Received: 3 February 2021 | Revised: 8 June 2021 | Accepted: 10 June 2021

DOI: 10.1111/ajt.16725

ORIGINAL ARTICLE

Circulatory follicular helper T lymphocytes associate with lower incidence of CMV infection in kidney transplant recipients

Patricia Suàrez-Fernández[1] | Alberto Utrero-Rico[1] | Virginia Sandonis[2] | Estéfani García-Ríos[2] | Daniel Arroyo-Sánchez[1,3] | Mario Fernández-Ruiz[1,4] | Amado Andrés[5] | Natalia Polanco[5] | Cecilia González-Cuadrado[1] | Patricia Almendro-Vázquez[1] | Pilar Pérez-Romero[2] | José María Aguado[1,4] | Estela Paz-Artal[1,3,6] | Rocío Laguna-Goya[1,3]

[1]Instituto de Investigación Biomédica Hospital 12 de Octubre (imas12), Madrid, Spain

[2]National Center for Microbiology, Instituto de Salud Carlos III, Madrid, Majadahonda, Spain

[3]Department of Immunology, Hospital Universitario 12 de Octubre, Madrid, Spain

[4]Unit of Infectious Diseases, Hospital Universitario 12 de Octubre, Madrid, Spain

[5]Department of Nephrology, Hospital Universitario 12 de Octubre, Madrid, Spain

[6]Department of Immunology, Universidad Complutense de Madrid, Madrid, Spain

Correspondence
Patricia Suàrez-Fernández, Instituto de Investigación Biomédica Hospital 12 de Octubre (imas12), Madrid, Spain.
Email: suarez.fernandez.imas12@h12o.es

Funding information
Instituto de Salud Carlos III, Grant/Award Number: PI17-01120, PI17CIII-00014, PI18/969 and PIE13/00045

Primary infection and/or reactivation of cytomegalovirus (CMV) in kidney transplant recipients (KTR) favor rejection and mortality. T follicular helper cells (TFH) could contribute to protection against CMV. Circulatory TFH (cTFH) were studied pretransplant and early posttransplant in 90 CMV seropositive KTR not receiving antithymocyte globulin or antiviral prophylaxis, followed-up for 1 year. Patients who presented CMV infection had significantly lower cTFH and activated cTFH pretransplant and early posttransplant. Pretransplant activated cTFH were also lower within patients who developed CMV disease. Pre- and 14 days posttransplant activated cTFH were an independent protective factor for CMV infection (HR 0.41, $p = .01$; and 0.52, $p = .02$, respectively). KTR with low cTFH 7 days posttransplant (<11.9%) had lower CMV infection-free survival than patients with high cTFH (28.2% vs. 67.6%, $p = .002$). cTFH were associated with CMV-specific neutralizing antibodies (Nabs). In addition, IL-21 increased interferon-γ secretion by CMV-specific $CD8^+$ T cells in healthy controls. Thus, we show an association between cTFH and lower incidence of CMV infection, probably through their cooperation in CMV-specific Nab production and IL-21-mediated enhancement of $CD8^+$ T cell activity. Moreover, monitoring cTFH pre- and early posttransplant could improve CMV risk stratification and help select KTR catalogued at low/intermediate risk who could benefit from prophylaxis.

KEYWORDS
basic (laboratory) research / science, flow cytometry, immunobiology, infection and infectious agents - viral: Cytomegalovirus (CMV), infectious disease, kidney transplantation / nephrology, translational research / science

Abbreviations: CMI, T cell–mediated immune response; CMV, cytomegalovirus; cTFH, circulatory T follicular helper cells; HR, hazard ratio; HSCT, hematopoietic stem cell transplant; IFN, interferon; IL-21, interleukin-21; KTR, kidney transplant recipients; Nab, neutralizing antibody; SOT, solid organ transplant; TFH, T follicular helper cells; Tx, transplantation.

1 | INTRODUCTION

Cytomegalovirus (CMV) causes one of the most relevant infections following transplantation, in certain cases triggering allograft rejection[1] and even death.[2,3] Despite efforts to define the risk after transplantation based on CMV donor-recipient paired serostatus, and the use of CMV prophylaxis in selected cases, an important number of patients develop CMV infection.

T cell–mediated immune response (CMI) has a major role in protection against CMV infection.[4,5] The correlation between CMI and protection from CMV infection in transplant recipients has already been shown.[6–8] However, the extended use of lymphocyte-depleting agents for induction therapy, or as a treatment for allograft rejection, causes a drug-induced deficiency in global and CMV specific immunity.[9,10] Although serum levels of these drugs diminish during the first few months posttransplant, profound T cell lymphopenia may persist for up to 1 year, increasing the risk of CMV reactivation.[11,12]

Recent evidence indicates that the humoral response, particularly neutralizing antibodies, may also be necessary for protection against CMV infection.[13] There are several studies supporting this protective role of antibodies against CMV in solid organ transplant (SOT) recipients. Gabanti et al[8] showed that a proportion of transplant patients with CMV-specific $CD8^+$ T cells were not protected against new CMV episodes and long-term protection from CMV infection was only reached in the presence of specific $CD4^+$ T cells, suggesting the need for other arms of the adaptive immunity to control CMV infection. Moreover, a study in SOT recipients demonstrated a correlation between neutralizing antibody (Nabs) titers and protection from infection.[14] In addition, the administration of CMV-specific hyperimmune globulin was found to prevent CMV disease[15] and to improve survival rates[16] in transplant patients. Similarly, in a hematopoietic stem cell transplant (HSCT) murine model depleted of all T cell subsets, transfer of immune serum prevented viral reactivation.[17]

T follicular helper lymphocytes (TFH) are a subset of $CD4^+$ T lymphocytes which express BCL6 and CXCR5 and were first identified in human tonsils.[18] High CXCR5 and low CCR7 expression enables TFH to leave the T-zone in lymphoid organs and migrate to germinal centers. By secreting interleukin (IL)-21 and IL-4, and through CD40-CD40L and OX40-OX40L interactions, TFH promote the differentiation of B cells into memory and antibody-secreting plasma cells.[19,20] Based on their expression of CXCR3 and CCR6 TFH can be divided in TFH1 ($CXCR3^+CCR6^-$), TFH2 ($CXCR3^-CCR6^-$) and TFH17 ($CXCR3^-CCR6^+$) subsets with different helper capacity.[21] TFH are also present in peripheral blood (circulating TFH, cTFH). cTFH exhibit TFH-like properties such as T:B cooperation, CXCR5 expression and IL-21 secretion and have been identified as memory counterparts of secondary-lymphoid organ TFH.[22,23] In addition to the promotion of neutralizing and non-neutralizing antibody production, TFH could enhance cellular responses through the secretion of IL-21, since IL-21 has been shown to increase the $CD8^+$ T cell effector functions.[24,25] According to these capacities, cTFH have been involved in immune responses against a variety of human infections such as influenza,[26,27] papillomavirus[28] and malaria,[29] and have been related to the production of Nabs in Human Immunodeficiency virus (HIV) and Hepatitis C Virus (HCV) infected patients.[30,31]

Due to the dual influence of IL-21 in humoral and cellular responses, we hypothesize that cTFH could have a protective role against CMV infection. The aim of this study was to evaluate the potential protective effect of cTFH against CMV infection in kidney transplant recipients (KTR), and the mechanisms that mediate protection.

