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Natural Killer cell function predicts severe infection in kidney transplant recipients

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Abbreviations

area under receiver operating characteristic (AUROC)

cytomegalovirus (CMV)

Epstein-Barr virus (EBV)

estimated glomerular filtration rate (eGFR)

fluorescent target cell marker (TLF4)

herpes simplex virus (HSV)

human papilloma virus (HPV)

interquartile range (IQR)

level of immunosuppression study (LOIS)

major histocompatibility complex (MHC)

natural killer (NK)

peripheral blood mononuclear cell (PBMC)

varicella-zoster virus (VZV)

ABSTRACT

The aim of this study was to determine if natural killer cell number ($CD3^-$ $CD56^+CD16^+$) and cytotoxic killing function predicts severity and frequency of infection in kidney transplant recipients.

A cohort of 168 kidney transplant recipients with stable graft function underwent assessment of natural killer cell number and functional killing capacity immediately prior to entry into this prospective study. Participants were followed for two years for development of severe infection, defined as hospitalization for infection. Areas under receiver operating characteristic (AUROC) curves were used to evaluate the accuracy of natural killer cell number and function for predicting severe infection. Adjusted odds ratios were determined by logistic regression.

Fifty-nine kidney transplant recipients (35%) developed severe infection and 7(4%) died. Natural killer cell function was a better predictor of severe infection than natural killer cell number: AUROC 0.84 and 0.75, respectively (p=0.018). Logistic regression demonstrated that after adjustment for age, transplant function, transplant duration, mycophenolate use and increasing natural killer function (OR 0.82 95% CI 0.74-0.90 p<0.0001) but not natural killer number (OR 0.96 95% CI 0.93-1.00 p=0.051) remained significantly associated with a reduced likelihood of severe infection.

Natural killer cell function predicts severe infection in kidney transplant recipients.

INTRODUCTION

Improvements in graft survival following solid organ transplantation have meant that the prevention of infection and malignancy have become a key focus in the care of transplant recipients.[1] Predicting the subset of transplant recipients at risk of infection is difficult using clinical factors alone and therefore requires biomarkers.[2] There is emerging evidence that natural killer (NK) cell quantification may be a predictor of infection in transplant recipients.[3,4,5] However, there are very few studies in this area and none that examine the relationship of NK cell function with infection.

NK cells are innate immune cells that are capable of immediate defense against infective pathogens and cancer.[6] In transplantation, the majority of current immunosuppressive medications target the adaptive immune system, allowing the innate immune system to have increasing importance in the protection against infections. NK cell subsets in the peripheral blood can be differentiated according to expression of surface molecules. [7 8 9] The two main subsets include NK ^{bright}, which represents an early stage of NK cell differentiation and lacks $CD16^{+}$ and NK ^{dim} cells which represent a later stage of differentiation and the majority are positive for $CD16^{+}$. [8] The $CD56^{bright}CD16^{-}$ NK subset have unique receptor characteristics such as negativity of killer-cell immunoglobulin-like receptors and higher levels of CD94/NKG2A. [8] Functionally, they subsets have different roles. NK bright cells predominantly secrete cytokines such as interferon gamma and/or tumor necrosis factor and NK dims cells have a more prominent role in cytotoxicity. [9] These subsets also express different activating and inhibitory receptors. [7 8] Evidence suggests that NK subsets expressing CD94/NKG2C^{bright} activating lectin-like receptor have a role in control of CMV infection in kidney transplant recipients. [10]

One of the most important immune functions of NK cells, learnt from observing patients with primary NK cell deficiency, is defense against herpes viral infections.[11] NK cells recognize and respond to cells without MHC class 1.[6,11,12] The particular susceptibility to herpes viruses may relate to the fact that herpes viruses can downregulate Major Histocompatibility Complex (MHC) class 1 in order to evade host cytotoxic T cell response.[13] Other infections that are associated with NK cell deficiency include human papilloma virus, fungal and mycobacterial infections. [3,7,14-13]

Patients with primary NK cell deficiency are susceptible to virally driven malignancies. [11,15-16] Twenty-one percent of a cohort of patients with Classical NK cell deficiency developed cancer, including an EBV-driven smooth muscle tumor, Human Papilloma Virus (HPV) associated cancers and leukemia. [11,15-19]

The aim of this study is to determine whether NK function is a predictor of severe infection risk in kidney transplant recipients. Our hypothesis was that NK cell function predicts severe infection in kidney transplant recipients.

MATERIALS AND METHODS

Setting

In April 2015, the Level of Immunosuppression Study (LOIS) was commenced. This prospective cohort study was performed at Monash Health, a 1500 bed academic health service in Melbourne, Australia.

All kidney transplant recipients were offered the opportunity to have their immune parameters measured at study entry and were then followed for two years for episodes of infection and malignancy. Consenting participants underwent outpatient blood collection for NK cell number and functional testing.

To determine the incidence of infection, the investigators received notification from the treating physicians whenever a subject was admitted to the hospital and they underwent clinical review by a specialist infectious diseases physician to determine the site of infection and to review the microbiological investigations.

Inclusion and exclusion criteria

Kidney transplant recipients were eligible to participate if they were ≥18 years, had stable graft function for a minimum of three months (median 4 years). Kidney transplant recipients were excluded if they recently augmented immunosuppression to treat rejection, or had an infectious illness just prior to or at the time of the study.

The study was approved by the human research ethics committee of Monash Health (Number 13085). Written informed consent was obtained from all participants.

Data collection

Patient clinical and demographic details were assessed at enrolment.

Laboratory methods

NK cell number and percentage

Lymphocyte subset testing was performed on freshly collected peripheral blood. 100µL aliquots of heparinized blood were labeled with appropriately titered monoclonal antibodies. Fluorescently labelled antibodies used in this study were obtained from Beckman Coulter and included combinations of CD3-APC, CD14-PE, CD16-FITC, CD56-PE and CD45-PC7. Following incubation, red blood cells were lysed using the Beckman-Coulter Q-Prep system and acquired on a FC500 flow cytometer (Beckman Coulter, Brea, California, USA). NK cells were identified by the immunophenotype $CD3^{-}/CD16^{\pm}/CD56^{\pm}$ and percentages determined after gating on lymphocytes by forward and side scatter characteristics. The following NK subsets were reported: $CD56^{+bright}CD16^{-}$, $CD56^{+dim}CD16^{+}$, $CD56^{+dim}CD16^{-}$ and and $CD56^{-}CD16^{+}$. A representation of the gating strategy for NK cells is presented in Figure 1. Absolute numbers were calculated using the lymphocyte count provided by full blood examination.

