients either positive (solid line) or negative (dotted line) for the KIR2DS3 gene. B) Mean creatinine values measured over time in patients with different recipient/donor combinations of KIR2DS3/HLA-C1.

KIR2DS3-, absence of KIR2DS3 gene; KIR2DS3+, presence of KIR2DS3 gene; HLA-C1-, absence of HLA-C1 gene; HLA-C1+, presence of HLA-C1 gene; HLA-C1±, both presence or absence of HLA-C1 gene.

$^\circ p<0.05$ for comparison between KIR2DS3-negative and KIR2DS3-positive patients.

$^* p<0.05$ for comparison between KIR2DS3-/HLA-C1± patients and KIR2DS3+/HLA-C1- patients.
REFERENCES


Additional files provided with this submission:

Additional file 1: La Manna_supplementary file 1.doc, 24K
http://www.biomedcentral.com/imedia/4203639907829316/supp1.doc
Additional file 2: Supplemental Digital Content 2.docx, 12K
http://www.biomedcentral.com/imedia/1585665782931666/supp2.docx
Additional file 3: La Manna_supplementary file 3.doc, 36K
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Additional file 4: La Manna_TABLE 1.docx, 11K
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