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Antibody-mediated allograft rejection is associated with an increase in peripheral differentiated CD28-CD8+ T cells — Analyses of a cohort of 1032 kidney transplant recipients

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Summary

Background CD28-CD8+ T cells represent a differentiated CD8+ T cell subset that is found to be increased in various conditions associated with chronic antigenic stimulation such as aging, chronic viral infections, autoimmune diseases, cancers, and allotransplantation.

Methods Using multivariate models, we analyzed a large cohort of 1032 kidney transplant patients in whom 1495 kidney graft biopsies were performed concomitant with a peripheral blood leukocyte phenotyping by flow cytometry. We investigated the association between the level of CD28-CD8+ T cells in the blood and the diagnosis of graft rejection according to the recent Banff classification of renal allograft pathology.

Findings We found that antibody-mediated rejection (ABMR) was associated with a significant increase in the percentage as well as the absolute number of CD28-CD8+ T cells in the peripheral blood of kidney transplant patients at the time of biopsy. The confounder-adjusted mean difference of log percentage and log absolute value between the ABMR group and the normal/subnormal histology group were 0.29 ($p=0.0004$) and 0.38 ($p=0.0004$), respectively. Moreover, we showed that CD28-CD8+ T cells from the patients diagnosed with ABMR responded more rigorously to TCR and FcγRIIIA (CD16) engagement compared to their CD28+ counterparts as evidenced by an increase in the expression of IFNγ, TNFα, and CD107a.

Interpretation Collectively, our data suggest that differentiated CD28-CD8+ T cells, with increased frequency, number, and function, may participate in the pathobiology of ABMR. Further studies are warranted to clarify the immunological role of this T cell subset in kidney graft rejection.

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Keywords: Kidney transplantation; Rejection; Antibody; T lymphocyte; CD8; CD28

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² SB and MG equally contributed as last authors.

Research in context

Evidence before this study

CD28-CD8+ T cells represent a differentiated CD8+ T cell subset that is found to be increased in various conditions associated with persistent antigen exposure such as chronic infection and allotransplantation. In 2006, we have reported that kidney graft patients with biopsy-proven chronic rejection had a significant increase in the percentage of CD28-CD8+ T cells in the peripheral blood compared to patients with long-term drug-free graft tolerance and healthy individuals. In searching in PubMed publications from 2000 to 2020 containing both key words “CD28-CD8+ T cells” and “transplantation”, we identified 6 other publications in which the percentage or number of CD28-CD8+ T cells was found to be associated with the risk of allograft rejection (Ref 21 to 26). All of them are small studies with a maximum of 200-300 patients per study.

Added value of this study

Using carefully designed multivariable model, we analyzed a large cohort of 1032 kidney transplant patients in whom 1495 kidney graft biopsies were performed and showed that antibody-mediated rejection (ABMR) was associated with a significant increase in the percentage as well as the absolute number of CD28-CD8+ T cells in the peripheral blood. Moreover, we showed that CD28-CD8+ T cells from the patients diagnosed with ABMR responded more rigorously to TCR and FcγRIIIA engagement compared to their CD28+ counterparts in terms of TH1 cytokine secretion and cytotoxicity. These findings suggest that differentiated CD28-CD8+ T cells participate in the pathobiology of ABMR.

Implications of all the available evidence

Our study sheds new insight into the understanding of a potential role of a “cellular component”, namely the CD28-CD8+ T cell population, in the pathobiology of ABMR. Future studies using more comprehensive lymphocyte phenotyping strategies and functional experiments should clarify the role of memory/effector CD8+ T cells in different forms of graft rejection.

Introduction

Kidney allograft rejection remains the major cause of renal damage and graft loss. The histological diagnosis of graft rejection is complemented by donor-specific antibody (DSA) identification¹ and more recently by molecular analyses.^{2,3} The Banff classification divides kidney graft rejections into two main categories: antibody-mediated rejection (ABMR) and T cell-mediated rejection (TCMR).^{1–3} Nevertheless, the biological mechanisms underlying the rejection process are more complicated than it appears in the pathological

classification and involve both the adaptive immune system, particularly CD4+ and CD8+ T cells and the innate immune system.⁴ Among the several cell types involved in graft rejection, CD8+ T cells play an important role, especially in allorecognition.⁵ Patients with high frequency of donor-specific CD8+ T cells before kidney transplant have increased risk of acute rejection after transplant.⁶ CD8+ T cells are found in kidney allograft undergoing rejection, both acute and chronic, and the majority of them are cytotoxic T cells.^{7–9} Both CD4+ and CD8+ T cell activation by alloantigens require the second or costimulatory signal, most importantly via the interaction between CD28 on T cells and B7 on antigen-presenting cells (APCs).¹⁰ Chronic antigenic stimulation of CD8+ T cells, however, can lead to a down-regulation of CD28. The CD28-CD8+ T cell population has been shown to be increased with age and in various diseases associated with chronic immune activation such as chronic viral infection, autoimmune diseases, cancer, and allotransplantation [reviewed in¹¹ and¹²]. In 2006, we reported that kidney graft patients with biopsy-proven chronic rejection according to the Banff 05 classification^{13,14} had a significant increase in the percentage of CD28-CD8+ T cells in the peripheral blood compared to patients with long-term drug-free graft tolerance and healthy individuals.¹⁵ Although CD28-CD8+ T cells may exhibit regulatory properties in some disease models,^{16,17} we have shown that CD28-CD8+ T cells from patients with chronic rejection express the cytotoxic molecules granzyme A and perforin and degranulate in response to stimulation with donor MHC.¹⁵ Following those observations, we set up a clinical protocol in which a peripheral blood lymphocyte phenotyping was performed each time a kidney graft biopsy was done at our center between 2008 and 2016. Kidney graft biopsies were carried out either for cause or for surveillance (at 3 months and 1 year post-transplantation). We hypothesized that there was an association between peripheral blood T cell phenotypes, especially CD28-CD8+ T cells and the histological diagnosis of kidney graft biopsies. Based on the large cohort of 1495 kidney graft biopsies established from our clinical protocol, we confirmed that ABMR diagnosis was associated with an increase in the percentage as well as the absolute number of CD28-CD8+ T cells in the peripheral blood at the time of graft biopsy. Using in vitro culture assays, we also showed that CD28-CD8+ T cells from recipients diagnosed with ABMR responded vigorously to TCR and CD136 stimulation, suggesting their active role in mediating graft rejection.

Patients, materials and methods

Study design

From 2008 to 2016, we established at our institution, Nantes University Hospital (Centre Hospitalier

Universitaire or CHU de Nantes), a cohort of 1645 kidney graft biopsies (protocol “Peribiopsy N° RC13_0251”) for which a peripheral lymphocyte phenotyping was performed at the time of biopsy. Renal biopsies were interpreted by our renal pathologist (K.R.) based on the last Banff classification² except for the diagnosis of chronic cellular rejection which was based exclusively on the presence of chronic allograft arteriopathy.¹ The full histological criteria were detailed in the Banff 2015 and 2017 Kidney Meeting Report.^{1,2} Briefly, ABMR was diagnosed based on the presence histologic evidence of tissue injury (microvascular inflammation [g>0 and/or ptc>0] and/or acute thrombotic microangiopathy and/or acute tubular injury in the absence of any other cause for acute ABMR or transplant glomerulopathy [cg>0] and/or chronic allograft vasculopathy for chronic ABMR), associated with evidence of current antibody interaction with vascular endothelium including significant linear C4d staining in peritubular capillaries or at least moderate microvascular inflammation ([g+ptc]≥2) and serologic evidence of DSA. The diagnosis of acute TCMR was based on a combination of interstitial inflammation (i≥2), tubulitis (t≥2), and/or arteritis (v≥1), whereas chronic TCMR was diagnosed mainly based on the presence of chronic allograft arteriopathy in the absence of DSA, C4d positivity or significant microvascular inflammation. After having taken into account the clinical decision, the histological diagnoses were organized into 6 biopsy groups (Figure 1):

- Group 1 (n=1060): normal/sub-normal or interstitial fibrosis and tubular atrophy (IFTA) grade 1.
- Group 2 (n=90): IFTA grade 2 or 3.
- Group 3 (n=211): ABMR or borderline rejection treated as ABMR with plasma exchanges and intravenous immunoglobulins (IVIg), with or without rituximab.
- Group 4 (n=51): TCMR or borderline rejection treated as TCMR with corticosteroids.