2 | MATERIALS AND METHODS

2.1 | Study design and patients

We included all KTR between November 2014 and June 2016 at Hospital Universitario 12 de Octubre ($N = 227$). All patients signed a written informed consent. The institutional medical ethical committee (reference 14/245) approved the study. Since the aim of the study was to evaluate the potential protective effect of cTFH against CMV in KTR, patients who received anti-CMV prophylaxis with (val)ganciclovir or the cell-depleting agent antithymocyte globulin as induction therapy were excluded. Pretransplant CMV seronegative recipients and patients lacking blood samples were also excluded. The final cohort for analysis included 90 patients (Figure 1). Blood samples were collected few hours before the surgery and at days 7 and 14 posttransplant. Clinical and immunological data were collected during 1 year

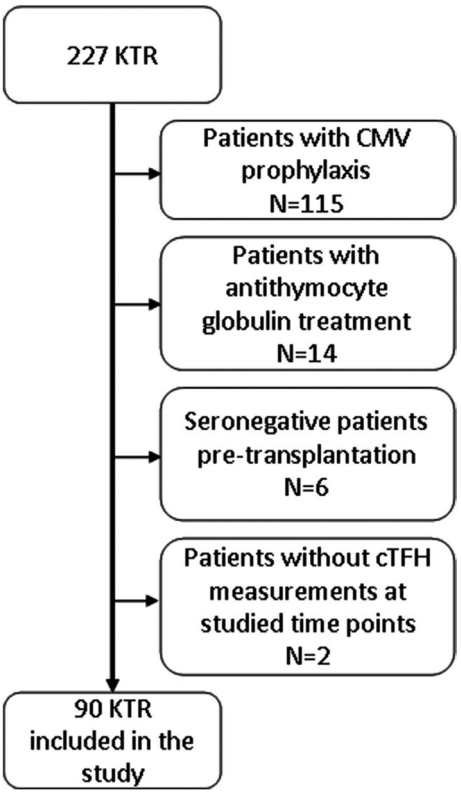


FIGURE 1 Flowchart of patients included in the study

of follow-up. Rejection was defined as biopsy-proven acute rejection (BPAR) according to the 2013 and 2015 Banff classification.[32,33]

CMV viral load was determined using real-time polymerase chain reaction (RT-PCR) every week or every 2 weeks during the first 2 months posttransplant, and monthly or at any time when clinically indicated until completing the follow-up period. CMV infection was defined when a new replication episode occurred with high-level CMV DNAemia (>1000 IU/ml). Patients without CMV replication and with low-level CMV DNAemia (<1000 IU/ml) were included in the "no infection" group. CMV disease was defined as CMV infection accompanied by consistent clinical signs and symptoms.[34] Intravenous ganciclovir (5 mg/Kg/12 h) or oral valganciclovir (900 mg/12 h) for at least 2 weeks was initiated in the presence of high-level or rapidly increasing CMV DNAemia.

2.2 | cTFH phenotyping by flow cytometry

cTFH were prospectively studied pretransplant and at days 7 and 14 posttransplant. Freshly isolated peripheral blood mononuclear cells were stained with the following monoclonal antibodies (mAb): CD4 FITC, CXCR5 APC, CCR7 PerCP-Cy 5.5 (all from BD) and PD1 PE (eBioscience). We considered cTFH as $CD4^+CXCR5^+$ and activated cTFH as $CD4^+CXCR5^+CCR7^{lo}PD1^{hi}$ cells (Figure S1A). Cells were acquired using a BD Canto II flow cytometer and the results were analyzed with the Flow Jo V10 software.

2.3 | Microneutralization assay

Nab titers were determined in the 73 pretransplant serum samples available by microneutralization assays as previously published.[14] Briefly, heat inactivated sera were two-fold serially diluted (from 1/5 to 1/2560), and each dilution incubated with the BADrUL131-Y4 strain during 2 h at 37°C with 5% CO2. Virus-serum mixture was added to confluent ARPE-19 cell monolayers. After 48 h of incubation at 37°C, cells were stained with p72-(immediate-early-1 protein) mAb (MAB810R CMV, Clone 8B1.2, Millipore) and HRP-conjugated horse anti-mouse IgG (Cell Signaling) as secondary antibody. After peroxidase substrate addition (3.3',5.5'-Tetrametyl-benzidine Liquid Substrate, Supersensitive for ELISA, Sigma-Aldrich) the reaction was stopped with 1N sulfuric acid. We defined the Nab titers as the serum titer that reduced virus infectivity by 50% or more compared with the infected control. Based on the work by Blanco-Lobo et al[14] we considered positive a 1/320 or higher Nab titer, assuming that a minimum Nab titer is necessary for protection.

2.4 | Functional assessment of CMV-specific $CD8^+$ T cells

Cytomegalovirus-specific CMI (CMV-CMI) was evaluated in nine seropositive healthy controls by a modified version of the QuantiFERON®-CMV test (Qiagen). One ml of heparinized whole blood was incubated in each of the 5 QTF-CMV tubes evaluated: one tube contained phytohemagglutinin (PHA) (positive control), two negative control tubes were used with or without 100 ng/ml of recombinant human IL-21 (RPB688Hu01, Cloud-Clone Corp) and two tubes contained a pool of CMV peptides, with or without recombinant IL-21 added. The five tubes were incubated for one hour at 37°C. Afterwards, 10 μg/ml of brefeldin A (Merck group) was added to each tube. Samples were incubated for 18 h at 37°C followed by treatment with 10 ml of FACS Lysis Solution (BD). For the assessment of $CD8^+$ T cell activation, cells were surface-stained with CD3-Pacific Blue (Beckman Coulter) and CD8-PerCP-Cy 5.5 (BD), and intracellularly stained with interferon (IFN)-γ-APC (BD), using the Miltenyi Inside Stain kit. Cells were acquired using a BD Canto II flow cytometer and the results were analyzed with Flow Jo V10 software. All subjects presented >10% of IFN- γ-producing $CD8^+$ T cells in PHA tube, and <0.5% IFN-γ-producing $CD8^+$ T cells in negative control tube. Results were presented as the frequency of IFN-γ-producing $CD8^+$ T cells in CMV-peptide tube (±IL-21) minus the frequency of IFN-γ-producing $CD8^+$ T cells in negative control tube (±IL-21).

2.5 | CMV-specific neutralizing antibody culture

The relationship between cTFH subsets and activation with anti-CMV Nabs induction was evaluated *in vitro* in 7 seropositive healthy donors. 2×10^6 PBMCs were cultured for 3 and 10 days in 1.5 ml of IMDM medium (ThermoFisher Scientific) supplemented with 1% penicillin-streptomycin and 2,5 μg/ml of anti-CD28 (Mabtech) in the presence and absence (negative control) of 1 μg/ml CMV-peptides (PepMixTM Pan-CMV, JPT Peptide Technologies). At day 3, cells were surface-stained with CD4-APC-H7, CXCR5-APC, CXCR3-PE, CCR6-BB515, ICOS-BV421 and PD1-PE-Cy7 (all from BD); acquired using BD Canto II flow cytometer and the results were analyzed with Flow Jo V10 software. cTFH subsets were defined as: cTFH1 ($CXCR3^+CCR6^-$), cTFH2 ($CXCR3^-CCR6^-$), and cTFH17 ($CXCR3^-CCR6^+$). At day 10 cell culture supernatants were collected and Nabs titers were determined by the microneutralization assay described above.

2.6 | Statistical analysis

Quantitative data in graphs were shown as the median with interquartile range (IQR) and compared by Mann Whitney U test or Kruskal–Wallis test when necessary. Paired analyses were performed using Wilcoxon signed-rank test. Categorical variables were represented as percentages and compared with the Fisher's exact test. The correlation between cTFH and CMV infection time was assessed with a Spearman test and graphically represented by scatter plot and regression line. The risk for CMV infection was analyzed using a univariate Cox proportional-hazards model for each

