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CMV driven CD8⁺ T-cell activation is associated with acute rejection in lung transplantation

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Abstract Lung transplantation is the definitive treatment for terminal respiratory disease, but the associated mortality rate is high. Acute rejection of the transplanted lung is a key determinant of adverse prognosis. Furthermore, an epidemiological relationship has been established between the occurrence of acute lung rejection and cytomegalovirus infection. However, the reasons for this association remain unclear. Here, we performed a longitudinal characterization of CMV-specific T-cell responses and immune activation status in the peripheral blood and bronchoalveolar lavage fluid of forty-four lung transplant patients. Acute rejection was associated with high levels of cellular activation in the periphery, reflecting strong CMV-specific CD8⁺ T-cell activity post-transplant. Peripheral and lung CMV-specific CD8⁺ T-cell responses were very similar, and related to the presence of CMV in the transplanted organ. These findings support that activated CMV-specific CD8⁺ T-cells in the lung may play a role in promoting acute rejection.

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Abbreviations: APC, allophycocyanin; AR, acute rejection; BAL, bronchoalveolar lavage; CMV, cytomegalovirus; IFN, interferon

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1. Introduction

Lung transplantation is the only curative therapeutic option for end-stage respiratory diseases such as cystic fibrosis, chronic obstructive pulmonary disease and pulmonary fibrosis. Despite general improvements in the management of lung transplantation allowing better survival, mortality remains high. Overall, survival is 88% at three month post-transplant, 79% after one year, 64% after 3 years and 53% after 5 years. Along with post-operative complications and infections, acute rejection is an important determinant of mortality during the first 2 year post-transplant [1]. Further understanding of the multiple co-factors that participate in the acute cellular rejection of transplanted lungs is critical to enhance the survival of patients. Cytomegalovirus (CMV) infection appears to play an important role in graft injury and patient mortality [2,3]. First, viral recrudescence can cause severe pathology (e.g. retinitis, neurological, gastrointestinal and pulmonary disease) in immunosuppressed individuals. Second, in solid organ transplantation, an epidemiological association between CMV infection and acute rejection has been established [2,4,5]. The CMV serostatus of donors (D) and recipients (R) has a direct impact on the occurrence of acute rejection: D⁺/R⁻ combinations present the highest risk of acute rejection [6]. Although the implementation of robust CMV prophylaxis has improved considerably the survival of seropositive donors after lung transplantation [7], the link between CMV and acute rejection remains unclear.

During acute lung rejection, peribronchiolar and perivascular mononuclear cell infiltration is often apparent histologically. At the mechanistic level, lymphocyte activation [8,9], direct (donor antigen-presenting cell) and indirect (recipient antigen-presenting cell) allorecognition are all implicated [10]. Moreover, such alloimmune responses typically display a predominant Th1 cytokine profile and cytotoxicity. These cellular factors may result in severe graft dysfunction (*i.e.* acute rejection) [11,12]. Cellular immune activation is thus thought to be an important co-factor for acute rejection in lung transplantation. CMV infection has been hypothesized to play a particular role in this context [13–16]. CMV tropism is mostly directed to endothelial and epithelial cells, and monocytes, which allow infection to occur at multiple sites and in multiple organs, including the lungs [17,18]. CMV infection is usually associated with strong immune activation, persistent antigen exposure and inflammation [19–21]. In the general population, the immune response against CMV is characterized by massive expansions of CMV-specific T-cells, in particular within the CD8⁺ T-cell compartment. CMV-specific CD8⁺ T-cells generally exhibit robust cytotoxic potential and a strong pro-inflammatory profile, reflected by their capacity to produce cytokines such as IFN γ and TNF α [19,22]. Moreover, these cells display a late differentiation phenotype (CD27⁻ CD28⁻ CD57⁺), associated with endovascular homing capacities, with the expression of receptors (e.g. CX3CR1) that enable migration to the perivascular space [20,23]. There, their presence may result in collateral damage favoring acute rejection. CMV-specific CD8⁺ T-cells may indirectly enhance alloreactive reactions as a consequence of MHC class II upregulation in response to IFN γ production and endothelial activation [24]. Activated CMV-specific CD8⁺ T-cells may also cause direct cytolytic or cytokine mediated damage to constitutive graft cells, either

due to the presentation of CMV antigens or through cross-alloreactivity, as suggested in *in vitro* studies [25–30]. However, there has been no *in vivo* study to further support the hypothesis that the presence of CMV-specific T-cells in the infected lung post-transplant may be associated with the process of acute rejection.

