Adaptive NKG2C+ NK cells and cytomegalovirus infection in kidney transplant recipients

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ABSTRACT

Cytomegalovirus (CMV) infection is frequent, persistent and, generally, asymptomatic in healthy individuals. In immunosuppressed kidney transplant recipients (KTR) CMV infection or its reactivation constitutes a frequent complication which may reduce graft and patient survival. T lymphocytes are essential to control CMV infection which, in addition, has been shown to promote the differentiation and expansion of an NK cell subset characterized by the expression of the CD94/NKG2C receptor. Currently, knowledge on the mechanisms underlying this adaptive response and its role in antiviral defense is partial. In this work we analyzed the relationship between adaptive NKG2C+ NK cells and the incidence of posttransplant infection, in a cohort of CMV seropositive KTR receiving a similar immunosuppressive regimen without antiviral prophylaxis.

First, we detected a significant association, independent of other relevant clinical variables, between pretransplant proportions of adaptive NKG2C+ NK cells and a reduced incidence of posttransplant symptomatic infection, as well as an inverse correlation with peak viral loads. In a study conducted in parallel, no relation between CMV-specific T cells and NKG2C+ NK cells was detected, indirectly pointing out that they may contribute to prevent posttransplant infection progression.

On the other hand, we analyzed the evolution of adaptive NKG2C+ NK cells following posttransplant CMV infection, at different time points for over two years. In a subgroup, adaptive NK cells and T

cell subsets were compared, pretransplant and at the end of follow up, assessing at that stage viremia and the frequencies of CMV-specific T cells. Posttransplant infection promoted the development of an adaptive NKG2C+ NK cell response, yet of variable magnitude and without apparent relation with the peak viral load detected along it, thus pointing out the influence of other factors in this process. Viremia was exceptionally detected at the end of follow up suggesting that the adaptive NK cell response might contribute to long-term CMV control, though its relative role cannot be appreciated due to the overlap of a marked impact of the infection on the T cell compartment. From a practical standpoint, a combined analysis of T lymphocytes and adaptive NKG2C+ NK cells might allow a more precise assessment of CMV infection in KTR.

RESUMEN

La infección por citomegalovirus (CMV) es frecuente, persistente y, generalmente, asintomática en individuos sanos. En los pacientes con un trasplante renal tratados con inmunosupresores la infección primaria por CMV o su reactivación constituye una complicación frecuente, que puede reducir la supervivencia del injerto y del paciente. Los linfocitos T son esenciales para el control de la infección por CMV que, por otra parte, promueve la diferenciación y expansión de una subpoblación de células NK caracterizada por la expresión del receptor CD94/NKG2C. El conocimiento de los mecanismos que controlan esta respuesta adaptativa y de su papel en la defensa antiviral es parcial. En este trabajo analizamos la relación de las células adaptativas NK NKG2C+ con la incidencia de la infección post-trasplante, en una cohorte de pacientes seropositivos para CMV que recibieron un tratamiento inmunosupresor similar sin profilaxis antiviral.

En primer lugar, detectamos una asociación significativa e independiente de otras variables clínicas, entre las proporciones de células adaptativas NK NKG2C+ pre-trasplante y una menor incidencia de infección sintomática post-trasplante, así como una correlación inversa con los picos de carga viral. En un estudio paralelo no se detectó relación entre los linfocitos T específicos para CMV y las células NK NKG2C+, indicando indirectamente que estas pueden contribuir a prevenir la progresión de la infección post-trasplante.

Por otra parte, analizamos la evolución a distintos tiempos de las células NK NKG2C+ en relación con la infección por CMV post-trasplante, con un seguimiento superior a dos años. En un subgrupo, se compararon las células NK adaptativas y las subpoblaciones de linfocitos T, pre-trasplante y al final del seguimiento, analizando en esa fase la viremia y la frecuencia de células T específicas para CMV. La infección post-trasplante promovió el desarrollo de la respuesta adaptativa de las células NK NKG2C+, si bien en grado variable y sin relación aparente con el pico de carga viral detectado durante la misma, apuntando la influencia de otros factores en el proceso. La ausencia de viremia detectable al final de seguimiento apunta que el desarrollo de la respuesta adaptativa post-trasplante puede contribuir al control del CMV, sin embargo su papel relativo no puede apreciarse al solaparse con un marcado impacto de la infección en el compartimento T. Desde un punto de vista práctico, un análisis combinado de los linfocitos T y de las células NK NKG2C+ permitiría una valoración más precisa de la infección por CMV en el trasplante renal.

PREFACE

CMV is a betaherpesvirus which causes a highly prevalent infection in all human populations. In healthy individuals, primary infection is often asymptomatic and the virus establishes life-long latency, undergoing occasional subclinical reactivations which allow its transmission through secretions. Several mechanisms participate in maintaining CMV under control, resulting in profound changes in the immune system. T cells play a pivotal role in the response to CMV and clinical evidence supports a contribution by NK cells. The virus has reciprocally developed mechanisms to counteract both arms of the immune response. The expansion of an NK cell subset with adaptive features, expressing the CD94/NKG2C activating receptor has been reported in CMV-infected healthy individuals, being particularly prominent in the immunocompromised. Thus far, the mechanisms underlying development of the adaptive NKG2C+ NK cell response to CMV and its contribution to antiviral defense have not been fully elucidated.

Kidney transplant recipients (KTR), receiving immunosuppressive therapy to avoid graft rejection, are especially susceptible to CMV infection, which has been associated with an increased risk of graft rejection and patient mortality. T cells play an important role in CMV control in KTR and preliminary evidence indicate an association of NKG2C+ NK cells with a reduced risk of posttransplant infection. In this work, we further explored this issue in two different scenarios. First, our results supported that

pretransplant NKG2C+ NK cells bearing adaptive markers may contribute to reduce the incidence of posttransplant symptomatic CMV infection in KTR. Second, analysis of the long-term evolution of NKG2C+ NK cells in KTR, revealed a wide variability in the magnitude and dynamics of the adaptive NKG2C+ NK cell response to posttransplant CMV infection. Moreover, the results suggested that it may exert antiviral action together with T cells, though their relative role could not be discerned. From a practical standpoint, monitoring NKG2C+ NK cells in renal transplant patients provides a broader insight on the individual host-pathogen interaction.

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PART I

INTRODUCTION AND OBJECTIVES

INTRODUCTION

1. Cytomegalovirus

Herpesviruses pack large double-stranded DNA genomes within a three-layered structure composed of an icosahedral capsid surrounded by a protein layer called the tegument, and further enveloped by a lipid bilayer membrane with embedded glycoproteins. Herpesviruses infect different cell types and are characterized by strict species specificity. The *betaherpesviridae* subfamily includes human and murine cytomegaloviruses. The Human CMV genome is approximately 235kb, and clinical isolates have been found to contain up to 252 classically annotated protein-coding open reading frames in addition to miRNAs (1). However, detailed genomic analysis reveals widespread use of alternative transcript start sites, suggesting hundreds of additional ORFs which combined with temporal regulation contribute to the large complexity of the CMV proteome (2).

CMV seroprevalence can reach 100% in children of southern Asia and sub-Saharan Africa, in line with cultural behaviors that result in CMV exposure through breast-feeding or secretions during group childcare. Infectious virus can be found in saliva, tears, breast milk, semen, urine, and cervical secretions. In countries without early ubiquitous infection, seroprevalence increases with age and is related to child-care practices, sexual activity and socio-economic status (3). In healthy individuals, primary CMV infection is generally subclinical, and the virus enters a life-long latent state. However, CMV infection during gestation may cause severe

congenital sensorineural disorders, and can result in important complications in individuals with immunodeficiencies or under immunosuppression (e.g. transplant recipients) (4).

Shedding during primary infection and upon reactivation allows its transmission in the population. Myeloid cells and their CD34+ progenitors in the bone marrow constitute an important reservoir for latency. Differentiation into macrophages and dendritic cells can reactivate lytic gene expression and virion production. Due to the challenge in studying CMV latency in vitro, the dynamics of latency versus periodic replication is incompletely understood (5,6). In vivo, CMV carries out successful viral replication in a broad range of cell types and may be detected in the circulation and in different organs during acute infection. Presence of capsids and late structural viral proteins supports that mucosal epithelial, smooth muscle, connective tissue and endothelial cells are permissive to infection (1).

Chronic infections are associated to immune senescence and development of age-related pathologies. Based on seroepidemiological studies, CMV has been related with an increased the risk of cardiovascular disease, and drives a decrease in the naïve T cell pool that may interfere with response to other pathogens and vaccination (7,8).

2. CMV and solid organ transplant (SOT)

SOT recipients receive different immunosuppression regimens that invariably increase their susceptibility to infections and related complications. Opportunistic infections are frequent, CMV being among the most common (9). Inflammation and activation of the DNA damage response induced by the process of transplantation is suggested to initiate viral replication, that is further enhanced by immunosuppression allowing dissemination (10). CMV and other viral infections stimulate the production of proinflammatory cytokines (e.g. IL-1, IL-6, IL-8 and TNFα) and growth factors that can result in allograft dysfunction and injury. Indirectly, reductions in immunosuppression to aid viral clearance and the shift towards a pro-inflammatory state in the graft may favor allograft rejection (11). The most common strategies for prevention of posttransplant CMV infection include prophylaxis, preemptive therapy or a combination of both. In European transplant centers, CMV DNA load is regularly monitored during the first 3-12 months in plasma or whole blood at regular intervals by RT-PCR assays. Preemptive therapy is indicated for patients at low and intermediate risk of disease (12) with analytical or clinical evidence of posttransplant infection.

CMV replication can be asymptomatic, accompanied by mild symptoms or result in tissue-invasive disease. According to CMV serology, the risk of viral replication and disease is high for D^+/R^- transplants, who commonly receive antiviral prophylaxis, being low and intermediate respectively for D^-/R^- and D^+/R^+ pairs. The former

are at greater risk of graft failure and mortality than D^-/R^-, and 20% higher risk of overall mortality compared to D^+/R^+. Additional important risk factors include the type of immunosuppressive regime, age and graft cold ischemia (13–16). In solid organ transplant of D^+/R^+, posttransplant CMV infection may result from reactivation of receptor-derived strain(s), a reinfection from a donor derived-strain(s) or both (17). CMV infection in kidney transplant recipients (KTR) is associated with reduced graft and patient survival (18,19). Antiviral prophylaxis lowers the risk of acute rejection in KTR and compared to pre-emptive therapy increases allograft failure-free survival indicating that even early low-level asymptomatic CMV replication has an impact on the graft evolution (11).

3. Natural Killer (NK) cells

Human peripheral blood NK cells have been conventionally defined by flow cytometry analysis as CD3- CD56+ cytotoxic lymphocytes, further subdivided in two major populations according to surface density of CD56, a neural cell adhesion molecule (NCAM) isoform. NK cells comprise 5-15% of circulating lymphocytes, of which ≈90% correspond to the $CD56^{dim}$ subset. $CD56^{dim}$ and $CD56^{bright}$ NK cell populations express distinct chemokine and adhesion molecules which determine their differential localization. $CD56^{bright}$ NK cells express CD94/NKG2A, CD62L, CXCR3 and CCR7, and are predominantly present in lymphoid and mucosal tissues while $CD56^{dim}$ NK cells preferentially migrate to sites of acute inflammation. $CD56^{bright}$ NK cells have limited cytotoxicity but

greater proliferative potential and efficiently produce cytokines, mainly interferon gamma (IFNγ), tumor necrosis factor alfa (TNFα), linfotoxin (TNFβ), and granulocyte macrophage colony-stimulating factor (GM-CSF). $CD56^{dim}$ NK cells display CD16A (FcγRIIIA) and variable expression of killer-cell immunoglobulin-like receptors (KIR), NKp46, NKp30, CD94/NKG2A and CD94/NKG2C (20–23) (Figure 1). CD16A is the strongest NK cell activating receptor, coupled to CD3ζ or FcεRIγ chain adapters, which triggers effector functions when encountering antibody-coated targets (24).

Variegated expression of an array of germ-line encoded activating and inhibitory receptors diversifies the NK cell compartment. Single cell RNA sequencing of the entire NK cell compartment of healthy individuals confirm previous phenotypic studies indicating that that the majority of circulating $CD56^{dim}$ $CD16^{bright}$ NK cells are closely related but can be further subdivided into subsets with different phenotypes and maturation levels, including a terminally differentiated population defined by CD57 expression (25). According to mass cytometry assessment of more than 30 parameters, the diversity of circulating NK cells from a single individual can be extremely large (26).

NK cells are considered part of the innate lymphoid cell (ILC) family, which stem from a precursor close to the common innate lymphoid progenitor (CLP). ILC2, ILC3 and lymphoid tissue-inducer cells (LTi cells) are non-cytotoxic. By contrast, ILC1 appears closely related to NK cells, and both depend on T-bet,

producing IFNγ and TNFα, though they follow different developmental pathways (27,28).

3.1 NK cell functions

NK cell effector functions against pathogens and tumor cells depend on cytokine production, release of cytotoxic granules and expression of pro-apoptotic ligands (29). The cross-talk between NK and dendritic cells influences the development of the adaptive immune response, and NK cells can also interact with T cells through cytokine production, antigen presentation and selective killing (30–33).

Direct NK cell cytotoxicity is mainly caused by the exocytosis of cytotoxic granules in the immune synapse (34). Molecules contained in these secretory granules include perforin, granzymes (Grz) and granulysin (35). Contact with the target cell and activation initiates cytoskeleton remodeling, aiding secretory granule migration towards the synaptic cleft where they fuse with the membrane and release their content (34). Perforin is safely stored in acidic granules, it activates at the neutral pH of the immune synapse and polymerizes, creating pore-forming structures on the target cell membrane (36). The best characterized Grz within NK cell secretory granules is GrzB but five different granzymes are expressed in humans (A, B, H, K, M) and all are transcribed in NK cells (37). Granzymes are serine proteases that induce the generation of reactive oxygen species, mitochondrial damage and DNA fragmentation via caspase-dependent and independent

processes (35). In addition, NK cells may express Fas ligand (FasL) and TNF-related apoptosis inducing ligand (TRAIL) on the plasma membrane; target cells are differentially susceptible to these ligands depending on the expression of their corresponding receptors. FasL and TRAIL engagement induce caspase-dependent death of the target cell (38,39). Serial killing by NK cells appears to switch between death receptor signaling and granule release (40).

NK cells produce cytokines (e.g. TNFα and IFNγ) independently from cytotoxic granules (41). Production of IFNγ by NK cells induces upregulation of HLA class I molecules (HLA-I), modulates the activity of other immune cells and establishes an anti-viral state (38,42,43). Furthermore, stimulation of $CD56^{dim}$ NK cells induces the early secretion of several chemokines, namely MCP-1 (CCL2), MIP-1α (CCL3), MIP-1β (CCL4), RANTES (CCL5), IL-8 (CXCL8), and IP-10 (CXCL10) leading to recruitment of other immune cells (44).

3.2. NK cell receptors

The interplay of inhibitory and activating receptor signaling, upon engagement by their ligands on target cells determines selective NK cell activation. KIR and CD94/NKG2A survey surface expression of HLA-I molecules preventing activation against normal autologous cells. Infected or transformed cells which downregulate HLA-I expression are susceptible to NK cell effector functions, as predicted by the missing-self hypothesis (45). Loss of inhibition is insufficient to induce NK cell activation, which requires

engagement of receptors triggering effector functions for tipping the balance.

Inhibitory receptor-ligand interactions not only modulate NK cell functions but regulate as well their development and maturation, establishing self-tolerance (46). Studies in sibling pairs have shown that KIR expression is primarily defined by their genotype but modulated by the HLA environment (47). The genetic diversity, stochastic expression, modulation by endogenous molecules and environmental factors determines NK cell functional maturation, shaping the individual NK cell compartment.

3.2.1 HLA-specific NK cell receptors

KIRs constitute a highly polymorphic family of over 12 activating and inhibitory receptors with two (KIR2D) or three (KIR3D) immunoglobulin (Ig)-like domains. Inhibitory KIR generally bind to classical HLA-I molecules (A, B, C loci) but, in some cases, their ligands as well as those of activating KIRs remain incompletely characterized. Inhibitory KIR have long cytoplasmic tails (KIRxD**L**x) with an immunoreceptor tyrosine-based inhibitory motif (ITIM) that recruits Src homology region 2 domain-containing tyrosine phosphatases (SHP-1 and -2). Activating members have short cytoplasmic tails (KIRxD**S**x) and are assembled with the DNAX activating protein of 12kb (DAP12). This adaptor bears immunoreceptor tyrosine-based activating motifs (ITAM), which connect the receptor to protein tyrosine kinase (PTK) activation pathways.

KIR genes span a ~150kb region of chromosome 19 and are distributed in different haplotypes, mostly generated by gene conversion and duplication events. Allelic polymorphisms further add another layer of heterogeneity (48,49). Haplotypes are broadly categorized in two groups. The A haplotype includes six genes encoding for inhibitory KIR and a single activating KIR gene, which is often truncated in Caucasian populations. By contrast, B haplotypes contain a variety of combinations of different activating and inhibitory KIRs, distributed in the telomeric and centromeric regions of the locus (50,51). KIRs generally recognize structural features conserved in groups of HLA-I alleles. KIR2DL2 and KIR2DL3 recognize HLA-C alleles containing an asparagine at position 80 (group C1), while those with a lysine at position 80 (group C2) interact with KIR2DL1. KIR3DL1 recognizes the Bw4 epitope which is present on a subgroup of HLA-B and some HLA-A alleles. KIR3DL2 has been so far only confirmed to interact with HLA-A*03 and HLA-A*11 (52).

The CD94/NKG2 family of C-type lectin receptors is composed of heterodimers formed by the CD94 common subunit covalently linked by a disulfide bond with different NKG2 molecules. (NKG2A, B, C, E- and F) (53) NKG2A/B and NKG2E/H are products of alternatively splicing of the corresponding genes. Formation of heterodimers with the CD94 subunit is necessary for signaling and stable surface expression (54). CD94/NKG2A and CD94/NKG2C receptors bind the non-classical class I molecule HLA-E (55–58). CD94/NKG2A inhibits NK cell activation and recognizes HLA-E with higher affinity than the activating

CD94/NKG2C receptor (59). The affinity of the interaction is modulated by the peptide bound to HLA-E (60). Akin to KIRs, CD94/NKG2A signals via the SHP-1 tyrosine phosphatase and CD94/NKG2C is coupled to DAP12 (61). HLA-E presents 9-mer leader sequence peptides from HLA-I molecules and is widely expressed (62). HLA-E has a low degree of polymorphism, eight alleles have been described of which only two are present at high frequency in the population (63). These major alleles differ in a single amino acid substitution not directly involved in peptide binding, but differences in their surface expression levels have been reported (64). Despite its limited polymorphism, HLA-E can present other peptides not derived from HLA-I molecules, and CD8+ T cell responses elicited by presentation of pathogen-derived peptides on HLA-E have been identified (65–67).

The family of leukocyte Ig-like receptors (LILR) also known as Ig-like transcripts (ILT), include activating (ILT1, ILT7, ILT8, ILT6) and inhibitory receptors (ILT2, ILT3, ILT4, ILT5, LIRE) predominantly expressed on myeloid cells. ILT2 (LILRB1, LIR-1 or CD85j) is also expressed on B cells, NK and T cell subsets at late stages of differentiation (68–72). Variation in ILT2 expression between different individuals is determined by polymorphisms that affect transcription and surface expression (73). Engagement of ILT2 results in inhibitory signaling by recruitment of SHP-1 tyrosine phosphatase (74). ILT2 recognizes a variety of HLA-I alleles, with particular affinity for HLA-G and HLA-I dimers (69,75–77).

3.2.2 Non-HLA specific NK cell receptors

CD16 (FcγRIIIA) is a type I transmembrane receptor with two extracellular Ig-like domains. CD16 recognizes the constant fragment (Fc) of IgG, triggering antibody-dependent cell-mediated cytotoxicity (ADCC) and cytokine production upon interaction with antibody coated cells (24,78). CD16 downstream signaling is mediated by association with CD3ζ or FcεRIγ chain adaptors (79). CD16 was shown to trigger NK cell activation in the absence of other costimulatory signals (24). Variants with a dimorphism at position 158 (T>G) have differential surface expression and affinity for IgG (80), with clinical implications for immunotherapy (81).

NKG2D is constitutively expressed by all NK cells, CD8+ and some CD4+ T lymphocytes and detects ligands expressed under cell stress conditions (e.g. tumor transformation, infection and DNA-damage), being additionally implicated in graft rejection and autoimmunity. NKG2D is a assembled as a homodimer with a short cytoplasmic tail and interacts with the DNAX-activating protein of 10kb (DAP10) adapter through charged amino acid residues in their transmembrane domains (82). DAP-10 contains a cytoplasmic YxxM motif coupling it to PI3K activation. NKG2D recognizes polymorphic MHC class I polypeptide-related chain (MIC)-A and -B molecules as well as members of the UL16-binding protein family (83). Engagement of NKG2D induces cytotoxicity in activated but not resting NK cells (24,84).