TABLE 1 Demographic and clinical characteristics

	Overall cohort, $n = 90$	No CMV infection, $n = 47$	CMV infection, $n = 43$	p values
Age in years (median, range)	63 (18–83)	57 (18–80)	66 (33–83)	**.01**
Gender (male)	72 (80.0%)	36 (76.6%)	36 (83.7%)	.76
Cause of end-stage renal disease	20 (22.2%)	9 (19.1%)	11 (25.6%)	.36
Diabetic nephropathy				
IgA nephropathy	8 (8.9%)	2 (4.3%)	6 (14.0%)	
Polycystic kidney disease	10 (11.1%)	7 (14.9%)	3 (7.0%)	
Hypertensive nephrosclerosis	9 (10.0%)	5 (10.6%)	4 (9.3%)	
Focal segmental glomerulonephritis	4 (4.5%)	3 (6.4%)	1 (2.3%)	
Membranoproliferative glomerulonephritis	3 (3.3%)	—	3 (7.0%)	
Other glomerulonephritis	7 (7.8%)	5 (10.6%)	2 (4.6%)	
Tubulointerticial nephropathy	5 (5.5%)	3 (6.4%)	2 (4.6%)	
Vasculitis	2 (2.3%)	2 (4.3%)	—	
Other	22 (24.4%)	11 (23.4%)	11 (25.6%)	
Previous kidney transplantation	3 (3.4%)	2 (4.3%)	1 (2.3%)	.61
Transplant type				.49
Renal	88 (97.7%)	45 (95.7%)	43 (100%)	
LKT	2 (2.3%)	2 (4.3%)	—	
Transplant source				**.04**
Brain death donor	73 (81.1%)	34 (72.4%)	39 (90.7%)	
Living donor	15 (16.7%)	11 (23.4%)	4 (9.3%)	
Cardiac death donor	2 (2.2%)	2 (4.2%)	—	
Induction therapy	62 (68.9%)	31 (66.0%)	31 (72.1%)	.73
Basiliximab				
Rituximab	1 (1.1%)	1 (2.1%)	—	
No induction	27 (30.0%)	15 (31.9%)	12 (27.9%)	
Maintenance immunosuppression				
Steroids + Tacrolimus + MPA	90 (100%)	47 (52.2%)	43 (47.8%)	>.99
Delayed graft function[a]	43 (47.8%)	21 (44.7%)	22 (51.2%)	.67
BPAR within 12 months of transplantation	7 (7.7%)	3 (6.4%)	4 (9.3%)	.70
CMV serostatus				.55
D+/R+	77 (85.6%)	39 (83.0%)	38 (88.4%)	
D−/R+	13 (14.4%)	8 (17.0%)	5 (11.6%)	
CMV infection	43 (47.8%)	—	43 (100%)	
Days from transplant to CMV infection onset (median, range)	53 (13–342)	—	53 (13–342)	
CMV disease	12 (13.3%)	—	12 (27.9%)	
Days from transplant to CMV disease onset (median, range)	57 (13–342)	—	57 (13–342)	

Note: Significant p values are represented in bold.

Abbreviations: BPAR, biopsy proven acute rejection; CMV, cytomegalovirus; D, donor; LKT, liver kidney transplant; MPA, mycophenolic acid; R, recipient.

[a]Dialysis requirement in first two postoperative weeks.

variable, and then adjusted including all significant variables in a multivariable Cox proportional-hazards model. We evaluated each cTFH measure as a potential biomarker for CMV infection by area under the receiver operating characteristic curves (AUROC). A cut-off selection for variables with values higher than 0.70 in the AUROC analysis, was made with Youden's index. Time-to-event curves were plotted by Kaplan–Meier estimator and compared with the long-rank test to evaluate the suitability of the cut-off. For the evaluation of IFN-γ-producing $CD8^+$ T cells, we performed a Wilcoxon signed rank test.

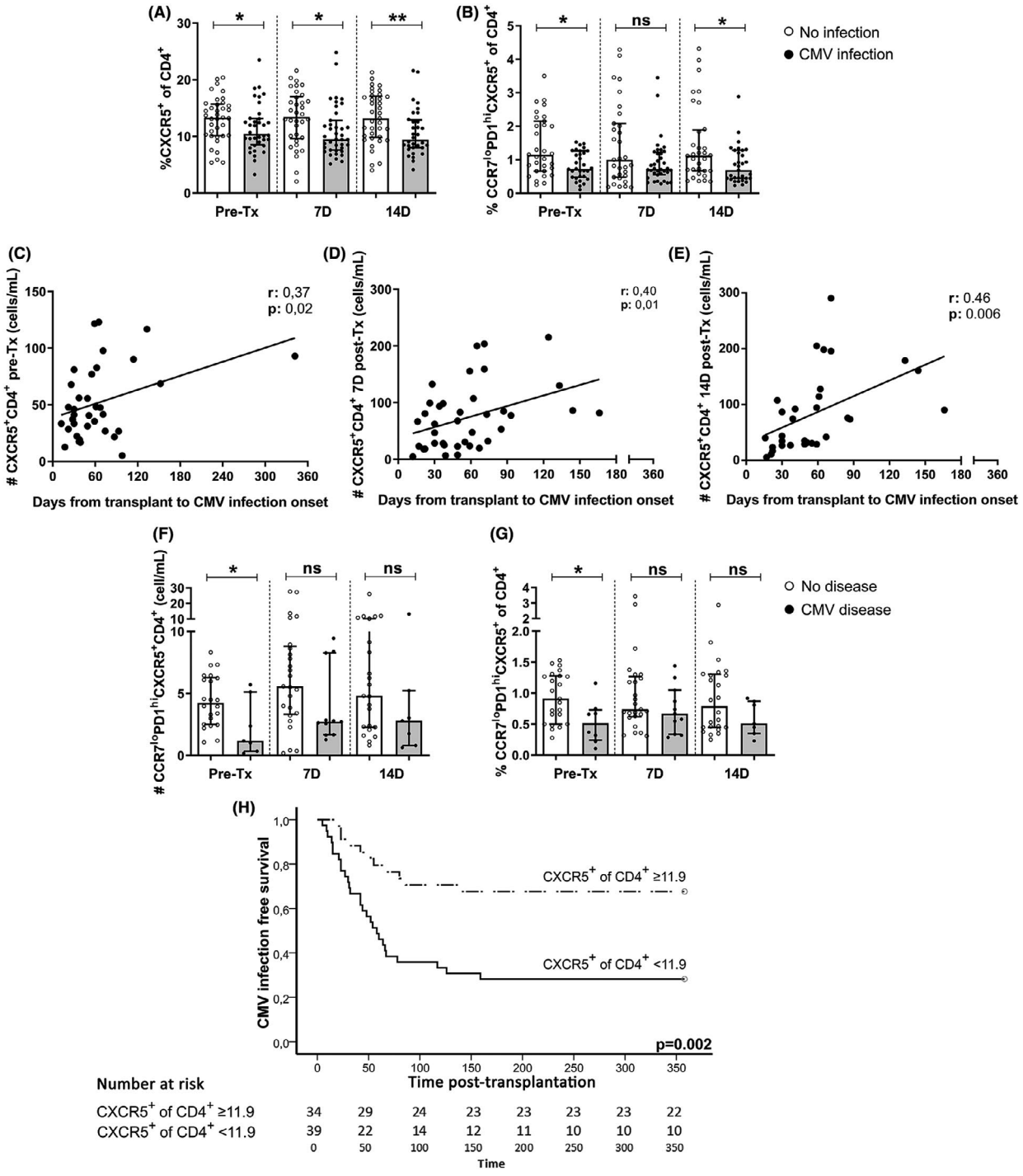


FIGURE 2 Higher pretransplant cTFH correlated with lower incidence of CMV infection and disease posttransplant. (A) Patients who did not present CMV infection during the first year posttransplant had higher cTFH than those patients who presented CMV infection ($p = .01$, $p = .01$ and $p = .008$, respectively; $N = 37$ vs. 36 pretransplant; 34 vs. 39 at 7 days; and 37 vs. 32, at 14 days posttransplant). (B) Patients without CMV infection also presented higher frequencies of activated cTFH pretransplant and 14 days posttransplant than those who presented CMV infection ($p = .01$ and $p = .02$, respectively; $N = 31$ vs. 31 pretransplant; 31 vs. 36 at 7 days; and 32 vs. 31 14 days posttransplant). (C-E) The higher cTFH numbers pretransplant ($N = 36$), 7 days ($N = 39$), and 14 days posttransplant ($N = 32$), the later CMV infection appeared. (F-G) Absolute numbers and frequencies of activated cTFH pretransplant were higher in KTR who did not develop CMV disease during the first year of follow-up, compared to CMV-infected patients who developed CMV disease ($p = .02$ and $p = .01$; $N = 23$ vs. 8; 25 vs. 11; and 24 vs. 7 respectively, in both panels). (H) Kaplan–Meier analysis based on Youden's index optimal cut-off showed a higher CMV infection-free survival rate in patients with high CXCR5+CD4+ T cell frequencies 7 days posttransplant (≥11.9%), compared to patients with low frequencies ($p = .002$). $^{*}p < .05$ $^{**}p < .01$ $^{***}p < .001$

The analysis was performed using SPSS version 23 (IBM Corp.) and GraphPad Prism version 6.0 software (GraphPad Software Inc). Differences were considered statistically significant when $p < .05$.