In the present study, we examined the relationship between CMV infection and acute rejection. For this purpose, we performed a prospective analysis of immunological parameters in lung transplant recipients, including levels of immune activation and CMV-specific CD8⁺ T-cell frequencies. These longitudinal analyses were performed in both peripheral blood and bronchoalveolar lavage fluid (BAL), and related to clinical events such as CMV replication and acute rejection for up to 24 months after lung transplantation. Our results highlight a relationship between acute rejection, peripheral immune activation and anti-CMV cellular immunity in the lung.

2. Methods

2.1. Study subjects

Forty-four patients undergoing lung transplantation were recruited at the Service de Pneumologie, Hôpital Foch, Suresnes, France. Four groups were designated according to donor/recipient CMV serostatus: D⁻/R⁻, D⁺/R⁻, D⁻/R⁺ and D⁺/R⁺ (Table 1). For the purposes of this study, we defined CMV seropositivity based on the presence of specific IgG in the blood; CMV replication denotes a positive virus-specific PCR in total blood, CMV reactivation denotes viral replication outside of primary infection, and CMV disease denotes viral replication with related organ dysfunction. A positive virus-specific PCR with (*i.e.* CMV disease) or without (*i.e.* CMV replication) clinical abnormalities defined a CMV event. Post-transplant immunosuppressive treatment consisted of an induction protocol followed by a maintenance regimen. The induction protocol included a high dose corticosteroid, a calcineurin inhibitor (cyclosporine or FK506) and a purine synthesis inhibitor (azathioprine or mycophenolate mofetil), with or without depleting antibodies (horse anti-thymocyte globulin; Thymoglobulin®) according to existing contraindications (CMV and EBV serostatus mismatch, pre-operative *Burkholderia cepacia* colonization, pre-transplant immunosuppression or re-transplantation). The maintenance regimen consisted of adapted doses of the corticosteroid, calcineurin inhibitor and purine synthesis inhibitor. Bronchoscopy with BAL, transbronchial biopsy (TBB) and CMV PCR in blood were performed either at scheduled visits (days 7 and 30; months 2, 4, 6, 9, 12, 18 and 24) or upon clinical decision (suspected infection or rejection; airway complication). Acute rejection was defined histologically on transbronchial biopsy according to the International Society of Heart and Lung Transplantation (ISHLT) classification [11]. Acute rejection episodes were graded A1 (n = 76) and A2 (n = 10), considering all patients. Acute rejection episodes were all treated with methylprednisolone bolus. Herpesvirus prophylaxis was adjusted according to CMV serostatus and comprised valgancyclovir (900 mg) or valacyclovir (8 g or 2 g) daily for 3–6 months for D⁺/R⁻, R⁺ and D⁻/R⁻ patients, respectively. All participants provided written informed consent. The study was approved by the Comité de Protection des Personnes of the Pitié Salpêtrière

Table 1 Study population with clinical characteristics according to CMV D/R serostatus.