Natural cytotoxicity receptors (NCR) include three different activating molecules (NKp46, NKp44, NKp30) of the Ig-superfamily coupled to signaling adaptors (85). NKp30 and NKp46 are expressed by resting NK cells and signal through CD3ζ-CD3ζ or CD3ζ-FcεRIγ dimers. NKp44, coupled to DAP12, is expressed in activated and decidual NK cells (86,87). NKp44 displays an ITIM motif in its cytoplasmic tail and has been found to have a dual role delivering inhibitory signals in response to recognition of proliferating cell nuclear antigen (PCNA) (88,89). NKp46 was the first receptor to be recognized as a mediator of natural cytotoxicity against HLA-I deficient cells (90) and has been directly implicated in the response against HCMV-infected cells (91,92). Since their discovery, NCRs have been reported to interact with a range of pathogen derived proteins of viral, parasite, fungal and bacterial origin as well as molecules expressed or released by cancer cells. The characterization of their ligands, in particular those of non-microbial origin upregulated on tumor cells, is incomplete (93,94). All three NCR have been reported to bind to heparan sulfate sequences with distinct specificities (95). NKp30 binds to B7-H6, HLA-B associated transcript (BAT3) and galectin-3. NKp44 has been found to interact with Enactin, platelet-derived growth factor (PDGF) and Nidogen-1 (NID-1). B7-H6 is absent in normal cells, BAT3 is released by cells under stress conditions and galectin-3 facilitates tumor growth. Pathogen derived molecules recognized by NCRs include hemagglutinin and hemagglutinin-neuraminidases (i.e. from influenza), flavivirus envelope proteins and vimentin on *M. tuberculosis*-infected cells (96).

Other NK cell receptors implicated in viral and tumor immunity include the activating DNAM-1 and the homologous inhibitory TIGIT. DNAM-1 and TIGIT share common ligands, binding to Nectin and Nectin-like molecules (e.g CD112, CD155) that are upregulated on stressed cells (97,98). DNAM-1 has been has been involved in triggering NK cell activity against CMV-infected monocyte-derived dendritic cells (moDCs), and related with the development of several autoimmune disorders (91,98,99). TIGIT competes with DNAM-1 for ligand binding, inhibiting NK cell cytotoxicity (100).

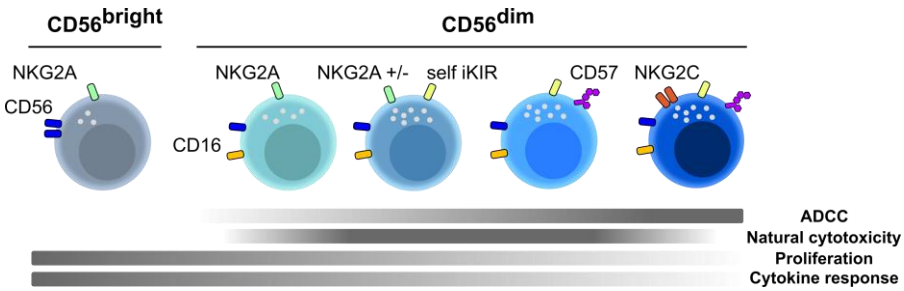

Figure 1. NK cell maturation stages. ADCC; antibody-dependent cell-mediated cytotoxicity. Adapted from *Pfferle A, et. al. 2020. Front Immunol.*(209).

4. Immune response to CMV

The pressure exerted to control acute CMV infection and reactivation from latency exerts a profound influence in the immune system. This was clearly illustrated by a study of CMV discordant monozygotic (MZ) twins, showing that over 50% of immune parameters measured differed between CMV negative/positive twins (101).

The ancestral pattern recognition receptors elicit the first alarms in response to infection by many pathogens (102), including CMV. Prior to replication events, recognition of viral envelope glycoproteins triggers an innate response promoting inflammatory cytokine and type I IFN production, as well as the induction of interferon-stimulated genes (ISG) and NF-kB-dependent pathways in the infected cell (103–107). Single nucleotide polymorphisms in Toll-like receptors (TLR) have been associated to risk of CMV replication in transplant recipients (108,109). Envelope glycoproteins B (gB), gH/gL and gM/gN are essential for replication and together mediate attachment, fusion and cell entry. The humoral response against CMV is mainly dominated by specificity towards these glycoproteins (1), and absence of neutralizing antibodies against glycoprotein B may increase risk of CMV disease in KTR (110).

4.1. TcRαβ and γδ T cell response to CMV

TcRαβ+ T cell responses play a fundamental role in the response against pathogens, and there is evidence that a substantial proportion of the compartment is engaged in CMV control. In a comprehensive peptide-based screening of all CMV ORFs, healthy adults were shown to have a CD4+ and CD8+ T cell response to CMV, as measured by IFNγ production, representing a median of 9-10% of T cells. Peptide mixes of 19 ORF were estimated to be required to approximate the whole CMV T cell response (111). The CD8+ T cell population undergoes memory inflation in response to CMV, as indicated by high frequencies of a few clonotypes. Both

reinfection and particularly reactivation have been proposed to drive these expansions (112,113).

Most studies of CMV-specific T cell immunity have been conventionally designed for assessing the response to peptides from immunodominant IE1 and pp65 antigens. Qualitative and quantitative features of the CMV-specific T cell response appear important for infection control. Functional characterization of CD8+ T cells has revealed a relationship between multifunctional (IFNγ+, TNFα+, MIP1β, IL-2+) pp65-specific CD8+ T cells and a lower risk of CMV reactivation following hematopoietic cell transplant (HCT)(114). Higher pretransplant frequencies of IE1-specific T cells in KTR were associated with a lower risk of posttransplant infection (115). In a small cohort of KTR at intermediate risk of CMV infection, pretransplant frequencies of IE1-specific CD8+ T cells appeared to be more protective than pp65-specific CD8+ and CD4+ responses to IE1 or pp65 (116). To escape recognition, CMV encodes a set of proteins (i.e. US2, US3, US6 and US11) which inhibit HLA-I expression in the infected cell along different phases of the lytic cycle (117). During the immediate-early phase of infection US3 forms oligomers, sequestering HLA-I. US2 and US11, expressed at the early phase, translocate HLA-I molecules to the cytosol for proteasomal degradation. US6, expressed during early and late phases, inhibits TAP-mediated peptide import into the ER (117).

CD4+ T cells also play a role in pathogen control. Impaired CD4+ T cell activation, as in patients with AIDS or HLA class II (HLA-II)

and CD40L deficiencies, is associated with increased susceptibility to CMV (118). Lower CD4+ T cell counts in D+/R- SOT patients have been associated with posttransplant CMV infection and disease (119). Moreover, frequencies of CD4+ pp65-specific T cells predicted absence of CMV replication in KTR (120). In D+/R- renal transplant pairs, a delayed CMV-specific effector-memory $CD4^+$ T cell response was associated to symptomatic CMV infection (121). In line with these results, US2 and US3 have been reported to also downregulate HLA-II molecules and, moreover, CMV interferes with IFNγ-induced HLA-II expression in infected cells (122). In addition, the viral IL-10 homologue leads to decreased cytokine production and surface expression of HLA-I and –II (123).

The majority of circulating TcRγδ+ T cells in adult blood are *Vγ9Vδ2+* and derive from a small population which undergoes expansion most likely after exposure to phosphoantigens during early childhood. The remaining γδ T cell subsets are predominantly V*δ*1+ and likely expand in response to antigenic challenge. Fetal non-Vγ9Vδ2+ T cells mount a response elicited by congenital CMV infection, and in CMV-seropositive healthy individuals $V\delta2^{neg}$ clonal expansions with a skewing towards V*δ*1+ repertoires have been observed. $V\delta2^{neg}$ expansions have a cytotoxic effector-memory phenotype and repertoire restriction, as compared to $V\delta2^{neg}$ T cells in CMV- individuals (124,125). Following CMV infection in KTR, $V\delta2^{neg}$ γδ T cell expansions correlated with resolution of CMV viremia, while their absence was related to recurrent CMV replication (126). A delayed $V\delta2^{neg}$ γδ T expansion was associated to a higher risk of symptomatic infection in KTR, regardless of

pretransplant serostatus (127). In CMV+ KTR immunosuppressed with anti-thymocyte globulin, frequencies of $V\delta2^{neg}$ γδ T cells were significantly increased in those without posttransplant CMV replication (126). Furthermore, patient-derived Vδ1/δ5/δ3 γδ T cell lines selectively targeted CMV-infected cells, limiting viral propagation *in vitro* in a T cell receptor-dependent manner (128).

4.2 NK cell response to CMV

NK cells respond to viral infections by direct recognition of the infected cells, but are also indirectly activated by pro-inflammatory cytokines, type I IFN (IFNβ/α) and specific antibodies (129). Type I IFN, IL-12, IL-18 and IL-15 contribute to promote the maturation, proliferation, effector functions and survival of NK cells (130–132). Human CMV immune evasion mechanisms inhibiting HLA-I expression hamper the CD8+ T cell response, but render infected cells susceptible to NK cells by reducing HLA-I engagement of inhibitory receptors.

To counteract this effect, CMV has developed additional immune evasion strategies based on interfering with expression in the infected cells of ligands for NK cell activating receptors or displaying ligands for inhibitory receptors. In this regard, CMV-encoded UL16 and UL142 (133,134) inhibit the expression of NKG2D ligands (e.g. MICA) in the infected cell. Surface expression of the adhesion molecule CD58, a ligand for the co-stimulatory receptor CD2, is inhibited by UL148 (135,136). Similarly, CD155 and CD112 ligands for DNAM-1 and CD96 are

sequestered by UL141 (137,138). NKp30 has been shown to be inhibited at the signaling level by UL83 which induces its dissociation from the CD3ζ adaptor (139). Expression of DNAM1 ligand is downregulated in HCMV-infected moDCs, and ligands for NKp30 and NKp46 are reduced in moDCs after CMV infection (91). The leader sequence of the UL40 CMV protein is identical to that derived from HLA-I molecules and stabilizes surface HLA-E during infection in a TAP-independent manner (140,141), thus leading to inhibition of NKG2A+ NK cells. Last but not least, NK cells expressing ILT2 are inhibited by UL18, a viral homologue of HLA-I which binds with the highest affinity to the inhibitory receptor (139).

4.3 Adaptive NK cell response to CMV

Persistent increased proportions of NK cells expressing high levels of the CD94/NKG2C receptor ($NKG2C^{bright}$), with other characteristic phenotypic and functional features, were reported in healthy CMV+ blood donors. The magnitude of NKG2C+ NK cells expansions is variable, ranging from >50% of total NK cells to levels indistinguishable from those of CMV- individuals (142). Hematopoietic stem cell transplant (HSCT) recipients undergoing CMV infection develop a rapid maturation of the NK cell compartment with an expansion of NKG2C+ CD57+ NK cells (143). NKG2C+ NK cells are increased in children with congenital CMV infection, and healthy infants infected in the first year of life undergo accelerated maturation of the NK cell compartment associated to NKG2C+ NK cell expansions (144,145). Of note, NK

cell populations with low surface expression of NKG2C ($NKG2C^{dim}$), are detectable in CMV- individuals.

The adaptive differentiation and expansion of NKG2C+ NK cells is reminiscent of the development in mice of memory NK cells in response to CMV infection. A memory NK cell response to CMV infection was first observed in C57BL/6 mice, which express the Ly49H receptor. Ly49H binds the MCMV-encoded protein m157 expressed on infected cells, leading to an expansion of NK cells bearing this receptor. Adoptive transfer of MCMV-primed Ly49H+ NK cells to Ly49H-/- mice infected with MCMV led to their antigen-driven expansion followed by a contraction phase once control of the infection was reached. These long-lived adaptive NK cells display a differentiated transcriptional and surface receptor signature and confer protection against viral re-challenge (146,147).

$NKG2C^{bright}$ NK cell expansions were originally reported to be independent of other herpesviruses, KIR haplotypes and HLA-E dimorphism. Their detection over the course of other infections appears dependent on CMV co-infection. The nature of a putative CD94/NKG2C ligand in CMV-infected cells has remained elusive and there is no evidence supporting NKG2C specific recognition of a viral molecule (142,148,149). Recently, it has been proposed that the adaptive NKG2C+ NK cell response may be driven by interaction of the receptor with HLA-E bound to UL40-derived peptides in the infected cell. As the affinity of the interaction with HLA-E was shown to depend on the sequence of HLA-I bound leader peptides, it has been hypothesized that differences in the

UL40-derived nonamers between different CMV strains might influence the adaptive NKG2C+ NK cell response (59,150).

Oligoclonal expansions of NKG2C- NK cells expressing activating KIR have been observed in CMV+ healthy individuals and following CMV infection in umbilical cord blood transplant (UCBT) recipients (151,152). NK cell populations with reduced expression of the FcεRIγ adaptor were associated to CMV infection (153). CMV adaptive NK cells have a particular epigenetic and transcriptional signature compared to conventional NK cells, reminiscent of cytotoxic T lymphocytes (154), including remodeling of the IFNγ locus. This results in an efficient cytokine and cytotoxic response to Ab-coated cells due to potent release of IFNγ and TNFα and high intracellular levels of granzyme B (155–157).

CMV-induced expansions of NKG2C+ NK cells are characterized by an oligoclonal pattern of inhibitory KIR specific for self HLA-I, reduced levels of NKp30, NKp46, Siglec7, CD161, and the FcεRIγ adaptor, acquisition of late differentiation markers such as CD57, and ILT2, and increased levels of CD2 (142,158,159). Additionally, they display variable epigenetic silencing of other adaptor molecules (ie SYK, EAT2) and transcription factors PLZF, IKZF2 and Helios (160,161) (Figure 2).

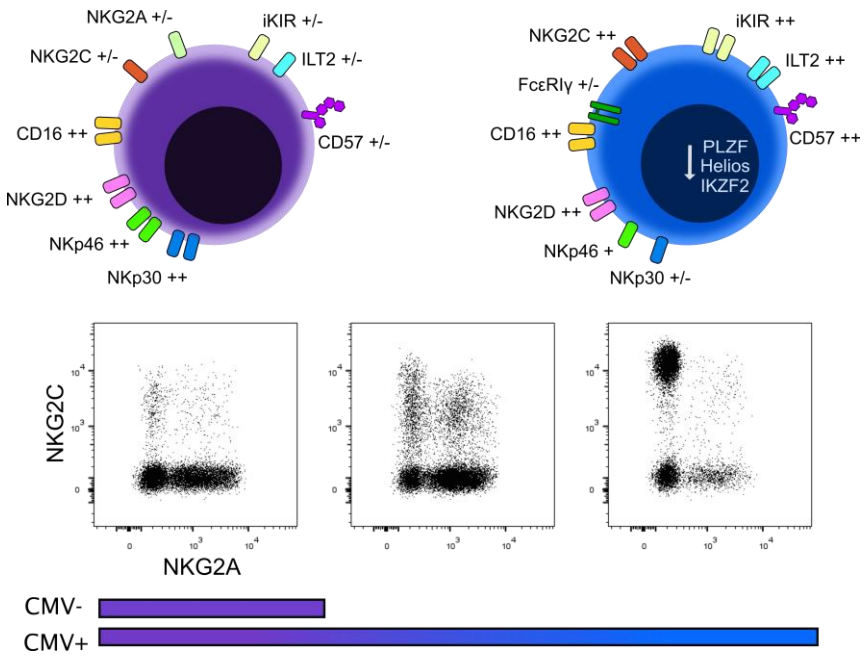

Figure 2. CMV modulation of the NK cell compartment.

5. NK cells and transplantation

5.1. Role of NK cells in transplant rejection

NK cells react against targets that provide activating signals in the absence of HLA-I expression and other inhibitory ligands. In certain settings, such as HSCT for patients with leukemia, the presence of KIR-HLA-I mismatch in the direction of the recipient results in significantly higher leukemia-free survival (162). In KTR, genetically predicted KIR-ligand mismatch has been reported to negatively impact short- and long-term outcomes (163,164), however conflicting reports have been published (165). Recently, recipients with higher number of predicted KIR-HLA-I mismatches

against the graft were found more likely to present donor-specific antibody-independent (DSA-) microvascular inflammation (MVI). Longer cold ischemia times and incidence of CMV infection were also overrepresented in the MVI+ DSA- group (166). These observations support that NK cells have alloreactive potential against solid organ transplants, determined by KIR-ligand mismatches and variables that promote graft expression of activating ligands. In this regard, NK cells are likely to have an impact in antibody mediated rejection (ABMR). NK cell-related transcripts, CD16A-induced transcripts and increased CD56+ cells in the graft are associated with ABMR (167,168). Furthermore, increased activated $CD56^{dim}$ NK cells and IFNγ-related transcripts are consistent with MVI in ABMR mediated by antibody-dependent NK cell activation (169,170).

5.2. NK cells and CMV in transplant recipients

Expansions of NK cells expressing activating KIR are found in healthy CMV+ individuals and cord blood graft recipients homozygous for the deletion of the *KLRC2* gene encoding NKG2C (151,152). Several studies show a relationship between KIR gene content and primary CMV infection, reactivation and/or severity in the context of transplantation. KIR haplotypes encoding activating KIR correlate with reduced CMV reactivation following HSCT (171,172). In CMV+ KTR, protection against CMV reactivation has been found to increase with the number of activating KIR genes. Patients with at least one haplotype B had a lower relative risk of infection. The telomeric region of haplotype B (Tel B), coding for

KIR2DS1, KIR3DS1, KIR2DS5 and KIR2DL5A is suggested to confer the protective effect (173–175). However, KIR B haplotype - associated control of CMV appears restricted to recipients with previous exposure (176). Telomeric KIR genes in B haplotypes in combination with the HLA-C2 ligand was more frequent in D+/R- KTR who experienced primary CMV infection, while telomeric KIR A genes in combination with the HLA-C1 ligand were increased in the group without severe infection (177). In a similar cohort, the KIR genotype appeared to have no effect on the incidence of CMV disease (178) . In a cohort of ~75% seropositive KTR, KIR2DL3, KIR2DL2-HLA-C1 or lack of KIR2DS2 were found to increase the likelihood of developing CMV infection (179).

CMV infection in immunocompromised individuals drives the long-term expansion of adaptive NKG2C+ NK cells. In cord blood transplant recipients, CMV reactivation leads to a long term increase in NKG2C+ NK cells (180,181). Similarly, in SOT, NKG2C+ NK cells proliferate during acute CMV infection and preferentially acquire CD57 over time (159). Clinical observations suggest NKG2C+ NK cells are protective against CMV replication. A case study of CMV infection in a patient with a severe T cell immunodeficiency showed development of an NKG2C+ NK cell expansion concomitant with reduced viremia (182). Increased proportions of NKG2C+ NK cells in the bronchoalveolar lavage of lung transplant recipients were correlated with lower blood CMV titers (183). In a population with near universal prevalence of CMV in childhood, *KLRC2*$^{del/del}$ children under 10 were found to present

elevated anti-CMV IgG titers, suggesting a relatively inefficient control of CMV infection (145). Preliminary evidence supporting an association of pre-transplant levels of circulating NKG2C+ NK cells with a reduced incidence of posttransplant CMV infection in KTR was reported (184).

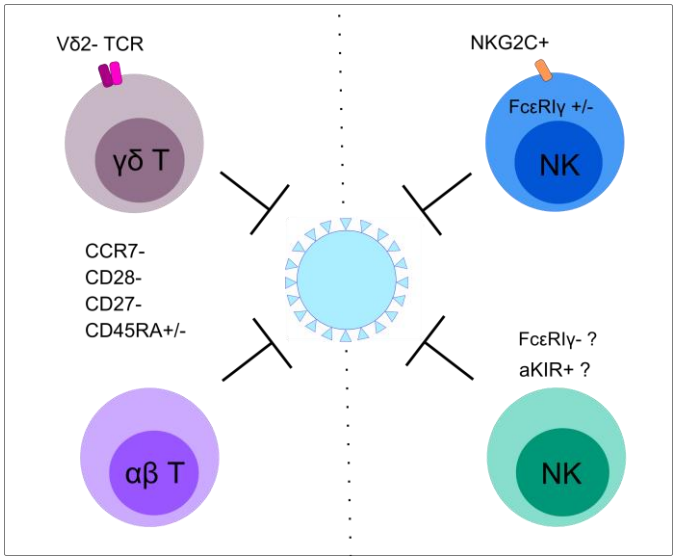

Figure 3. Contribution of NK and T cell subsets to CMV control.
Adapted from López-Botet M, et. al. 2017. Front Immunol (191).

OBJECTIVES

Cytomegalovirus (CMV) infection induces widespread changes in the immune system of healthy individuals and becomes clinically important in immunocompromised patients. Immunosuppressed kidney transplant recipients (KTR) are at risk of suffering CMV infection or reactivation, associated with detrimental effects on graft and patient survival. CMV infection promotes the adaptive differentiation and expansion of NK cells expressing the CD94/NKG2C receptor together with characteristic phenotypic and functional features, but evidence supporting their role in antiviral defense is limited. Based on preliminary observations we hypothesized that adaptive NKG2C+ NK cells may contribute with CMV-specific T lymphocytes to control CMV reactivation/reinfection in KTR. To address this, a cohort of CMV+ KTR was studied with the following objectives:

1. To explore the relationship between preexisting adaptive NKG2C+ NK cells, CMV-specific T cells and the incidence of posttransplant CMV infection.