3 | RESULTS

3.1 | Characteristics of the patient cohort

The main characteristics of the 90 KTR included patients are shown in Table 1. Median age was 63 years. Most patients were male, had their first kidney transplant and received basiliximab as induction therapy. The incidence of CMV infection was in 43 out of 90 patients (47.8%), with a median time from transplantation to CMV infection onset of 53 days (ranging from 13 to 342 days). Patients who developed CMV infection were significantly older than those who did not (66 vs. 57 years, $p = .01$) and were more likely to receive a kidney from a brain death donor versus from a living donor (90.7% vs. 72.4%, $p = .04$). Although CMV serostatus is an established factor influencing the frequency of CMV infection in early posttransplant, in our cohort the incidence of infection between D+/R+ versus D-/R+ did not reach statistically significant difference ($p = .55$). Only 12 out of 90 patients presented CMV disease (13.3%), diagnosed at a median time of 57 days after transplantation. All KTR received steroids, tacrolimus, and mycophenolic acid as maintenance immunosuppression. Nine patients who presented CMV infection were converted to mTORi, in all cases weeks after the first CMV replication episode. Seven patients (7.8%) were diagnosed with BPAR during the 1-year follow-up. Four BPAR episodes (57%) occurred in the CMV-infected group, in one case the BPAR occurred prior to viral replication. No association was found between CMV infection and rejection (data not shown).

3.2 | Patients with higher cTFH had lower incidence of CMV infection

The frequency of cTFH and activated cTFH remained stable from pretransplant to the first and second weeks posttransplant (cTFH: 12.1%, 11.6%, and 11.4%, $p = .81$; and activated cTFH: 0.91%, 0.82%, and 0.89%, $p = .87$, Figure S1B-C) as previously published in patients with no induction or basiliximab, and in distinction to patients who received antithymocyte globulin.[35] In this work, we compared cTFH between KTR who presented CMV infection ($N = 43$) and those who did not ($N = 47$). Patients who developed CMV infection had significantly lower proportions of cTFH at pretransplant, and 7 and 14 days posttransplant, than those without infection (10.45% vs. 13.3% $p = .04$, 9.49% vs. 13.45%, $p = .01$ and 9.42% vs. 13.2%, $p = .008$, respectively; Figure 2A). Similarly, activated cTFH frequencies were consistently lower in patients who subsequently developed CMV infection as compared to patients who did not develop CMV infection, with a statistically significant difference at pretransplant (0.73% vs. 1.15%, $p = .01$) and 14 days posttransplant (0.69% vs. 1.12%, $p = .02$, Figure 2B). Moreover, time from transplant to CMV infection onset positively correlated with pretransplant, and 7 and 14 days posttransplant cTFH absolute numbers ($p = .02$, $p = .01$ and $p = .006$, Figure 2C-E), indicating that viral replication was detected later in patients with higher cTFH.

3.3 | CMV-infected patients with higher pretransplant cTFH were less likely to present CMV disease

We analyzed whether cTFH also contributed to a reduced progression from CMV infection to disease. The 43 patients with CMV infection episodes were divided into those who presented CMV disease ($N = 12$) and those who did not ($N = 31$). The absolute numbers and frequency of pretransplant activated cTFH cells were higher in KTR without CMV disease, compared to those with CMV disease (4.23 vs. 1.18 cells/μL $p = .02$, and 0.91% vs. 0.51% $p = .01$, respectively, Figure 2F-G).

3.4 | cTFH were a protective factor against CMV infection and could be used to stratify infection risk

Since cTFH have been correlated with protection against multiple infections,[26,27,30] we tested the association between cTFH and CMV infection risk in KTR. Using a Cox regression model we found that higher frequencies of activated cTFH at pretransplant and 14 days after transplant were a protective factor against CMV infection with a hazard ratio (HR) of 0.44 (95% CI: 0.23–0.83, $p = .01$) and 0.53 (95% CI: 0.29–0.95, $p = .03$) respectively (Table 2). As we observed significant differences in age and type of donor between patients with and without CMV infection (Table 1), we performed a Cox regression model corrected by these two variables. Pre- and 14 days posttransplant activated cTFH remained as an independent protective factor for CMV infection (HR 0.41, 95% CI 0.21–0.80, $p = .009$; and HR 0.52, 95% CI: 0.30–0.90, $p = .02$) (Table 2).

The proportion of cTFH ($CXCR5^+$ of $CD4^+$ T cells) at 7 and 14 days posttransplant also showed a significant protective effect for CMV infection (HR 0.92, 95% CI: 0.86–0.99, $p = .03$; and HR 0.89, 95% CI: 0.89–0.97, $p = .01$). However, the magnitude of the effect was lower than for activated cTFH and the statistical significance was lost when adjusted by age and type of donor ($p = .07$ and $p = .05$) (Table 2).

To evaluate the potential of cTFH as an additional marker to stratify CMV infection risk, we performed an AUCROC analysis for the frequency of cTFH and of activated cTFH at different time points: pretransplant, at 7 and 14 days posttransplant (Table 3). The frequency of cTFH at 7 days posttransplant presented an

TABLE 2 Univariate and multivariate hazard ratios for CMV infection risk

		Univariate analysis		Multivariate analysis	
		Crude HR[a]		Adjusted HR	Adjusted
	Variable	(95% CI)	*p* value	(95% CI)	*p* value
Pre-Tx	Age	1.03 (1.00–1.05)	**.01**		
	Transplant source *(living donor)*	0.37 (0.14–0.99)	**.04**		
	%CXCR5$^+$ of CD4$^+$ T cells	0.93 (0.86–1.01)	.12		
	%CCR7^{lo}PD1^{hi}CXCR5$^+$ of CD4$^+$ T cells	0.44 (0.23–0.83)	**.01**	0.41 (0.21–0.80)	**.009**
7 days post-Tx	Age	1.03 (1.00–1.05)	**.01**		
	Transplant source *(living donor)*	0.37 (0.14–0.99)	**.04**		
	%CXCR5$^+$ of CD4$^+$ T cells	0.92 (0.86–0.99)	**.03**	0.93 (0.87–1.00)	.07
	%CCR7loPD1hiCXCR5$^+$ of CD4$^+$ T cells	0.68 (0.45–1.03)	.07		
14 days post-Tx	Age	1.03 (1.00–1.05)	**.01**		
	Transplant source *(living donor)*	0.37 (0.14–0.99)	**.04**		
	%CXCR5$^+$ of CD4$^+$ T cells	0.89 (0.82–0.97)	**.01**	0.91 (0.84–1.00)	**.05**
	%CCR7^{lo}PD1^{hi}CXCR5$^+$ of CD4$^+$ T cells	0.53 (0.29–0.95)	**.03**	0.52 (0.30–0.90)	**.02**

Note: Significant *p* values are represented in bold.

Abbreviations: CI, confidence interval; HR, hazard ratio; Tx, transplantation.

[a]Numerical variables are continuous and studied as increments.

TABLE 3 Variables analyzed as potential biomarkers for CMV infection

		ROC curve		Cox analysis[a]	
	Variable	AUC (95% CI)[b]	Cut-off	Adjusted HR (95% CI)	*p* value
Pre-Tx	%CCR7^{lo}PD1^{hi}CXCR5$^+$ of CD4$^+$ T cells	0.68 (0.54–0.81)			
	%CXCR5$^+$ of CD4$^+$ T cells	0.64 (0.51–0.76)			
7 days post-Tx	%CCR7^{lo}PD1^{hi}CXCR5$^+$ of CD4$^+$ T cells	0.58 (0.45–0.72)			
	%CXCR5$^+$ of CD4$^+$ T cells	0.71 (0.58–0.84)	≤11.9	3.21 (1.59–6.49)	**.001**
14 days post-Tx	%CCR7^{lo}PD1^{hi}CXCR5$^+$ of CD4$^+$ T cells	0.67 (0.53–0.80)			
	%CXCR5$^+$ of CD4$^+$ T cells	0.68 (0.56–0.81)			

Note: Significant *p* values are represented in bold.