NK cell function

NK-cell function was calculated as the percent of target cells capable of cleaving Pantoxilux substrate. Peripheral blood mononuclear cells (PBMCs) were used as a source of NK cells and it was anticipated that cytotoxic activity would be lower than if isolated NK cells were used. PBMCs were isolated by density gradient centrifugation using Leucosep tubes pre-filled with Ficoll-Paque Plus (Greiner Bio-One, Austria). PBMCs were cryopreserved in liquid nitrogen. To measure NK cytotoxicity, PBMCs (effector cells) were exposed to a known quantity of target cells (K562 cells) and cytotoxicity was determined using a commercially available kit, Pantoxilux ™ (OncoImmunin, Inc, Gaithersburg, MD). Target cell were K562 cells which are a human immortalised myelogenous leukemia line derived from a patient with chronic myelogenous leukemia patient in blast crisis. The cells can be killed by natural killer cells as they lack the Major Histocompatibility complex required to inhibit NK activity.[20] The Pantoxilux ™ assay is based on the hydrolysis of a cell-permeable fluorogenic peptide substrate containing a sequence recognized by the serum protease Granzyme B and upstream caspase activity.[21] Following kit instructions, target cells were counted and resuspended in RPMI 1640 medium containing the fluorescent target cell marker (TLF4) and incubated at 37℃ for 30 minutes and then washed twice with RPMI media. $2x10^4$ TLF4 labelled target cells per well were co-cultured with effector PBMCs at different concentrations:0, $5x10^5$, 1x10^6 and 2x10^6 cells per well (effector:target ratios; 25:1, 50:1 and 100:1 respectively) together with pantoxilux ™ substrate (75µL). Cells were incubated in 5% CO_2 at 37°C for 60 minutes. Co-cultured cells were spun down and washed in Pantoxilux ™ wash buffer and resuspended in 250µL of wash buffer for acquisition. Samples were acquired on a Navios flow cytometer (Beckman Coulter, Brea, CA) and data analyzed using Kaluza software (Beckman Coulter, Brea, CA).

NK cytotoxic function was reported as the percentage of K562 target cells that were dead following 60-minute incubation with effector cells (NK cells) at a ratio of effector: target of 100:1.

Normal ranges for NK number and function were derived from cuts offs derived using 10th to 90th percentile measurements from a random cohort of staff controls who were well, with no known medical, inflammatory or infectious conditions.

Clinical outcomes

Severe infection was defined as any infection requiring admission to hospital after the date of study enrolment. Details of all severe infections were collected. All infections reported in this study (including viral and urinary tract infections) were associated with hospitalization.

Infectious episodes were classified as: microbiologically defined (whereby a microorganism related to the clinical presentation was isolated) or clinically defined (whereby no microorganism related to the clinical presentation was isolated but a clinical diagnosis of the site of infection could be determined by the study investigators).

CMV infection was defined as virus isolation, or detection of viral nucleic acid in any body fluid or tissue specimen. CMV disease was defined as the presence of appropriate clinical symptoms and/or signs are required together with documentation of CMV in tissue from the relevant organ by histopathology, virus isolation, rapid culture, immunohistochemistry, or DNA hybridization.[22] Polyoma virus replication was defined by increasing polyoma viral loads. Probable polyoma virus disease was defined as viral replication >10^4 copies per ml or together with compatible symptoms and signs of viral syndrome or organ disease, but without histological confirmation. Proven polyoma virus disease was defined as evidence of virus replication plus corresponding specific histopathology.[23,24] Fungal infections was categorized as possible, probable and proven, and blood stream infections and sepsis were defined by internationally recognized criteria.[25,26,27]

Each episode of infection was classified according to source .

NK-associated infections or malignancies was a definition created by the authors. It is based on literature that suggests the types of organisms and malignancies to which patients with NK cell deficiency are susceptible. NK-associated infections were defined as any viral or fungal infection causing admission to hospital or probable or proven polyoma virus disease. NK-associated malignancies included any malignancies with a documented association with viral infections.[18] Details of the inclusions of the definition of NK associated infections or malignancies are described in Table 1.

Statistical analysis

We assumed an expected rate of severe infection of 20% based on previous data from our centre[28] and other data in the literature.[4,29] The minimum required sample size for a AUC ROC of 0.80 was 64 patients assuming a 90% power and a alpha at 0.05.

Categorical variables were summarised using frequency and percentage. Continuous variables were summarised using mean ± standard deviation or median and interquartile range (IQR) as appropriate. Categorical variables were compared using the Chi-square test and continuous variables were compared using the Mann Whitney U-test.

The extent to which NK cell number influenced NK cell function was analysed by calculating the ratio of the number of K562 cells per well to the number of NK cells/per well at an effector to target ratio of 100:1. This was defined as the absolute NK cell function.

The ability of baseline NK cell number and NK cell function to predict the first episode of severe infection within the study period was assessed using logistic regression. Receiver operator curves (ROC) were calculated from the logistic regression models for both baseline NK cell number and NK cell function and compared using the method from Hanley and McNeil.[30] Youdens index was determined to find the cut-off to maximize sensitivity and specificity. Variables were input into the multivariable logistic regression model if they had a *p* value of greater than 0.2 in the univariable logistic regression model. Age, time from transplant, mycophenolate use and eGFR were included regardless of their p value on the univariable analysis given the known influence of each factor on overall infection risk. [31,32] Adjustment for other factors was not performed due to the sample size. Statistical significance was set at a p-value of <0.05. Analyses were conducted on STATA (version 15, College Station, Texas, USA) and Graph Pad Prism (Version 7 La Jolla, California, USA).