2. To assess the long-term evolution of the adaptive NKG2C+ NK cell response to posttransplant CMV infection and its relation with T cell immunity

PART II

Pretransplant adaptive NKG2C+ NK cells protect against cytomegalovirus infection in kidney transplant recipients

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ORIGINAL ARTICLE

Pretransplant adaptive NKG2C+ NK cells protect against cytomegalovirus infection in kidney transplant recipients

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Cytomegalovirus (CMV) infection constitutes a complication for kidney transplant recipients (KTR) and CMV-specific T cells reduce the risk of viral replication in seropositive patients. CMV promotes the adaptive differentiation and expansion of an NK cell subset, hallmarked by expression of the CD94/NKG2C receptor with additional characteristic features. We previously reported an association of pretransplant NKG2C+ NK cells with a reduced incidence of CMV infection. We have strengthened the analysis in cryopreserved peripheral blood mononuclear cells from an enlarged KTR cohort (n = 145) with homogeneous immunosuppression, excluding cases at low risk of infection (ie, CMV D−R−) or receiving antiviral prophylaxis. Moreover, adaptive NKG2C+ NK cell–associated markers (ie, NKG2A, CD57, Immunoglobulin-like transcript 2 [LIR1 or LILRB1], FcεRI γ chain, and Prolymphocytic Leukemia Zinc Finger transcription factor) as well as T lymphocyte subsets were assessed by multicolor flow cytometry. The relation of NKG2C+ NK cells with T cells specific for CMV antigens was analyzed in pretransplant patients (n = 29) and healthy controls (n = 28). Multivariate Cox regression and Kaplan-Meier analyses supported that NKG2C+ NK cells bearing adaptive markers were specifically associated with a reduced incidence of posttransplant symptomatic CMV infection; no correlation between NKG2C+ NK cells and CMV-specific T cells was observed. These results support that adaptive NKG2C+ NK cells contribute to control CMV infection in KTR.

KEYWORDS

basic (laboratory) research/science, clinical research/practice, immunobiology, infection and infectious agents - viral: cytomegalovirus (CMV), infectious disease, kidney transplantation/nephrology, natural killer (NK) cells/NK receptors

Abbreviations: FcεRIγ, FcεRI γ chain; HCT, hematopoietic cell transplantation; ILT2, Immunoglobulin-like transcript 2 (LIR1 or LILRB1); KTR, kidney transplant recipients; PLZF, Prolymphocytic Leukemia Zinc Finger transcription factor; t-SNE, vi-stochastic neighbor embedding multidimensional analysis.

Michelle Ataya and Dolores Redondo-Pachón shared credit for equal contribution.

Marta Crespo and Miguel López-Botet share credit for senior authorship.

1 | INTRODUCTION

Cytomegalovirus (CMV) causes a prevalent and persistent infection, generally asymptomatic in immunocompetent individuals. The immune response contains the infection but fails to eradicate the pathogen, which enters a latency state, undergoing occasional reactivation.[1-3] Primary CMV infection, reactivation, or reinfection in immunosuppressed kidney transplant recipients (KTR) has been associated with graft loss and reduced patient survival.[4-7] Antiviral prophylaxis is administered to prevent primary infection in CMV-seronegative KTR transplanted from CMV+ donors, as well as in patients undergoing intensive immunosuppression (eg, thymoglobulin induction).[8] The incidence of CMV infection has been reported to decrease in patients treated with mammalian target of rapamycin–targeting drugs.[9,10] On the other hand, posttransplant surveillance of viremia in CMV+ KTR allows early detection of viral replication and administration of preemptive therapy.[11,12]

Ultimately, control of CMV replication in KTR depends on the fitness of the individual immune response to keep the pathogen at bay under immunosuppressive conditions. In CMV+ individuals, baseline frequencies of T cells specific for the IE-1 and pp65 immunodominant antigens have been associated with a reduced risk of posttransplant infection.[13-16] CMV exerts a broad impact in the immune system,[17] including the adaptive differentiation and persistent expansion of a mature NK cell subset, hallmarked by high expression of the CD94/NKG2C activating receptor specific for HLA-E, in the absence of its inhibitory CD94/NKG2A counterpart.[18-23] Additional adaptive NK cell–associated features include expression of the CD57 terminal differentiation marker and of inhibitory receptors for HLA class I molecules, that is, KIRs (killer immunoglobulin-like receptors) and ILT2 (immunoglobulin-like transcript 2, also termed LIR1, LILRB1). This is encompassed by downregulation of activating receptors (ie, NKp30, NKp46), signaling adaptors (eg, FcεRIγ [FcεRI γ chain]), and transcription factors (eg, Prolymphocytic Leukemia Zinc Finger transcription factor [PLZF]), partially resulting from epigenetic regulation.[24,25] In the same line, hypomethylation of the interferon γ (IFNγ) promoter in adaptive NKG2C+ NK cells enhances their production of this cytokine.[26] Of note, NKG2C+ NK cells lacking this characteristic profile are detectable in CMV− as well as in a fraction of CMV+ individuals[27]; thus, these markers, which may be sequentially displayed,[28] contribute to precisely identify the adaptive phenotype. Remarkably, adaptive NK cells proficiently mediate antibody-dependent effector functions activated through FcγR-IIIA (CD16), which can be triggered by IgG specific for CMV antigens on the surface of infected cells.[29-31]

The development of adaptive NKG2C+ NK cells is reminiscent of the specific response of murine memory Ly49H+ NK cells to CMV-infected cells.[32] The contribution of human NKG2C+ NK cells to control CMV infection is supported by some clinical observations. In this regard, CMV infection in a patient with a severe combined immunodeficiency lacking T cells was encompassed by a massive expansion of NKG2C+ NK cells and a reduction in CMV replication, detected prior to antiviral therapy.[33] Moreover, development of adaptive NKG2C+ NK cells in allogeneic hematopoietic cell transplantation (HCT) recipients from sibling but not umbilical cord blood grafts were related to protection against CMV reactivation.[34] Finally, in a pilot study we reported an association of pretransplant levels of total NKG2C+ NK cells with a reduced incidence of CMV viremia in KTR.[35] In the present study we extended the analysis, enlarging the cohort and introducing additional adaptive NK cell markers, to discern their role in protection against CMV infection and their relation to the T cell response.

2 | MATERIALS AND METHODS

2.1 | Patient population and clinical data

A retrospective study was carried out in patients who underwent kidney transplantation at Hospital del Mar, between March 2013 and December 2017. Patients were transplanted according to negative complement-dependent cytotoxicity cross-match with donor lymphocytes, as described.[36] KTR receiving the same immunosuppressive regimen (ie, tacrolimus, mycophenolic acid, and steroids) and anti-CD25 mAb induction were enrolled, excluding patients at low risk of CMV infection (D−R−) and those receiving antiviral prophylaxis (ie, CMV D+R−; thymoglobulin induction). Cases with available pretransplant cryopreserved peripheral blood mononuclear cells (PBMC) were studied; samples were obtained within 24 hours prior to transplantation and stored in liquid nitrogen until analysis (2018-2019). The study was conducted following the Declaration of Helsinki guidelines and approved by the CEIC Parc de Salut Mar Ethical Research Board (2018/7873I). All patients and healthy volunteers signed written informed consent for the use of peripheral blood samples for research purposes.

2.2 | CMV viremia screening

CMV DNA (copies/mL) in blood samples was measured by a standardized diagnostic quantitative polymerase chain reaction assay (qPCR; COBAS AmpliPrep, Cobas TaqMan; Roche Diagnostics, Indianapolis, IN). Patients were systematically monitored for CMV replication every 2 weeks for the first 3 months posttransplant. In addition, CMV DNA was assessed whenever infection was clinically suspected, administering preemptive therapy upon viremia detection. A general limitation of this setting is that an eventual incidence of subclinical CMV reactivation/reinfection after completing qPCR surveillance would be overlooked.

CMV infection was defined based on detection by qPCR of viral replication in blood. According to clinical guidelines, infected cases were stratified by nephrologists as asymptomatic and symptomatic; the latter category included cases with viral syndrome or with histopathological evidence of tissue-invasive disease.

2.3 | Immunophenotypic analysis

PBMC samples obtained by venous puncture in EDTA tubes were separated by Ficoll-Hypaque density gradient centrifugation and

cryopreserved. Phenotypic analysis by flow cytometry was performed in thawed samples as previously described.[37] Cells were pretreated with human aggregated IgG (100 μg/mL) to block FcR and surface stained using the following monoclonal antibodies: anti-CD3-APCCy7 (clone OKT3, BioLegend, San Diego, CA), anti-CD45-Alexa Fluor 700 (clone 2D1, eBioscience-Thermo Fisher, Waltham, MA), anti-CD56-APC-Cy7(clone NCAM, BioLegend, San Diego, CA), anti-CD3-PerCP (clone SK7, BD), anti-TCR pan gamma/delta-PE/Dazzle594 (clone B1, BioLegend, San Diego, CA), anti-TCR Vdelta2-FITC (clone IMMU389, Beckman-Coulter, Indianapolis, IN), anti-NKG2C-PE (clone FAB138P, R&D systems, Bio-Techne, Minneapolis, MN), anti-NKG2A-Pacific Blue (clone Z199; provided by Dr A. Moretta), anti-ILT2-PeCy7 (clone GHI/75, BioLegend, San Diego, CA), anti-CD57-FITC (clone HCCD57, BioLegend, San Diego, CA), anti-CD57-APC (clone HCD57, BioLegend, San Diego, CA), anti-CD56-APC (clone CMSSB, eBioscience-Thermo Fisher, Waltham, MA), anti-CD4-APC (clone RTA-T4, BD Biosciences, San Jose, CA), anti-CD8-BV510 (clone RPA-T8, BD, San Jose, CA). For intracellular staining, cells were fixed and permeabilized (FIX & PERM Cell Fixation & Cell Permeabilization Kit, Invitrogen, Thermo Fisher, Waltham, MA), according to manufacturer instructions, followed by intracellular staining with anti-FcεRIγ–FITC (polyclonal, Merck Millipore, Burlington, MA) and anti-PLZF-PECF594 (clone R17-809, BD). Data were acquired on an LSR Fortessa flow cytometer (BD Biosciences) and analyzed using FlowJo software (10.0.7, TreeStar, Ashland, OR). Absolute cell numbers were calculated based on whole blood cell counts obtained in the same puncture.

Multidimensional flow cytometry analysis using vi-stochastic neighbor embedding (SNE) was implemented in manually gated NK cells (CD45+CD3− CD56+), as previously described.[24,38] Raw cytometry data were compensated in FlowJo and imported using flowCore and openCyto. Parameters corresponding to FcεRIγ, PLZF, and ILT2 were normalized using flowStats to reduce technical variation between samples in fluorescence intensity, and for each parameter the highest and lowest percentile were reduced to their less extreme border. Five hundred cells per sample were randomly selected from normalized data and Barnes-Hut vi-stochastic neighbor embedding multidimensional analysis (t-SNE) was conducted in R (3.5.1, http://www.r-project.org) using the Rtsne package[39] (Krijthe, JH 2015. Rtsne: T-Distributed Stochastic Neighbor Embedding using a Barnes-Hut Implementation; https://github.com/jkrijthe/Rtsne). Graphics were generated using ggplot2 and RcolorBrewer.

2.4 | Detection of CMV-specific T cells

PBMCs were incubated overnight at 37°C in polypropylene tubes with complete medium (RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, 100 U/mL penicillin, 100 μg/mL streptomycin, and 1 mmol/L sodium pyruvate) and placed at 1×10^6 cells/well in 96-well plates. T cell activation was assessed by flow cytometry detection of cytokine production, as previously described.[40,41] Briefly, samples were incubated with either RPMI alone, anti-CD3 mAb as a positive control, or 1 μg/mL of peptide libraries from CMV pp65 or IE-1 antigens (Peptivator, Miltenyi Biotec, Bergisch Gladbach, Germany). Plates were incubated at 37°C in a 5% CO_2 atmosphere for 6 hours; during the last 5 hours brefeldin A (Sigma-Aldrich, Saint Louis, MO) and anti-CD49d (BD Biosciences, San Jose, CA) were added to a final concentration of 10 μg/mL and 1 μg/mL, respectively. Subsequently, cells were stained as described in the previous section with the following antibodies: anti-CD3-PerCP (clone SK7, BD), anti-CD56-APC (clone CMSSB, eBioscience-Thermo Fisher, Waltham, MA), anti-CD4-PeCy7 (clone OKT4, BioLegend, San Diego, CA), anti-CD8-BV510 (clone RPA-T8, BD), anti-IFNγ-PE (clone B27, BD Pharmingen), anti-tumor necrosis factor α (TNFα)-Pacific Blue (infliximab); a minimum of 10^5 T cells were analyzed by flow cytometry. Cell viability was assessed with the LIVE/DEAD fixable green stain (Invitrogen, Thermo Fisher, Waltham, MA) according to manufacturer instructions.

2.5 | Statistical analysis

Statistical analysis was performed in R version 3.5.1. Categorical variables are expressed as percentages, and continuous variables as mean and standard deviation or median and interquartile range. Relationship between characteristics of the population and time to CMV viremia was analyzed univariately by Cox proportional hazard regression (Cox). Comparison of immune cell populations between KTR stratified according to the incidence of posttransplant viremia was carried out by Mann-Whitney *U* test. Multivariable analysis to determine the relation between NK cell populations and CMV infection was performed by Cox proportional hazard modeling, including significant risk factors identified by univariate analysis (recipient age, living donor, and %T CD4). The proportional hazards assumption of included variables was tested. Correlations between CMV-specific T cells and NKG2C+ NK cells were assessed by Spearman analysis. The cutoff for the percentage of NK NKG2C+ NKG2A− cells that best discriminate risk of CMV infection in KTR after receiver operating characteristic (ROC) curve analysis was defined by bootstrapping (2000 iterations) the Youden Index using the cutpointr package (Thiele C. 2019. Cutpointr: Determine and Evaluate Optimal Cutpoints in Binary Classification Tasks. R package version 0.7.6. URL https://CRAN.R-project.org/package=cutpointr). *P* values were 2-sided and *P* values lower than 0.05 were considered significant.

3 | RESULTS

3.1 | Patients' demographic and clinical characteristics

Studies were conducted in a selected cohort of KTR (n = 145) (Table 1), undergoing a similar immunosuppression regimen. Cases at low risk of infection (ie, CMV D−/R−) and those at high risk (ie, CMV D+/R−; thymoglobulin induction) receiving antiviral prophylaxis were excluded; all selected KTR were CMV+ (D+/R+ 85.5%; D−/R+ 14.5%). During

TABLE 1 Characteristics of the study population

	All cases (n = 145)	No CMV Viremia (n = 97)	CMV[b] Viremia (n = 48)	P[a,c]
Recipient age (y), mean (SD)	56 ± 12.8	53 ± 12.4	61 ± 12.19	**<.001**
Recipient sex (female) n (%)	46 (31.8)	28 (28.9)	18 (37.5)	.597
Pre-KT CMV serostatus, n (%)				
D+/R+	124 (85.5)	79 (81.4)	45 (93.7)	.084
D−/R+	21 (14.5)	18 (18.6)	3 (6.3)	
Donor age (y), mean (SD)	57.6 ± 15.3	54.3 ± 15.4	63.9 ± 12.8	**<.001**
Donor sex (female), n (%)	71 (49)	49 (50.5)	22 (45.8)	.353
Living donor, n (%)	32 (22.1)	28 (28.9)	4 (8.3)	**.014**
Retransplantation, n (%)	11 (7.6)	9 (9.3)	2 (4.2)	.301
Peak PRA > 5%, n (%)	21 (14.5)	16 (16.5)	5 (10.4)	.897
Pretransplant PRA > 5%, n (%)	9 (6.2)	5 (5.2)	4 (8.3)	.372
Pretransplant DSA, n (%)	4 (2.6)	3 (3.1)	1 (2.1)	.682
Delayed graft function, n (%)	30 (20.7)	18 (18.5)	12 (25)	.604
Biopsy-proven acute rejection, n (%)	15 (10.3)	11 (11.3)	4 (8.3)	.193
Cold ischemia time (h), mean (SD)	12.01 (7)	11.5 (7.1)	13.2 (6.7)	.24
Symptomatic CMV, n (%)	—	—	26 (54.2)	—
CMV PCR at diagnosis (copies/mL), median (IQR)	—	—	465 (213.5-981)	—
Peak CMV PCR (copies/mL), median (IQR)	—	—	824 (272.5-2428)	—
CMV infection time after KT (d), median (IQR)	—	—	54.5 (38.75-73.75)	—

CMV, cytomegalovirus; DSA, donor-specific Abs; IQR, interquartile range; KT, kidney transplantation; PCR, polymerase chain reaction; PRA, panel reactive antibodies (by complement-dependent cytotoxicity); SD, standard deviation.

[a]*P* values correspond to univariate Cox proportional hazards regression analysis.

[b]CMV viremia was monitored biweekly for 3 months and successively assessed in case of clinical suspicion (n = 6).

[c]Bold indicates *P* values < .0 5

periodic monitoring, CMV infection was detected in 42 KTR between days 29 and 97; in addition, clinically suspected infection was confirmed in 5 cases between days 110 and 210 and in 1 patient at day 420. CMV infections were categorized as asymptomatic (n = 22) or symptomatic (n = 26), with the latter including 5 cases with tissue-invasive disease. In line with previous reports, the incidence of CMV viremia (33%) was significantly related to increasing age[35] and appeared to be decreased in the group transplanted from living donors (22.1%).[42]

3.2 | Adaptive NK cells are associated with a reduced incidence of CMV viremia

Pretransplant cryopreserved PBMC samples were analyzed by flow cytometry with a gating strategy suitable to assess total NK cells, NKG2C+ and NKG2A+ subsets, as well as their expression of adaptive NK cell–associated markers including CD57, ILT2, and FcεRIγ loss (Figures 1 and 2, Supplementary Figure S1). In parallel, major T cell subsets (ie, CD4+, CD8+, TcRγδ Vδ2+, and Vδ2-) and their expression of NKG2A, NKG2C, and CD57 were also assessed (Figure 3).

These parameters were compared between KTR categorized according to the incidence of posttransplant viremia (Table 1). The proportions and absolute numbers of total NK cells were similar in both groups, and no differences in their expression of ILT2, CD57, or FcεRIγ were observed (Table 2 and Figure 1). In contrast, proportions of NKG2C+ and NKG2C+ NKG2A− NK cells were significantly increased in KTR without viremia who, reciprocally, displayed reduced proportions of the NKG2A+ NKG2C− subset (Table 2 and Figure 2). Proportions of NKG2C+ NK cells with adaptive-associated markers (ie, CD57+, ILT2+, or FcεRIγ−), as well as of the minor NKG2C+ CD57− and NKG2C+ ILT2− subsets, were also inversely related to the incidence of viremia; the association remained significant for the absolute numbers of NKG2C+ NKG2A− and NKG2C+ CD57− NK cells. Of note, the CMV infection rate was unrelated to the proportions of NKG2C− FcεRIγ− and ILT2+ NK cells, but was directly associated with NKG2C− CD57+ NK cells.

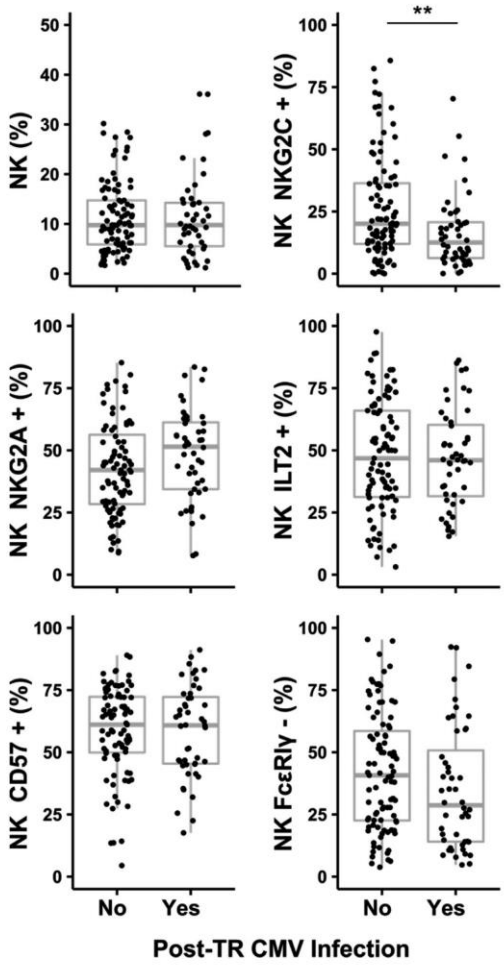

FIGURE 1 Association of NKG2C+ NK cells with reduced incidence of CMV viremia in KTR. Pretransplant frequencies of total NK cells and NKG2C+, NKG2A+, ILT2+, CD57+, and FcεRIγ− NK cell subsets were assessed by flow cytometry in PBMC samples from KTR, stratified according to the incidence of posttransplant CMV viremia. $^{**}P < .01$; Mann-Whitney *U* test. CMV, cytomegalovirus; FcεRIγ, FcεRI γ chain; KTR, kidney transplant recipients; PBMC, peripheral blood mononuclear cells

t-SNE analysis of NK cell marker distribution was performed in a subset of samples from the KTR cohort (n = 60), including PLZF downregulation as an additional adaptive NK cell feature (Figure 4). Comparison between groups with (n = 17) and without (n = 43) CMV viremia further evidenced substantial differences in their NK cell phenotypic profile. This was particularly evident for the distribution of adaptive NK cell markers, as illustrated by the prominent NKG2C+ NKG2A− ILT2+ CD57+ FcεRIγ− PLZF1− population detected in the absence of infection. Altogether, the data suggested an implication of adaptive NKG2C+ NK cells in the control of posttransplant CMV infection, despite its incidence in some patients with increased adaptive NKG2C+ NK cells.