Abbreviations: AUC, area under ROC curve; ROC, receiver operating characteristic.

[a]Numerical variables are continuous and studied as increments.

[b]Only variables with AUC higher than 0.70 were considered as potential biomarkers for CMV infection. Further analyses were not performed when AUC was lower than 0.70.

AUC of 0.71 (95% CI: 0.58–0.84). The optimal cut-off based on Youden´s index was calculated in order to classify patients into high or low risk of developing CMV infection. KTR with low cTFH frequencies at 7 days posttransplant, below the cut-off of 11.9%, had lower CMV infection-free survival during the first year post-transplant than patients with high cTFH frequencies (28.2% vs. 67.6%, $p = .002$, Figure 2H). Those patients with a low frequency of cTFH (<11.9% at 7 days posttransplant had 3.21 times more risk of developing CMV infection [HR 3.21, 95% CI: 1.59–6.49, $p = .001$]) (Table 3). We also studied the suitability of cTFH as a CMV disease-stratifying tool with AUCROC analysis (Table S1). Despite acceptable AUC values, mostly above 0.70, optimal cut-offs did not allow for the classification of patients into low and high risk of CMV disease.

In summary, for every 1% decrease in pretransplant and 14 days posttransplant activated cTFH frequencies, patients were 2.44 and 1.92 times more likely to present CMV infection. Additionally, cTFH at 7 days posttransplant helped to identify patients with higher risk of presenting CMV infection during the first year posttransplant.

FIGURE 3 Assessment of potential cTFH-mediated mechanisms involved in reducing CMV infection and disease incidence. (A) KTR positive for pretransplant CMV-specific Nabs (titer ≥1/320) had higher pretransplant cTFH frequencies than KTR without Nabs ($p = .004$) ($N = 48$ and 25, respectively). (B) Pretransplant cTFH frequencies above the median (≥12.1%) were related with presence of pretransplant CMV-specific Nabs ($p = .01$). (C) PBMCs from 7 seropositive healthy donors were cultured with and without CMV peptides. cTFH subsets were analyzed at day 3, and Nab production in the supernatants at day 10. Only assays in which Nabs were detected (six out of seven) were analyzed. Frequencies of activated cTFH1 (D), cTFH2 (E), and cTFH17 (F) were increased in wells stimulated with CMV-peptides compared with negative controls (all $p = .03$). (G) Nab titers correlated with activated cTFH17 frequencies ($p = .01$) but not with activated cTFH1 or cTFH2 ($N = 6$, all panels). (H) cTFH17 subset included more activated cells than cTFH1 or cTFH2 subsets ($p < .01$. $N = 6$). (I) The frequency of IFN-γ-producing CD8+ T cells upon stimulation with CMV peptides was increased by the addition of IL-21 ($p = .007$), in CMV seropositive healthy volunteers ($N = 9$). (J) Representative example of IFN-γ production by CD8+ T cells recorded by flow cytometry in the modified QuantiFERON®-CMV assay

3.5 | cTFH could associate with lower incidence of CMV infection by enhancement of CMV-specific Nabs and CD8$^+$ T cells

We first studied the association between cTFH and CMV-specific Nabs, to determine if the apparent protective role of cTFH could be mediated by collaboration in the anti-CMV defensive humoral response. We defined a cut-off titer ≥1/320 for Nab positivity based on previous work.[14] In our cohort of CMV seropositive KTR, 46 patients (63%) had Nabs pretransplant, while 27 (37%) did not have Nabs. Although interpersonal differences in cTFH frequencies are relatively high, KTR with Nabs pretransplant had higher proportions of pretransplant cTFH than patients without Nabs (13.2% vs. 10.1%, $p = .004$, Figure 3A). The median frequency of cTFH pretransplant (12.1%) was used to divide the cohort into KTR with high (≥12.62%) and low (<12.1%) cTFH. 29/38 (76.3%) KTR with high cTFH pretransplant (above the median) had CMV-specific Nabs while 17/35 (48.6%) KTR with low cTFH had them ($p = .01$, Figure 3B). In an *in vitro* assay (Figure 3C) we found a CMV-specific activation of the three cTFH subsets, namely cTFH1 ($p = .03$, Figure 3D), cTFH2 ($p = .03$, Figure 3E), cTFH1 ($p = .03$, Figure 3F). We found no correlation between activated cTFH1 and cTFH2 and CMV-specific Nabs, while activated cTFH17 positively correlated with Nab titers ($r^2 = .82$, $p = .01$, Figure 3G). In fact, the cTFH17 subset included significantly more activated cells than cTFH1 or cTFH2 subsets was the most activated subset ($p = .002$, Figure 3H).

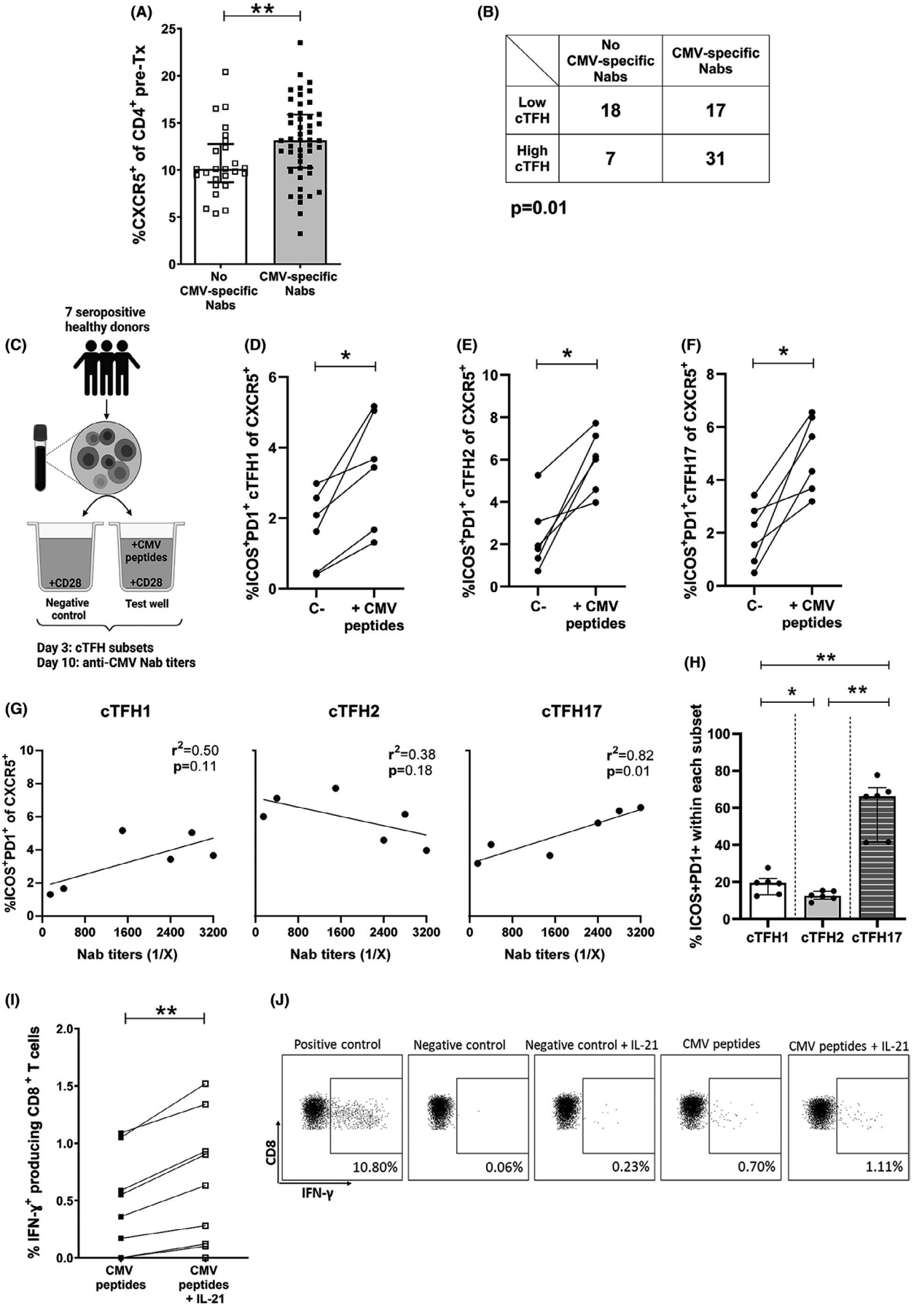
In addition, we asked if cTFH could reduce CMV infection incidence by enhancing the CMV-specific CD8$^+$ T cell effector response through IL-21 production, being TFH the most important cell source of this cytokine.[36] A modified Quantiferon test was performed in nine CMV seropositive healthy volunteers, in order to test the specific cellular response of CD8$^+$ T cells to CMV peptides in the presence or absence of IL-21. The frequency of IFN-γ-producing CD8$^+$ T cells increased significantly when IL-21 was added to the stimulus with CMV peptides ($p = .007$, Figure 3I), suggesting that this mechanism could mediate the observed relationship between cTFH and reduction in CMV infection incidence.