	D ⁺ /R ⁻	D ⁻ /R ⁺	D ⁺ /R ⁺	D ⁻ /R ⁻	P
Number of patient (total = 44)	17	10	9	8	
Age at transplantation (median/quartile)	29, 3 (36,6–23,7)	50 (55,5–29,2)	50 (56,5–42,1)	26 (47–24,4)	0.014
Sex F (%)	6 (35%)	4 (40%)	4 (44%)	3 (33%)	ns
Initial disease, CF/COPD/IPF/others	14/2/0/1	4/3/3/0	2/3/4/0	5/2/1/0	0.036
Follow up period, month (median/quartile)	24, 6 (30,75–20,2)	24, 4 (36,2–21,8)	21, 9 (28,8–18,3)	26 (30,2–24,3)	ns
Bilateral LT/unilateral LT (%)	17 (100%)/ 0 (0%)	8 (80%)/ 2 (20%)	4 (45%)/ 5 (55%)	8 (100%)/ 0 (0%)	0.0012
<i>CMV</i>					
CMV replication/patient, nb = mean (SD)	0, 76 (1, 14)	0, 4 (0, 51)	1, 66 (1, 65)	0	0.039
Patient with ≥ 1 react nb (%)	7 (41%)	4 (40%)	6 (66%)	0	0.045
CMV viral load (median, 25% quartile)	5350 (7190–1660)	3162 (46,000–1150)	27,400 (51,250–9771)	0	0.013
<i>AR</i>					
AR episode/patient, nb = mean (SD)	2, 05 (1, 29)	1, 9 (1, 1)	1, 44 (0, 88)	1, 8 (1, 12)	ns
Patient with ≥ 1 AR nb (%)	14 (82%)	9 (90%)	8 (88%)	8 (88%)	ns
<i>ARpostM2</i>					
ARpostM2 episode/patient, nb = mean (SD)	1, 5 (1, 2)	1, 1 (0, 7)	1 (0, 7)	1 (1, 06)	ns
Patient with ≥ 1 ARpostM2 nb (%)	14 (82%)	8 (80%)	7 (77%)	6 (64%)	ns
<i>BOS</i>					
Nb, % population	5 (29%)	1 (10%)	0 (0%)	1 (12%)	ns
<i>Mortality</i>					
Nb, % population	2 (11%)	1 (10%)	0 (0%)	0 (0%)	
<i>Initial T cell depletion</i>					
Thymoglobulin	1 (5, 8%)	2 (20%)	3 (33%)	4 (50%)	ns
<i>Match D/R HLA A/B</i>					
Nb, mean (SD)	0, 6 (0, 73)	0, 62 (0, 74)	0, 8 (0, 83)	0, 75 (0, 7)	ns
<i>Distribution</i>					
(No match/1 match/2 match/≥ 2 match/unknown)	8/5/2/0/2	4/3/1/0/2	2/2/1/0/4	3/4/0/0/1	ns
<i>Mismatch D/R sex</i>					
Distribution (identical; F/M; M/F; unknown)	8/2/1/5	6/1/1/2	6/0/1/2	4/2/1/1	ns
Cold ischemic time minutes (med, 25% quartile)	330 (375–277)	305 (347–247)	360 (390–330)	310 (468–252)	ns

The Kruskal–Wallis test was used to establish differences between groups. AR: acute rejection; BOS: bronchiolitis obliterans syndrome; CF: cystic fibrosis; COPD: chronic obstructive pulmonary disease; IPF: idiopathic pulmonary fibrosis; M2: Month 2; Nb: number.

Hospital, Paris. Blood and BAL samples were obtained regularly for up to 2 years (at months 2, 4, 6, 9, 12 and 18 or 24) following transplantation. PBMCs (isolated by density gradient centrifugation) and bronchoalveolar mononuclear cells (BMCs) (isolated from 40 mL of BAL by filtration) were cryopreserved until use.

2.2. Reagents

Monoclonal antibodies were obtained from the following vendors: (i) αCD8-V450, αCCR7-PE-Cy7, αCD38-APC, αCD45RA-

FITC and αIFNγ-Alexa700 (BD Biosciences); (ii) αCD27-Alexa700 (BioLegend); and (iii) αCD3-ECD (Beckman Coulter). The viability dye Aqua (Invitrogen) was used to eliminate dead cells from the analysis. Fluorescent tetrameric antigen complexes (tetramers) were produced as described previously [31], and included: HLA-A*0201 CMV-pp65-NV9, HLA-A*0201 EBV-BMLF1-GL9 and BRLF1-YV9, HLA-A*1101 EBV-EBNA3B-AK10 and EBNA3B-IK9, HLA-B*0701 CMV-pp65-TM10, HLA-B*0801 EBV-BZLF1-RL8 and EBNA3A-FL9. Overlapping CMV peptides were kindly provided by Dr. Daniel Olive (Centre Paoli Calmettes, Marseille, France).