3.3 | NKG2C+ NK cells are unrelated to CMV-specific T cells

The rate of viremia was unrelated to total T cells and NKG2C+, NKG2A+, ILT2+, CD57+, TcRγδ (Vδ2+, Vδ2−) T cell subsets (Table 2 and Figure 3), yet differences between proportions of CD8+ and CD4+ cells were noticed when both groups were compared. Baseline frequencies of T cells specific for CMV immunodominant antigens have been shown to predict a reduced risk of posttransplant infection. Thus, the possibility that our observations might indirectly reflect a relation of adaptive NKG2C+ NK cells with CMV-specific T lymphocytes was considered. Addressing this issue in the same KTR cohort was not feasible given the limited availability of cryopreserved pretransplant samples. To circumvent this limitation, a parallel study was conducted with PBMC from CMV+ chronic renal disease patients on the KT waiting list (n = 29) and a group of CMV+ healthy controls (n = 28). The response to peptide libraries corresponding to IE-1 and pp65 CMV antigens was assessed by flow cytometry, measuring the frequencies of CD4+ and CD8+ T cells producing IFNγ and TNFα. A wide interindividual variability in the frequencies of CMV antigen-specific T cells detected in both KTR and controls was noticed, without correlation with the proportions of adaptive NKG2C+ NK cells (Figure 5 and Supplementary Figure S2, Table 3). These data render it unlikely that the association of NKG2C+ NK cells with a reduced incidence of viremia may be attributable to the protective effect of specific T cells.

3.4 | NKG2C+ NK cells independently predict risk of CMV infection

Multivariate Cox regression analysis was carried out considering clinical and analytical variables individually associated with CMV viremia (Tables 1 and 2) (eg, recipient age, deceased donor, and % CD4+ T cells). As shown in Table 4, we observed an independent inverse relation between posttransplant infection and the proportions of total NKG2C+ (HR 0.978, *P* = .022) and NKG2C+ NKG2A− NK cells (HR 0.974, *P* = .018), as well as of NKG2C+ CD57+ (HR 0.977, *P* = .028), and NKG2C+ FcεRIγ− (HR 0.977, *P* = .04) NK cells; this was mirrored by a direct association of viremia with total NKG2A+ (HR 1.022, *P* = .007) NK cell subsets. In contrast, the relation of NKG2C+ CD57− NK cells with a reduced infection rate observed in univariate analysis did not reach statistical significance (Supplementary Table S1), pointing to the influence of other variables, particularly recipient age, that may also partially account for the direct relation of NKG2C− CD57+ NK cells with viremia.

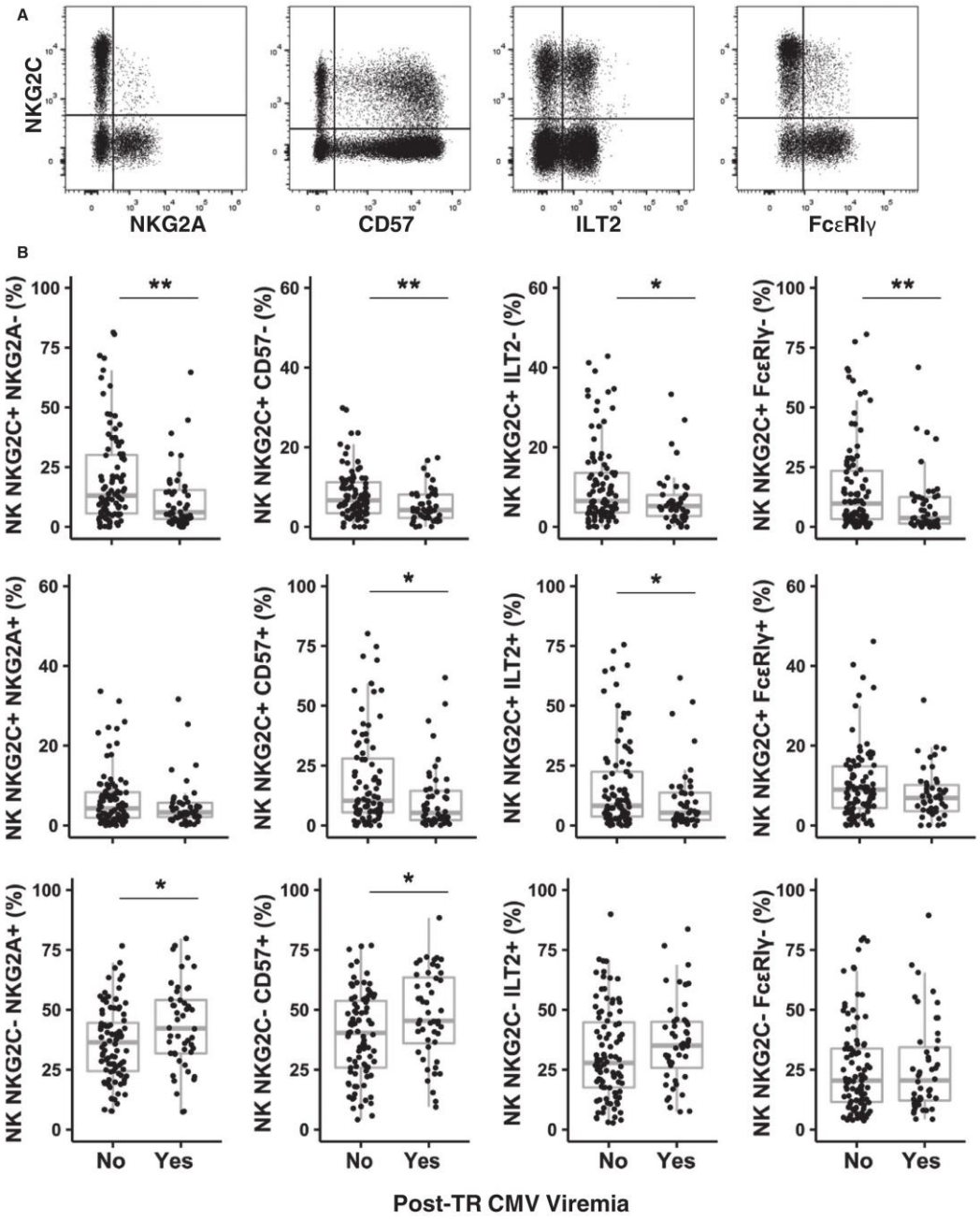

FIGURE 2 Pretransplant NKG2C+ NK cells with an adaptive phenotypic profile specifically associate with a lower rate of CMV viremia in KTR. NKG2C+ NK cell subsets bearing different adaptive NK cell-associated markers were analyzed by flow cytometry. A, Representative gating of NKG2C+ cell subpopulations according to CD57, ILT2, and FcεRIγ expression. B, Frequencies of adaptive NKG2C+ NK cells in KTR, categorized according to the incidence of posttransplant CMV viremia. $*P < .05$, $**P < .01$; Mann-Whitney *U* test. CMV, cytomegalovirus; FcεRIγ, FcεRI γ chain; KTR, kidney transplant recipients; ILT2, immunoglobulin-like transcript 2

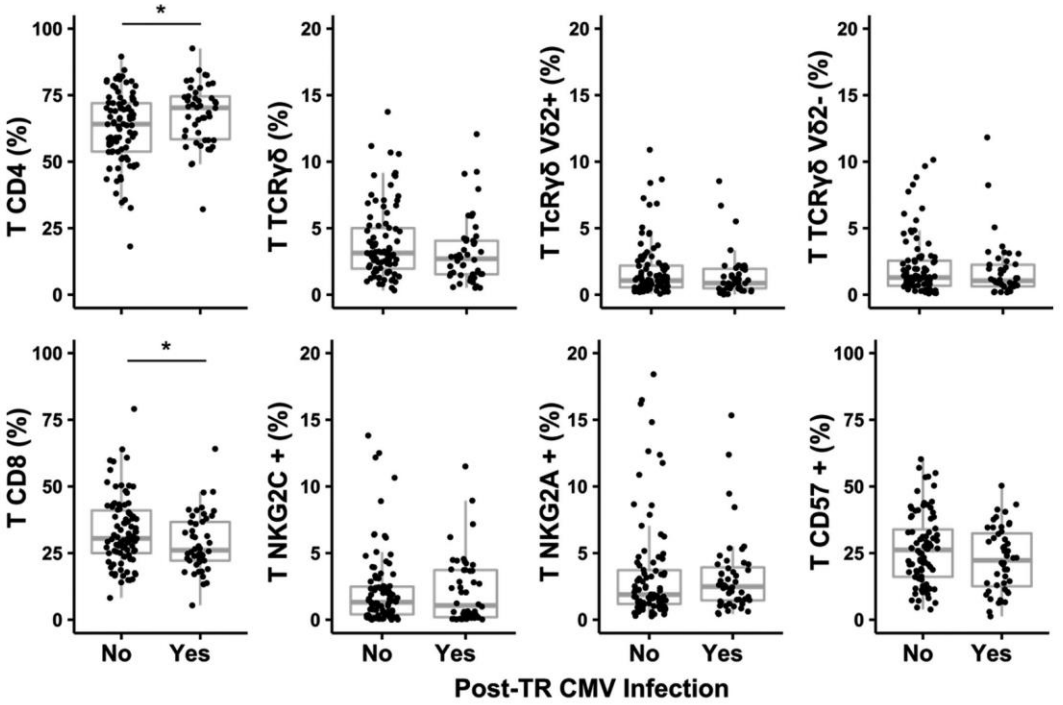

FIGURE 3 Pretransplant distribution of T cell subsets according to the incidence of CMV viremia in KTR. Frequencies of CD4+, CD8+, NKG2C+, CD57+, TCRγδ (Vδ2+ and Vδ2−) T cell subsets were assessed in PBMC samples from KTR with or without posttransplant CMV viremia. ∗P < .05, Mann-Whitney U test. CMV, cytomegalovirus; KTR, kidney transplant recipients; PBMC, peripheral blood mononuclear cells

To explore whether results might be influenced by clinical expression of CMV infection, multivariate Cox regression analysis was carried out in infected cases stratified as described in Materials and Methods. Remarkably, reduced proportions of total NKG2C+ NKG2A− adaptive NK cells appeared significantly associated with symptomatic (HR 0.951, P = .016) but not with asymptomatic (HR 0.985, P = .235) infection; similar differences were confirmed for the other adaptive markers studied (Supplementary Table S2). These results were in accordance with univariate analysis (Figure 6A); moreover, a significant inverse correlation between NKG2C+ NKG2A− NK cells and peak viral loads was detected only in symptomatic cases (Figure 6B), which tended to display greater CMV loads (Figure 6B,C).

ROC curve analysis was used to define cutoff values for % NKG2C+ NKG2A− NK cells and Kaplan-Meier analyses were performed (Figure 7). ROC analysis for predicting posttransplant CMV disease showed an AUC of 0.71 compared to 0.65 for CMV viremia. Cutoff values yielded a sensitivity/specificity of 0.63/0.58 and 0.73/0.60, respectively, for global viremia and symptomatic infection, whose incidences were significantly lower in the groups with greater proportions of NKG2C+ NKG2A− NK cells in log-rank test comparisons. These results further illustrate the protective antiviral effect of NKG2C+ adaptive NK cells.

4 | DISCUSSION

CMV reactivation and/or reinfection in immunosuppressed CMV+ KTR constitutes a frequent event addressed by monitoring viral replication and preemptive therapy administration. Pretransplant T cells specific for pp65 and IE-1 CMV antigens have been shown to reduce the risk of viremia.[13-16] The present report consistently supports that adaptive NKG2C+ NK cells in CMV+ KTR also contribute to reduce the incidence of posttransplant viremia which, in multivariate Cox regression analysis, was inversely related with the proportions of NKG2C+ NKG2A−, NKG2C+ CD57+, and NKG2C+ FcεRIγ− NK cell subsets. These features are also displayed by NKG2C− NK cell subsets,[37,43,44] and epigenetic downregulation of FcεRIγ has been considered a marker for a broader spectrum of adaptive NK cells.[25,30] Yet, phenotypic similarities between different NK cell subsets do not imply that they share functional competences; in fact, differences in KIR expression and antibody-mediated activation have been reported between NKG2C+ FcεRIγ− and NKG2C− FcεRIγ− NK cells.[37] Our observations strengthen this view showing that the rate of viremia in KTR was unrelated to NKG2C− FcεRIγ− NK cells. It is uncertain whether this NK cell subset may fully compensate for the lack

TABLE 2 Pretransplant distribution of NK and T cell subsets stratified according to posttransplant CMV detection by qPCR

	CMV viremia	No CMV viremia		CMV viremia	No CMV viremia	
	%	Median (IQR)	$P^{b,c}$	Cells/μL[a]	Median (IQR)	$P^{b,c}$
NK	9.8 (6-14)	9.7 (6-15)	.961	154.9 (92-231)	141.6 (89-218)	.804
NKG2C+	12.5 (6-21)	20.1 (12-36)	**.003**	17.1 (6-31)	24.6 (10-70)	.068
NKG2A+	51.4 (34-61)	42.1 (28-56)	.058	67 (35-107)	48.9 (32-83)	.151
CD57+	60.8 (45-72)	61.1 (50-72)	.881	79.9 (44-159)	82.5 (40-142)	.944
ILT2+	46 (32-60)	46.8 (31-66)	.829	60.7 (29-119)	58.5 (24-136)	.911
FcεRIγ-	28.7 (14-51)	40.8 (23-59)	.057	36.8 (16-86)	56.7 (20-108)	.177
NKG2C+ NKG2A+	3.3 (2-6)	4.3 (2-8)	.241	4.7 (3-8)	4.7 (3-10)	.566
NKG2C+ NKG2A−	6.1 (3-15)	13.1 (6-30)	**.004**	8.8 (3-26)	17.1 (6-45)	**.042**
NKG2C− NKG2A+	42.3 (32-54)	36.4 (24-45)	**.018**	61.6 (30-102)	43 (26-74)	.092
NKG2C+ CD57+	5.3 (2-14)	10.3 (5-28)	**.010**	8.6 (2-24)	12.3 (5-46)	.068
NKG2C+ CD57−	4.3 (2-8)	6.7 (3-11)	**.008**	6.7 (3-10)	9.4 (5-15)	**.013**
NKG2C− CD57+	45.4 (36-64)	40.4 (26-54)	**.016**	70.3 (20-131)	51.5 (24-87)	.236
NKG2C + ILT2+	5.3 (2-14)	8.2 (4-22)	**.049**	7.8 (2-23)	11.1 (3-34)	.248
NKG2C+ ILT2−	5.3 (3-8)	6.5 (4-14)	**.036**	7.5 (4-13)	8.6 (3-21)	.164
NKG2C− ILT2+	35.1 (26-45)	27.8 (18-45)	.087	54.5 (19-94)	30.4 (16-69)	.189
NKG2C+ FcεRIγ+	6.9 (4-10)	9 (4-15)	.123	8.7 (4-15)	11 (5-21)	.390
NKG2C+ FcεRIγ−	3.7 (1-13)	9.8 (3-23)	**.009**	6 (1-21)	10.9 (3-41)	.072
NKG2C− FcεRIγ−	20.5 (12-34)	20.5 (12-34)	.957	21 (11-63)	27.7 (11-52)	.908
T	78.3 (65-82)	78.6 (70-84)	.247	1143 (855-1416)	1095.7 (888-1461)	.888
CD4+	70.3 (58-75)	64.1 (54-72)	**.020**	711.2 (604-907)	715.7 (536-944)	.748
CD8+	26.1 (22-37)	30.5 (25-41)	**.047**	299.3 (238-476)	330.3 (244-555)	.193
T NKG2C+	1.1 (0-4)	1.3 (0-2)	.765	11.9 (2-37)	13.4 (4-25)	.906
T NKG2A+	2.5 (1-4)	1.9 (1-4)	.430	28.7 (17-45)	21.6 (12-52)	.345
T CD57+	22.3 (13-32)	26.5 (16-36)	.138	255.8 (110-335)	281.3 (177-409)	.205
T ILT2+	24.2 (12-34)	23 (13-33)	.961	241.2 (133-387)	253 (114-431)	.886
T TcRγδ	2.7 (2-4)	3.1 (2-5)	.117	29.4 (18-48)	34.9 (20-57)	.128
T TcRγδ Vδ2	0.9 (0-2)	1.1 (1-2)	.275	10.5 (4-20)	12.4 (6-25)	.219
T TcRγδ Vδ2−	1.1 (1-2)	1.3 (1-3)	.449	12.6 (7-24)	14.7 (6-25)	.711

CMV, cytomegalovirus; FcεRIγ, FcεRI γ chain; ILT2, immunoglobulin-like transcript 2; IQR, interquartile range; qPCR, quantitative polymerase chain reaction.

[a]Calculated based on total lymphocyte counts.

[b]*P*-values by 2-sided Mann-Whitney *U* test.

[c]Bold indicates *P* values < .0 5.

of conventional adaptive NKG2C+ NK cells in CMV+ individuals with a homozygous *NKG2C* deletion.[43,44] *NKG2C* copy number has been reported to influence the expansion of adaptive NKG2C+ NK cells, which tends to be greater in $NKG2C^{+/+}$ than in $NKG2C^{+/del}$ individuals.[27,45] No significant differences were detected between the frequencies of *NKG2C* genotypes reported in healthy donors and those detected in the KTR cohort, regardless of symptomatic/asymptomatic CMV infection (data not shown).

CD57 expression in NK cells has been related to terminal differentiation and reduced proliferative potential, illustrated by their shorter telomere length.[46-48] Increased circulating CD57+ NK cells are associated with immune senescence, a process that may develop independently of chronological age as shown for patients with end-stage renal disease (ESRD).[49] In multivariate Cox regression analysis, the incidence of viremia in KTR was confirmed to be inversely related to NKG2C+ CD57+ NK cells but, in contrast, the apparent protective effect of the NKG2C+ CD57− subset vanished, likely reflecting its dependence on age. The direct relation of NKG2C− CD57+ NK cells with viremia did not reach statistical significance (Supplementary Table S1), although the trend ($P < .06$) suggests the concomitant influence of age-independent factors.

The association of adaptive NKG2C+ NK cells with a reduced risk of infection suggests that they may exert antiviral activity, in line with other clinical observations in HCT[34] and a severe T cell immunodeficiency.[33] Recently, the frequency of NKG2C+ NK cells

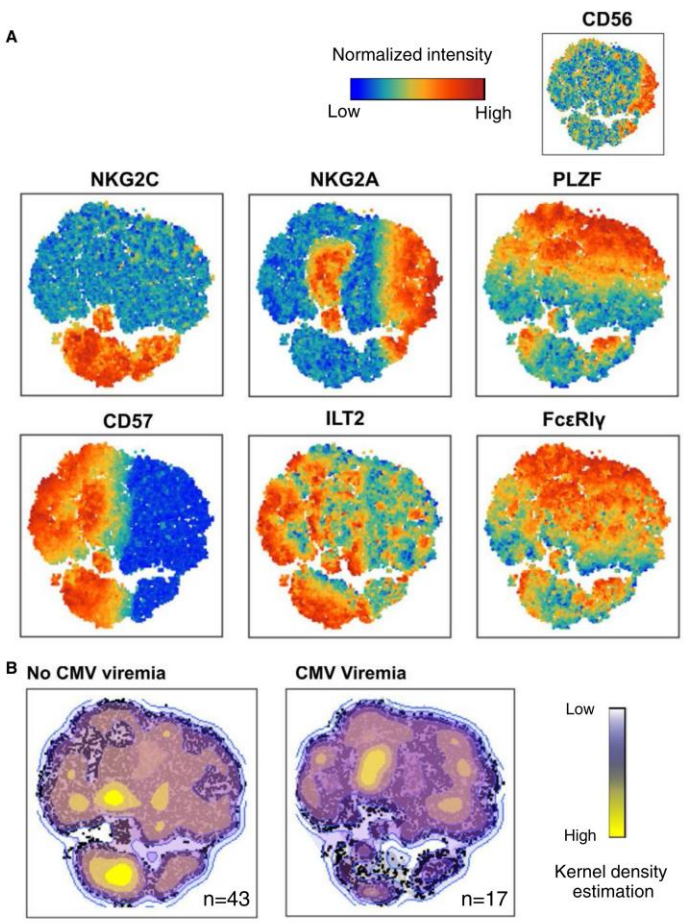

FIGURE 4 Multidimensional analysis of the distribution of adaptive NK cell markers in KTR categorized according to CMV viremia. Multidimensional Barnes-Hut t-SNE analysis of 7 parameters was performed on CD45+ CD3− CD56+ manually gated NK cells from pretransplant PBMC samples (n = 60). A, Single-parameter plots showing protein expression levels on the t-SNE field. B, Kernel density plots showing events in the t-SNE field compiled separately for PBMC samples from patients stratified according to detection of posttransplant CMV viremia. The integrated data reveal increased NKG2C+ CD57+ FcεRIγ− PLZF− NK cells in nonviremic cases. CMV, cytomegalovirus; FcεRIγ, FcεRI γ chain; ILT2, immunoglobulin-like transcript 2; KTR, kidney transplant recipients; PBMC, peripheral blood mononuclear cells; PLZF, Prolymphocytic Leukemia Zinc Finger transcription factor; t-SNE, vi-stochastic neighbor embedding multidimensional analysis

in bronchoalveolar lavage from lung allograft recipients was reported to be inversely correlated with CMV blood titers.[50] CD94/NKG2C is an activating receptor specific for HLA-E, whose engagement triggers in vitro NK cell effector functions and proliferation in response to IL-2 and IL-15. It was hypothesized that CD94/NKG2C might activate NK cells upon specific recognition in CMV-infected cells of either a viral molecule or HLA-E-bound peptide(s).[19] This was indirectly supported by in vitro proliferation of NKG2C+ NK cells in response to CMV-infected cells.[51,52] However, attempts to demonstrate NKG2C-mediated activation of NK cell effector functions against CMV-infected cells were unsuccessful, and similar negative results have been obtained with a CD94/NKG2C+ reporter cell line.[53] Recently, HLA-E presentation of leader peptide sequences from the UL40 CMV protein was reported to activate NKG2C+ NK cells under certain experimental conditions.[54] Moreover, adaptive NKG2C+ NK cells proficiently mediate specific antibody-dependent effector functions, particularly TNF-α and IFN-γ production, triggered through CD16 in a CD2-dependent manner against different targets, including CMV-infected cells.[29-31,43] This mechanism might contribute to the antiviral role of adaptive NK cells in KTR, yet is likely conditioned by the level and properties (eg, isotype, affinity) of IgG specific for CMV antigens displayed on the surface of infected cells.