4 | DISCUSSION

Several studies have identified cTFH as being crucial for the production of antibodies that protect against viral infection.[26–28,30,31] We demonstrate here that cTFH associates with a lower incidence in CMV infection and disease in KTR. The potential mechanisms for this protective effect may include the TFH cooperation for the production of CMV-specific Nabs and the IL-21-mediated strengthening of CD8$^+$ T cell effector function.

The protective immune response against CMV was initially thought to be mediated essentially by CMV-specific CD8+ T cells, and many studies focused on demonstrating that a robust CD8$^+$ T cell reconstitution was associated with a reduction in CMV reactivation and disease after HSCT.[37–39] However, during the last decade, the importance of CD4$^+$ T cells in CMV immune response has been highlighted. The study by Gabanti et al,[8] described SOT recipients whose CMV viral load did not drop until CMV-specific CD4$^+$ T cells appeared, despite optimal levels of CMV-specific CD8$^+$ T cells, indicating that in these patients the help provided by CD4$^+$ T cells was necessary for a complete protection. Similarly, in a large multicenter cohort of KTR, the presence of lower counts of CMV-specific CD4$^+$ (but not CD8$^+$) T cells at days 60 and 180 were associated with a higher incidence of late-onset CMV events.[40] Regarding the CD4$^+$ T cell subset of TFH, there is only one work published studying cTFH and CMV infection in SOT recipients, which showed an increase in the number of activated cTFH in the early stages of primary infection, suggesting an active role of cTFH in the immune response against CMV.[41] In the current study, we show for the first time in a large cohort of KTR that cTFH could be particularly important in reducing CMV infection incidence.

Different studies have addressed the importance of antibodies in CMV infection with differing results. While some studies have shown that Nabs were not associated with protection against CMV infection in SOT recipients[6,7] and HSCT recipients,[7] most literature supports a role of Nabs in protection against this infection in transplant patients.[14,15,41,42] Disparities in these results could be explained by differences in experimental methods for measuring Nabs, as well as in the characteristics of the patient cohort or in the timing of the measurement. In high-risk SOT patients treated with T cell-depleting agents, Blanco-Lobo et al found that Nab titers measured in ARPE-19 cells rose in successive CMV episodes and the authors defined a minimum Nab titer cut-off that correlated with protection from CMV infection.[14] Likewise, in primary infected SOT recipients, Nabs, also measured in ARPE-19 cells, and titers of IgG antibodies targeting the viral pentameric complex correlated with activated cTFH.[41] In the present study, in a CMV seropositive KTR cohort, we observed an association between cTFH and Nabs ≥1/320 pretransplant. Patients with high cTFH pretransplant were more likely to have Nabs above


(A)
**
%CXCR5+ of CD4+ pre-Tx
No CMV-specific Nabs
CMV-specific Nabs
(B)
No CMV-specific Nabs
CMV-specific Nabs
Low cTFH
18
17
High cTFH
7
31
p=0.01
(C)
7 seropositive healthy donors
+CD28
+CMV peptides
+CD28
Negative control
Test well
Day 3: cTFH subsets
Day 10: anti-CMV Nab titers
(D)
*
%ICOS+PD1+ cTFH1 of CXCR5+
C-
+ CMV peptides
(E)
*
%ICOS+PD1+ cTFH2 of CXCR5+
C-
+ CMV peptides
(F)
*
%ICOS+PD1+cTFH17 of CXCR5+
C-
+ CMV peptides
(G)
cTFH1
r2=0.50
p=0.11
%ICOS+PD1+ of CXCR5+
Nab titers (1/X)
cTFH2
r2=0.38
p=0.18
Nab titers (1/X)
cTFH17
r2=0.82
p=0.01
Nab titers (1/X)
(H)
**
*
**
% ICOS+PD1+ within each subset
cTFH1
cTFH2
cTFH17
(I)
**
% IFN-γ+ producing CD8+ T cells
CMV peptides
CMV peptides + IL-21
(J)
Positive control
Negative control
Negative control + IL-21
CMV peptides
CMV peptides + IL-21
CD8
IFN-γ
10.80%
0.06%
0.23%
0.70%
1.11%

the cut-off. Moreover, although the three cTFH subsets responded against CMV *in vitro*, the specific subset involved in Nab synthesis in our assay seemed to be cTFH17. A similar result was found by Locci et al who found a correlation between PD1+CXCR3-CXCR5+ TFH cells (namely cTFH2 and cTFH17) and the development of broadly neutralizing antibodies in HIV infection.[30]

An increasing number of studies are demonstrating that IL-21 may enhance $CD8^+$ T cell function during viral infections.[43,44] In fact, during hepatitis B and C virus infections, as well as in HIV, increased IL-21 levels and IL-21-producing $CD4^+$ T cells have been associated with better $CD8^+$ T responses and viral control.[45,46] TFH cells are producers of high levels of IL-21, and a recent study presented an increase in cytolytic properties when $CD8^+$ T cells were co-cultured with cTFH.[24] Similarly, van Leeuwen et al showed that antigen-specific $CD8^+$ T cells expand when stimulated with cognate peptides and IL-21.[47] In our present study, we corroborate the effect of IL-21 in enhancing CMV-specific $CD8^+$ T cell activity.

CMV-seropositive KTR who did not receive antithymocyte globulin or CMV prophylaxis were selected for this study. This design allowed us to assess the function of cTFH in the natural course of protection against CMV. These patients are usually regarded as at a low/intermediate risk of developing CMV infection or disease, however, a non-negligible proportion of them suffered this complication. We have shown that the frequency of cTFH at 7 days posttransplant could be used to stratify patients at low and high risk of CMV infection. Approximately 70% of our KTR with cTFH below the cut-off developed CMV infection (Figure 2H), which suggests that these patients could benefit from prophylactic treatment.

Given the inclusion criteria for this study, the conclusions may not apply to patients receiving induction with T cell-depleting agents or anti-CMV prophylaxis, or to patients without previous contact with CMV. The results are also limited by the number of events, especially regarding CMV disease. Finally, in this cohort we did not analyze CMV-specific, but global cTFH, which could be influenced by previous vaccinations or subclinical infections.[48] The analysis of CMV-specific cTFH could also offer valuable biomarkers for CMV-infection reactivation in renal recipients. Future studies addressing these considerations would be valuable.

In conclusion, the present study shows an association between TFH and lower incidence of CMV infection in KTR, probably through TFH cooperation for CMV-specific Nab production and IL-21-mediated enhancement of $CD8^+$ T cell activity. Moreover, the results highlight that monitoring cTFH pre- and early posttransplant could improve the stratification of CMV risk infection and help assess the need of prophylaxis administration in patients not catalogued as high-risk by serology.