RESULTS

Characteristics of kidney transplant recipients

One hundred and sixty-eight of a possible 850 (20%) kidney transplant recipients accepted the offer to participate in this study (Figure 2). All participants were followed for 24 months. The demographics details are presented in table 2. The participants in this study were similar in age and other demographic details to the cohort of kidney transplant recipients at our institution.[33]

NK cell number and function of healthy controls

Nineteen healthy controls underwent testing for NK cell number and function. The normal range for NK cell number among healthy controls was 61 to 776 cells/μL and NK cell percent was 7 to 28%, based on the normal range for laboratory controls. The median percentage cytotoxicity with an effector to target ratio of 100:1 was 21.6% (IQR 13.7-30.4). The 10^{th} centile was 11.6% and the 90^{th} centile was 39.2%.

NK number and function of kidney transplant recipients

The median NK cell number was 140 cells/μL (IQR 77-211.0) and the median NK cell percentage was 9.0% (IQR 6.0-15.0). There were 31 (19%) participants who had an NK cell number below the normal range for healthy controls.

Percentage NK cytotoxicity activity increased dose dependently and was maximal at an effector to target ratio of 100:1. For participants, the median percentage cytotoxicity for an effector to target ratio of 100:1 was 13.4 (IQR 9.4-18.8) this was significantly lower than that for healthy controls (*p*=0.0009) (figure 3.)

126 participants had both NK number and function tested. Using the cut offs for healthy controls, 62 (47%) participants had NK number and function within the normal range, 16 (13%) had normal NK number but reduced function and 37 (29%) had reduced NK number but normal NK function. Eleven participants (9%) had both reduced NK number and function.

Description of severe infections

Fifty-nine of the 168 participants (35%) had at least one severe infective episode, with a total of 141 episodes, during the 24 months follow-up. Overall 23 (39%) experienced three or more infections in the 24-month follow up period.

Of the 141 episodes of severe infection, 68 (48.2%) were microbiologically proven, 72 (51.1%) were clinically defined and 1 (0.7%) was fever without focus.

The microbiology of the severe infections is summarized in table 3. Of the 141 severe infections, 33 (48%) were bacterial, 29 (43%) were viral, 5 (7%) were fungal and 1 (2%) was parasitic.. There were 4 proven fungal infections (3 cases of invasive pulmonary aspergillosis and one case of disseminated cryptococcosis), one probable fungal infection (*Pneumocystis jirovecii* pneumonia (PJP).

The most common source of infection was respiratory, accounting for 53 (38%) severe infections. There were 8 (6%) episodes of blood stream infection and 10 (7%) episodes of sepsis.

48 (29%) participants developed NK-associated infection or malignancy. There were nine episodes of herpes virus infections (6 CMV, 1 EBV, 1 VZV and 1 HSV), 40 probable or proven polyoma virus infections, 16 respiratory virus infections, and five invasive fungal infections. Three participants developed virally driven malignancy. One participant developed post-transplant lymphoproliferative disorder, which is associated with latent EBV infection in the setting of immunosuppression. One patient developed Merkel cell carcinoma which is associated with polyoma virus infection and another participant developed cervical intraepithelial neoplasia, which is associated with human papilloma virus infection.

Seven (9%) participants died during the follow-up period, four from malignancy and three from infection.

Table 4 compares the clinical characteristics and other immune cells in infected and uninfected patients.

NK cell number, NK cell function and severe infection

Figure 4 illustrates NK cell number and function between participants who developed infection and those who did not. Median NK cell number at entry into the study was lower in participants who developed severe infection compared to those who did not (p=0.026, figure 4A). Likewise, the proportion of NK targeted killing of K562 cells at entry into the study was lower in those who developed severe infection (p=<0.001, figure 4B).

When taking into account the influence of NK cell number on NK cell function by assessing the absolute NK cell function (ratio of killed K562 cells per well to the absolute number of NK cells per well), participants with severe infection had significantly lower absolute NK cell cytotoxic activity compared to those without infection (p=0.044, figure 4C).

Prediction of a severe infection

Results of the logistic regression models are presented in table 5. On univariate analysis both increasing NK cell function (OR 0.83 per 1% increase 95% CI 0.76-0.91 p<0.0001) and number (OR 0.96 95% CI 0.93-0.99 p=0.049) were associated with a lower likelihood of severe infection over the 24-month follow-up. After adjustment for age, renal function (eGFR), mycophenolate use and transplant duration, increasing NK function (OR 0.82 95% CI 0.74-0.90 p<0.0001) but not NK number (OR 0.96 95% CI 0.93-1.00 p=0.051) remained significantly associated with a reduced likelihood of severe infection.

Figure 5A presents the ROC curves for NK cell number and NK function derived from the adjusted models described above. The model incorporating NK cell number demonstrated moderate predictive ability with an area under the curve ROC curve of 0.75 (95% CI 0.67 - 0.84). The model incorporating NK cell function as opposed to NK number demonstrated improved model discrimination (ROC AUC 0.84 (95% CI 0.77-0.91, p=0.0183 for the difference between the two AUC).

For NK cell function, using the unadjusted model, the most appropriate cut off to maximize sensitivity and specificity was 13.5%. The sensitivity and specificity of this cut off value for predicting the occurrence of severe infection were 80% and 67%, respectively.

Figure 5B illustrates the probability of developing severe infection relative to NK cell function.

Of the 46 (36%) participants with NK cell function ≤13.5% of K562 target cells that were dead following 60-minute incubation with effector cells (NK cells) at a ratio of effector: target of 100:1, 37 (58%) developed severe infections compared with 9 (14%) with NK cell function >13.6% (*p*<0.0001, figure 5C).