We addressed whether the association of adaptive NKG2C+ NK cells with the risk of CMV infection might indirectly reflect the action of other immune cells.[55] Assessing the relation of adaptive NKG2C+ and CMV-specific T cells in the same KTR cohort was not feasible due to the insufficient availability of cryopreserved samples. This limitation was partially circumvented by comparing both parameters in PBMC from ESRD patients on a waiting list for transplantation and from healthy donors. Our results revealed a lack of correlation between adaptive NKG2C+ NK and CMV-specific T cells, ruling out that the latter might be responsible for the reduced incidence of viremia associated with adaptive NK cells. Furthermore, neither TcRγδ Vδ2− T cells, which have been associated with the control of active CMV infection,[56] nor NKG2C+ T cells were related

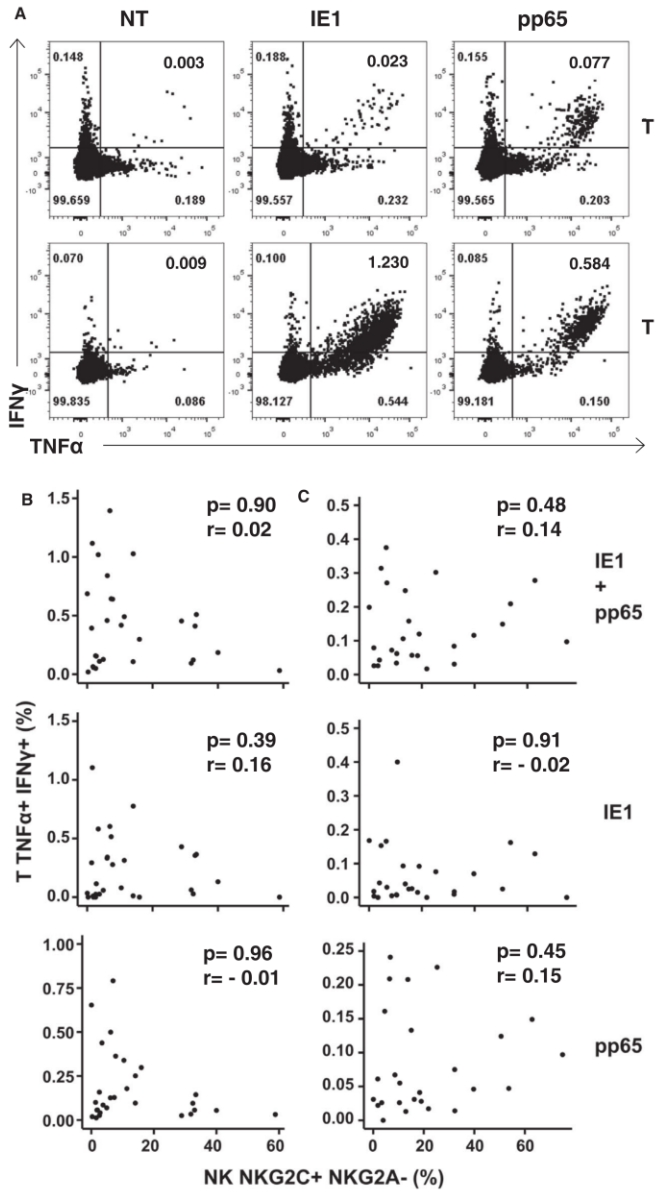

FIGURE 5 Frequencies of adaptive NKG2C+ NKG2A− NK cells and of CMV-specific T lymphocytes are unrelated. Specific CD4+ and CD8+ T cell responses following PBMC stimulation with peptide pools from immunodominant CMV antigens (ie, IE-1 and pp65) were assessed by flow cytometry analysis of intracellular IFNγ and TNFα production. A, Data corresponding to a representative individual are displayed. B-C, The relation between the frequencies of NKG2C+ NK cells and pp65/ IE1-specific T cells was analyzed in samples from (B) patients (n = 29) and (C) healthy donors (n = 28); *r* and *P* values correspond to Spearman analysis. CMV, cytomegalovirus; IFNγ, interferon γ; NT; not treated; PBMC, peripheral blood mononuclear cells; TNFα, tumor necrosis factor α

to posttransplant CMV replication. On the other hand, telomeric activating KIR genes present in KIR B haplotypes have been associated with a reduced incidence of CMV infection in KTR.[57] It is of note that adaptive NKG2C+ NK cells do express inhibitory KIR,[20] and their expansions were reported in the presence of both KIR A/B haplotypes,[18] including blood donors with no functional activating KIR (Vilches and López-Botet, unpublished). These observations render it unlikely that the observed protective effect against CMV might be subsidiary to the action of NK cells displaying activating KIR in patients with KIR-B haplotypes.

The present data support a protective role played by NKG2C+ NK cells in the control of posttransplant CMV infection; yet, viremia was detected in some patients with high levels of NKG2C+ NK cells. Moreover, the sensitivity calculated by ROC analysis (63%) was below that reported for IE-1-specific T cells analyzed by Elispot in KTR not receiving prophylaxis (80%),[14] whereas specificity was similar in both

TABLE 3 Correlation analysis of pretransplant proportions of NKG2C+ NKG2A− NK cells with CMV-specific T cells[a]

	Peptide specificity		
	pp65	IE1	pp65+ IE1
T	−0.01 (0.96)[b]	0.16 (0.93)	0.02 (0.90)
T CD4+	−0.03 (0.88)	−0.13 (0.51)	−0.004 (0.98)
T CD8+	−0.05 (0.80)	0.12 (0.54)	0.02 (0.90)

CMV, cytomegalovirus.

[a]Correlation analysis between proportions of NKG2C+ NKG2A− NK cells and peptide-specific total T cells, CD4+ and CD8+ T cell subsets from patients with end-stage renal disease (n = 29).

[b]Rho (*P* value) corresponding to Spearman correlation analysis.

TABLE 4 Multivariate Cox proportional hazard modeling of posttransplant CMV infection risk

	HR	95% CI	*P*[b]
[a]% NK NKG2C+	0.978	0.96-0.997	**.022**
% NK NKG2C+ NKG2A−	0.974	0.954-0.995	**.018**
% NK NKG2C+ FcεRIγ−	0.977	0.955-0.999	**.04**
% NK NKG2C+ CD57+	0.977	0.957-0.997	**.028**
% NK NKG2A+	1.022	1.006-1.038	**.007**

CMV, cytomegalovirus; CI, confidence interval; FcεRIγ, FcεRI γ chain; HR, hazard ratio.

[a]Multivariate Cox results for each variable in models adjusted for receptor age, donor type, and percentage CD4 T cells. Full results of the analysis are displayed in Supplementary Table S1.

[b]Bold indicates *P* values < .0 5

studies. Remarkably, the effect of adaptive NK cells was perceived for symptomatic but not asymptomatic infection, suggesting that they may contribute to controlling clinical progression rather than impairing viral replication. Given the small group sample sizes, further studies are required to confirm this clinically relevant observation. Several interpretations for these results may be proposed. First, adaptive NK cells likely cooperate with specific T cells, but may on their own be insufficient to fully control viral replication. Moreover, should the antiviral function of adaptive NK cells involve antibody-dependent activation, as discussed above, qualitative/quantitative differences in the levels of IgG specific for CMV antigens expressed on the surface of infected cells might be relevant; yet, this information is not provided by conventional serological tests. Finally, both pretransplant T cells and adaptive NKG2C+ NK cells may be less efficient in controlling CMV reinfection as compared to CMV reactivation events,[58] a variable not controlled in the majority of CMV D+/R+ KTR.

Adaptive NKG2C NK cells are absent in CMV− individuals and thus cannot prevent primary infection in CMV D+ R− cases. However, whether they play an antiviral function following their expansion in response to posttransplant CMV infection[21,35] and in CMV+ KTR treated with lymphocyte-depleting antibodies deserves attention.

In summary, our results support that assessment of pretransplant adaptive NK cells, together with detection of CMV-specific T lymphocytes, may contribute to more precisely predict the risk of posttransplant CMV infection in KTR. Prospective multicenter studies combining standardized analytical techniques are warranted to validate this assumption.

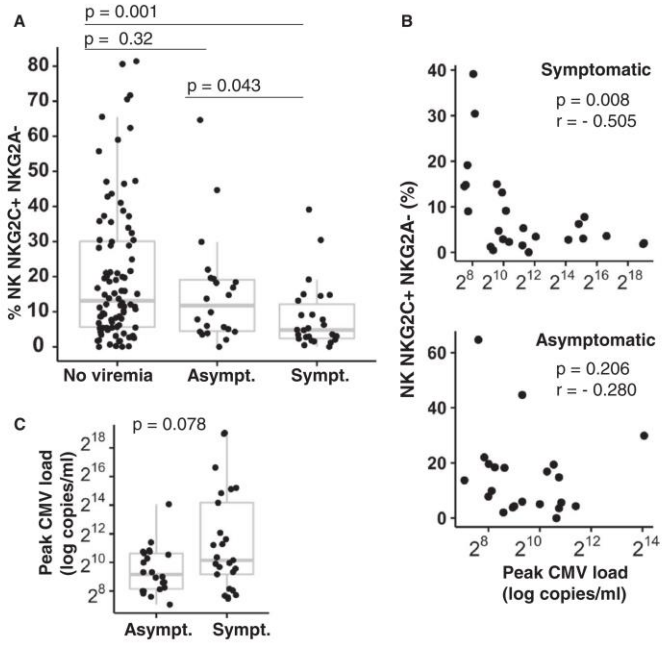

FIGURE 6 Adaptive NK cells associate with the incidence of symptomatic CMV infection in KTR correlating inversely with peak viral load. A, Comparison of the proportions of NKG2C+ NKG2A− NK cells in noninfected and CMV-infected KTR, categorized as asymptomatic or symptomatic (Mann-Whitney *U* test). B, Analysis of correlation between NKG2C+ NKG2A− NK cells and peak viral loads (copies/mL) detected in asymptomatic or symptomatic CMV-infected cases (Spearman). C, Comparison of peak viral loads detected in CMV-infected symptomatic and asymptomatic cases (Mann-Whitney *U* test) CMV, cytomegalovirus; KTR, kidney transplant recipients
[Correction added on 29 November 2019, after first online publication: Figure 6 has been updated]

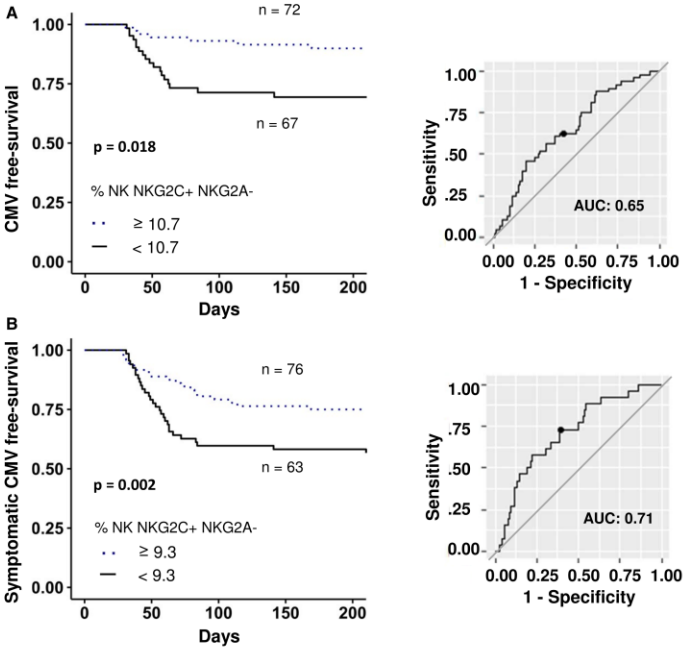

FIGURE 7 CMV viremia- and symptomatic infection-free survival in KTR stratified according to the proportions of pretransplant NKG2C+ NKG2A− NK cells. ROC estimation of sensitivity/specificity and Kaplan-Meier curves with log-rank test comparisons were calculated for predicting the influence of adaptive NK cells on the risk of (A) CMV viremia and (B) CMV symptomatic infection. Proposed thresholds are indicated and cutoff values were calculated as indicated in Materials and Methods. AUC, area under the curve; CMV, cytomegalovirus; KTR, kidney transplant recipients; ROC, receiver operating characteristic. The case diagnosed at day 420 is not included in the figure

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DISCLOSURE

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

DATA AVAILABILITY STATEMENT

Data supporting the findings of this study are available on reasonable request from the corresponding authors. Data on KTR are not publicly available due to privacy or ethical restrictions, and are safely kept at our institution.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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PART III

Long-term evolution of the adaptive NKG2C+ NK cell response to cytomegalovirus infection in kidney transplantation: an insight on the individual host-pathogen interaction

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Long-term evolution of the adaptive NKG2C+ NK cell response to cytomegalovirus infection in kidney transplantation: an insight on the individual host-pathogen interaction[1]

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FOOTNOTES

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(2) shared credit for equal contribution.

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(4) Abbreviations: FcεRIγ, FcεRI gamma chain; ILT2, Immunoglobulin-like transcript 2 (LIR1 or LILRB1); IQR, interquartile range; KTR, kidney transplant recipients; LLOQ, lower limit of quantification; PLZF, Prolymphocytic Leukemia Zinc Finger transcription factor; QNAT, quantitative nucleic acid amplification testing; t-SNE, vi- t distributed stochastic neighbor embedding multidimensional analysis.

ABSTRACT

Cytomegalovirus (CMV) infection is a frequent event in kidney transplant recipients (KTR). Pretransplant antigen-specific T cells and adaptive NKG2C+ NK cells are associated with a reduced incidence of infection in CMV+ KTR. Expansions of NKG2C+ NK cells have been reported in posttransplant CMV-infected KTR. To explore this issue, NKG2C+ NK cells were analyzed pretransplant and at different time points posttransplant for >24 months in a cohort of CMV+ KTR (n=112), stratified according to CMV infection. In a subgroup (n=49), adaptive NKG2C+ NK cell markers and T cell subsets were compared after a longer follow-up (median 56 months), assessing the frequencies of CMV-specific T cells and DNAemia at the last time point. CMV-infected KTR showed increased proportions of NKG2C+ NK cells, yet a substantial individual variability in the magnitude and kinetics of the response was noticed, without relation with the peak viral load and *KLRC2* zygosity. KTR categorized as non-infected maintained steady levels of adaptive NKG2C+ NK cells, however expansions were exceptionally noticed likely reflecting subclinical viral replication events. Increased proportions of adaptive NKG2C+ NK cells (CD57+, ILT2+, FcεRIγ-) were observed in CMV-infected KTR with undetectable DNAemia long-term posttransplant, coinciding with increased CD8+ and TcRγδ Vδ2- T cells, as well as CMV-specific T cells comparable to non-infected cases. These data suggest that adaptive NKG2C+ NK and T cells participate in the long-term control of CMV, though their relative role cannot be

discerned. Monitoring the adaptive NKG2C+ NK cell response may contribute to survey the evolution of CMV infection in KTR.

INTRODUCTION

Cytomegalovirus (CMV) establishes a prevalent and life-long infection, generally asymptomatic in healthy individuals. Different immune mechanisms contribute to control the pathogen, which enters a latency state undergoing sporadic reactivations (1,2). CMV becomes an important threat in congenital infection and in immunocompromised patients. In kidney transplant recipients (KTR) immunosuppression favors CMV infection, which has been associated to reduced graft and patient survival (3-5). Antiviral prophylaxis is indicated in high-risk CMV- recipients transplanted from CMV+ donors (CMV D+/R-), as well as in patients undergoing intensive immunosuppression (6). CMV+ KTR at intermediate risk may experience viral reactivation or reinfection. In that case, monitoring DNAemia along the first months posttransplant allows selective preemptive antiviral therapy (6). Low DNAemia values of uncertain clinical significance may be detected by sensitive quantitative nucleic acid amplification testing (QNAT), but there is no consensus on a precise threshold for therapeutic intervention (6,7).

In CMV+ KTR the risk of posttransplant infection and restoration of CMV control depend on the individual fitness of the immune system (8). In this regard, pretransplant frequencies of T cells specific for IE-1 and pp65 CMV antigens have been associated with

a reduced risk of posttransplant infection (9-12), whose resolution was reported to correlate with increased cytotoxic TcRγδ Vδ2- T cells (13). Together with T cells, NK cells are known to contribute to immune defense against CMV infection, which promotes the adaptive differentiation and persistent expansion of a mature CD94/NKG2C+ NK cell population with a distinctive phenotypic and functional profile (14-18). Common adaptive NK cell-associated features include: a) lack of the homologous CD94/NKG2A inhibitory receptor; b) expression of CD57 and inhibitory receptors for HLA class I molecules i.e. KIRs (Killer immunoglobulin-like receptors) and ILT2 (Immunoglobulin-like transcript 2, also termed LIR1, LILRB1); c) downregulation of activating receptors (i.e. NKp30, NKp46), signaling adaptors (e.g. FcεRI γ chain) and transcription factors (e.g. PLZF). This pattern of response to the viral infection is detected to a variable degree in healthy blood donors, being particularly prominent in immunocompromised individuals (15,19-21). A deletion of the *KLRC2* gene encoding NKG2C has been identified in populations of different ethnic origins (22-24), and the numbers of NKG2C+ NK cells have been reported to be greater in *KLRC2* homozygous compared to hemizygous CMV+ healthy individuals (24,25). We reported that pretransplant adaptive NKG2C+ NK cells in KTR were associated with a reduced incidence of symptomatic CMV infection (26,27), suggesting that they may contribute with T cells to contain infection progression, rather than impairing initial viral replication. Development of adaptive NKG2C+ NK cells in allogeneic hematopoietic stem cell transplantation (HSCT) was

related with protection against CMV reactivation (28,29). Expansions of adaptive NKG2C+ NK cells in response to posttransplant CMV infection in KTR have been described, yet information on that process is limited (30-32). In the present study we analyzed the evolution of adaptive NKG2C+ NK cells for >24 months in a cohort of CMV+ KTR (n=112), stratified according to the incidence of posttransplant CMV infection. In a subgroup (n=49), additional adaptive NKG2C+ NK cell markers, T cell subsets, frequencies of CMV-specific T cells and DNAemia were assessed at the end of a longer follow-up period (median 56 months).

METHODS

Patient population

Patients undergoing kidney transplantation at Hospital del Mar (Barcelona) between February 2013 and June 2017 were enrolled. KTR at intermediate risk of CMV infection (D+R+ or D-R+) followed for at least 24 months, and receiving the same maintenance immunosuppressive regimen (tacrolimus, mycophenolic acid and steroids) with anti-CD25 mAb induction were included (n=112) (26). Cases at low risk of CMV infection (D-R-) and those at high risk receiving antiviral prophylaxis (i.e. CMV D+R-) were excluded. Patients were transplanted according to negative complement-dependent cytotoxicity cross-match with donor lymphocytes, as previously described (33). Peripheral blood mononuclear cells (PBMC) cryopreservation and

immunophenotyping was routinely performed prior to transplantation and at different time points afterwards (≈3, 6, 12, 24 and 36 months). In a subgroup (n=49), an additional analysis was carried out coinciding with a later clinical visit (median 56 months; IQR: 46-62) assessing adaptive NK cell markers, T cell subpopulations, CMV-specific T cell frequencies and DNAemia. The study was conducted following the Declaration of Helsinki guidelines and approved by CEIC Parc de Salut Mar Ethical Research Board (2018/7873I). All patients signed written informed consent for the use of peripheral blood samples for research purposes.

CMV DNAemia analysis

CMV DNAemia was systematically monitored following transplantation by standardized diagnostic quantitative nucleic acid amplification testing (QNAT) (COBAS AmpliPrep, Cobas TaqMan; Roche Diagnostics) every two weeks, for the first three months posttransplant and whenever infection was clinically suspected. CMV infection was defined based on QNAT detection in plasma samples of viral loads above the lower limit of quantification (LLOQ) (137 IU/ml) or by histopathological examination. Infected patients were stratified as asymptomatic or symptomatic according to clinical guidelines (6). The latter category included cases with viral syndrome or with histopathological evidence of invasive disease. Symptomatic cases and those asymptomatic with DNAemia >500 copies/ml (455 IU/ml) received antiviral therapy. In the subgroup studied in more

detail (n=49), DNAemia was surveyed at the end of follow up (median 56 months posttransplant) employing a different technique (Abbot Diagnostics, IL, USA) with a lower LLOQ (31.2 IU/ml), adopted by the clinical diagnostics laboratory during the study (2018).

Immunophenotypic analysis and assessment of *KLRC2* (*NKG2C*) gene zygosity

As previously described (27), PBMC samples obtained by venous puncture in EDTA tubes were separated by Ficoll-Hypaque density gradient centrifugation and cryopreserved. Routine analysis of NKG2C expression was performed by indirect immunofluorescence using anti-NKG2C (MAB1381, R&DSystems) and PE-conjugated F(ab')2 goat anti-mouse secondary antibody, data were acquired on a FACS Canto II cytometer.