- Group 5 (n=83): borderline rejection without treatment.
- Group 6 (n=150): other changes not considered to be caused by rejection.

Since our study focused on the association between lymphocyte phenotypes and rejection, 150 biopsies from group 6 (other changes) were excluded, leaving 1495 biopsies for the analysis, including 527 for cause biopsies and 448 and 520 three-month and 1-year surveillance biopsies, respectively. The designation of those groups was done a priori before all statistical analyses, which were then performed by an independent biostatistician (FLB). The main objective of our study was to investigate whether there was an association between the percentage and the absolute number of CD28-CD8+ T cells and the five biopsy groups (the principal analysis). In addition, we also analyzed the subgroup of patients with lymphocyte phenotyping at 1 year post-transplantation (patients with 1-year surveillance biopsy) to look for an association between the percentage and absolute number of CD28-CD8+ T cells at 1 year and graft survival (the graft survival analysis).

Lymphocyte phenotyping by flow cytometry

Each time a patient underwent a kidney graft biopsy at CHU de Nantes, a blood sample was drawn for routine laboratory analyses and an EDTA tube containing about 5 ml of blood was sent to the center for immunomonitoring (Centre d'Immunomonitorage Nantes-Atlantique or CIMNA) at CHU de Nantes for lymphocyte phenotyping. Flow cytometry was performed on fresh whole blood within 24 h after sampling and cells were analyzed on a BD FACS Canto II flow cytometer. Several lymphocyte surface markers were stained but in this report, we focused our study on the CD28-CD8+ T cell subset. To determine the percentage of CD28-CD8+ T cells, blood was incubated with the following

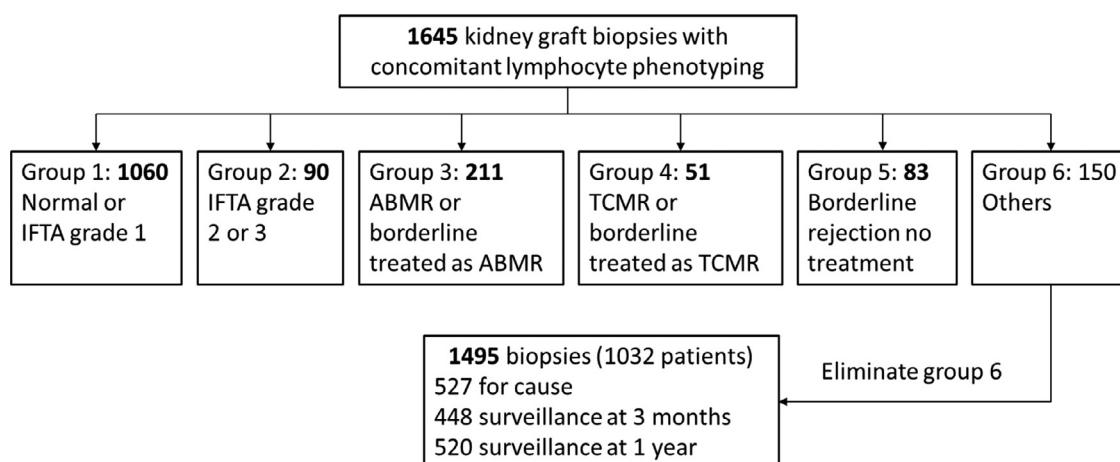


Figure 1. Histological diagnoses of kidney graft biopsies.

fluorescence-conjugated monoclonal antibodies: anti-CD45-PerCP Cy5.5 (clone 2D1), anti-CD3-FITC (clone SK7), anti-CD28-APC (clone CD28.2), and anti-CD8-PE (clone B9.11) (all from BD Biosciences except anti-CD8 from Beckman Coulter), lysed with FACS lysing solution, and washed with PBS. CD28-CD8⁺ T cells were identified as CD45⁺CD3⁺CD8⁺CD28⁻ and the percentage of CD28-CD8⁺ T cells in total lymphocytes was reported (see Supplementary Figure 1 for gating strategy). CD8⁺ T cell absolute number was also measured by flow cytometry by staining whole blood with the four-color monoclonal antibody reagent BD Multitest™ CD3/CD8/CD45/CD4 in BD Trucount™ Tubes (BD Biosciences) according to the manufacturer's instruction. The absolute number of CD28-CD8⁺ T cells was calculated by multiplying the absolute number of CD8⁺ T cells by the percentage of CD28-CD8⁺ T cells in CD8⁺ T cells and expressed in thousand/ μ L.

Functional analysis of CD8⁺ T cells

Frozen PBMCs of 24 and 46 kidney transplant recipients belonging to group 3 (ABMR) and group 1 (normal/subnormal biopsies), respectively, were retrieved from Centre de Ressources Biologiques of Nantes (CRB). Frozen PBMCs were thawed and rested overnight in complete RPMI medium. 10^6 PBMCs were cultured for 4h in 24-well flat bottom plates at 37°C in 5% CO₂ and, when indicated, treated with plate-bound anti-CD3 (clone OKT3; 2 μ g/mL), plate-bound anti-CD16 (clone 3G8; 10 μ g/mL), soluble anti-CD28 (clone CD28.2; 2 μ g/mL) and/or soluble IL-15 (10 ng/mL; Miltenyi Biotec). Anti-CD107a PE (clone H4A3) and Brefeldin A (5 μ g/mL, Sigma) were added at culture initiation. After stimulation, cells were first stained for cell surface markers (CD3 [clone UCHT1], CD8 [clone HIT8a], CD45RA [clone HI100], CD28 [clone CD28.2] and CCR7 [clone Go43H7]), followed by fixation and permeabilization (Intracellular Fixation & Permeabilization Buffer Set, Thermo Fisher) and stained for intracellular cytokines TNF α [clone Mab11] and IFN γ [Clone B27]). Cells were acquired with a Celesta flow cytometer (BD Immunocytometry Systems) and the data analyzed using FlowJo Version 10.8.0 (TreeStar). The percentage of cells expressing TNF α /IFN γ and CD107a after stimulation were compared using non-parametric tests.

Clinical data

Clinical data required for the analysis were extracted from the DIVAT (for "Données Informatisées et Validées en Transplantation") database which was carried out prospectively, exhaustively, and independently by clinical research associates on key dates during post-operative follow-up of clinical and biological data of all incident transplanted patients at our institute. The data are subject to an annual audit to warrant quality and completeness. Recipient characteristics include age,

gender, transplantation rank (first transplantation or re-transplantation), year of transplantation, the initial kidney disease (possibly recurrent or not), and the cytomegalovirus (CMV) serology, history of diabetes, history of arterial hypertension, history of cardiovascular disease, CMV serology, number of HLA-A-B-DR incompatibilities, ABO mismatch, donor-specific antibodies (DSA), anti-HLA class I and II immunization. Donor features include age, gender, and donor type (living or deceased). Baseline transplantation parameters were cold ischemia time, delayed graft function (DGF), induction therapy, and maintenance treatments [including cyclosporine A (CSA), tacrolimus, mammalian target of rapamycin (mTOR) inhibitors, mycophenolate mofetil (MMF), and corticosteroids]. Parameters collected at the time of biopsy were the reason for biopsy – for cause or surveillance biopsy (at 3 months or 1 year post-transplantation), serum creatinine, the rank of biopsy, post-transplantation time, histological diagnosis according to 2015 Banff classification and the whole details of the Banff elementary lesions, DSA, and type of treatment for each rejection episode, and CD28-CD8⁺ T cells both in percentage and in absolute value. The follow-up and the collection of data were stopped upon graft failure (defined as return to dialysis or retransplantation) or death.