DISCUSSION

The key finding of this study was that reduced NK cell function was an independent predictor of severe infection in kidney transplant recipients. We identified that participants with NK cell function below 13.5% were at a higher risk of severe infection compared to those with NK cell function above this cut off. This is the first study, to our knowledge, in solid organ transplant recipients that have specifically examined the use of NK cell function to predict infection. Impairment of NK cell function by iatrogenic immunosuppression has been examined in animal and in-vitro models with conflicting results. Some studies have suggested minimal effects of calcineurin inhibitors on NK cell function[34,35,36] whilst others have suggested dose dependent inhibition.[35,37,38]

NK cells are capable of several effector functions, the most important of which is the ability to mediate contact-dependent killing of target cells. This process is mediated by lytic granules contained within the NK cells that contain the pore-forming molecule perforin and death-inducing enzymes, such as granzymes.[39] The lytic granules are deposited at the interface of the NK cell with target cells resulting in cytotoxic killing. NK cells also release inflammatory cytokines (such as interferon gamma) to amplify the immune response.[40] The Pantoxilux ™ assay measures cytotoxicity by Granzyme B and caspase activity. NK cells express Fas Ligand which when cross-linked by Fas on K562 cells results in granzyme B release by degranulation, which in turn activates caspase to induce apoptosis.[41,42] NK induced cytotoxicity is dependent upon both phenotypic and functional characteristics of NK cells.[42] The amount of granzyme and caspase produced by NK cells is dependent upon the interaction of activating and inhibitory receptors on the NK cell surface.[6] In this study, we did not phenotype the NK cells, therefore the degree to which phenotype has influenced cytotoxicity is unknown.

We demonstrated that NK cell function was superior to NK cell number in the ability to predict infection. Although there is no literature on NK cell function as a predictor of infection, several studies have found NK cell number to be predictive, either independently or as part of a composite immune score.[3,4,28,29,43,44,45] A recent study examined NK cell number one-month post-transplant to predict CMV disease and opportunistic infections infections in 92 liver transplant recipients. NK cell number at one month was a better predictor for opportunistic infection compared with $CD3^+$, $CD4^+$ and $CD8^+$ cell number. In the multivariate models, an NK cell count of $0.05x10^3$ cells/μL at one month post-transplant was an independent risk factor for CMV disease and opportunistic infection.[46] Another study found NK cell number seven-days post-transplant was an independent predictor of infection in heart transplant recipients, after adjustment of total IgG.[4] Our findings cannot be directly compared with these studies, which were performed in the first post-transplant year (compared with a median duration of four years post-transplant in our study), in different organ transplant recipients and using different definitions of infection.

Reduced NK function was associated with an increased risk of all severe infections, NK associated infections and malignancies but not bacterial infections. The lack of association of NK cell function with bacterial infection is consistent with the observation that bacteria are infrequent pathogens in those with primary NK cell deficiency.[11] In solid organ transplant recipients, Fernandez-Ruiz *et al* also found that although reduced NK cell number predicted viral and fungal infections, it did not predict bacterial infections.[3] In our study there were too few invasive fungal infections to perform a subgroup analysis however NK cell number has been demonstrated to predict fungal infection in haematopoetic stem cell transplant[5] and solid organ transplant recipients.[3] Interestingly, in our study, the NK bright subset ($CD56^{bright}CD16^-$) were lower in patients who developed infection compared to those who did not, and this was approaching statistical significance (p= 0.065). This is consistent with emerging evidence that certain NK subsets are involved in control of infections such as CMV, as well as signatures of allo-reactive humoral responses in in kidney transplant recipients. [8,10,47]

In this study, 24% of participants developed the composite endpoint of NK associated infections or malignancies. This definition was designed by the authors due to the paucity of literature describing the spectrum of infections and malignancies in patients with acquired NK deficiency.

In this study, eGFR was a predictor of severe infection. We found older participants with poor renal function had reduce NK cell function and an increased probability of infection than younger participants with good renal function. This reinforces previous literature showing that advancing age and reduced graft function increases the risk of infection[31,32] and also suggests that poor NK function may be a factor associated with this increased risk.

This study was performed at a single transplant centre, which may limit its generalizability. Although less than half of the infections were microbiologically proven, all patients admitted with infection were reviewed by an infectious diseases physician to confirm the diagnosis of severe infection. All patients with a severe infection received inpatient antimicrobial therapy so it is unlikely that the number of clinically relevant infections were overestimated. It is however, possible that patients developed infections that did not require admission such as labial herpes or shingles, and therefore the burden of infection may have been underestimated. PBMCs were used as a source of NK cells, rather than purified NK cells. This may influence results in that the proportions of NK cells in each assay. To accurately measure NK cytotoxic activity, we took into account the fact that comparison of cytotoxicity among participants' PBMCs must consider both the number of NK cells and the functional capacity. To do this, we report cytotoxic capacity on target cells on a per cell basis as well as total circulating numbers of NK cells. PBMC killing of K562 target cells predominately reflects NK cell cytotoxicity as other PBMCs have little or no cytotoxicity for K562 cells.[42] This is because K562 cells do not express MHC and the observation that monocytes require activation and/or prolonged co-incubation (18hrs) than the 1 hr required by NK cells as used in this study. [48] This study did not measure cytokine secretion, only cytotoxicity[7]. In this study, we did not analyze the relationship between NK number and function and other immune cells (such as T cell number and function). Future research could examine the interaction between various measured components of the immune system, NK cell function and the relationship with infection. The authors acknowledge that although the definition of NK-associated infection or malignancies contains conditions associated with NK cell deficiency, there are also conditions associated with excess immunosuppression. This definition was applied in a priori study and we believe this categorization is useful in trial design of targeted prophylactic strategies in patient with isolated NK cell deficiency.

NK cell function may predict severe infection in patients, allowing its potential use as biomarker to identify the subset of transplant recipients at risk for infection. These findings should be validated in different transplant settings and other transplant organ groups and to correlate NK cells with other cell types however it would be important to develop standardized protocols for NK cytotoxic testing. The potential implications of finding reduced NK function may lead to enhanced anti-viral prophylaxis, targeted vaccinations, intensified monitoring for viral infections, and tailoring of immunosuppressive regimens however further research is required. NK functional assays are relatively simple to perform and interpret and may represent an important strategy to identify and manage those transplant patients at risk of infection.