Extensive immunophenotyping was performed in thawed samples as described (26). Cells were pre-treated with human aggregated IgG (100µg/ml) to block FcR and surface stained using the following monoclonal antibodies: anti-CD3-APCCy7(OKT3, BioLegend), anti-CD45-Alexa Fluor 700 (2D1, eBioscience), anti-CD56-APC-Cy7(NCAM, BioLegend), anti-CD3-PerCP(SK7, BD), anti-TCR pan γ/δ-PE/Dazzle594 (B1, BioLegend), anti-TCR Vδ2-FITC (IMMU389, Beckman-Coulter), anti-NKG2C-PE (FAB138P, R&D systems), anti-NKG2A-Pacific Blue (Z199; provided by Dr. A. Moretta), anti-ILT2-PeCy7 (GHI/75, BioLegend), anti-CD57-FITC (HCCD57, BioLegend), anti-CD57-APC (HCD57, BioLegend), anti-CD56-APC (CMSSB, eBioscience), anti-CD4-

APC (RTA-T4, BD Pharmingen), anti-CD8-BV510 (RPA-T8, BD) anti-CD56-BV510 (NCAM16.2, BD Bioscience). For intracellular staining, cells were fixed and permeabilized (FIX & PERM Cell Fixation & Cell Permeabilization Kit, Invitrogen), according to manufacturer instructions, followed by intracellular staining with anti-FcεRIγ–FITC (polyclonal, Merck Millipore) and anti-PLZF-PECF594 (R17-809, BD). Data were acquired on an LSR Fortessa flow cytometer (BD Biosciences) and analyzed using FlowJo software (10.0.7, TreeStar). Gating strategy is shown in Supplementary Figure 1.

Multidimensional flow cytometry analysis using vi-stochastic neighbor embedding (SNE) was implemented in manually gated NK and T cells as described (17,26,34). *KLRC2* (NKG2C) gene deletion was assessed on genomic DNA samples as described (23).

Detection of CMV-specific T cells

PBMCs were incubated overnight at 37 ºC in polypropylene tubes with complete medium (RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, 100 µg/ml streptomycin and 1mM sodium pyruvate) and placed at 1x106 cells per well in 96-well plates. T cell activation was assessed by flow cytometry detection of cytokine production, as described (26) Briefly, samples were incubated with RPMI alone, anti-CD3 mAb or 1µg/ml of peptide libraries from CMV pp65 or IE-1 antigens (Peptivator, Miltenyi Biotec) for 6 hours. During the last 5h brefeldin A (Sigma-Aldrich) and anti-CD49d (BD Biosciences) were added to a final concentration of 10 µg/ml and 1µg/ml,

respectively. Subsequently, cells were stained with fluorochrome conjugated antibodies and a minimum of 10^5 T cells were analysed on a LSR Fortessa flow cytometer (26).

Statistical analysis

Statistical analysis was performed in R version 3.5.1. Categorical variables are expressed as percentages, and continuous variables as mean and standard deviation (SD) or median and interquartile range (IQR). Univariate analysis was performed by Chi-square test, Spearman correlation, Wilcoxon or Fisher's exact test, as appropriate. Two-sided p-values <0.05 were considered significant.

RESULTS

Evolution of adaptive NKG2C+ NK cells in response to posttransplant CMV infection in KTR

An immunophenotypic analysis was sequentially carried out, pretransplant and at different time points posttransplant, in PBMC from a cohort of CMV+ KTR (n=112), who did not receive antiviral prophylaxis and were followed up for at least 24 months. Demographic and clinical information is summarized in Table I. NKG2C+ NK cells were assessed in patients stratified according to the incidence of CMV infection (see Methods). Given the scope of the study, 6 cases (3 infected and 3 non-infected) with undetectable NKG2C+ NK cells and a confirmed homozygous *KLRC2* deletion were excluded from subsequent analyses. As compared to pretransplant levels, the proportions of NKG2C+ NK cells were

significantly increased in infected cases at 12 and 24 months posttransplant (Figure 1A-B). Yet, a substantial individual variability in the evolution of the NKG2C+ NK cell subset was noticed (Figure 1C).

By regression analysis the magnitude of NKG2C+ NK cell expansions did not correlate with the CMV DNAemia peak detected (Figure 1D). The putative influence of *KLRC2* copy number on the magnitude of posttransplant NKG2C+ NK cell expansions was considered. In line with previous observations (27), greater proportions of NKG2C+ NK cells in $KLRC2^{wt/wt}$ as compared to $KLRC2^{wt/del}$ KTR were detected pretransplant, but no differences were observed at 24 months posttransplant (Figure 1E). Moreover, variations of NKG2C+ NK cells in cases with posttransplant infection were comparable in *KLRC2* homozygous and hemizygous individuals (Figure 1F).

Pretransplant NKG2C+ NK cells have been associated with a reduced incidence of symptomatic infection (26,27), therefore KTR displaying high pretransplant proportions of this subset (>30%, n=26) were separately analysed (Figure 1G). An overall reduction of NKG2C+ NK cells was observed in non-infected KTR (n=23, solid lines). Yet, at the individual level, steady levels or minimal variations of NKG2C+ NK cells, as well as increased or decreased proportions were noticed. Of note, two of the three infected cases (dashed lines) displayed marked late decrements of NKG2C+ NK cells (Figure 1G).

Table I. Characteristics of the studied KTR cohort

	all cases (n=112)	No CMV Infection (n=71)	CMV Infection (n=41)	p-value
Recipient age (years), mean(SD)	55.3 (13)	52.5 (12.4)	60.1 (12.7)	0.003
Female recipient, n(%)	40 (35.7)	23 (32.4)	17 (41.5)	0.447
Donor age (years), mean(SD)	57.5 (14.4)	54.2 (14.3)	62.9 (12.9)	0.004
Female donor, n(%)	57 (50.9)	35 (49.3)	22 (53.7)	0.860
Live donor, n(%)	23 (20.5)	19 (26.8)	4 (9.8)	0.050
Retransplant, n(%)	9 (8)	8 (11.3)	1 (2.4)	0.151
Pre-KT CMV serostatus, n(%)				0.128
D+/R+	99 (88.4)	60 (84.5)	39 (95.1)	
D-/R+	13 (11.6)	11 (15.5)	2 (4.9)	
Peak PRA >5%, n(%)	16 (14.3)	12 (16.7)	4 (9.8)	0.262
Pretransplant PRA >5%, n(%)	9 (8)	5 (7)	4 (9.8)	0.779
Pretransplant DSA, n(%)	2 (1.8)	2 (2.8)	0	0.532
Delayed graft function, n(%)	25 (22.3)	15 (21.1)	10 (24.4)	0.870
Biopsy-proven acute rejection, n(%)	16 (14.3)	10 (14.1)	6 (14.6)	1
Symptomatic CMV, n(%)	-	-	25 (61)	-
CMV PCR at diagnosis (IU/ml), median (IQR)	-	-	430 (197-736)	-
Peak CMV PCR (IU/ml), median (IQR)	-	-	576 (258-1789)	-
CMV infection time after KT (days), median (IQR)	-	-	56 (40-72)	-

CMV, cytomegalovirus; DSA, donor-specific Abs; IQR, interquartile range; KT, kidney transplantation; PCR, polymerase chain reaction; PRA, panel reactive antibodies (by complement-dependent cytotoxicity); SD, standard deviation; IU/ml, international units per ml.

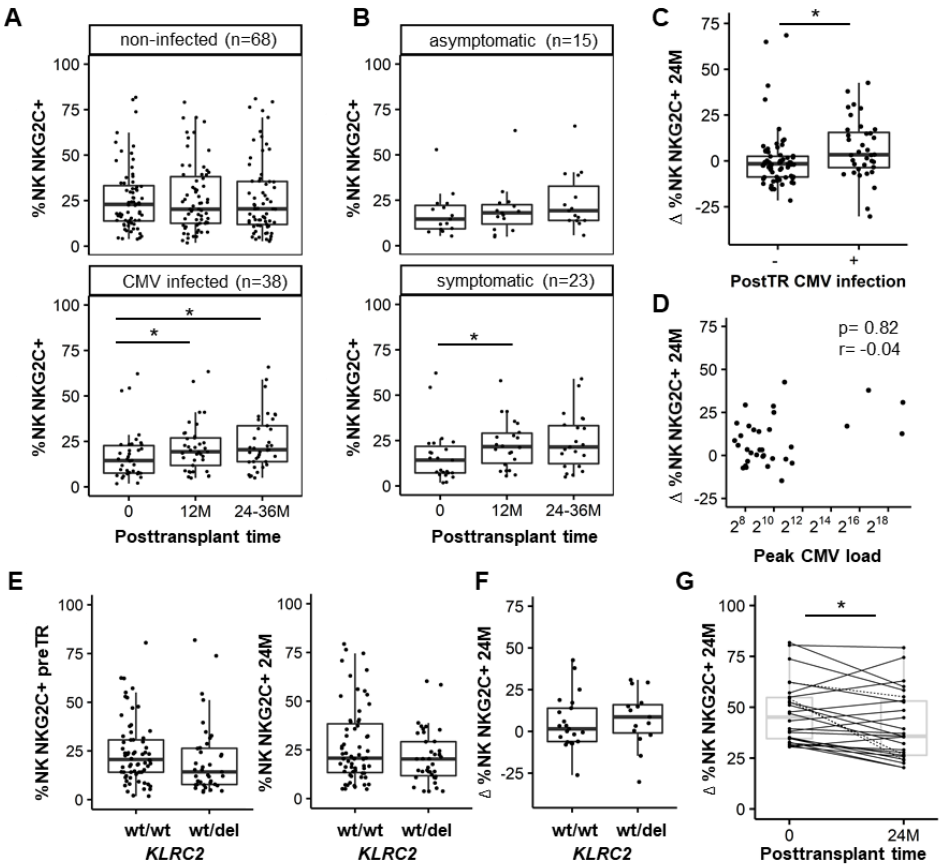

Figure 1. Evolution of NKG2C+ NK cells in KTR. **(A)** Frequencies of NKG2C+ NK cells were assessed pretransplant and at 12 and 24-36 months posttransplant in PBMC from a cohort of CMV+ KTR (n=106) stratified according to the incidence of posttransplant CMV infection. **(B)** NKG2C+ NK cells in infected KTR (n=41) stratified according to the incidence of symptomatic or asymptomatic infection. **C)** Individual variations between NKG2C+ NK cell frequencies pretransplant and at 24 months posttransplant in CMV-infected and non-infected KTR. **D)** Regression analysis of the relation between the variations in NKG2C+ NK cells at 24 months and peak CMV DNAemia (IU/ml) detected in CMV infected KTR. **E)** Frequencies of NKG2C+ NK cells detected pretransplant and 24 months posttransplant in KTR segregated according to *KLRC2* zygosity.

F) Variation of the proportions of NKG2C+ NK cells at 24 months, referred to pretransplant levels, in infected KTR segregated according to *KLRC2* zygosity. **G)** Frequencies of NKG2C+ NK cells pretransplant and 24 months posttransplant in KTR with >30% NKG2C+ NK cells pretransplant (n=26); dashed lines correspond to infected cases. Graphs include median and IQR. Paired (A,B,G) and unpaired Wilcoxon (C,E,F), * = $p \leq 0.05$.

A more detailed analysis was conducted in a KTR subgroup, including infected (n=23) and non-infected (n=26) cases, who were further studied at a late regular clinical visit (median 56 months; IQR: 46-62) (Supp. Tables I and II). Consistent with observations in the whole cohort, NKG2C+ NK cells in CMV-infected KTR were significantly increased at 24 months (Figure 2A), as compared to pretransplant levels, but the differences tended to decline at the latest time point (median 56 months). A group of KTR with marked increases of NKG2C+ NK cells was clearly differentiated from cases showing minimal changes or decreased proportions. Representative evolution profiles of NKG2C+ NK cells in infected cases are illustrated in Figure 2B. These included early/delayed increases, steady levels similar to the common profile in non-infected KTR or late decrements. Of note, NKG2C+ NK cell increments were also exceptionally observed in 5 out of 68 KTR cases categorized as non-infected (e.g. Supp. Table II; c.1199).

To further evaluate the impact of posttransplant infection on the development of adaptive NKG2C+ NK cells, expression of differentiation markers (i.e. lack of NKG2A; CD57 and ILT2 expression and FcεRγ downregulation) was assessed in PBMC samples obtained pretransplant and at the last follow-up time point,

analyzing also in the latter PLZF downregulation. Figure 3 displays the flow cytometry analysis of two representative infected KTR. The evolution of adaptive NKG2C+ NK cell markers in infected and non-infected KTR was compared. Given the scope of the analysis, cases already displaying high pretransplant proportions of adaptive NK cells (>30%) (Figure 1G) that would mask the changes were not included.

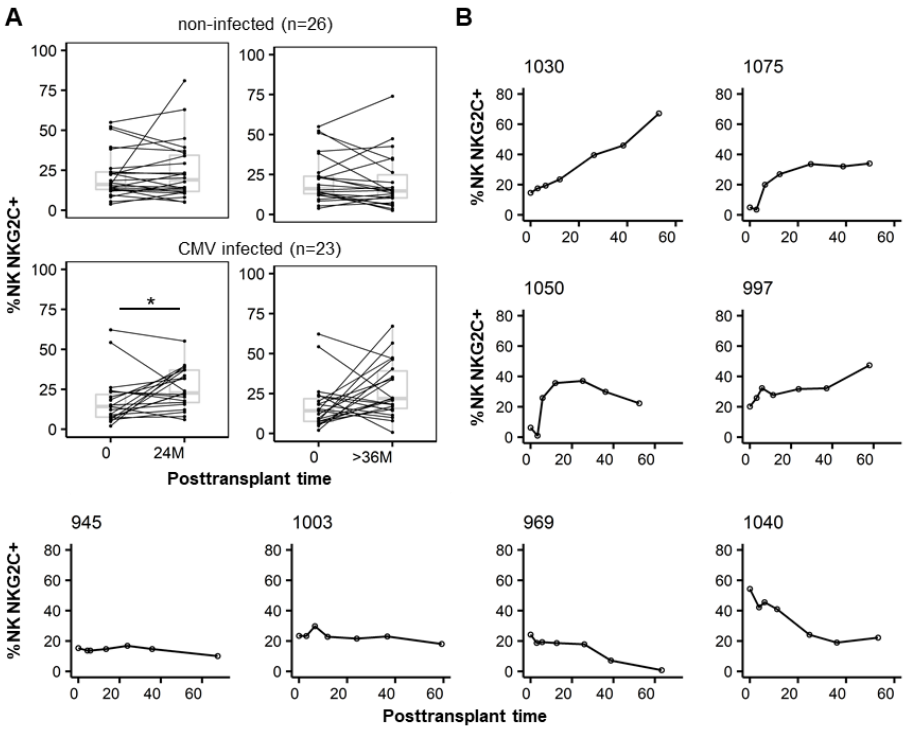

Figure 2. Long-term evolution patterns of NKG2C+ NK cells in CMV-infected KTR. A) Frequencies of NKG2C+ NK cells detected pretransplant and >36 months (median 56, IQR: 46-62m) posttransplant in KTR (n=49) stratified according to posttransplant CMV infection. **B)** Line graphs showing representative evolution patterns of the frequencies of NKG2C+ NK cells in infected KTR. Paired Wilcoxon, * = $p \leq 0.05$.

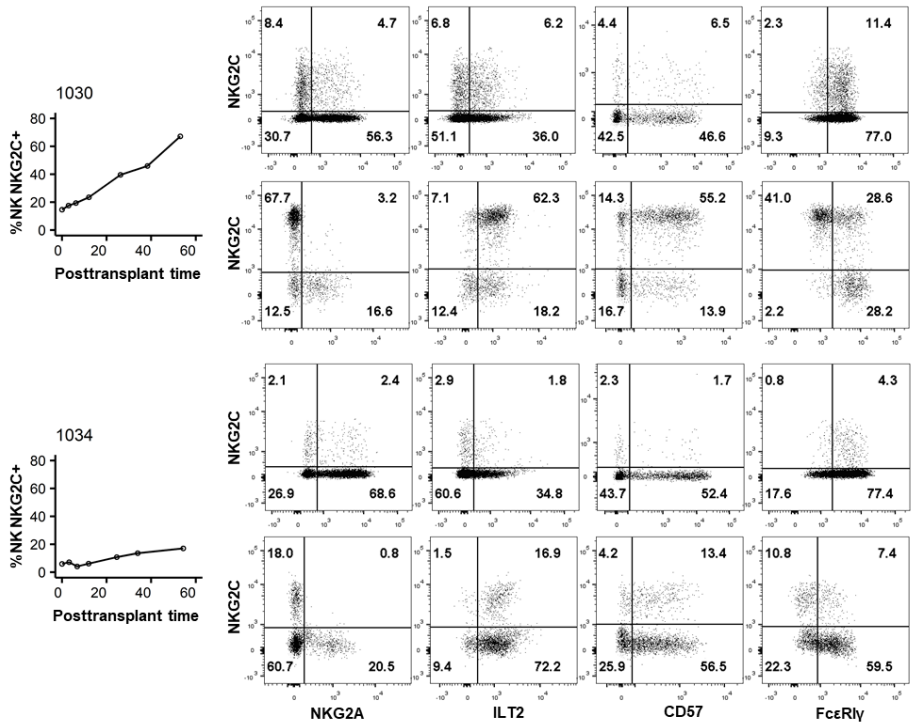

Figure 3. Expression of adaptive NKG2C+ NK cell differentiation markers in CMV infected KTR. Flow cytometry plots comparing the expression of adaptive NKG2C+ NK cell markers analyzed pretransplant and at the end of the long-term follow-up in infected KTR. Cases are representative of two different evolution profiles, displayed in the accompanying line graphs.

As shown in Figure 4A, proportions of NKG2C+ NK cells with adaptive phenotypic features (i.e. NKG2A-, CD57+, FcεRIγ and ILT2+) were significantly increased posttransplant in infected KTR. Conversely, their pretransplant levels were greater in non-infected cases, consistent with the reported association with a reduced risk of CMV infection (26). Altogether, these results revealed a marked individual variability in the long-term evolution of canonical

NKG2C+ adaptive NK cells following posttransplant CMV infection. By tSNE analysis, three clusters of NKG2C+ NKG2A- NK cells, prominent in infected cases, could be discriminated according to CD57, ILT2, FcεRγ and PLZF expression, likely representing distinct adaptive NK cell differentiation stages (Figure 4B). By contrast, NKG2C- NKG2A+/- subsets expressing PLZF and FcεRγ as well as different levels of CD57 and ILT2 predominated in non-infected cases.

Although no clinical evidence of CMV infection was recorded at the end of the long-term follow-up, DNAemia was assessed in parallel to the last immunophenotypic analysis. In this survey, out of the 49 KTR studied, low DNAemia levels were only detected in three initially infected cases (Supp. Table 1; 982, 1034, 1224) and one non-infected (Supp.Table 1; 1105) all displaying scarce proportions of NKG2C+ cells, as well as in a patient with early increased NKG2C+ NK cells (Table 1; 1106). Though limited to a single point analysis, these data did not support an association between adaptive NKG2C+ NK cell expansions and late DNAemia, but rather suggested their involvement in long-term CMV control, a process conventionally attributed to T cells.

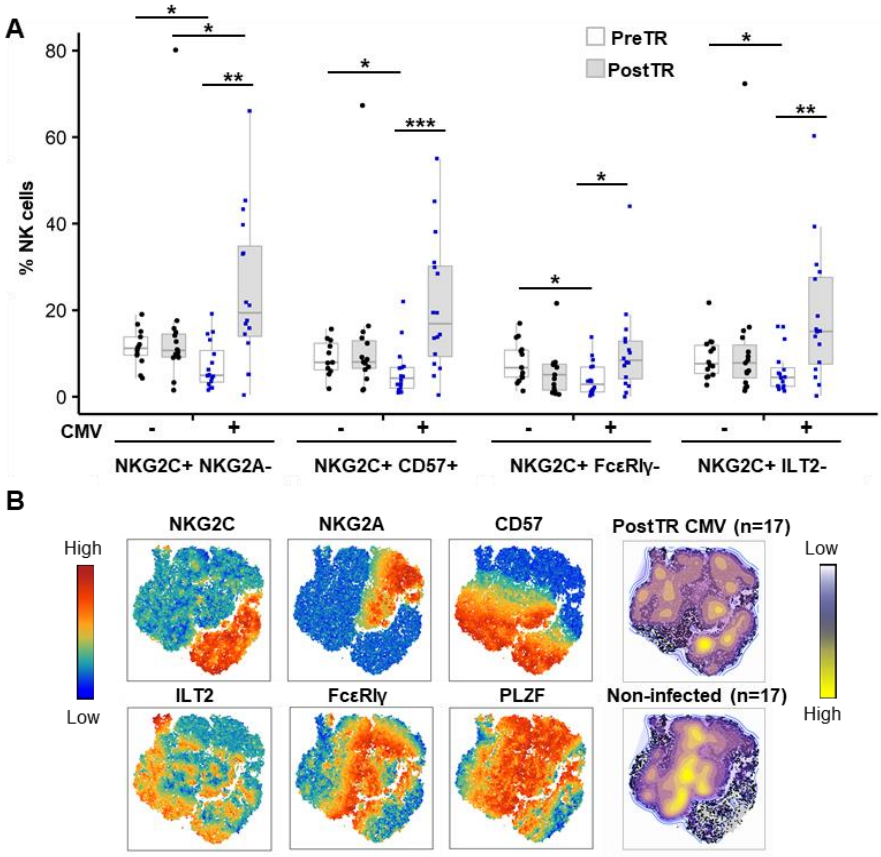

Figure 4. Effect of CMV infection on the long-term distribution of adaptive NKG2C+ NK cell markers. A) Frequencies of NKG2C+ NK cells displaying adaptive NK cell-associated markers, pretransplant and at the end of the follow-up, in KTR with available pretransplant information (n=31), segregated according to posttransplant CMV infection. Graphs include median and IQR. **B)** Multidimensional Barnes-Hut t-SNE analysis of 6 parameters was performed on manually gated NK cells at the end of the follow-up in PBMC samples from KTR (n= 34). Single-parameter plots show protein expression levels on the t-SNE field. Kernel density plots show events in the t-SNE field compiled separately for samples from patients stratified according to detection of posttransplant CMV DNAemia. Cases with high baseline levels (>30%) of NKG2C+ NK cells were excluded from both analyses. Paired Wilcoxon for pretransplant and

posttransplant comparisons and unpaired Wilcoxon for comparison between infected and non-infected patients, * = $p \leq 0.05$; * $p \leq 0.01$; *** $p \leq 0.001$.