Statistical analyses

For the principal analysis, the characteristics at the time of biopsy between the five biopsy groups were described using median and quartiles for continuous variables and frequency and proportion for categorical data. Two features were studied: the percentage and the absolute value of CD28-CD8⁺ T cells. Since these two features were non-Gaussian, they were transformed using the natural logarithm function (Supplementary Figure 2). Linear mixed-effects models (random intercept per transplantation) were used to study the association between the biopsy groups and each feature.¹⁸ We systematically included in multivariate models the following clinically important variables: recipient and donor age, recipient and donor sex, re-transplantation, recipient and donor CMV serology, cold ischemia time, time from transplantation to biopsy, reason for biopsy (for cause vs surveillance biopsy), induction therapy at transplantation, creatinine at biopsy, HLA-A, -B and -DR incompatibilities and anti-HLA class I and II immunization. We also included into the models all the covariates significantly associated with the features in univariate models (p -value < 0.2). We did not consider interaction. The residuals' analyses were performed to check the models' validities. In each model, we first tested if the outcome was significantly different in at least one of the biopsy groups using a likelihood ratio test. If significant, we explored which groups differed by performing the six following comparisons: group 3 versus 1, group 4 versus 1, group 5 versus 3, group 5 versus

4, group 2 versus 1, and group 5 versus 1. Corrected *p*-values were determined using the Holm-Bonferroni method to control for the inflation of the type I error rate associated with multiple testing.

For the graft survival analysis, in order to determine if there was an association between the log percentage and/or absolute number of CD28-CD8+ T cells at 1 year post-transplantation and graft survival, we included recipients with a 1-year post-transplantation surveillance biopsy and a concomitant lymphocyte phenotyping. The baseline of this analysis was the time at which the 1-year biopsy was performed. The outcome was the graft survival defined by the time to the first event of either return to dialysis or pre-emptive re-transplantation (all-cause death with a functioning graft were right-censored). The median time of follow-up was estimated by using the reverse Kaplan-Meier estimator. The survival curve was obtained by the Kaplan-Meier estimator. To take into consideration confounding factors, we used multivariable cause-specific Cox models. We included in multivariable models all the covariates significantly associated with the outcomes in univariate Cox models (*p*-value <0.2). The proportional hazard assumption was tested from the Schoenfeld residuals.¹⁹ The log-linearity assumption has been checked in unadjusted analysis if the Bayesian Information Criterion was not reduced using natural spline transformation compared to the inclusion of the covariate in its natural scale. In case of violation, variables were transformed. We used R version 3.6.3 and the package 'base', 'dplyr', 'survival', 'etm', 'plotrix', 'prodlm', 'data.table', 'splines', 'officer', 'flextable', 'lattice', 'forestplot', 'gttools', 'lme4' and 'lmerTest' packages for all data analyses (see <https://www.r-project.org/>).

Ethical statement

This study on organ transplantation was conducted in accordance with the declaration of Istanbul, the declaration of Helsinki, and local regulations. DIVAT has received ethical committee authorization (CNIL decision DR-2015-087, N°914184). All participants gave their informed consent (www.divat.fr).

Role of funders

Funders had no role in study design, data analyses, interpretation, or writing of reports.

Results

Patient characteristics

A total of 1495 kidney graft biopsies performed on 1032 patients having received kidney or combined kidney/pancreas allografts were included in the principal analysis. On average, we observed 1.5 biopsies per recipient (range from 1 to 5, median at 1). The median time from transplantation to biopsy was one year (range from 0 to

31 years). Seventy percent (1060) of biopsies were normal or sub-normal (group 1), 6% (90) were grade 2/3 IFTA (group 2), 14% (211) were ABMR (group 3), 3.4% (51) were TCMR (group 4), and 5.6% (83) of biopsies and untreated borderline rejection (group 5). The characteristics of the whole sample (1495 biopsies) as well as of each histological group from 1 to 5 were presented in Table 1.

ABMR is associated with an increase in the percentage and absolute number of peripheral blood CD28-CD8+ T cells

The distribution of the log percentage and log absolute number of CD28-CD8+ T cells in the 5 biopsy groups are shown in Figure 2A and 2B, respectively. We first analyzed the unadjusted effect of baseline covariates on the log percentage of CD28-CD8+ T cells using linear mixed models and found that biopsy groups were significantly associated with the log percentage of CD28-CD8+ T cells (*p*<0.0001) (Supplementary Table 1). In addition to the predetermined clinically important variables as mentioned in Material and Methods (Statistical Analyses), the following covariates were also included into the multivariate model because they were found to be associated with the log percentage of CD28-CD8+ T cells (*p*<0.2) in the linear mix model: transplantation after 2008, delay graft function (DGF), history of cardiovascular diseases, deceased donor, ABO mismatch, cyclosporine A (CSA), tacrolimus, and biopsy rank. Next, we performed multivariate linear mixed model (Table 2) and confirmed a significant association between biopsy groups and the log percentage of CD28-CD8+ T cells (*p*=0.0003). The confounder-adjusted mean difference of log percentage of CD28-CD8+ T cells between group 3 (ABMR) and group 1 (normal or sub-normal) was 0.29 (*p*=0.0004) (Figure 3A). In other words, it means that the percentage of CD28-CD8+ T cells is 34% higher in ABMR patients compared with patients with normal/sub-normal histology. Other comparisons were not statistically significant (Figure 3A).

Similarly, we analyzed the unadjusted effect of baseline covariates on the log absolute number of CD28-CD8+ T cells using linear mixed models and found that biopsy groups were also significantly associated with the log absolute number of CD28-CD8+ T cells (*p*<0.0001) (Supplementary Table 2). The following covariates were found to be associated with the log absolute number of CD28-CD8+ T cells (*p*<0.2) in the linear mix model and were included into the multivariate model (in addition to the predetermined clinically important variables): transplantation after 2008, renal transplantation, recurrent initial disease, DGF, deceased donor, ABO mismatch, CSA, tacrolimus, steroid, positive DSA, and biopsy rank. Next, we performed multivariate linear mixed model (Table 3) and observed a significant association between biopsy groups and the log absolute number of CD28-CD8+ T cells (*p*=0.0006). The