DISCLOSURE

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

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Table 1. Definition of NK associated infections or malignancies

NK associated infections[7,11, 20, 45-49]
Viral infection
Proven or probable polyoma viral infection*
Mycobacterial infection
Fungal infection

NK associated malignancies[11,11-15]
EBV associated malignancies (Burkitts lymphoma, immunoblastic lymphoma, post-transplant lymphoproliferative disease, smooth muscle tumor) Polyoma virus associated malignancies (Merkel cell carcinoma) Human papilloma virus associated malignancies (cervical and anogenital malignancy)
*Polyoma virus may not require hospital admission NK=Natural killer EBV= Epstein Barr Virus

Table 2. Demographic, clinical and immunological details of kidney transplant recipients

Characteristic		**n (%) median (IQR)**
Age (years)		54.6 (47.1-63.9)
Sex	Male	106 (63)
Ethnicity	Caucasian	106 (63)
	Asian	20 (12)
	Other	42 (25)
Cause of ESRF	Diabetes	37 (22)
	IgA	35 (21)
	Glomerulonephritis	31 (18)
	PCKD	18 (11)

	Reflux	15 (9)
	Other	32 (19)
No. of previous grafts	0	150 (89)
	≥1	18 (11)
Transplant duration (years)		4.1 (1.6-7.8)
Medications	Tacrolimus	137 (81)
	Mycophenolate	140 (83)
	Azathioprine	22 (13)
	Prednisolone	144 (86)
	mTORi	11 (6)
Tacrolimus level (μg/l)		4.6 (3.5-5.5)
Mycophenolate dose (mg/day)		1375 (1000-1500)
Serum creatinine μmol/L		113.0 (91.0-153.1)
eGFR mls/min/m^3		54.9 (41.0-73.2)
CMV donor/recipient serostatus	D-R-	21(12)
	D-R+	60 (36)
	D+R+	68 (40)
	D+R-	19 (11)
NK numbers and percent of total lymphocytes in the peripheral blood	$CD3^-56\pm16\pm$ number	140 (77-211)
	$CD3^-56\pm16\pm$ %	9 (6-15)
	$CD3^-16^-56^{+bright}$ number	11(6.35-20)
	$CD3^-16^-56^{+\,bright}$ %	1(1-1)
	$CD3^-56^{+dim}16^+$ number	106 (47-167)
	$CD3^-56^{+dim}16^+$ %	7 (4-12)
	$CD3^-56^{+dim}16^-$ number	1 (1-2)
	$CD3^-56^{+dim}16^-$ %	0 (0-0)
	$CD3^-16^-56^+$ number	17(11-26)
	$CD3^-16^+56^-$ %	1(1-1)

NK cytotoxic function (% cytotoxicity)*	100:1	
Trimethoprim/sulphamethoxazole		62 (37)

IQR = interquartile range ESRF = end-stage renal failure IgA = IgA nephropathy PCKD = polycystic kidney disease mTORi = Mammalian Target of rapamycin inhibitor eGFR = estimated glomerular filtration rate CMV= cytomegalovirus D = Donor R = Recipient

Table 3. Microbiology of severe infections

Organism	Number	Site
Bacterial infection		
Staphylococcus aureus	3	Wound (3)
Staphylococcus epidermidis	2	BSI (2)
Streptococcus pneumoniae	1	BSI (1)
Streptococcus agalactiae	1	MSU (1)
Enterococcus faecium	2	MSU (2)
Escherichia coli	14	BSI (4), sputum (2), urine (14)
Klebsiella spp.	1	Urine (1)
Serratia spp.	1	BSI (1)
Morganella sp.p	1	Urine (1)
Pseudomonas spp.	2	Urine (2), sputum (1)
Campylobacter spp.	2	Faeces (2)
Shigella spp.	1	Faeces (1)
Nocardia spp.	1	BAL (1)
Viral infection		
Adenovirus	1	Blood (1), urine (1)
Cytomegalovirus	7	Blood (7), BAL (1)
Influenza	9	NPA (9)
Picornavirus	4	NPA (4)

Respiratory syncytial virus	3	NPA (3)
Human metapneumovirus	1	NPA (1)
Herpes Simplex 1	1	CSF (1)
Varicella Zoster	1	Wound (1)
Epstein Barr Virus	3	Blood (3), CSF (1)
Fungal infection		
Aspergillus spp.	3	BAL culture (3)
Cryptococcus spp.	1	Skin biopsy (1), Blood (1),CSF (1)
Pneumocystis jirovecci	1	BAL PCR (1)
Parasitic infection		
Microsporidia spp.	1	Kidney biopsy (1)

BSI; blood stream infection, MSU: mid-stream urine, BAL: bronchoalvoelar lavage, NPA: nasopharyngeal aspirate, PCR: polymerase chain reaction, CSF: cerebrospinal fluid, *spp*: species

Table 4. Comparison of the clinical characteristics and immune tests in patients with and without severe infection

Characteristic		**No severe infection n (%) median (IQR)**	**Severe infection**	***p* value**
Age (years)		55.7(46.9-63.1)	57.9(48.6-65.1)	0.262
Sex	Male	67(61)	39(66)	0.553
Ethnicity	Caucasian	73(67)	33 (56)	
	Asian	14 (13)	3 (5)	
	Other	22 (20)	23 (39)	
Cause of ESRF	Diabetes	22 (20)	15 (25)	0.233
	Glomerulonephritis	33 (30)	20 (34)	
	PCKD	11 (11)	7 (12)	
	Reflux	10 (9)	5 (8)	

	Other	33 (30)	12 (20)	
No. of previous grafts	0 ≥1	96 (88) 13 (12)	54 (92) 5 (8)	0.537
Transplant duration (years)		4.0 (1.7-8.3)	3.7(1.1-7.5)	0.261
Immunosuppressive regimen of mycophenolate plus tacrolimus plus prednisolone		69 (63)	44 (74)	0.137
Medications	Tacrolimus	88 (81)	49 (83)	
	Mycophenolate	86 *(79)*	54 (91)	0.249
	Azathioprine			
	Prednisolone	92 (84)	52 (88)	
	mTORi	8 (7)	3 (5)	
Tacrolimus level (µg/l)		4.6(3.9-5.5)	5.1(3.9-5.7)	0.373
Mycophenolate dose (mg/day)		1500(1000-1500)	1000(1000-1500)	0.249
eGFR mls/min/m^3		58.6(45.5-76.2)	44.5(27.3-65.0)	**0.002**
CMV donor/recipient serostatus	D-R-	16 (15)	5 (8)	0.466
	D-R+	35 (35)	25 (42)	
	D+R+	41 (38)	27 (46)	
	D+R-	17 (16)	2 (3)	
NK numbers and percent of total lymphocytes in the peripheral blood	CD3$^-$56±16± number	143(97-226)	112(54-194)	**0.045**
	CD3$^-$56±16± percent of lymphocytes in the peripheral blood	9 (6-14)	10(6-15)	0.962
	CD3$^-$16$^-$56$^{+bright}$ number	13 (7-21)	10 (6-14)	0.373
	CD3$^-$16$^-$56$^{+ bright}$ %	1(1-1)	1 (0-1)	0.065