2.3. Flow cytometry and assessment of T-cell function

Staining for cell surface markers was performed as described previously [21]. To detect virus-specific CD8⁺ T-cells, 10⁶ PBMCs or BMCs were sequentially stained with pre-titrated tetramers conjugated to phycoerythrin and a panel of pre-titrated mAbs for 15 min at room temperature. To measure CMV-specific CD8⁺ T-cell responses, 10⁶ PBMCs were stimulated in the presence of 15 mer peptides (5 μM), overlapping by 10 amino acids, spanning the CMV proteins pp65 and IE1. After 1 h, the secretion inhibitor brefeldin A (5 μg/mL; Sigma-Aldrich) was added and the incubation was continued overnight at 37 °C in a 5% CO₂ atm. Cytofix/Cytoperm™ (BD Biosciences) was used to fix/permeabilize the cells prior to staining for intracellular IFNγ. Data were acquired using an LSRII flow cytometer (BD Biosciences).

2.4. Clonotypic analysis

Viable HLA-A*0201 CMV pp65-NV9 tetramer⁺ CD8⁺ T-cells were sorted directly *ex vivo* at >98% purity using a FACSAria flow cytometer (BD Biosciences). Molecular analysis of *TRB* gene expression was conducted using a template-switch anchored RT-PCR as described previously [32]. TCR nomenclature was directly translated from the IMGT database using web-based alignment of molecular TRB transcripts (<http://imgt.cines.fr>).

2.5. Statistical analysis

Acute rejection was considered as a categorical variable (*i.e.* 0 or ≥1, independently of the grade). Among CMV exposed patients, CMV infection and disease were considered as categorical variables (*i.e.* 0 or ≥1), or as continuous variables (*i.e.* number of CMV replication events) for correlations. Flow cytometry data were considered as continuous variables. Groups were compared using the non-parametric Kruskal–Wallis or Mann–Whitney tests. Spearman's rank test was used to determine correlations. A parametric survival model with exponential survival distribution was also used to assess the association between changes in CD38 expression on CD8⁺ T cells and acute lung rejection considering multiple events during the course of follow-up. *P* values < 0.05 were considered significant.

3. Results and discussion

3.1. Clinical description of study population

A total of 44 patients undergoing lung transplantation between 2008 and 2009 were included in this study (Table 1). The differences observed between D/R groups based on CMV serostatus groups (with respect to pre-transplantation lung disease, type of lung transplantation and age) were expected and reflect the normal distribution of patients according to D/R CMV serostatus. The frequency of CMV replication and viral load also differed between CMV serostatus groups, with D⁺/R⁺ patients experiencing the highest number of CMV replication episodes with the highest CMV loads. This is likely due to the fact that CMV prophylaxis for D⁺/R⁺ patients did not include

valgancyclovir. No differences were observed for other clinical parameters. We first aimed to assess the main clinical factor associated with acute rejection in our set of patients. The variables mentioned above (*i.e.* age, initial disease, type of transplantation and number of CMV replication events) as well as donor/recipient mismatch for HLA-A and B (previously associated with acute rejection) were considered in a multivariate model (*i.e.* likelihood ratio test) of acute rejection risk factor. None of these factors appeared to be linked to the total number of acute rejection events. However, this model yielded a significant and independent association between numbers of CMV replication events and numbers of post-month 2 (M2) acute rejection events (*P* = 0.002). This links to the timing of clinical event occurrence: while acute rejection events occurred both very early and throughout the post-transplant period, CMV replication events were commonly observed only later after transplantation (*i.e.* from month 2 onwards) (Fig. 1). On this basis, we reasoned that CMV replication may influence acute rejection only later after transplantation (*i.e.* after the first 2 months). This might be related to the time necessary to reconstitute or mount a CMV specific cellular response. Accordingly, we distinguished pre-M2 and post-M2 acute rejection events in our analyses. Moreover, since epidemiological studies have highlighted an increased risk of acute rejection in D⁺/R⁻ graft recipients [6] (*i.e.* patients undergoing primary CMV infection after lung transplantation, typically associated with extensive CMV replication), we concentrated our initial analysis on D⁺/R⁻ patients in comparison to D⁻/R⁻ patients.