Categorical variables	Whole sample (n=1495)			Biopsy group 1 (n=1060)			Biopsy group 2 (n=90)			Biopsy group 3 (n=211)			Biopsy group 4 (n=51)			Biopsy group 5 (n=83)		
	NA	n	%	NA	n	%	NA	n	%	NA	n	%	NA	n	%	NA	n	%
Transplantation after 2008	0	1174	78.5	0	899	84.8	0	55	61.1	0	117	55.5	0	36	70.6	0	67	80.7
Male recipient	0	927	62.0	0	670	63.2	0	48	53.3	0	130	61.6	0	28	54.9	0	51	61.4
Retransplantation	0	238	15.9	0	165	15.6	0	13	14.4	0	51	24.2	0	3	5.9	0	6	7.2
Renal transplantation	0	1343	89.8	0	946	89.2	0	86	95.6	0	192	91.0	0	44	86.3	0	75	90.4
Recurrent initial disease	0	350	23.4	0	241	22.7	0	20	22.2	0	57	27.0	0	9	17.6	0	23	27.7
DGF	29	484	33.0	19	325	31.2	2	39	44.3	5	68	33.0	2	20	40.8	1	32	39.0
History of diabetes	0	356	23.8	0	260	24.5	0	14	15.6	0	44	20.9	0	15	29.4	0	23	27.7
History of hypertension	0	1328	88.8	0	946	89.2	0	81	90.0	0	185	87.7	0	47	92.2	0	69	83.1
History of cardiovascular disease	0	524	35.1	0	350	33.0	0	38	42.2	0	84	39.8	0	23	45.1	0	29	34.9
Recipient/Donor CMV serology	6			0			2			3			0			1		
0		514	34.5		383	36.1		22	25.0		65	31.2		18	35.3		26	31.7
1		324	21.8		220	20.8		33	37.5		45	21.6		10	19.6		16	19.5
2		330	22.2		234	22.1		14	15.9		49	23.6		13	25.5		20	24.4
3		321	21.6		223	21.0		19	21.6		49	23.6		10	19.6		20	24.4
Male donor	1	861	57.6	1	617	58.3	0	61	67.8	0	110	52.1	0	28	54.9	0	45	54.2
Deceased donor	0	1340	89.6	0	941	88.8	0	85	94.4	0	190	90.0	0	47	92.2	0	77	92.8
HLA-A-B-DR mismatches > 4	0	330	22.1	0	233	22.0	0	11	12.2	0	52	24.6	0	15	29.4	0	19	22.9
ABO mismatch	10	30	2.0	7	25	2.4	1	0	0.0	2	4	1.9	0	0	0.0	0	1	1.2
Depleting induction	0	601	40.2	0	424	40.0	0	31	34.4	0	112	53.1	0	16	31.4	0	18	21.7
CSA	0	137	9.2	0	63	5.9	0	13	14.4	0	38	18.0	0	8	15.7	0	15	18.1
Tacrolimus	0	1354	90.6	0	995	93.9	0	77	85.6	0	173	82.0	0	41	80.4	0	68	81.9
Steroid	0	1256	84.0	0	886	83.6	0	78	86.7	0	182	86.3	0	38	74.5	0	72	86.7
Positive anti-class I immunization	1	445	29.8	0	309	29.2	1	23	25.8	0	94	44.5	0	9	17.6	0	10	12.0
Positive anti-class II immunization	1	390	26.1	0	264	24.9	1	18	20.2	0	87	41.2	0	10	19.6	0	11	13.3
Positive DSA	1	100	6.7	1	62	5.9	0	2	2.2	0	34	16.1	0	1	2.0	0	1	1.2
Biopsy rank	0			0			0			0			0			0		
1		982	65.7		733	69.2		41	45.6		120	56.9		36	70.6		52	62.7
2		425	28.4		293	27.6		40	44.4		55	26.1		10	19.6		27	32.5
3		79	5.3		32	3.0		9	10.0		30	14.2		4	7.8		4	4.8
4		8	0.5		2	0.2		0	0.0		5	2.4		1	2.0		0	0.0
5		1	0.1		0	0.0		0	0.0		1	0.5		0	0.0		0	0.0
For cause biopsy	0	527	35.3	0	264	24.9	0	53	58.9	0	155	73.5	0	37	72.5	0	18	21.7
Continuous variables	NA	Md	Q1 Q3	NA	Md	Q1 Q3	NA	Md	Q1 Q3	NA	Md	Q1 Q3	NA	Md	Q1 Q3	NA	Md	Q1 Q3
Log of CD28-CD8+ T cell percentage	17	1.9	1.1 2.8	12	1.8	1.0 2.6	1	2.5	1.6 3.0	4	2.3	1.4 3.2	0	2.2	1.4 2.9	0	2.3	1.2 2.9
Log of CD28-CD8+ T cell absolute value	19	-2.7	-3.6 -1.7	14	-2.9	-3.7 -1.9	1	-2.1	-3.0 -1.4	4	-2.1	-3.2 -1.3	0	-2.6	-3.3 -1.6	0	-2.4	-3.4 -1.4

Table 1 (Continued)

Categorical variables	Whole sample (n=1495)			Biopsy group 1 (n=1060)			Biopsy group 2 (n=90)			Biopsy group 3 (n=211)			Biopsy group 4 (n=51)			Biopsy group 5 (n=83)		
	NA	n	%	NA	n	%	NA	n	%	NA	n	%	NA	n	%	NA	n	%
Recipient age (years)	0	51.0	39.0 62.0	0	51.0	40.0 62.0	0	51.0	37.0 63.0	0	49.0	33.0 60.0	0	51.0	35.0 63.5	0	51.0	38.5 61.5
Cold ischemia time (hours)	0	15.5	12.0 20.1	0	15.1	11.7 19.3	0	18.3	12.8 24.7	0	17.5	13.5 23.9	0	16.4	13.2 18.8	0	15.9	13.0 19.9
Donor age (years)	0	52.0	40.0 64.0	0	52.0	42.0 63.0	0	56.0	43.0 65.0	0	51.0	35.0 62.5	0	50.0	36.0 65.0	0	51.0	40.5 64.5
Creatininemia at biopsy (μmol/l)	22	144	113 193	14	136	108 176	4	178	145 247	2	172	134 238	0	225	156 360	2	129	108 168
Time from transplant to biopsy (years)	0	1.0	0.3 1.1	0	0.9	0.3 1.0	0	1.1	1.0 5.2	0	2.0	1.0 6.6	0	0.6	0.3 2.3	0	1.0	0.3 1.0

Table 1: Characteristics of 1495 renal biopsies included in the analysis according their biopsy groups, including categorical and continuous variables.

CMV, cytomegalovirus; CSA, cyclosporin A; DGF, delayed graft function; DSA, donor specific antibodies; HLA, human leucocyte antigens; Md, median; Q, quartile; Q1, 25th percentile; Q3, 75th percentile; NA: not available (missing). Recipient/Donor CMV serology definition, 0: negative donor and positive recipient; 1: negative donor and negative recipient; 2: positive donor and negative recipient; 3: positive donor and recipient; Log: natural logarithm.

confounder-adjusted mean difference between group 3 and group 1 was 0.38 ($p = 0.0004$) (Figure 3B), meaning that the absolute number of CD28-CD8+ T cells is 46% higher in the ABMR group compared to the normal/sub-normal histology group. The confounder-adjusted differences between the others groups were not statistically significant (Figure 3B). Taken together, those analyses show that ABMR is associated with a significant increase in the percentage and absolute number of CD28-CD8+ T cells in the patient's blood at the time of biopsy.

Functional analysis of CD8+ T cells in ABMR patients

In order to decipher the mechanisms underlying the association between CD28-CD8+ T cells and ABMR, we retrieved 24 frozen PBMC samples from 24 patients belonging to biopsy group 3 (ABMR) to be used for further functional studies. A detailed flow cytometry analysis of those samples revealed that CD28-CD8+ T cells contained higher percentage of the effector-memory CD8+ T cells expressing CD45RA (TEMRA) defined as CCR7-CD45RA+ compared to CD28-CD8+ T cells (Figure 4A). We stimulated PBMCs with plate-bound anti-CD3 mAb and either soluble anti-CD28 or IL-15 and then stained for intracellular TNF α and IFN γ or surface CD107a, a marker of cytotoxic degranulation. As shown in Figure 4B, compared to CD28+CD8+ T cells, significantly higher percentages of CD28-CD8+ T cells were double-positive for TNF α and IFN γ (left) and positive for CD107a (right), suggesting that CD28-CD8+ T cells produce more TH1 cytokines and have stronger cytotoxic potential in response to TCR stimulation. Because the CD28-CD8+ T cells can include a subpopulation of T cells that expresses Fc γ RIIIA (CD16),²⁰ we replaced anti-CD3 by anti-CD16 mAb and obtained the same result, i.e. significantly higher percentages of CD28-CD8+ T cells were double-positive for TNF α and IFN γ (left) and positive for CD107a (right) (Figure 4C).