	$CD3^-56^{+dim}16^+$ number	114 (52-169)	101 (30-162)	0.505
	$CD3^-56^{+dim}16^+$ %	7 (4-12)	7 (3-13)	0.840
	$CD3^-56^{+dim}16^-$ number	1 (0-1)	1 (1-2)	0.438
	$CD3^-56^{+dim}16^-$ %	0 (0-0)	0 (0-0)	0.942
	$CD3^-16^-56^+$ number	18 (11-28)	16 (10-23)	0.429
	$CD3^-16^+56^-$ %	1(1-2)	1(1-2)	0.663
NK cytotoxic function (% cytotoxicity)*	K562 alone	15.6 (12.1-20.1)	9.8(8.2-13.1)	<0.0001

IQR = interquartile range ESRF = end-stage renal failure IgA = IgA nephropathy PCKD = polycystic kidney disease mTORi = Mammalian Target of rapamycin inhibitor eGFR = estimated glomerular filtration rate CMV= cytomegalovirus D = Donor R = Recipient

Table 5. Regression analysis for predictors of a first severe infectious episode

	Univariable			Multivariable		
	OR	**95% CI**	***p* value**	**OR**	**95% CI**	***P* value**
NK cell number model n=126						
Age (per 10 years)	1.10	0.83-1.45	0.510	0.99	0.69-1.40	0.968
Sex	0.81	0.37-1.73	0.581	-	-	-
Transplant duration (per year)	0.94	0.86-1.02	0.173	0.94	0.85-1.03	0.226
Number of previous grafts	0.71	0.34-1.48	0.368	-	-	-
Mycophenolate	3.53	0.97-12.84	0.056	3.88	0.98-15.33	0.540
eGFR (per ml/min/m^3)	0.71	0.60-0.86	**<0.0001**	0.69	0.56-0.86	**0.001**
NK cell number (per 10 cells/µL)	0.96	0.93-0.99	**0.049**	0.96	0.91-1.00	0.051
NK cell function model n=126						
Age (per 10 years)	1.10	0.83-1.45	0.510	0.99	0.95-1.02	0.579
Sex	0.81	0.37-1.73	0.581	-	-	-
Transplant duration (per year)	0.94	0.86-1.02	0.173	0.95	0.86-1.05	0.354
Number of previous grafts	0.71	0.34-1.48	0.368	-	-	-
Mycophenolate	3.53	0.97-12.84	0.056	4.12	0.86-19.72	0.076
eGFR (per ml/min/m^3)	0.71	0.60-0.86	**<0.0001**	0.67	0.54-0.83	**<0.0001**
NK cell function (per 1% increase)	0.83	0.76-0.91	**<0.0001**	0.82	0.74-0.90	**<0.0001**

OR=Odds ratio CI=Confidence interval eGFR=Estimated glomerular filtration rate NK=Natural killer cell

*NK cytotoxic function was reported as the percentage of K562 target cells that were dead following 60-minute incubation with effector cells at a ratio of effector: target of 100:1"

FIGURE LEGENDS

Figure 1. Representative flow cytometry plots from human peripheral blood mononuclear cells to demonstrate natural kill cell gating strategy.

Figure 2. Flow chart of kidney transplant recipients

Figure 3. Percentage cytotoxicity according to effector cell: target cell ratios in healthy controls and kidney transplant recipients. Bars represent median. Mann-Whitney was used to compare healthy controls and kidney transplant recipients at each of the three target: effector ratios.

Figure 4. Study entry (A) NK cell number, (B) NK cell function (C) Absolute NK cell function (ratio of the absolute number of killed K562 cells per well to the absolute number of NK cells per well). Mann-Whitney was used to compare kidney transplant recipients with and without infection for NK number, NK function and absolute NK function.