3.2. Acute lung rejection is associated with cellular immune activation

CMV replication is associated with elevated levels of immune activation and systemic inflammation [22], especially during

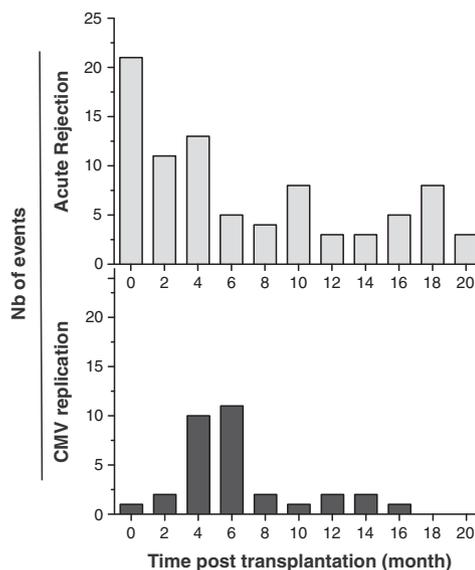


Figure 1 Timing of acute rejection and CMV replication events. Cumulative numbers of events of acute rejection and CMV replication (*i.e.* positive PCR in total blood) across all patients are shown for serial 2 month periods post-transplantation.

primary infection. To explore whether this may influence stable organ engraftment, we performed a longitudinal assessment of immune activation levels in relation to post-M2 acute rejection and CMV replication events. The expression of CD38 on memory CD8⁺ T-cells (*i.e.* excluding CD45RA⁺ CCR7⁺ naive cells) is a

robust marker of general cellular activation [33,34]. We therefore quantified CD38 expression in PBMC samples by flow cytometry. CD38 expression on total memory CD8⁺ T-cells from two representative donors (without or with CMV replication episodes) is shown in Fig. 2A. Mean CD38