We asked whether the finding that CD28-CD8+ T cells respond more vigorously to TCR or Fc γ RIIIA stimulation compared to their CD28+CD8+ counterparts is specific to patients diagnosed with ABMR. To answer this question, we retrieved 46 PBMC samples from patients belonging to biopsy group 1 (normal or subnormal) and performed the same functional tests. We found that this finding is not restricted to ABMR because CD28-CD8+ T cells from patients with normal/subnormal biopsies also showed higher responses to TCR or Fc γ RIIIA stimulation compared to their CD28+CD8+ counterparts (Supplementary Figure 3). Next, we compared the functional responses of the CD28-CD8+ T cell subpopulation between these two groups of patients, ABMR and normal/subnormal biopsies. Interestingly, we found that CD28-CD8+ T cells from patients with ABMR showed higher cytotoxic potential in response to TCR stimulation than those from

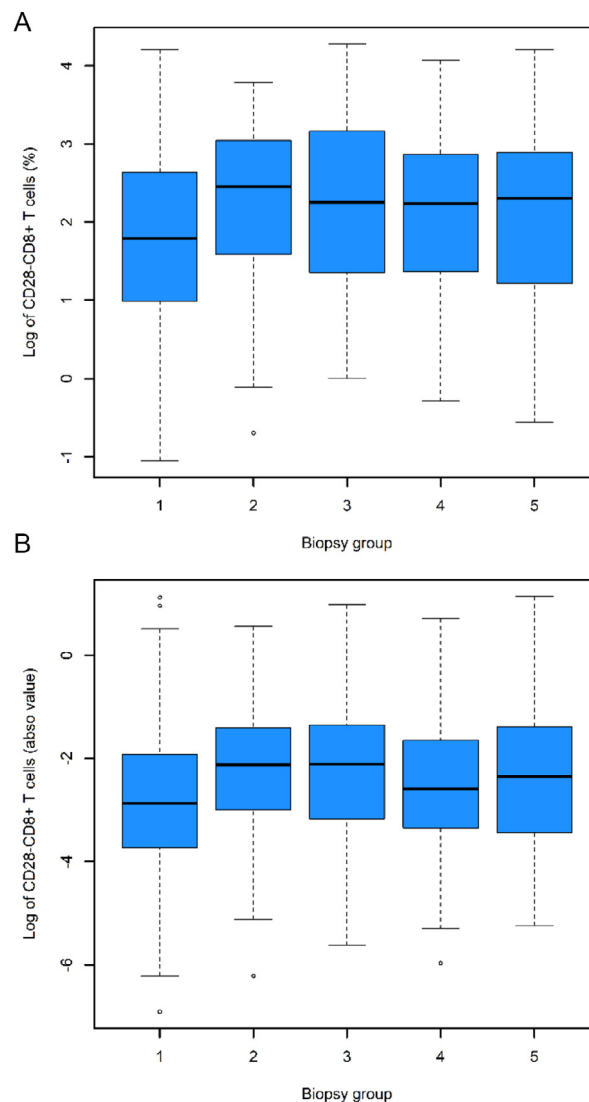


Figure 2. Distribution of the natural logarithm of the percentage (A) and absolute number (B) of CD28-CD8+ T cells according to histological groups. In each boxplot, the central line indicates the median of the data set, the shaded box represents the interquartile range (IQR) which means 25 percentile to 75 percentile or quartile 1 (Q1) to quartile 3 (Q3), and the lower and upper bars indicate Q1 - 1.5 IQR and Q3 + 1.5 IQR, respectively. Biopsy group 1: normal/sub-normal, group 2: IFTA grade 2/3, group 3: ABMR, group 4: TCMR, and group 5: borderline rejection. See also Supplementary Tables 2 and 3 for the unadjusted mean difference in the log percentage and log absolute number, respectively, between biopsy groups.

patients with normal/subnormal biopsies, as evidenced by significantly higher percentages of cells with CD107a expression (Figure 4D, upper right). On the other hand, the percentages of CD28-CD8+ T cells expressing both TNF α and IFN γ in response to TCR stimulation (Figure 4D, upper left) or expressing CD107a in response to Fc γ RIIIA stimulation (Figure 4D, lower right) were not different between these two groups of patients. Surprisingly, lower percentages of CD28-CD8+ T cells from ABMR patients expressed TNF α and IFN γ in response to Fc γ RIIIA stimulation compared

to those from patients with normal/subnormal biopsies (Figure 4D, lower left).

The percentage and absolute number of CD28-CD8+ T cells at 1 year are not associated with graft survival

For the graft survival analysis, we asked whether there was an association between the percentage and absolute number of CD28-CD8+ T cells at 1 year and graft survival. To this end, we used data from the subgroup of 510 patients who underwent a 1 year surveillance biopsy

	Adjusted mean difference	95% CI	p-value
Biopsy group			0.0003
2 (vs. 1)	0.09	[-0.09; 0.28]	
3 (vs. 1)	0.29	[0.15; 0.43]	
4 (vs. 1)	0.27	[0.05; 0.49]	
5 (vs. 1)	0.19	[0.01; 0.36]	
Transplantation after 2008	-0.25	[-0.41; -0.08]	0.0033
Recipient age (years)	0.00	[-0.00; 0.01]	0.5457
Male recipient	0.01	[-0.11; 0.12]	0.9157
Retransplantation	0.35	[0.16; 0.53]	0.0003
DGF	0.03	[-0.09; 0.15]	0.6582
Cold ischemia time (hours)	0.00	[-0.00; 0.01]	0.2846
History of cardiovascular disease	0.00	[-0.11; 0.12]	0.9378
Recipient/Donor CMV serology			<0.0001
1 (vs. 0)	1.19	[1.04; 1.33]	
2 (vs. 0)	0.66	[0.51; 0.80]	
3 (vs. 0)	1.24	[1.09; 1.38]	
Donor age (years)	0.01	[0.00; 0.01]	0.0305
Male donor	0.02	[-0.09; 0.14]	0.6653
Deceased donor	-0.02	[-0.26; 0.22]	0.8721
HLA-A-B-DR mismatches > 4	-0.01	[-0.14; 0.13]	0.9103
ABO mismatch	-0.50	[-0.92; -0.08]	0.0194
Depleting induction	0.00	[-0.13; 0.14]	0.9541
CSA	0.14	[-0.49; 0.77]	0.6632
Tacrolimus	-0.02	[-0.65; 0.62]	0.9542
Positive anti-class I immunization	0.01	[-0.13; 0.14]	0.9216
Positive anti-class II immunization	-0.12	[-0.27; 0.03]	0.1151
Biopsy rank			<0.0001
2 (vs. 1)	0.32	[0.24; 0.39]	
3 (vs. 1)	0.38	[0.22; 0.53]	
4 (vs. 1)	0.85	[0.40; 1.29]	
5 (vs. 1)	0.46	[-0.74; 1.65]	
For cause biopsy	-0.03	[-0.14; 0.07]	0.5267
Creatininemia at biopsy (μmol/l)	-0.00	[-0.00; 0.00]	0.1940
Post-transplantation time of the biopsy (years)	0.01	[-0.02; 0.03]	0.5772

Table 2: Results of the multivariable linear mixed model of log percentage of CD28-CD8+ T cells measured at time of biopsy.