Long-term effects of early posttransplant CMV infection on the T cell compartment

TcRγδ Vδ2- T cells have been associated with control of posttransplant CMV infection in KTR (13), and expansions of specific CD8+ T cells were reported even in the absence of CMV disease (35). Thus, TcRαβ (CD4+, CD8+), TcRγδ (Vδ2-, Vδ2+) and a minor NKG2C+ T cell subset reported in CMV+ blood donors (14), were analysed in parallel to NK cells.

Pretransplant and posttransplant phenotypes were compared according to the incidence of CMV infection. As shown in Figure 5, significantly increased CD8+ and reduced CD4+ T cell subsets, as well as raised proportions of TcRγδ Vδ2- T cells and a minor NKG2C+ Vδ2- subset were detected in infected cases long-term after transplant. By contrast, no significant differences in TcRγδ Vδ2+ nor total NKG2C+ T cells were observed. Of note, some differences were also noticed comparing the levels of CD4+ and TcRγδ Vδ2- in non-infected cases. tSNE analysis graphically discriminated differences between infected and non-infected cases in the distributions of discrete clusters of CD4+, CD8+ and TcRγδ Vδ2- T cell populations, as well as of a minor NKG2C+ TcRγδ Vδ2- subset (Figure 5B).

The specific T cell response to pp65 and IE-1 immunodominant CMV antigens was also assessed at the end of the follow-up. As shown in Figure 6B, no differences in the frequencies of pp65- and

IE1-specific T cells were perceived between infected and non-infected KTR. Altogether, these data illustrated the complex influence exerted by CMV infection on the NK and T cell compartments long-term after transplantation, which likely cooperate in controlling CMV, rendering difficult to ascertain the individual contribution of adaptive NKG2C+ NK cells in this process.

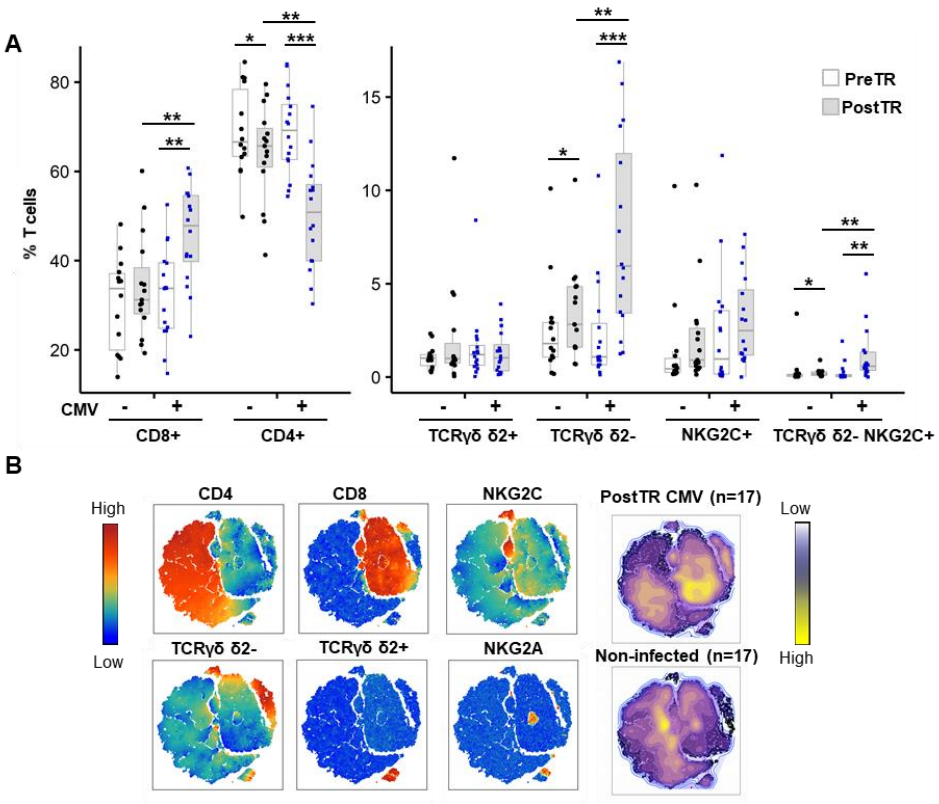

Figure 5. Effects of CMV infection on the long-term distribution of T cell subsets. A) The frequencies of CD4+, CD8+, NKG2C+, TCRγδ (Vδ2+ and Vδ2−) T cell subsets were assessed by flow cytometry, pretransplant and at the end of the follow-up (median 56 months) in KTR (n=31), stratified as CMV infected and non-infected. Graphs include median and IQR. **B)** Multidimensional

Barnes-Hut t-SNE analysis of 6 parameters was performed on manually gated T cells at the end of the follow-up in PBMC samples from KTR (n=34), as described in Figure 4. Paired Wilcoxon for pretransplant and posttransplant comparisons and unpaired Wilcoxon for comparison between infected and non-infected patients, * = $p \leq 0.05$; * $p \leq 0.01$; *** $p \leq 0.001$.

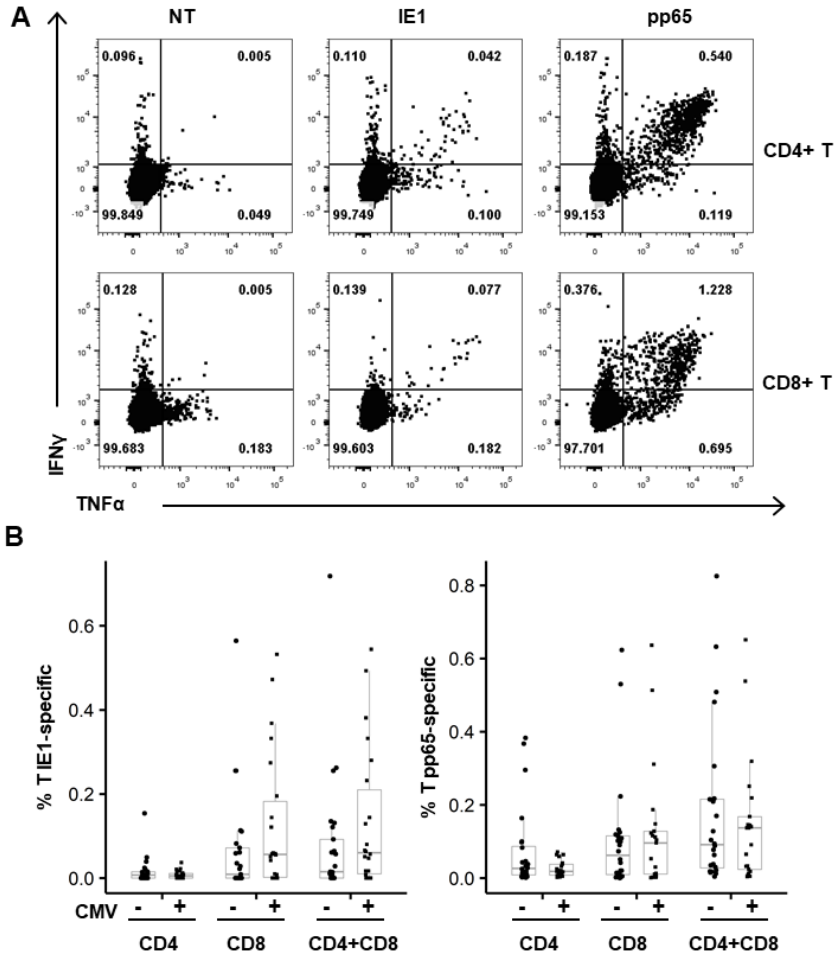

Figure 6. Detection of T cells specific for IE1 and pp65 CMV antigens in KTR long-term after transplantation. **A)** Flow cytometry data showing the CD4+ and CD8+ T cell response of a representative individual to peptide pools from IE-1 and pp65 CMV antigens, assessed by intracellular IFNγ and TNFα production. **B)** Frequencies of CD4+ and CD8+ T cells responding to peptide

pools from IE-1 and pp65 CMV antigens, detected in KTR stratified according to the incidence of CMV infection. Graphs include median and IQR.

DISCUSSION

Control of CMV replication in seropositive KTR is altered posttransplant, often leading to viral reactivation and/or reinfection. Factors related with the transplantation procedure (e.g. cold ischemia) may trigger viral replication, while immunosuppression promotes infection progression (36). The risk of posttransplant infection and its control ultimately depend on the individual fitness of the immune system. Frequencies of T cells specific for CMV antigens (i.e. pp65 and IE-1) have been related with a lower incidence of posttransplant infection, consistent with their central role in keeping the pathogen at bay (9-12). Recently, pretransplant adaptive NKG2C+ NK cells were also associated with a reduced incidence of symptomatic infection in KTR (26,27), suggesting that they may contribute with T cells to contain infection progression, rather than hampering initial viral replication. On the other hand, CMV infection has been shown to promote posttransplant expansions of adaptive NKG2C+ NK cells (20,21,30) that might contribute with T cells to restore CMV replication control.

In this study, adaptive NKG2C+ NK cells were analyzed in a cohort of CMV+ KTR undergoing a similar immunosuppressive regimen without antiviral prophylaxis, followed at different times posttransplant. Expansions of adaptive NKG2C+ NK cells were detected in CMV-infected KTR, though marked individual differences regarding their magnitude and kinetics were noticed. In

some cases, proportions of NKG2C+ NK cells sharply increased early after infection, persisting elevated along the follow-up, whilst increments appeared delayed and progressive in others. We interpret that these evolution profiles primarily reflected an inefficient T cell-mediated control of CMV replication. This might take place during the first months after transplant, when DNAemia was systematically monitored, but also at any time along posttransplant evolution. Conversely, minor changes or steady levels of adaptive NKG2C+ NK cells, comparable to the common profile observed in non-infected KTR, likely indicated a prompt and stable resolution of CMV infection. Of note, NKG2C+ NK cell expansions were exceptionally detected in some cases categorized as non-infected. This unexpected finding presumably reflected the incidence of subclinical CMV replication events occurring beyond the early DNAemia monitoring period.

The association of pretransplant adaptive NKG2C+ NK cells with a reduced incidence of symptomatic CMV infection, suggested that they contribute to contain infection progression, but may not prevent early viral replication (26). Consistent with this view, DNAemia values <LLOQ were detected in some KTR with high baseline proportions of adaptive NKG2C+ NK cells, who were categorized as non-infected, did not receive any antiviral therapy and did not display DNAemia in the late survey years after transplant (Supp. Table II, cases 1002, 1056, 1079).

As pretransplant levels of adaptive NKG2C+ NK cells have been associated with a reduced incidence of symptomatic infection (26), it is plausible that development of the adaptive NK cell response in

infected KTR may also contribute to restore CMV control. Consistent with this hypothesis, DNAemia was undetectable at the late follow-up time point in most infected KTR, including cases with adaptive NKG2C+ NK cell expansions. Moreover, in that survey 4 out of 5 cases with positive DNAemia values displayed low proportions (< 20%) of NKG2C+ NK cells. Although these data suggested that NKG2C+ NK cell expansions might contribute to maintain CMV control, concomitant changes were detected in the T cell compartment of infected KTR who, moreover, displayed frequencies of IE1- and pp65-pecific T cells comparable to non-infected patients. Late effects of posttransplant CMV infection in the T cell compartment have been previously reported in KTR (37). The complex influence of CMV infection on the immune system, which includes the T cell response to different viral antigens not assessed in clinical studies (38,39), did not allow to discern the relative role played by different lymphocyte subsets that likely cooperate in controlling the pathogen.

As reported in healthy individuals (25), adaptive NKG2C+ NK cell expansions generally persisted long-term in KTR, but their proportions declined at late time points in some cases. The interpretation that decrements of NKG2C+ NK cells detected long-term after transplant might reflect a balanced control of CMV is tempting. Yet, the possibility that immunosuppression may alter the adaptive NK cell turnover (40), resulting in a reduction of circulating adaptive NK cells warrants attention.

In CMV+ KTR, the adaptive NKG2C+ NK cell response to posttransplant viral infection may result from expansion of a

preexisting pool of this subset, and/or from *de novo* differentiation, proliferation and survival. These events may be dissociated, as illustrated by detection in some infected KTR of moderate increases of NKG2C+ NK cells with an adaptive phenotype. Evidence for *de novo* generation of adaptive NKG2C+ NK cells has been obtained in a group of high-risk CMV seronegative KTR suffering posttransplant infection (Ataya et al. unpublished observations), indicating that this process is, at least partially, resistant to standard immunosuppression.

CMV infection appears the main determinant for triggering an adaptive NKG2C+ NK cell response. Thus, its magnitude may be modulated by factors indirectly related with restoration of pathogen control, as well as with the underlying molecular mechanisms, which remain incompletely defined. In this regard the following considerations deserve attention: a) The adaptive NK cell response did not correlate with the DNAemia peak. Remarkably, it was not perceived in three out of six cases with invasive disease in the whole cohort, coinciding with low DNAemia peak values (e.g. Supp. Table II, c. 945, 1054). In this regard, low viral loads have been reported in gastrointestinal CMV infection (41) suggesting that development of the adaptive NK cell response might be also influenced by the tissue location and extension of viral replication (e.g. epithelial vs hematopoietic tissues). b) It has been proposed that preemptive antiviral therapy may reduce antigenic stimulation of the immune system, eventually favoring the recurrence of CMV infection (42), and thus might also blunt adaptive NKG2C+ NK cell development. c) Control of CMV reinfection may be less efficient

than that of reactivation (43) potentially favoring adaptive NK cell development. A similar situation would predictably occur in case of resistance to antiviral therapy (6). d) Despite that adaptive NK cells can develop in KTR, there is no precise information on their relative sensitivity to the different immunosuppressive drugs shown to influence NK cell functions (44). e) Stimulation through FcγRIIIA (CD16) has been reported to induce the proliferation of adaptive NKG2C+ NK cells (18), which proficiently mediate ADCC against CMV-infected cells (45-47). This process, predictably sensitive to immunosuppression, might be influenced by the concentrations of IgG specific for CMV antigens (e.g. gB), as well as by polymorphisms of the FcγR-IIIA (CD16) influencing its affinity for IgG (48). f) A putative influence of the relation of *KLRC2* copy number with the proportions of NKG2C+ NK cells, reported in healthy CMV+ individuals (24,25) and pretransplant in KTR (27) was considered. Yet, the magnitude of the adaptive NK cell response to posttransplant infection appeared similar in *KLRC2* homozygous and hemizygous KTR. g) The adaptive NKG2C+ NK cell response has been proposed to be triggered by CD94/NKG2C recognition of HLA-E bound to peptides from the CMV UL40 molecule (49). These mimic endogenous HLA-I-derived leader sequence nonamers, whose polymorphism influences the affinity for HLA-E (50). Prospective studies on DNAemia samples are required to assess whether variability of the UL40 leader sequence modulates the magnitude of the adaptive NKG2C+ NK cell development in KTR.

In summary, our observations provide a perspective on the evolution of the adaptive NKG2C+ NK cell response to CMV infection in KTR, illustrating its individual variability. Expansions of adaptive NKG2C+ NK cells following infection primarily reflect an inefficient/delayed control of the pathogen. Thus, their detection in KTR clinically considered non-infected should warn on subclinical viral replication events. On the other hand, following their development, adaptive NKG2C+ NK cells may contribute with T cells to restore CMV control. From a practical standpoint, the observations support the putative interest of this parameter to monitor the individual host-pathogen interaction at different stages posttransplant, complementing other immune correlates involved in protection against CMV (8).

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Supplementary table I. Characteristics of the study population subgroup

	all patients (n=49)	No CMV Infection (n=26)	CMV Infection[a] (n=23)
Recipient age (years), mean(SD)	57 (12)	55 (10.2)	60 (13.6)
Female recipient, n (%)	17 (34.7)	10 (38.5)	7 (30.4)
Live donor, n (%)	9 (18.4)	7 (26.9)	2 (8.7)
Retransplantation, n (%)	6 (12.2)	5 (19.2)	1 (4.3)
Pre-KT CMV serostatus, n (%)			
D+/R+, n (%)	41 (83.7)	20 (76.9)	21 (91.3)
D-/R+, n (%)	8 (16.3)	6 (23.1)	2 (8.7)
Pretransplant PRA >5%, n (%)	5 (10.2)	3 (11.5)	2 (8.7)
Pretransplant DSA, n (%)	1 (2)	1 (3.8)	0
Delayed graft function, n (%)	10 (20.4)	5 (19.2)	5 (21.7)
Biopsy-proven rejection, n (%)	6 (12.2)	3 (11.5)	3 (13)
Symptomatic CMV, n (%)	-	-	18 (85.7)
CMV PCR at diagnosis (IU/ml), median (IQR)	-	-	376 (202-634)
Peak CMV PCR (IU/ml), median (IQR)	-	-	435 (245-926)
CMV infection time after KT (days), median (IQR)	-	-	57 (44-75)

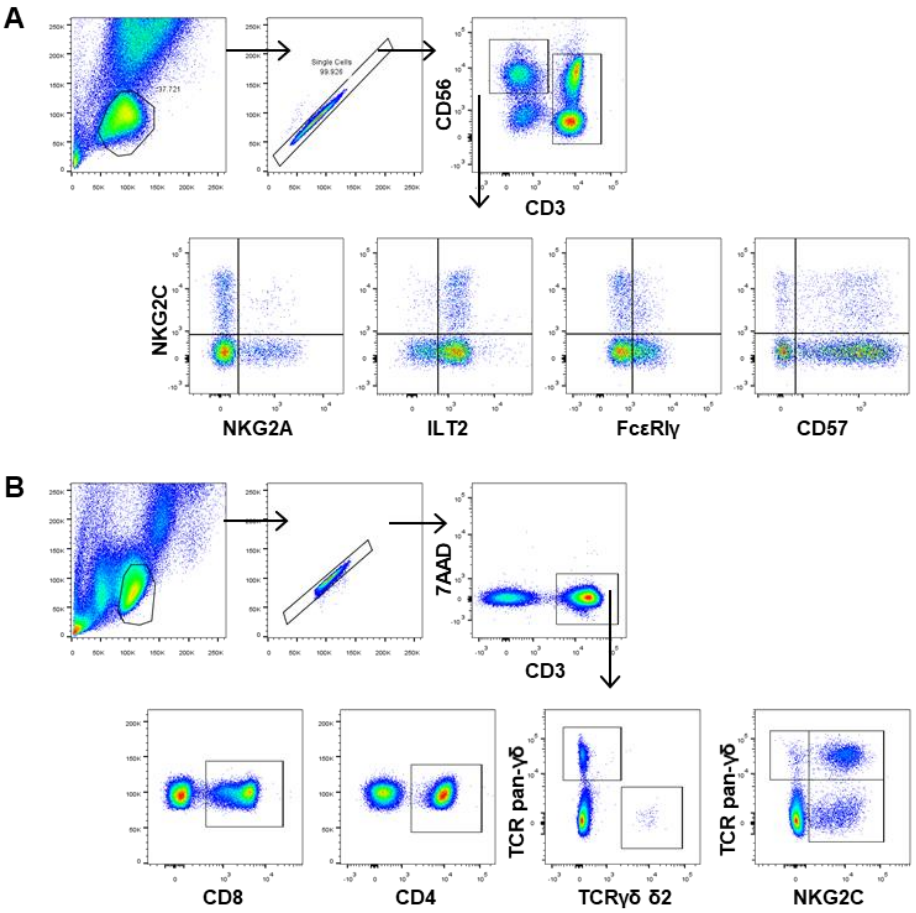
CMV, cytomegalovirus; DSA, donor-specific Abs; IQR, interquartile range; KT, kidney transplantation; PCR, polymerase chain reaction; PRA, panel reactive antibodies (by complement-dependent cytotoxicity); SD, standard deviation; IU/ml, international units per ml.