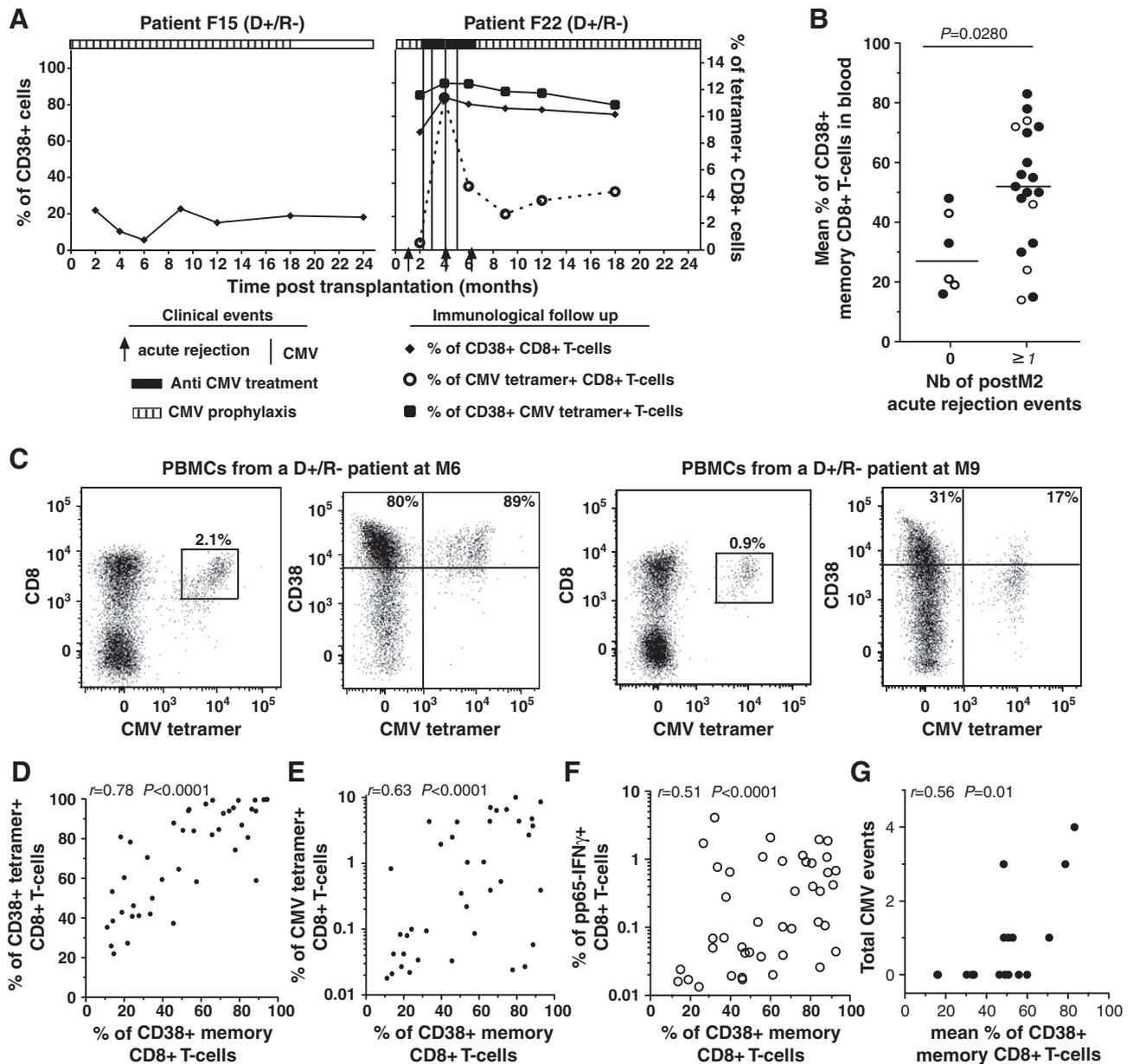


Figure 2 Acute rejection and activation of memory CD8⁺ T-cells. (A) Changes in immunological markers and occurrence of clinical events during the post-transplant follow-up period for two D⁺/R⁻ patients with either no signs of AR (F15 patient), or with recurrent AR events (F22 patient). (B) Association between mean percentages of CD38⁺ memory CD8⁺ T-cells in PBMCs and total number of post-M2 acute rejection events (none or at least one) for D⁺/R⁻ (full circles) and D⁻/R⁻ (open circles) patients. Mean percentages of CD38⁺ memory CD8⁺ T-cells for each patient represent the average value of all time points. Bars indicate median values. (C) Representative examples of flow cytometry dot plots showing percentages of pp65 tetramer⁺ CD8⁺ T-cells (left panels) and percentages of CD38⁺ in total and tetramer⁺ CD8⁺ T-cells (right panels) from one HLA-A*0201 D⁺/R⁻ patient at two different time points. (D) Correlation between activation levels (CD38 expression) in total memory and CMV tetramer⁺ CD8⁺ T-cell populations considering all time points for D⁺/R⁻ patients. (E) Correlation between CD38 expression on total memory CD8⁺ T-cells and frequency of CMV tetramer⁺ CD8⁺ T-cells considering all time points for D⁺/R⁻ patients. (F) Correlation between CD38 expression on total memory CD8⁺ T-cells and percentages of IFN γ -producing CD8⁺ T-cells upon stimulation with overlapping pp65 peptides considering all time points for D⁺/R⁻ patients. (G) Correlation between peripheral cellular activation (*i.e.* mean percentages of CD38⁺ memory CD8⁺ T-cells) and cumulative numbers of CMV replication events in D⁺/R⁻ patients. The Spearman's rank test was used to determine correlations.

expression over the multiple time points examined was calculated and considered in our analyses as the average activation level for each patient. Notably, regardless of CMV serostatus, we observed a strong association between the occurrence of acute rejection and elevated levels of peripheral CD8⁺ T-cell activation (Fig. 2B). Although the pathophysiological nature of acute rejection is complex and multifactorial, this data supports the relationship between the incidence of acute rejection and high cellular activation levels in lung transplant patients [34,35].