CI, confidence interval; CMV, cytomegalovirus; CSA, cyclosporin A; DGF, delayed graft function; HLA, human leucocyte antigens. Recipient/Donor CMV serology definition, 0: negative donor and recipient; 1: negative donor and positive recipient; 2: positive donor and negative recipient; 3: positive donor and recipient.

because they also had a lymphocyte phenotyping at the time of biopsy. The median follow-up time was 5.0 years (range from 0 to 11.1 years). During follow-up, we observed 61 returns to dialysis and 51 deaths with a functioning graft. The characteristics of the 510 patients included in this analysis are shown in Supplementary Table 3. As in the principle analysis, the percentage and absolute number of CD28-CD8+ T cells also underwent log transformation. First, we established the death-censor graft survival curve using the Kaplan-Meier method (Supplementary Figure 4). Survival rates at 5 and 8 years (the baseline being the date of the 1-year screening biopsy) were 88.4% (95% CI: 85.2%-91.8%) and 78.3% (95% CI: 72.6%-84.5%), respectively. For robustness

analysis, we estimated the cumulative incidence curves of the outcome from both the Kaplan-Meier estimator and the Aalen-Johansen estimator considering deaths with functioning graft as competing events and found that the two curves were close (Supplementary Figure 5). Unadjusted Cox models did not find any significant association between the log percentage and log absolute number of CD28-CD8+ T cells and graft survival (Supplementary Table 4). The multivariable Cox models confirmed the absence of significant association between the log percentage (Table 4) and the log absolute number (Table 5) of CD28-CD8+ T cells and graft survival (HR = 1.15, 95% CI: 0.88-1.51, $p = 0.30$ and HR = 1.17, 95% CI: 0.93-1.46, $p = 0.18$, respectively).

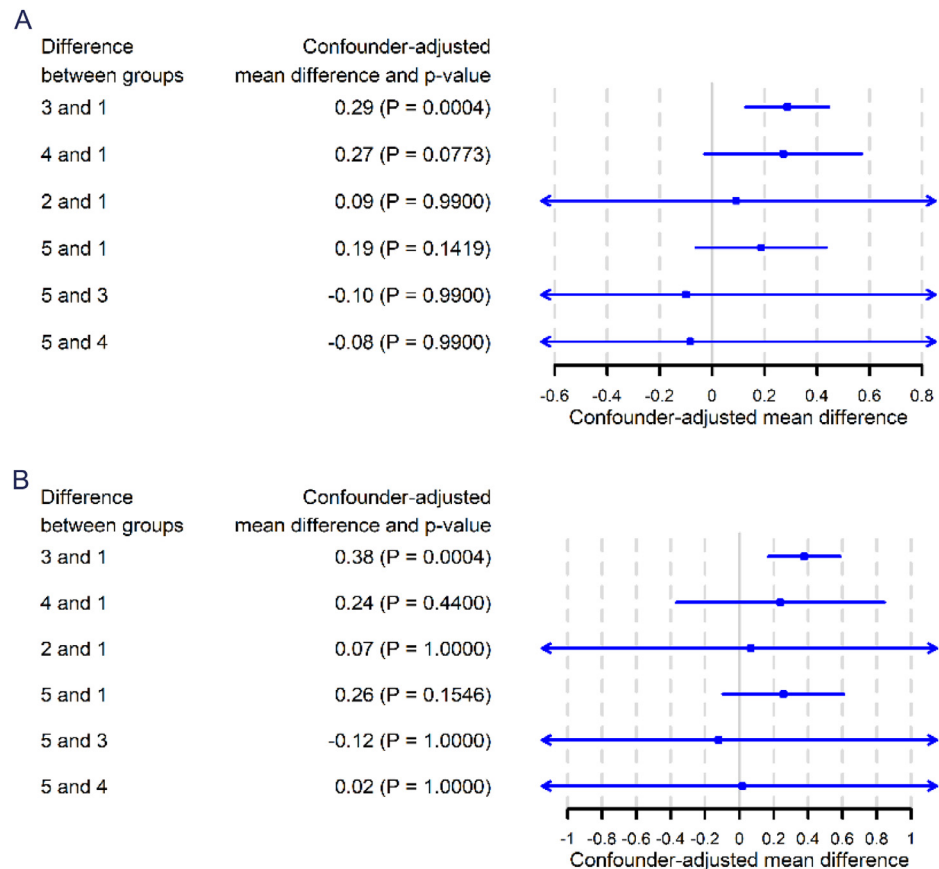


Figure 3. Confounder-adjusted mean difference, 95% confidence intervals and p-value adjusted by Holm–Bonferroni method for the natural logarithm of the percentage (A) and the absolute number (B) of CD28-CD8+ T cells.

Discussion

Our analyses of a cohort of 1495 kidney graft biopsies performed on 1032 patients have shown that ABMR is significantly associated with an increase in the percentage as well as the absolute number of the differentiated CD28-CD8+ T cells in the peripheral blood measured at the time of graft biopsy. This large cohort study corroborates the findings of our previous study performed on a small number of kidney graft recipients showing an increase in the percentage of CD28-CD8+ T cells in patients with chronic rejection.¹⁵ Increased CD28-CD8+ T cells have been shown to be associated with graft rejection in several studies by other groups based on smaller numbers of patients. In a cross-sectional study of 121 kidney transplant recipients, CD28-CD57+CD8+ T cells were found to be increased in patients with TCMR compared to those with normal biopsy.²¹ In another study including 25 living donor kidney transplant recipients, patients with borderline or acute rejection were found to have higher pretransplant frequencies of CD28-CD8+ T cells compared to rejection-free kidney graft recipients.²² CD28-CD8+ T cells were also shown to be increased in patients with

bronchiolitis obliterans syndrome (BOS) compared to stable patients in a study including 68 lung transplant recipients.²³ On the contrary, some studies showed that high numbers of CD28-CD8+ T cells before transplant were associated with a lower risk of early²⁴ and late kidney graft rejection and graft loss.²⁵ Since the interaction between CD28 on T cells and B7 on APC is the principal target for the costimulatory blocking drug belatacept, CD28-CD8+ T cells have also been implicated in belatacept-resistant kidney graft rejection.²⁶

However, CD28-CD8+ T cells are likely a heterogeneous population containing both cells with effector/memory properties and those with regulatory capacities. In vitro expanded CD28-CD8+ T cells were shown to have suppressive effect on CD4+ T cell proliferation in response to allogenic stimulation.²⁷ On the contrary, we and others have shown that CD28-CD8+ T cells in patients with allograft rejection have effector properties as they express cytotoxic molecules such as granzyme A, granzyme B, and perforin, as well as inflammatory cytokines such as IFN- γ , TNF- α , and IL-2.^{8,15,28} The result of our analyses showing an association between an increase in CD28-CD8+ T cells and antibody-