ACKNOWLEDGMENTS

This work was supported by projects to EPA from Instituto de Salud Carlos III, Spanish Ministry of Science and Innovation (PI18/969, PIE13/00045), cofinanced by the European Regional Development Fund "A way to achieve Europe", and from Sociedad Madrileña de Trasplante (PI17/082), and projects to PPR from Instituto de Salud Carlos III (PI17CIII-00014, PI17-01120). R.L.G. holds a research contract "Rio Hortega" (CM19/00120) and M.F.R. a research contract "Miguel Servet" (CP18/00073), both from the Instituto de Salud Carlos III, Spanish Ministry of Science and Innovation. We would like to thank all patients, nurses and medical colleagues who contributed to the study.

DISCLOSURE

The authors of this manuscript have conflicts of interest to disclose as described by the *American Journal of Transplantation*. PPR is founder and shareholder of Vaxdyn, S.L., a biotechnology company developing vaccines. The other authors have no conflicts of interest to disclose.

ORCID

Patricia Suàrez-Fernández https://orcid.org/0000-0003-1601-6464
Alberto Utrero-Rico https://orcid.org/0000-0001-8814-8410
Estéfani García-Ríos https://orcid.org/0000-0001-9028-055X
Mario Fernández-Ruiz https://orcid.org/0000-0002-0315-8001
Amado Andrés https://orcid.org/0000-0003-0238-1364
Pilar Pérez-Romero https://orcid.org/0000-0003-3026-7905
José María Aguado https://orcid.org/0000-0002-9520-8255
Estela Paz-Artal https://orcid.org/0000-0002-0646-2375
Rocío Laguna-Goya https://orcid.org/0000-0003-4265-8567

REFERENCES

1. Martin-Gandul C, Mueller NJ, Pascual M, Manuel O. The impact of infection on chronic allograft dysfunction and allograft survival after solid organ transplantation. *Am J Transplant*. 2015;15(12):3024–3040. https://doi.org/10.1111/ajt.13486
2. Razonable RR, Humar A. Cytomegalovirus in solid organ transplant recipients—guidelines of the american society of transplantation infectious diseases community of practice. *Clin Transplant*. 2019;33(9):e13512. https://doi.org/10.1111/ctr.13512
3. Harvala H, Stewart C, Muller K, et al. High risk of cytomegalovirus infection following solid organ transplantation despite prophylactic therapy. *J Med Virol*. 2013;85(5):893–898. https://doi.org/10.1002/jmv.23539
4. Crough T, Khanna R. Immunobiology of human cytomegalovirus: from bench to bedside. *CMR*. 2009;22(1):76–98. https://doi.org/10.1128/CMR.00034-08
5. Polić B, Hengel H, Krmpotić A, et al. Hierarchical and redundant lymphocyte subset control precludes cytomegalovirus replication during latent infection. *J Exp Med*. 1998;188(6):1047–1054.
6. Lilleri D, Zelini P, Fornara C, et al. Human cytomegalovirus (HCMV)-specific T cell but not neutralizing or IgG binding antibody responses to glycoprotein complexes gB, gHgLgO, and pUL128L correlate with protection against high HCMV viral load reactivation in solid-organ transplant recipients. *J Med Virol*. 2018;90(10):1620–1628. https://doi.org/10.1002/jmv.25225
7. Giménez E, Blanco-Lobo P, Muñoz-Cobo B, et al. Role of cytomegalovirus (CMV)-specific polyfunctional CD8+ T-cells and antibodies neutralizing virus epithelial infection in the control of CMV infection in an allogeneic stem-cell transplantation setting. *J Gen Virol*. 2015;96(9):2822–2831. https://doi.org/10.1099/vir.0.000203
8. Gabanti E, Bruno F, Lilleri D, et al. Human Cytomegalovirus (HCMV)-Specific CD4+ and CD8+ T Cells Are Both Required for Prevention

of HCMV Disease in Seropositive Solid-Organ Transplant Recipients. Spencer J, ed. *PLoS One*. 2014;9(8):e106044–https://doi.org/10.1371/journal.pone.0106044