3.3. Anti-CMV immunity is a strong component of cellular activation post-transplant

We next explored the potential link between cellular immune activation and CMV infection in D⁺/R⁻ patients. To this end, total CD8⁺ T-cell immune activation levels in PBMC samples were analyzed in relation to the attributes of CMV-specific CD8⁺ T-cells. The magnitude and activation of CMV-specific CD8⁺ T-cells in 9 D⁺/R⁻ patients (HLA-A*0201 or HLA-B*0701) were assessed using HLA-A*0201 pp65-NV9 or HLA-B*0701 pp65-TM10 tetramers (Fig. 2C). Although the use of tetramers does not permit the detection of all CMV-specific CD8⁺ T-cells due to HLA and epitope constraints, it provides a representative reflection of CMV-specific CD8⁺ T-cell response kinetics. Strong expansions of CMV tetramer⁺ cells were observed in most D⁺/R⁻ patients a few months after transplantation, corresponding to primary CMV infection and usually closely associated with episodes of detectable viral replication (see Fig. 2A – right panel for a representative example). Of note, CD38 expression on total memory CD8⁺ T-cells was strongly correlated with CD38 expression on CMV-specific CD8⁺ T-cells (Fig. 2D). Likewise, total CD8⁺ T-cell activation levels were correlated with the frequency of CMV tetramer⁺ cells (Fig. 2E). In contrast, CD38 expression on EBV-specific CD8⁺ T-cells, also identified using tetramers, did not correlate with overall CD8⁺ T-cell activation (Fig. S1). We also measured the magnitude of CD8⁺ T-cells specific for the immunodominant CMV pp65 protein (*i.e.* IFN- γ producing cells upon stimulation

with overlapping pp65 peptides), which has been shown to correlate with the total CMV-specific CD8⁺ T-cell response [36]. This response was significantly correlated with total CD8⁺ T-cell immune activation levels (Fig. 2F). Finally, CD38 expression levels on memory CD8⁺ T-cell were correlated with the number of CMV replication events in D⁺/R⁻ patients (Fig. 2G). Our observations are in line with reports of large CMV-specific immune responses impacting on the CD8⁺ CD38⁺ compartment of patients after kidney or liver transplantation [37–40]. Altogether, our data indicate that CMV infection, and in particular the CMV-specific CD8⁺ T-cell response, is a major driver of immune activation, which is associated with acute rejection in D⁺/R⁻ lung transplant patients.

3.4. Acute rejection is linked to local immune activation and CMV replication

To comprehend further the relationship between CMV-driven peripheral immune activation and acute rejection in the lung, we next analyzed local immune correlates (*i.e.* directly in the lungs). The human lung transplant model allows for relatively straightforward exploration of in-graft pathophysiology through the analysis of BAL, which is representative of the alveolar compartment. The expression of CD38 was therefore assessed on total memory CD8⁺ T-cells from BAL. Patients displaying acute rejection (*i.e.* at least one event post-M2) had significantly higher memory CD8⁺ T-cell activation levels in BAL (Fig. 3A). This was reminiscent of the observations made in the blood. Furthermore, CD38 levels on memory CD8⁺ T-cells were strongly correlated in blood and BAL from D⁺/R⁻ patients, in contrast to D⁻/R⁻ patients (Fig. 3B). Thus, cellular activation levels in the periphery and in the lung of CMV-infected transplant patients are closely related.

To assess the importance of local CMV replication on immune activation and acute rejection, we then compared CMV seropositive patients with evidence of acute rejection who were transplanted either with CMV-infected (*i.e.* D⁺/R⁻ or R⁻) or uninfected (*i.e.* D⁻/R⁺) lungs. D⁺ patients differed from D⁻/R⁺ patients by showing higher immune activation levels in