	Adjusted mean difference	95% CI	p-value
Biopsy group			0.0006
2 (vs. 1)	0.07	[-0.18; 0.31]	
3 (vs. 1)	0.38	[0.19; 0.56]	
4 (vs. 1)	0.24	[-0.05; 0.53]	
5 (vs. 1)	0.26	[0.02; 0.49]	
Transplantation after 2008	-0.40	[-0.61; -0.19]	0.0002
Recipient age (years)	-0.01	[-0.02; -0.00]	0.0215
Male recipient	0.03	[-0.11; 0.17]	0.6869
Retransplantation	0.41	[0.16; 0.65]	0.0011
Renal transplantation	-0.03	[-0.31; 0.24]	0.8055
Recurrent initial disease	0.07	[-0.09; 0.24]	0.3728
DGF	0.01	[-0.14; 0.16]	0.8637
Cold ischemia time (hours)	0.01	[-0.00; 0.02]	0.2286
Recipient/Donor CMV serology			<0.0001
1 (vs. 0)	1.30	[1.12; 1.49]	
2 (vs. 0)	0.72	[0.53; 0.90]	
3 (vs. 0)	1.38	[1.19; 1.56]	
Donor age (years)	0.01	[0.00; 0.02]	0.0089
Male donor	0.05	[-0.10; 0.19]	0.5286
Deceased donor	-0.06	[-0.36; 0.24]	0.7066
HLA-A-B-DR mismatches > 4	-0.04	[-0.20; 0.13]	0.6558
ABO mismatch	-0.88	[-1.41; -0.36]	0.0010
Depleting induction	-0.61	[-0.80; -0.42]	<0.0001
CSA	0.29	[-0.49; 1.08]	0.4622
Tacrolimus	0.14	[-0.65; 0.93]	0.7305
Steroid	-0.04	[-0.23; 0.15]	0.6902
Positive anti-class I immunization	-0.02	[-0.18; 0.15]	0.8527
Positive anti-class II immunization	-0.15	[-0.34; 0.04]	0.1262
Positive DSA	-0.04	[-0.34; 0.25]	0.7783
Biopsy rank			<0.0001
2 (vs. 1)	0.50	[0.39; 0.61]	
3 (vs. 1)	0.64	[0.42; 0.86]	
4 (vs. 1)	1.06	[0.44; 1.68]	
5 (vs. 1)	1.02	[-0.65; 2.69]	
For cause biopsy	-0.05	[-0.19; 0.09]	0.4612
Creatininemia at biopsy (μmol/l)	-0.00	[-0.00; -0.00]	0.0110
Post-transplantation time of the biopsy (years)	0.04	[0.01; 0.07]	0.0202

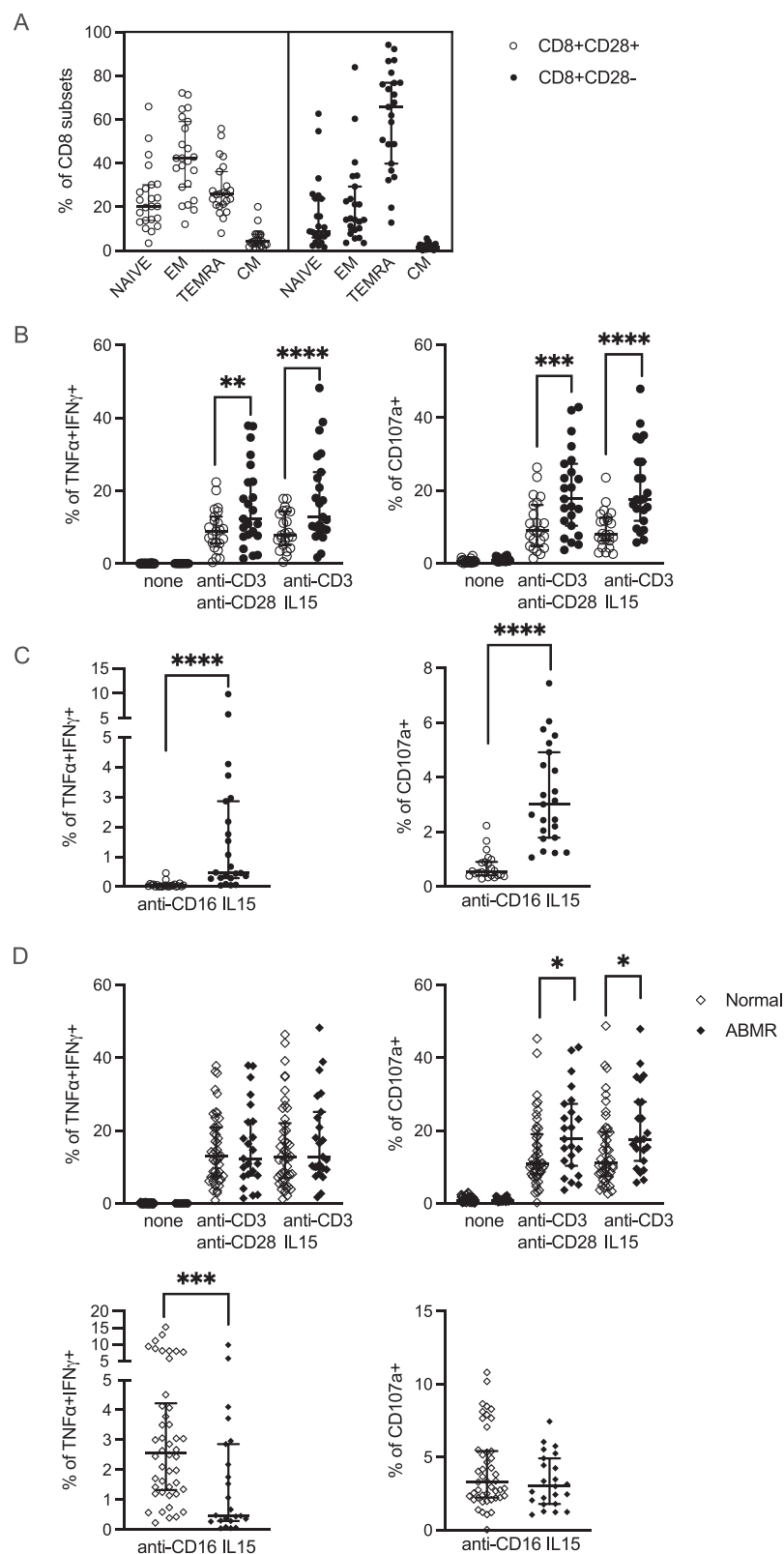
Table 3: Results of the multivariable linear mixed model of log absolute number of CD28-CD8+ T cells measured at time of biopsy.

CI, confidence interval; CMV, cytomegalovirus; CSA, cyclosporin A; DGF, delayed graft function; HLA, human leucocyte antigens; NA: not available (missing). Recipient/Donor CMV serology definition, 0: negative donor and recipient; 1: negative donor and positive recipient; 2: positive donor and negative recipient; 3: positive donor and recipient.

mediated rejection is interesting but not surprising. The Banff classification of renal allograft pathology might give an impression that kidney transplant rejections are caused by 2 distinct underlying mechanisms, either T cell-mediated or antibody-mediated. In reality, both mechanisms are usually operational at the same time. For instance, T cell relative frequency among graft infiltrating cells is similar between ABMR and TCMR.²⁹ Since MHC as well as minor histocompatibility antigens are T cell-dependent antigens, recipient B cell activation and differentiation leading to the generation of donor-specific antibodies (DSA) requires T cell help. In

addition, cytotoxic CD8+ T cells are found in renal biopsies with ABMR, either by immunohistological staining or gene expression analyses.^{8,9,30,31}

Moreover, we have demonstrated in the current study that compared to their CD28+ counterparts, CD28-CD8+ T cells produce more TH1 cytokines and have stronger cytotoxic potential in response to TCR stimulation or FcγRIIIA (CD16) engagement. Those results are in concordance with our previous report on TEMRA CD8+ T cells.²⁰ The potent effector functions of the CD28-CD8+ T cell subpopulation is not specific to ABMR because we obtained the same results in



	HR	95% CI	p-value
Log of CD28-CD8+ T cells (%)	1.15	[0.88; 1.51]	0.2963
Male recipient	1.57	[0.84; 2.94]	0.1605
Retransplantation	1.14	[0.48; 2.70]	0.7731
DGF	1.17	[0.66; 2.09]	0.5954
Cold ischemia time (hours)			0.0548
Between 12 and 24 h (vs. less than 12 h)	3.76	[1.15; 12.31]	
24 h or more (vs. less than 12 h)	2.34	[0.58; 9.49]	
History of hypertension	0.68	[0.31; 1.48]	0.3267
Donor older than 55 years	0.97	[0.55; 1.71]	0.9077
HLA-A-B-DR mismatches > 4	1.92	[1.01; 3.62]	0.0453
CSA	1.98	[0.79; 4.97]	0.1451
Positive anti-class II immunization	1.60	[0.82; 3.10]	0.1683
Creatininemia at biopsy ($\mu\text{mol/l}$)	1.02	[1.01; 1.02]	<0.0001

Table 4: Results of the multivariable cause-specific Cox model studying the associations between the percentage of CD28-CD8+ T cells and graft survival.