Supplementary Table II. Evolution of NKG2C+ NK cells in the KTR subgroup (n=49) in relation with CMV infection

# [a]	Age [b]	F.U [c]	PostTR CMV [d]	Peak IU/ml [e]	CMV treatment	% NKG2C+ NK				End F.U CMV [g]
						PreTR	6-12M	24-36M	End F.U [f]	
913	54	72	Asymp [h]	233	No	10	22	42	46	UD [i]
926	68	70	Viral S. [j]	750	VGC [k]	8	12	26	21	UD
945	64	67	Disease [l]	308	GCV [m]	15	15	15	10	-
962	44	64	Asympt.	349	No	8	17	14	15	UD
964	45	63	Viral S.	163	VGC	26	41	33	18	UD
969	73	63	Viral S.	691	VGC	24	19	7	1	LLOQ [j]
982	78	60	Viral S.	260	VGC	24	30	12	14	53
997	75	58	Viral S.	187	VGC	20	28	32	47	UD
1003	67	57	Viral S.	875	VGC	23	23	23	18	UD
1006	36	56	Disease	10443	VGC	9	11	59	57	UD
1008	39	56	Viral S.	26614	VGC	18	22	-	12	UD
1030	63	53	Asympt.	932	VGC	15	24	46	67	LLOQ
1034	77	53	Viral S.	2165	VGC	6	6	14	17	159
1040	49	51	Viral S.	242	VGC	54	41	19	22	UD
1050	55	51	Disease	488331	VGC	6	36	30	22	UD
1054	74	50	Disease	LLOQ [j]	VGC	14	6	5	8	UD
1075	42	47	Viral S.	910	VGC	5	27	32	34	UD
1082	71	46	Viral S.	154	No	19	35	25	34	UD
1139	69	38	Viral S.	242	VGC	62	58	52	47	UD
1148	48	37	Asympt.	468	VGC	9	9	20	22	UD
1163	41	35	Asympt.	254	No	12	24	35	35	UD
1196	67	29	Viral S.	91924	GCV	2	21	-	40	UD
1224	62	26	Viral S.	403	VGC	7	8	-	8	281
912	55	72	No	UD	No	12	15	17	6	LLOQ
914	61	72	No	UD	No	52	39	39	26	-
927	45	70	No	UD	No	13	13	7	4	UD
933	60	69	No	UD	No	4	9	10	12	UD
934	57	69	No	UD	No	13	15	11	11	UD
957	58	65	No	UD	No	19	26	12	15	UD
976	42	62	No	LLOQ	No	24	24	23	13	UD
979	48	61	No	UD	No	22	44	31	35	UD
998	66	58	No	LLOQ	No	14	10	13	15	UD

999	62	58	No	UD	No	23	24	18	20	UD
1002	63	57	No	LLOQ	No	51	41	32	34	LLOQ
1009	32	56	No	UD	No	10	12	15	13	UD
1010	63	56	No	UD	No	14	7	6	5	UD
1012	46	56	No	LLOQ	No	14	8	3	3	UD
1027	50	56	No	UD	No	9	7	9	5	UD
1043	65	51	No	UD	No	15	20	17	17	UD
1044	55	51	No	LLOQ	No	23	27	15	16	UD
1056	40	50	No	LLOQ	No	55	55	67	74	UD
1079	71	47	No	LLOQ	No	40	44	39	43	UD
1091	51	45	No	UD	No	38	39	25	14	UD
1101	39	43	No	UD	No	9	13	9	10	UD
1105	69	43	No	UD	No	5	7	11	7	129
1106	43	42	No	LLOQ	No	26	44	42	47	192
1199	63	29	No	LLOQ	No	16	63	-	81	UD
1247	55	24	No	UD	No	14	12	-	13	UD
1267	57	22	No	UD	No	17	15	-	11	UD

[a] Patient identifier, [b] Recipient age in years at transplantation, [c] End of follow up in months posttransplant, [d] Posttransplant CMV viremia was monitored biweekly in plasma for 3 months and successively assessed in case of clinical suspicion, [e] peak CMV viral load in international units per ml determined during biweekly monitoring in the first 3 months posttransplant or in case of clinical suspicion, [f] NKG2C+ NK phenotype performed at the last follow-up time point, [g] CMV DNAemia assessed at the last follow-up time point in international units per ml, [h] asymptomatic, [i] undetectable, [j] viral syndrome, [k] valganciclovir, [l] end-organ disease, [m] ganciclovir, [j] below the LLOQ (lower limit of quantification).



Supplementary Figure 1. Representative gating strategy for NK (A) and T (B) cells.

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PART IV

DISCUSSION AND CONCLUSIONS

DISCUSSION

Immunosuppression in kidney transplantation is often accompanied by opportunistic CMV infection prompting detrimental side-effects to graft and patient survival (15,185). Clinical expression of posttransplant CMV replication may vary from a mild viral syndrome to tissue invasive disease (186). The risk of infection can be influenced by the type or intensity of immunosuppression, and is reduced for younger CMV seropositive patients and recipients of live donors grafts (187,188); moreover end-stage renal disease may cripple the immune system (189). In CMV- KTR the progression of primary infection depends on the fitness of the developing immune response to control viral replication after withdrawal of antiviral therapy. In CMV+ KTR the quality of pretransplant antiviral immunity is of paramount importance to prevent and control viral reactivation/reinfection. CMV+ KTR are surveyed by periodical DNAemia monitoring and preemptive antiviral therapy is administered when indicated by clinical criteria. However, the host-pathogen *statu quo* may be altered at any point following transplantation; in that case only patients under clinical suspicion, confirmed by DNAemia or histopathological detection, are diagnosed and treated.

In this context, surveying the host antiviral immunity is relevant for decision making on CMV prevention and treatment strategies. Detection of T cells specific for CMV immunodominant antigens (i.e. pp65 and particularly IE1) provide good correlates of antiviral immunity and the incidence of posttransplant infection (115).

Increasing evidence supports that the NK cell response also contributes to keep CMV at bay. In particular, differentiation and expansion of a subpopulation expressing the CD94/NKG2C activating receptor specific for HLA-E and with a characteristic phenotypic and functional profile has been described in CMV+ healthy individuals and immunocompromised patients (190). Circulating proportions of these cells remain stable for years, accounting in some healthy donors for over 50% of the NK cell compartment. This unconventional adaptive response to the viral infection is reminiscent of the development of virus-specific cytotoxic memory T cells. Based on a number of studies in different pathological conditions and healthy individuals at different ages, it has been hypothesized that development of adaptive NKG2C+ NK cells compensates an insufficient T cell mediated control (191). This may occur following primary infection (144,192), but also upon reactivation of latent infection in immunocompromised subjects (e.g. HIV+ individuals)(148). A deletion of the gene encoding NKG2C (*KLRC2*) has been identified in different populations at a relatively high frequency (approx. 5% homozygous). These observations indicate that NKG2C+ NK cells are dispensable for controlling the infection in healthy individuals, but may confer an advantage when other immune mechanisms fail . Despite a number of studies developed since its first description, molecular and cellular mechanisms underlying the differentiation and expansion of the adaptive NKG2C+ NK cell response remain incompletely unraveled. *In vitro* evidence pointed out the involvement of CD94/NKG2C together with cytokine stimulation

(i.e. IL-12, IL-15, and IL-18) in promoting their expansion (193,194). Recently, data suggesting that recognition of HLA-E presenting peptides derived from the CMV UL40 leader sequence may drive the adaptive NKG2C+ NK cell response have been reported (150). Nevertheless, there is no consistent evidence supporting that interaction with CMV infected cells directly triggers their effector functions, which can be otherwise efficiently activated by CMV-specific antibodies through the CD16 receptor.

NKG2C+ NK cells were shown to expand following CMV infection in transplant recipients, and preliminary evidence indicating they may reduce the risk of CMV infection was reported (184,195). In this work we further explored both issues in a cohort of CMV+ KTR receiving a similar immunosuppressive regimen. DNAemia was regularly monitored during the first three months posttransplant and antiviral therapy was administered in case of symptomatic infection or DNAemia values exceeding a clinically established threshold. First, we addressed whether pretransplant adaptive NKG2C+ NK cells influence the risk of CMV infection. Our observations indicated that NKG2C+ NK cell subsets with an adaptive phenotype (NKG2A-, CD57+ and FcεRIγ-) were associated to a reduced incidence of posttransplant infection, independently of other related variables such as age. These features are also displayed by NKG2C- NK cell subsets (152,158,196), and epigenetic downregulation of FcεRIγ has been proposed as a broad marker of adaptive NK cells by some authors (157,160). However, functional differences have been reported between NKG2C+ FcεRIγ- and NKG2C- FcεRIγ- NK cells (196) and, according to our

observations, the latter appeared unrelated with the rate of CMV infection in KTR.

Multivariate Cox regression analysis indicated that the incidence of CMV infection in KTR was inversely associated with NKG2C+ CD57+ NK cells. Whereas a relation with NKG2C- CD57+ NK cells was observed, it did not reach statistical significance in multivariate analysis. CD57 expression in NK cells has been related to terminal differentiation, reduced proliferative potential, and shorter telomere lengths (197–199). Increased circulating CD57+ NK cells are associated with immune senescence, a process that may develop independently of chronological age, as shown for end-stage renal disease patients (189). Together these results pointed out a specific association between reduced risk of CMV infection and adaptive NKG2C+ NK cells, but not with other NK cell subsets sharing these differentiation markers.

Some KTR with high pretransplant levels of NKG2C+ NK cells experienced CMV infection. Moreover, the sensitivity calculated by ROC analysis (63%) was below that reported for IE-1-specific T cells analyzed by ELISpot in KTR (80%) (115), while specificity was similar in both studies. The possibility that the association of adaptive NKG2C+ NK cells with CMV infection might indirectly reflect the action of other immune cells was considered (191). Unfortunately, assessing the relation of adaptive NKG2C+ NK cells and CMV-specific T cells with CMV infection in the same KTR cohort was not feasible due to insufficient cryopreserved samples. This limitation was partially circumvented by comparing both

parameters in PBMC from patients with chronic renal disease in waiting list for transplantation and from healthy donors. No correlation between adaptive NKG2C+ NK cells and CMV-specific T cells was found, indirectly ruling out that the latter might account for the reduced incidence of viremia associated with adaptive NK cells. Moreover, no relation was observed between posttransplant CMV infection and TcRγδ Vδ2- T cells, whose expansions have been associated with the control of posttransplant CMV infection in KTR (200), nor with the NKG2C+ T cell subset which is also increased in CMV+ individuals albeit with unknown significance.

Additional NK cell populations have been reported to play a role in the response to CMV infection. Telomeric genes in KIR B haplotypes encoding activating receptors have been related to a reduced incidence of CMV infection (174). Furthermore, KIR3DL1+ NK cells have been reported to expand at the time of acute CMV infection and contract within 6 months (201). Analyzing KIR expression in NK cells was beyond the scope of this study but a relationship with our observations was considered. Adaptive NKG2C+ NK cells are known to display an oligoclonal pattern of inhibitory KIR, mainly specific for self HLA-C molecules, whereas KIR3DL1 (specific for Bw4+ HLA-B alleles) is infrequent. Moreover, NKG2C+ NK cell expansions were reported to be independent of KIR haplotypes (142,151), being observed in blood donors with no functional activating KIR (Vilches and López-Botet, unpublished). On that basis the possibility that activating KIRs might underlie the relation between NKG2C+ NK cells and a reduced risk of CMV infection is unlikely. Studies are

warranted to assess at the phenotypic level the relation of activating KIR with posttransplant CMV infection.

Of note, the effect of adaptive NK cells was perceived for symptomatic but not asymptomatic infection. A plausible interpretation is that adaptive NK cells contribute to prevent infection progression rather than impairing initial viral replication, which is controlled by specific T cells (202). Should the antiviral function of adaptive NK cells involve antibody-dependent activation, qualitative/quantitative differences in IgG specific for CMV antigens and/or reinfection with an antigen mismatched strain might be determinant (203).

In the second part of the study, we followed the evolution of the adaptive NKG2C+ NK cell response to posttransplant CMV infection. In contrast to the stability observed in healthy individuals after primary infection, posttransplant expansions of adaptive NKG2C+ NK cells were detected in CMV-infected KTR, though with marked individual differences regarding their magnitude and kinetics. In some patients NKG2C+ NK cells raised progressively over the years, while in others sharp increases were noticed promptly after detection of infection, persisting elevated along the follow-up. We interpret that these profiles reflect poor control of CMV replication, which might take place during the first months after transplant when DNAemia was systematically monitored, but also at any time along posttransplant evolution. Conversely, minor changes or steady levels of adaptive NKG2C+ NK cells, comparable to the common profile observed in non-infected KTR,

likely indicated prompt and stable resolution of CMV infection. NKG2C+ NK cell expansions were exceptionally detected in some cases categorized as non-infected; this finding presumably reflects subclinical CMV replication events occurring beyond the early DNAemia monitoring period.

In CMV+ KTR, posttransplant NKG2C+ NK cell expansions may derive from proliferation of a preexisting differentiated population, and/or *de novo* differentiation. These events may be dissociated, as illustrated by detection in some infected KTR of moderate increases of NKG2C+ NK cells with an adaptive phenotype, stressing the importance of analyzing other markers, particularly FcεRIγ downregulation. Of note, *de novo* generation of adaptive NKG2C+ NK cells has been observed in a group of high-risk CMV seronegative KTR suffering posttransplant infection despite antiviral prophylaxis (Ataya et al. unpublished observations), indicating that this process is, at least partially, resistant to standard immunosuppression.

CMV infection appears the primary determinant for triggering an adaptive NKG2C+ NK cell response, and thus its magnitude may be indirectly modulated by factors related with pathogen control. In this regard, the timing and efficacy of preemptive antiviral therapy may influence viral burden and thus antigenic stimulation of the immune system (204), blunting adaptive NKG2C+ NK cell development. In the same line, control of donor-derived CMV reinfection may be less efficient than that of viral reactivation (205), potentially favoring adaptive NK cell development. In addition,

despite that adaptive NK cells can develop in KTR, information is lacking on their sensitivity to the different immunosuppressive drugs shown to influence NK cell functions (206). It cannot be excluded that changes in the immunosuppressive regimen introduced along clinical management may modulate the adaptive NK cell response.

On the other hand, the weight of host/viral variables directly related with the underlying mechanisms is incompletely defined. *KLRC2* zygosity was reported to influence the numbers of circulating adaptive NKG2C+ NK cells, which tend to be greater in $KLRC2^{+/+}$ than in $KLRC2^{+/del}$ individuals (145,184,207). Yet, no association was found between *KLRC2* zygosity and the magnitude of posttransplant NKG2C+ NK cell expansions.

Activation through CD16 has been reported to induce the proliferation of adaptive NKG2C+ NK cells (160), which proficiently mediate ADCC against CMV-infected cells (155–157). This process, might be influenced by the concentrations of IgG specific for CMV antigens (e.g. gB), as well as by CD16 polymorphisms conditioning its affinity for IgG. Finally, the adaptive NKG2C+ NK cell response has been proposed to be triggered by CD94/NKG2C recognition of HLA-E bound to peptides from the CMV UL40 molecule (150). These mimic endogenous HLA-I-derived leader sequence nonamers, whose polymorphism influences the affinity for HLA-E (59). Prospective studies are required to assess whether variability of the UL40 leader sequence modulates the magnitude of the adaptive NKG2C+ NK

cell development in KTR; work is underway to test this hypothesis by sequencing UL40 from CMV in KTR plasma samples.

Additional observations in this study which contribute to better understand adaptive NK cell development in KTR deserve attention. A) The adaptive NK cell response did not correlate with the DNAemia peak and, remarkably, it was not perceived in three out of six cases with invasive disease present in the whole cohort. The lack of a response coincided with low DNAemia peak values, previously reported in gastrointestinal CMV infection (208). These data suggest that development of the adaptive NK cell response may be dependent on the tissue location and extension of viral replication (i.e. epithelial vs hematopoietic tissues). B) <LLOQ DNAemia levels were detected early in some KTR categorized as non-infected and with high pretransplant proportions of adaptive NKG2C+ NK cells, who did not receive antiviral therapy and did not display DNAemia in the late survey years after transplant. These observations are consistent with the association of pretransplant adaptive NKG2C+ NK cells with reduced incidence of symptomatic CMV infection, suggesting that they contribute to contain infection progression, but may not prevent early viral replication. C) As reported in healthy individuals (207), adaptive NKG2C+ NK cell expansions generally persisted long-term in KTR, but their proportions declined at late time points in some cases. The interpretation that NKG2C+ NK cells decrements detected long-term after transplant reflect CMV control does not correspond with the stability observed in healthy individuals. Alternatively, it is plausible that these late reductions may reflect an

altered turnover (209), promoted by the combined effects of sustained immunosuppression and CMV pressure.

Pretransplant levels of adaptive NKG2C+ NK cells were associated with reduced incidence of symptomatic infection, and therefore it is conceivable that development of the adaptive NK cell response in infected KTR may also contribute to restore CMV control. Consistent with this hypothesis, DNAemia was undetectable at the late follow-up time point in most infected KTR, including cases with adaptive NKG2C+ NK cell expansions. Moreover, in that survey 4 out of 5 cases with positive DNAemia values displayed low proportions (<20%) of NKG2C+ NK cells. Although these data suggest that NKG2C+ NK cell expansions might contribute to maintain CMV control, concomitant changes were detected in the T cell compartment of infected KTR, particularly increased proportions of CD8+ and TcRγδ Vδ2- T cells. An impact of posttransplant CMV infection on T cells has been previously reported (210). Moreover, frequencies of IE1- and pp65-specific T cells were comparable to non-infected patients. Such complex influence of CMV infection on the immune system, together with the diversity of the T cell response to viral antigens, not assessed in clinical studies (111,211), did not allow to discern the relative role played by the different lymphocyte subsets which likely cooperate in controlling the pathogen.

Altogether, these observations provide a broader perspective on the evolution of the adaptive NKG2C+ NK cell response to CMV infection in KTR, illustrating its individual variability. Expansions

of adaptive NKG2C+ NK cells following infection likely reflect an inefficient or delayed control of the pathogen. Therefore, their detection in KTR considered non-infected should warn on subclinical viral replication events. On the other hand, following their development, adaptive NKG2C+ NK cells may contribute with T cells to restore CMV control. From a practical standpoint, our results indicate that analysis of adaptive NKG2C+ NK cells in combination with CMV-specific T cells, may allow a more accurate assessment of the risk and evolution of CMV infection in KTR.

CONCLUSIONS

Objective 1

1. Pretransplant frequencies of adaptive NKG2C+ NK cells (i.e. NKG2A-, CD57+, FcεRIγ-) were associated with a reduced incidence of posttransplant CMV infection in KTR, independently of other variables related with this event (e.g. age).

2. Pretransplant adaptive NKG2C+ NK cells were significantly associated with reduced incidence of symptomatic infection, inversely correlating with peak viral loads.

3. The risk of CMV infection was unrelated with the frequencies of NKG2C- NK cell populations sharing adaptive-associated markers (i.e. CD57+, FcεRIγ-).

4. No correlation between adaptive NKG2C+ NK cells and CMV-specific T lymphocytes was detected in healthy donors and in chronic renal disease patients awaiting transplantation.

5. These results support that adaptive NKG2C+ NK cells may contribute to control CMV infection progression, rather than preventing initial viral replication.

Objective 2

1. NKG2C+ NK cell expansions were associated to posttransplant infection, but exceptionally detected in non-infected cases presumably reflecting the effect of subclinical viral replication events.

2. Individual variability was observed in the dynamics and magnitude of the adaptive NKG2C+ NK cell response to CMV infection. This was unrelated with peak viral load, pointing out the influence of additional factors.

3. Adaptive NK cell expansions generally persisted long-term after transplantation in CMV infected KTR, coinciding with increased proportions of CD8+ and TcRγδ Vδ2− T cells, thus their respective contribution to long-term CMV control cannot be ascertained.

4. A combined analysis of adaptive NKG2C+ NK and CMV-specific T cells may allow a more accurate assessment of the risk and evolution of CMV infection in KTR.

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Abbreviations

ABMR	Antibody mediated rejection
ADCC	Antibody-dependent cellular cytotoxicity
CD	Cluster of differentiation
CMV	Cytomegalovirus
DSA	Donor-specific antibodies
FcR	Fc-receptor
FcεRIγ	FcεRI gamma chain
Grz	Granzyme
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HSCT	Hematopoietic stem cell transplant
IFN	Interferon
IgG	Immunoglobuling G
IL	Interleukin
ILC	Innate lymphoid cells
ILT	immunoglobulin-like transcript
IQR	Interquartile range
ITAM	Immunoreceptor tyrosine-based activating motif
ITIM	Immunoreceptor tyrosine-based inhibitory motif
KIR	Killer Immunoglobulin-like receptors
KTR	Kidney transplant recipient
LILR	Leukocyte Immunoglobulin-like Receptor
LLOQ	Lower limit of quantification
MHC	Major histocompatibility complex
MVI	Microvascular inflammation

NCR	Natural cytotoxicity receptors
NK	Natural Killer cells
PBMC	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction
PLZF	Promyelocytic leukaemia zinc finger
PRA	Panel reactive antibodies
SOT	Solid organ transplant
TCR	T cell receptor
t-SNE	t-stochastic neighbor embedding
Tel B	Telomeric region of haplotype B
TLR	Toll-like receptor
TNF	Tumor necrosis factor
QNAT	Quantitative nucleic acid amplification testing
t-SNE	t-distributed stochastic neighbor embedding

Appendix

Publications not included in the thesis:

1. Costa-García M, Ataya M, Moraru M, Vilches C, López-Botet M, Muntasell A. Human Cytomegalovirus Antigen Presentation by HLA-DR+ NKG2C+ Adaptive NK Cells Specifically Activates Polyfunctional Effector Memory CD4+ T Lymphocytes. *Front Immunol*. 2019; 10:687. doi:10.3389/fimmu.2019.00687