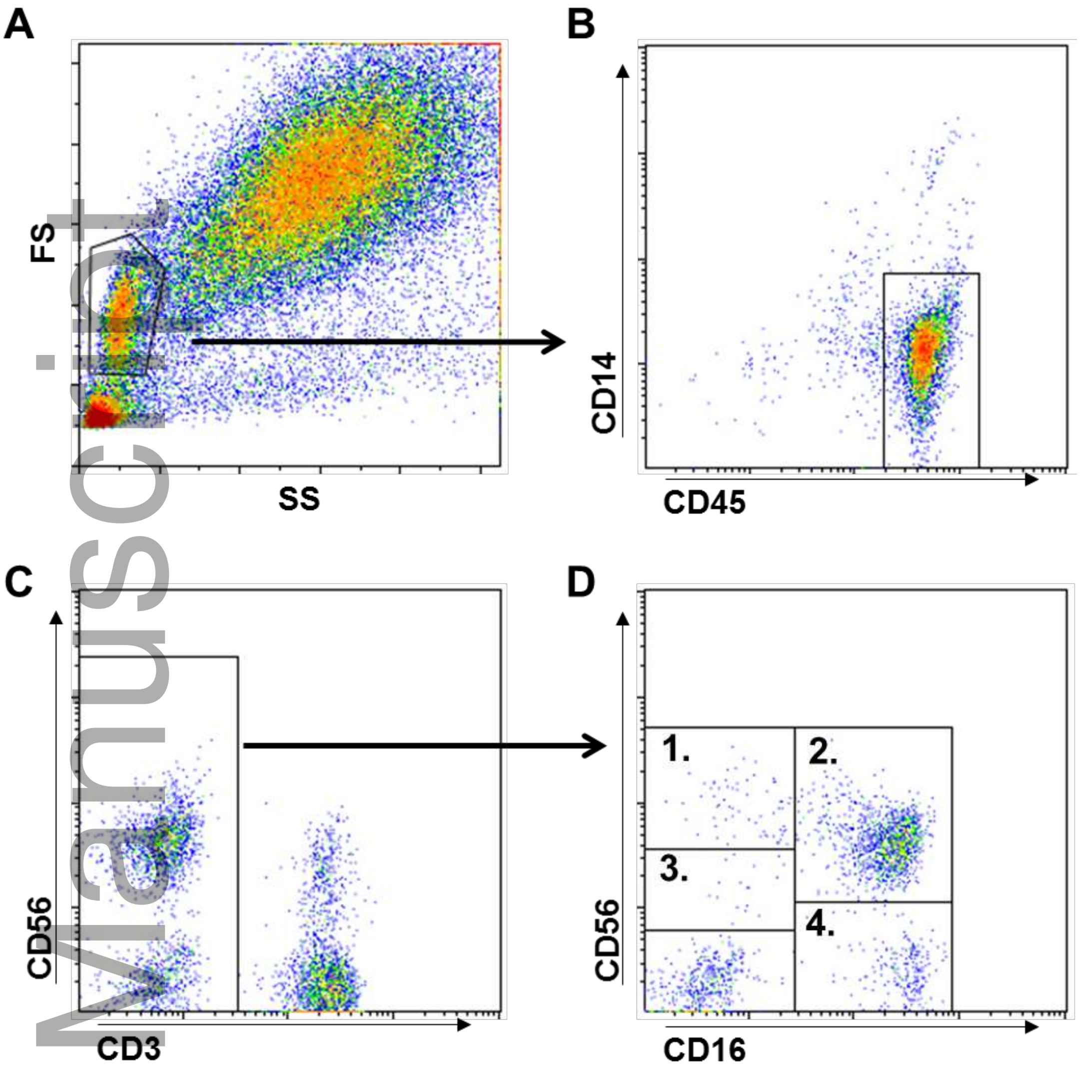
Figure 5. Natural Killer Cell number and cytotoxic function and the association with infection.

5A. Comparison of the ROC curve of the natural killer cell function model (NK cell function, age, time from transplant, mycophenolate used and eGFR) and the natural killer cell number model (natural killer cell number, age, time from transplant, mycophenolate use and eGFR) as a test to predict the development of severe infection in the following 24 months. p=0.0183

5B. The probability of developing severe infection relative to NK cell function. Overall, a steep increase in the probability of severe infection occurs once NK cell function falls below approximately 20%. Two theoretical patients are illustrated on this graph. For an older patient with poor renal function, the curve is shifted to the right, demonstrating that the probability of severe infection occurs at higher levels of NK function. The opposite is true in younger patients with better renal function (probability curve shifts to the left).

5C. Outcomes of severe infections and death over the two-year period according to whether NK cell function as normal or reduced. NK cytotoxic function was reported as the percentage of K562 target cells that were dead following 60-minute incubation with effector cells at a ratio of effector: target of 100:1. Patients with an NK cytotoxic function below 13.5% were defined as having reduced NK function. NK-associated infections were defined as any viral or fungal infection causing admission to hospital or probable or proven polyoma virus disease. NK-associated malignancies included any malignancies with a documented association with viral infections. *** p <0.002

Figure 1. NK-cell distribution in kidney transplant recipients.



Representative flow cytometry plots from human peripheral blood mononuclear cells to demonstrate natural kill cell gating strategy. Natural killer cell subsets were identified by sequentially gating on lymphocytes (A), then excluding monocytes by gating on $CD14^{-}$ $CD45^{+}$ population (B), then $CD3^{-}$ expressing CD56 (C). NK cells were identified as $CD3^{-}$ lymphocytes that were (D) 1. $CD56^{bright}CD16^{-}$, 2. $CD56^{dim}CD16^{+}$, 3. $CD56^{dim}CD16^{-}$ and 4. $CD56^{-}$ $CD16^{+}$

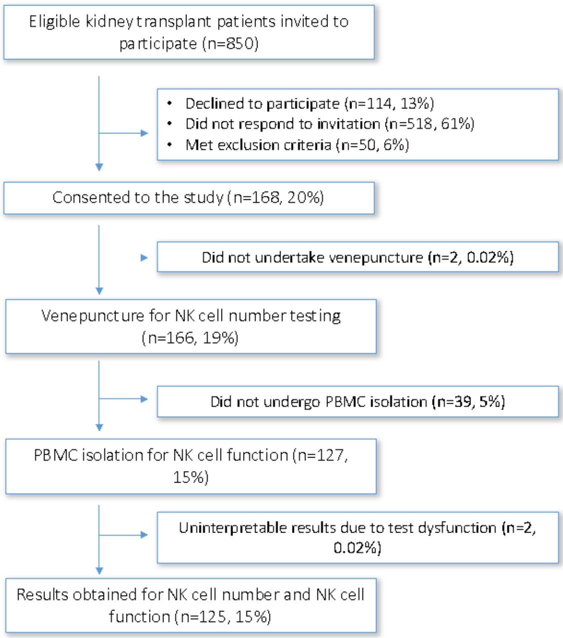

Eligible kidney transplant patients invited to participate (n=850)
Declined to participate (n=114, 13%)
Did not respond to invitation (n=518, 61%)
Met exclusion criteria (n=50, 6%)
Consented to the study (n=168, 20%)
Did not undertake venepuncture (n=2, 0.02%)
Venepuncture for NK cell number testing (n=166, 19%)
Did not undergo PBMC isolation (n=39, 5%)
PBMC isolation for NK cell function (n=127, 15%)
Uninterpretable results due to test dysfunction (n=2, 0.02%)
Results obtained for NK cell number and NK cell function (n=125, 15%)

Figure 2.