CI, confidence interval; CSA, cyclosporin A; DGF, delayed graft function; HLA, human leucocyte antigens; HR, hazard ratio; Log, natural logarithm.

	HR	95% CI	p-value
Log of CD28-CD8+ T cells (absolute value)	1.17	[0.93; 1.46]	0.1791
Male recipient	1.55	[0.82; 2.90]	0.1742
Retransplantation	1.20	[0.51; 2.83]	0.6730
DGF	1.16	[0.65; 2.08]	0.6128
Cold ischemia time (hours)			0.0536
Between 12 and 24 h (vs. less than 12 h)	3.76	[1.15; 12.31]	
24 h or more (vs. less than 12 h)	2.32	[0.57; 9.42]	
History of hypertension	0.67	[0.31; 1.47]	0.3201
Donor older than 55 years	0.95	[0.54; 1.69]	0.8692
HLA-A-B-DR mismatches > 4	1.86	[0.99; 3.52]	0.0555
CSA	1.97	[0.79; 4.95]	0.1483
Positive anti-class II immunization	1.61	[0.83; 3.14]	0.1617
Creatininemia at biopsy ($\mu\text{mol/l}$)	1.02	[1.01; 1.02]	<0.0001

Table 5: Results of the multivariable cause-specific Cox model studying the associations between the absolute number of CD28-CD8+ T cells and graft survival.

CI, confidence interval; CSA, cyclosporin A; DGF, delayed graft function; HLA, human leucocyte antigens; HR, hazard ratio; Log, natural logarithm.

recipients with normal biopsies. However, we found that CD28-CD8+ T cells from ABMR patients have stronger cytotoxicity in response to TCR stimulation than those from patients with normal biopsies. Although the increase in CD28-CD8+ T cells might be a

marker of the overall strength of alloimmune reaction, taken together, our experimental data suggest that CD28-CD8+ T cells may also participate in the pathobiology of ABMR, especially through their potent cytotoxic response upon TCR stimulation.

Figure 4. Functional studies of CD8+ T cells in ABMR. Frozen PBMCs (n=24) from patients diagnosed with ABMR (biopsy group 3) was retrieved from our biocollection for functional studies. (A) Flow cytometry staining for CD45RA and CCR7 divides CD8+ T cells into 4 subpopulations: naïve (CD45RA+CCR7+), central memory (CM) (CD45RA-CCR7+), effector memory (EM) (CD45RA-CCR7-), and effector memory expressing CD45RA (TEMRA) (CD45RA+CCR7-). The percentage of these 4 subpopulations in CD28+CD8+ and CD28-CD8+ T cells are shown. (B) and (C) PBMCs were stimulated with plate-bound anti-CD3 mAb, soluble anti-CD28 mAb or IL-15, and plate-bound anti-CD16 mAb as indicated for 4 h. Cells were stained for surface CD8, CD28, and CD107a, followed by intracellular staining for TNF α and IFN γ and analyzed by flow cytometry. The percentage of cells double positive for TNF α and IFN γ (left) and positive for CD107a (right) among CD28+CD8+ (open circles) and CD28-CD8+ (filled circles) T cells are shown. (D) Frozen PBMCs (n=46) were also retrieved from patients with normal/subnormal biopsies (group 1) and stimulated as in (B) and (C). The functional responses of the CD28-CD8+ T cell subpopulation from patients with ABMR and normal/subnormal biopsies were compared (see also Supplementary Figure 3). *: p<0.05; **: p<0.01; ***: p<0.001; ****: p<0.0001.

We next performed multivariable Cox models to investigate whether an increase in CD28-CD8+ T cells can help to predict kidney graft failure. We did not find any significant association between the percentage and the absolute number of CD28-CD8+ T cells at 1 year post-transplantation and graft survival. Nevertheless, in one of our previous studies using a more sophisticated gating strategy, we have shown that increased levels of TEMRA CD8+ T cells defined as CD45RA+CCR7- CD8+ were associated with a 2-fold higher risk of long-term graft dysfunction.³²

Our study has some limitations. First of all, it is a single-center study. Secondly, based on our publication in 2006,¹⁵ we selected only a few markers so that the test could be performed routinely on fresh blood samples at the immunophenotyping platform of our hospital at the time of kidney graft biopsy and the results of this study could validate the findings of our previous study. This lymphocyte phenotyping strategy was designed more than a decade ago, now it becomes clear that more lymphocyte markers are necessary to better identify effector/memory CD8+ T cells.^{20,32–34} Thirdly, polyclonal TCR stimulation was used in our functional studies of CD8+ T cells, stimulation with donor antigens could further confirm our findings. Fourthly, although we have included many covariates in the multivariable statistical models, we cannot exclude unobserved confounders, for example, ethnicity and socioeconomic status, which were not collected in our database.

Finally, in multivariable analyses, we did not consider individuals with missing data on the covariates, which might reduce the statistical power leading to non-significant results. However, our effort to limit missing data was considerable. As shown in Table 1, 20/30 variables studied contained no missing data. Of the 10 variables with missing data, 4 variables contained only 1 case with missing data. Overall, about 5% of total cases were excluded from the multivariable analysis because of at least 1 missing data. We have looked at variables with missing data and found no overt reason to suspect the presence of « missing not at random » (MNAR), where the cause of missing data is directly linked with the value of those data. Given the small percentage of cases with missing data and the probable absence of MNAR, our approach to analyze only cases with complete data would unlikely induce significant bias.

In summary, this is the first time the differentiated CD28-CD8+ T cell phenotype is shown to be associated with ABMR in a large cohort of more than 1000 kidney graft recipients with nearly 1500 kidney graft biopsies. Our study sheds new insight into the understanding of an intriguing subpopulation of CD8+ T cells in the transplant setting. Future studies using more comprehensive lymphocyte phenotyping strategies on large cohorts of renal transplant patients together with

functional experiments using preserved donor cells as stimulators are needed to clarify the role of memory/effector CD8+ T cells in different forms of graft rejection.

Contributors

HLM, ND, MR, KR, FLB, SB, and MG participate in the writing and revision of the manuscript; SB, MG, HLM, ND, SLB, RD, and FLB participate in the conceptualization and methodology; MR provides and verifies lymphocyte phenotyping data; KR interpretes kidney graft biopsies; FD and AW provide and verify patients' laboratory data; FLB perform statistical analyses; ND, GT, and AV design and perform in vitro functional studies; CK perform data extraction from the DIVAT database; SLB, RD, CK, HLM, SB, and MG verify the extracted data; SB and MG acquire funding and supervise the study. All authors approved the final version of the manuscript.

Data sharing statement

All the data supporting the findings of this study are available within the article and its Supplemental Information files or from the corresponding author upon reasonable request.

Declaration of interests

All authors declared no conflict of interest.

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Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.ebiom.2022.104226.

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