9. Manuel O, Husain S, Kumar D, et al. Assessment of cytomegalovirus-specific cell-mediated immunity for the prediction of cytomegalovirus disease in high-risk solid-organ transplant recipients: a multicenter cohort study. *Clin Infect Dis*. 2013;56(6):817–824. https://doi.org/10.1093/cid/cis993
10. Meesing A, Razonable RR. Pharmacologic and immunologic management of cytomegalovirus infection after solid organ and hematopoietic stem cell transplantation. *Expert Rev Clin Pharmacol*. 2018;11(8):773–788. https://doi.org/10.1080/17512433.2018.1501557
11. Issa NC, Fishman JA. Infectious complications of antilymphocyte therapies in solid organ transplantation. *Clin Infect Dis*. 2009;48(6):772–786. https://doi.org/10.1086/597089
12. Fernández-Ruiz M, López-Medrano F, Allende LM, et al. Kinetics of peripheral blood lymphocyte subpopulations predicts the occurrence of opportunistic infection after kidney transplantation. *Transpl Int*. 2014;27(7):674–685. https://doi.org/10.1111/tri.12321
13. Sandonís V, García-Ríos E, McConnell MJ, Pérez-Romero P. Role of neutralizing antibodies in CMV infection: implications for new therapeutic approaches. *Trends Microbiol*. 2020;28(11):900–912. https://doi.org/10.1016/j.tim.2020.04.003
14. Blanco-Lobo P, Cordero E, Martín-Gandul C, et al. Use of antibodies neutralizing epithelial cell infection to diagnose patients at risk for CMV Disease after transplantation. *J Infect*. 2016;72(5):597–607. https://doi.org/10.1016/j.jinf.2016.02.008
15. Bonaros N, Mayer B, Schachner T, Laufer G, Kocher A. CMV-hyperimmune globulin for preventing cytomegalovirus infection and disease in solid organ transplant recipients: a meta-analysis. *Clin Transplant*. 2008;22(1):89–97. https://doi.org/10.1111/j.1399-0012.2007.00750.x
16. Snydman DR, Kistler KD, Ulsh P, Bergman GE, Vensak J, Morris J. The impact of CMV prevention on long-term recipient and graft survival in heart transplant recipients: analysis of the Scientific Registry of Transplant Recipients (SRTR) database. *Clin Transplant*. 2011;25(4):E455–E462. https://doi.org/10.1111/j.1399-0012.2011.01459.x
17. Martins JP, Andoniou CE, Fleming P, et al. Strain-specific antibody therapy prevents cytomegalovirus reactivation after transplantation. *Science*. 2019;363(6424):288–293. https://doi.org/10.1126/science.aat0066
18. Schaerli P, Willimann K, Lang AB, Lipp M, Loetscher P, Moser B. Cxc chemokine receptor 5 expression defines follicular homing T cells with B cell helper function. *J Exp Med*. 2000;192(11):1553–1562. https://doi.org/10.1084/jem.192.11.1553
19. Crotty S. T follicular helper cell biology: a decade of discovery and diseases. *Immunity*. 2019;50(5):1132–1148. https://doi.org/10.1016/j.immuni.2019.04.011
20. Vinuesa CG, Cook MC. Blood relatives of follicular helper T cells. *Immunity*. 2011;34(1):10–12. https://doi.org/10.1016/j.immuni.2011.01.006
21. Schmitt N, Bentebibel S-E, Ueno H. Phenotype and functions of memory Tfh cells in human blood. *Trends Immunol*. 2014;35(9):436–442. https://doi.org/10.1016/j.it.2014.06.002
22. Morita R, Schmitt N, Bentebibel S-E, et al. Human blood CXCR5+CD4+ T cells are counterparts of T follicular cells and contain specific subsets that differentially support antibody secretion. *Immunity*. 2011;34(1):108–121. https://doi.org/10.1016/j.immuni.2010.12.012
23. de Graav GN, Dieterich M, Hesselink DA, et al. Follicular T helper cells and humoral reactivity in kidney transplant patients. *Clin Exp Immunol*. 2015;180(2):329–340. https://doi.org/10.1111/cei.12576
24. Shi W, Dong L, Sun Q, Ding H, Meng J, Dai G. Follicular helper T cells promote the effector functions of CD8+ T cells via the provision of IL-21, which is downregulated due to PD-1/PD-L1-mediated suppression in colorectal cancer. *Exp Cell Res*. 2018;372(1):35–42. https://doi.org/10.1016/j.yexcr.2018.09.006
25. Kasaian MT, Whitters MJ, Carter LL, et al. IL-21 limits NK cell responses and promotes antigen-specific T cell activation: a mediator of the transition from innate to adaptive immunity. *Immunity*. 2002;16(4):559–569. https://doi.org/10.1016/S1074-7613(02)00295-9
26. Bentebibel S-E, Lopez S, Obermoser G, et al. Induction of ICOS+CXCR3+CXCR5+ TH cells correlates with antibody responses to influenza vaccination. *Sci Transl Med*. 2013;5(176):176ra32. https://doi.org/10.1126/scitranslmed.3005191
27. Koutsakos M, Wheatley AK, Loh L, et al. Circulating TFH cells, serological memory, and tissue compartmentalization shape human influenza-specific B cell immunity. *Sci Transl Med*. 2018;10(428):eaan8405. https://doi.org/10.1126/scitranslmed.aan8405
28. Matsui K, Adelsberger JW, Kemp TJ, Baseler MW, Ledgerwood JE, Pinto LA. Circulating CXCR5+CD4+ T follicular-like helper cell and memory B cell responses to human papillomavirus vaccines. Ho PL, ed. *PLoS One*. 2015;10(9):e0137195–https://doi.org/10.1371/journal.pone.0137195
29. Obeng-Adjei N, Portugal S, Tran T, et al. Circulating Th1-Cell-type Tfh cells that exhibit impaired B cell help are preferentially activated during acute malaria in children. *Cell Rep*. 2015;13(2):425–439. https://doi.org/10.1016/j.celrep.2015.09.004
30. Locci M, Havenar-Daughton C, Landais E, et al. Human circulating PD-1+CXCR3−CXCR5+ memory Tfh cells are highly functional and correlate with broadly neutralizing HIV antibody responses. *Immunity*. 2013;39(4):758–769. https://doi.org/10.1016/j.immuni.2013.08.031
31. Zhang J, Liu W, Wen BO, et al. Circulating CXCR3+ Tfh cells positively correlate with neutralizing antibody responses in HCV-infected patients. *Sci Rep*. 2019;9(1):10090. https://doi.org/10.1038/s41598-019-46533-w
32. Loupy A, Haas M, Solez K, et al. The Banff 2015 kidney meeting report: current challenges in rejection classification and prospects for adopting molecular pathology. *Am J Transplant*. 2017;17(1):28–41. https://doi.org/10.1111/ajt.14107
33. Haas M. The revised (2013) Banff classification for antibody-mediated rejection of renal allografts: update, difficulties, and future considerations. *Am J Transplant*. 2016;16(5):1352–1357. https://doi.org/10.1111/ajt.13661
34. Ljungman P, Boeckh M, Hirsch HH, et al. Definitions of cytomegalovirus infection and disease in transplant patients for use in clinical trials: table 1. Snydman DR, ed. *Clin Infect Dis*. 2017;64(1):87–91. https://doi.org/10.1093/cid/ciw668
35. Cano-Romero FL, Laguna Goya R, Utrero-Rico A, et al. Longitudinal profile of circulating T follicular helper lymphocytes parallels anti-HLA sensitization in renal transplant recipients. *Am J Transplant*. 2019;19(1):89–97. https://doi.org/10.1111/ajt.14987
36. Oliver B, Jackson E, Soliman H. *Chapter 17 - immunostimulators and immunomodulators in cancer treatment*. (Singh M, Salnikova M, eds.). Academic Press; 2015. https://doi.org/10.1016/B978-0-12-416603-5.00017-1
37. Cwynarski K, Ainsworth J, Cobbold M, et al. Direct visualization of cytomegalovirus-specific T-cell reconstitution after allogeneic stem cell transplantation. *Blood*. 2001;97(5):1232–1240. https://doi.org/10.1182/blood.V97.5.1232
38. Reusser P, Riddell SR, Meyers JD, Greenberg PD. Cytotoxic T-lymphocyte response to cytomegalovirus after human allogeneic bone marrow transplantation: pattern of recovery and correlation with cytomegalovirus infection and disease. *Blood*. 1991;78(5):1373–1380.
39. Gratama JW, van Esser JWJ, Lamers CHJ, et al. Tetramer-based quantification of cytomegalovirus (CMV)–specific CD8+ T

lymphocytes in T-cell–depleted stem cell grafts and after transplantation may identify patients at risk for progressive CMV infection. *Blood*. 2001;98(5):1358–1364. https://doi.org/10.1182/blood.V98.5.1358
40. Fernández-Ruiz M, Giménez E, Vinuesa V, et al. Regular monitoring of cytomegalovirus-specific cell-mediated immunity in intermediate-risk kidney transplant recipients: predictive value of the immediate post-transplant assessment. *Clin Microbiol Infect*. 2019;25(3):381.e1–381.e10. https://doi.org/10.1016/j.cmi.2018.05.010
41. Bruno F, Fornara C, Zelini P, et al. Follicular helper T-cells and virus-specific antibody response in primary and reactivated human cytomegalovirus infections of the immunocompetent and immunocompromised transplant patients. *J Gen Virol*. 2016;97(8):1928–1941. https://doi.org/10.1099/jgv.0.000488
42. Snydman DR, Werner BG, Heinze-Lacey B, et al. Use of cytomegalovirus immune globulin to prevent cytomegalovirus disease in renal-transplant recipients. *N Engl J Med*. 1987;317(17):1049–1054. https://doi.org/10.1056/NEJM198710223171703
43. Tian Y, Cox MA, Kahan SM, Ingram JT, Bakshi RK, Zajac AJ. A context-dependent role for IL-21 in modulating the differentiation, distribution, and abundance of effector and memory CD8 T cell subsets. *JI*. 2016;196(5):2153–2166. https://doi.org/10.4049/jimmunol.1401236
44. Yi JS, Du M, Zajac AJ. A vital role for interleukin-21 in the control of a chronic viral infection. *Science*. 2009;324(5934):1572–1576. https://doi.org/10.1126/science.1175194
45. Li L, Liu M, Cheng L-W, et al. HBcAg-specific IL-21-producing CD4+ T cells are associated with relative viral control in patients with chronic hepatitis B. *Scand J Immunol*. 2013;78(5):439–446. https://doi.org/10.1111/sji.12099
46. Chevalier MF, Julg B, Pyo A, et al. HIV-1-specific interleukin-21+ CD4+ T cell responses contribute to durable viral control through the modulation of HIV-specific CD8+ T cell function. *J Virol*. 2011;85(2):733–741. https://doi.org/10.1128/JVI.02030-10
47. van Leeuwen EM, Gamadia LE, Baars PA, Remmerswaal EB, ten Berge IJ, van Lier RA. Proliferation requirements of cytomegalovirus-specific, effector-type human CD8+ T cells. *J Immunol*. 2002;169(10):5838–5843. https://doi.org/10.4049/jimmunol.169.10.5838
48. He J, Tsai L, Leong Y, et al. Circulating precursor CCR7(lo)PD-1(hi) CXCR5$^+$ CD4$^+$ T cells indicate Tfh cell activity and promote antibody responses upon antigen reexposure. *Immunity*. 2013;39(4):770–781. https://doi.org/10.1016/j.immuni.2013.09.007

SUPPORTING INFORMATION

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

How to cite this article: Suàrez-Fernández P, Utrero-Rico A, Sandonis V, et al. Circulatory follicular helper T lymphocytes associate with lower incidence of CMV infection in kidney transplant recipients. *Am J Transplant*. 2021;21:3946–3957. https://doi.org/10.1111/ajt.16725