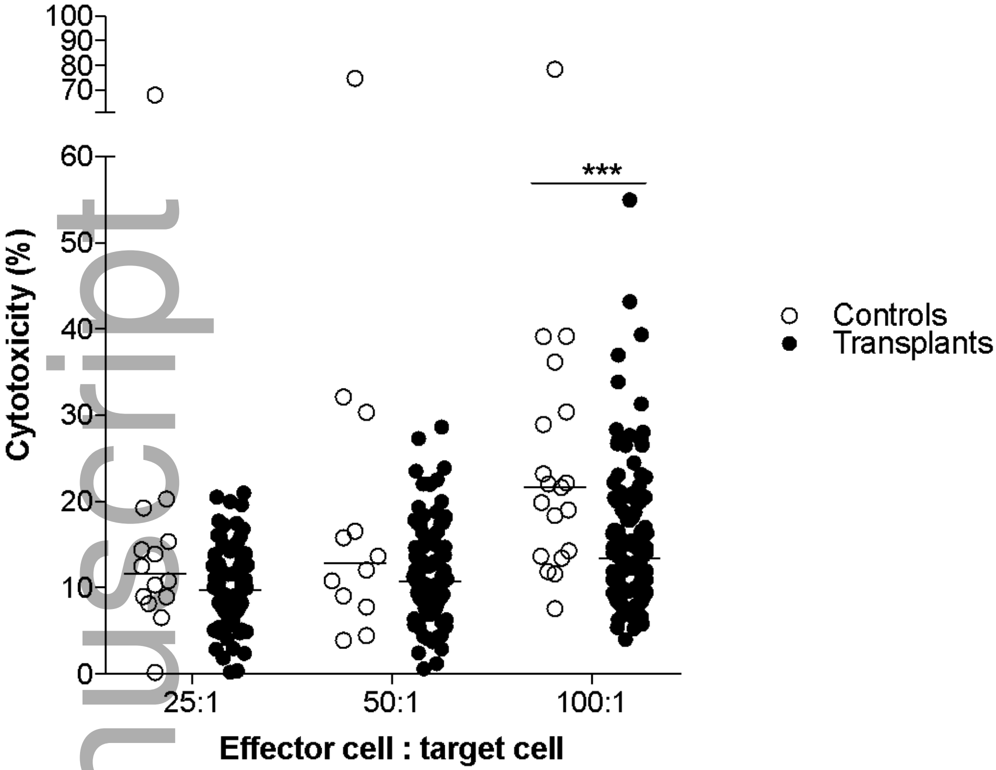


Figure 3.

Figure 4.

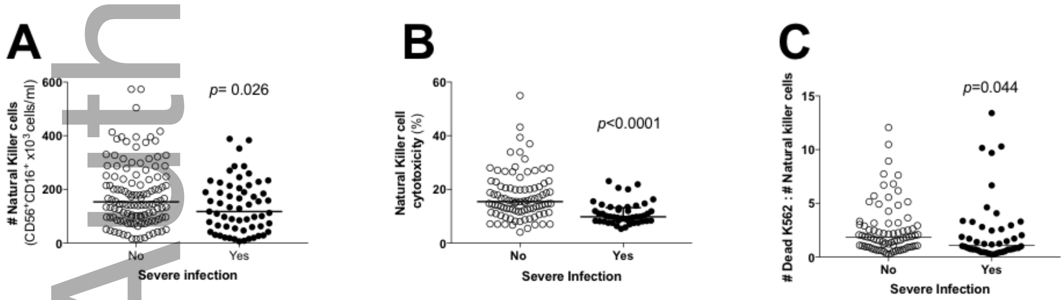
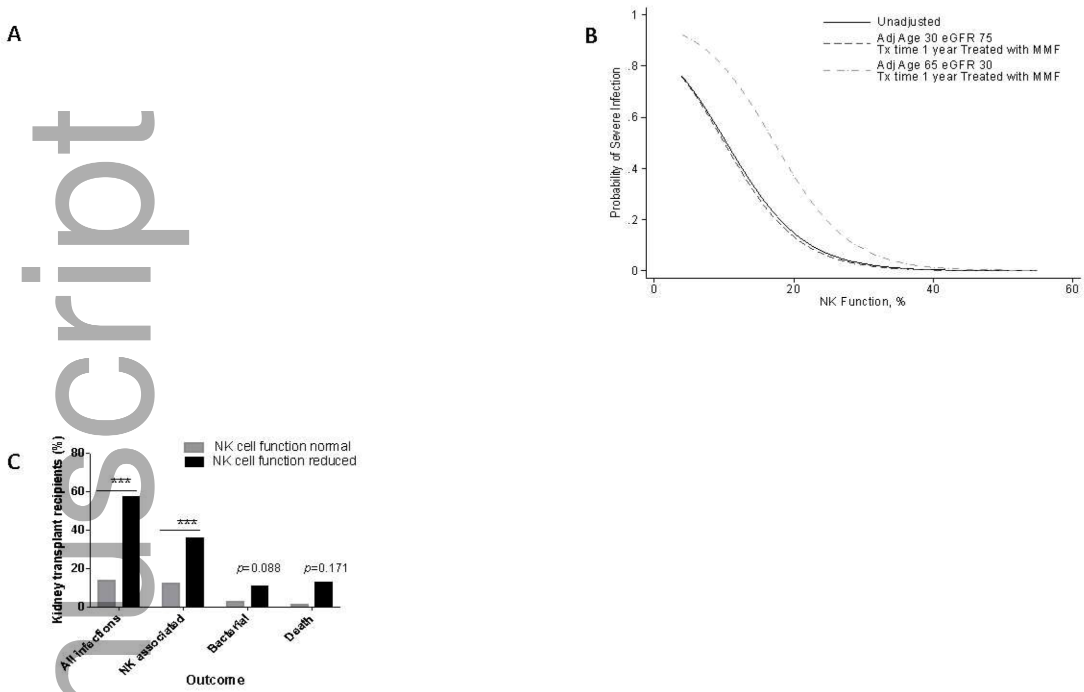


Figure 5.


A
B
Unadjusted
Adj Age 30 eGFR 75
Tx time 1 year Treated with MMF
Adj Age 65 eGFR 30
Tx time 1 year Treated with MMF
Probability of Severe Infection
NK Function, %
C
NK cell function normal
NK cell function reduced
Kidney transplant recipients (%)
***
***
p=0.088
p=0.171
All infections
NK associated
Bacterial
Death
Outcome