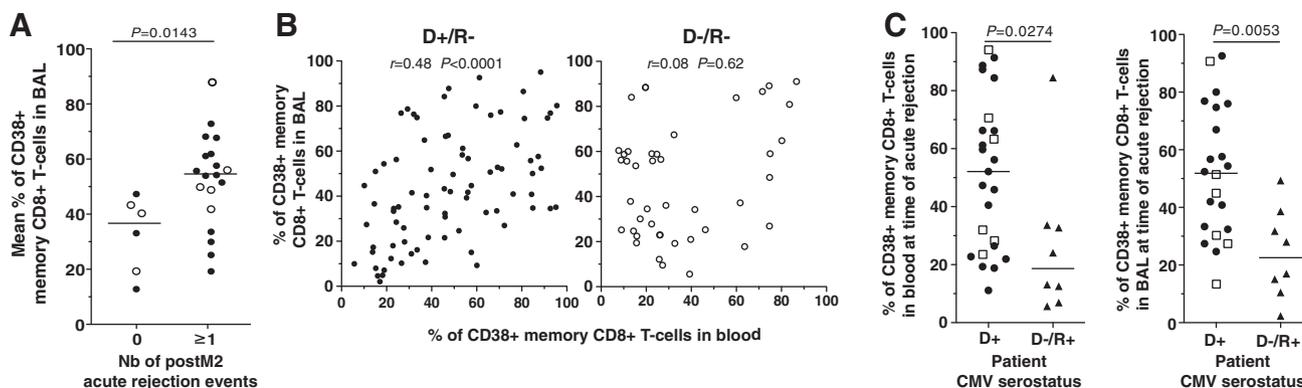


Figure 3 Memory CD8⁺ T-cell activation in lung versus blood. (A) Association between mean percentages (*i.e.* average value of all time points) of CD38⁺ memory CD8⁺ T-cells in BAL and total number of post-M2 acute rejection events (none or at least one) for D⁺/R⁻ (full circles) and D⁻/R⁻ (open circles) patients. (B) Correlations between CD38 expression on total memory CD8⁺ T-cells in blood and in BAL considering all time points for D⁺/R⁻ (left panel) or D⁻/R⁻ (right panel) patients. The Spearman's rank test was used to determine correlations. (C) Association between percentages of CD38⁺ memory CD8⁺ T-cells in blood (left panel) or BAL (right panel) at time points of acute rejection and the presence (*i.e.* D⁺ patients; full circles for D⁺/R⁻ and open squares for D⁻/R⁺) or absence (*i.e.* D⁻/R⁺ patients) of CMV in the transplanted lungs. The Mann–Whitney test was used to compare groups. Bars indicate median values.

blood and BAL at the time of acute rejection events (Fig. 3C). The differences between D^+/R^+ and D^-/R^+ patients are reminiscent of our observations in D^+/R^- versus D^-/R^- patients. These data suggest that the presence of CMV in the lungs (independent of recipient CMV serostatus) results in high levels of immune activation locally and in the periphery, and is likely an important factor in acute rejection.

3.5. Similar CMV-specific CD8⁺ T-cell attributes in blood and lung

We reasoned that the presence of CMV in the transplanted organ may lead to the induction of strong CMV-specific CD8⁺ T-cell responses in the host, regardless of primary infection, and the migration of these cells from the periphery to the infected lungs, thereby explaining the high levels of cellular activation locally. Accordingly, we performed direct analyses of CMV-specific CD8⁺ T-cells, comparing their frequency and activation status in both lung and blood. For this purpose, CMV-specific CD8⁺ T-cells were identified in these compartments using tetramer stainings, as exemplified in Fig. 4A (left panel). Although CMV tetramer analyses in BAL were only possible for a few donors ($n = 14$), the kinetics of the CMV-specific CD8⁺ T-cell response (considering both the frequency and CD38 expression levels on the cells) in the lung and blood of D^+ patients were generally very comparable: Fig. 4A (right panel) shows representative kinetics in one patient. Overall, there were significant correlations between the magnitude and activation levels (*i.e.* CD38

expression) of CMV-specific CD8⁺ T-cells in both compartments considering our set of patients (Figs. 4B and C). This was not the case for D^-/R^+ patients (data not shown). These results suggest that the virus-specific CD8⁺ T-cell responses in the lung and blood are very similar in patients transplanted with CMV-infected lungs.

To strengthen this parallel, we performed comparative clonotypic analyses of CMV tetramer⁺ cells in BAL and PBMC samples from two D^+/R^- patients and one D^-/R^+ patient at acute rejection time points. Individual CD8⁺ T-cell clonotypes (*i.e.* characterized by a single TCR) originates from the same initial cell, which has proliferated and generated multiple daughter cells. We observed the same dominant clonotypes in CMV-specific CD8⁺ T-cell populations from lung and blood in all cases (Fig. 5). These data suggest that CMV-specific CD8⁺ T-cells generated in the periphery upon CMV infection migrate readily to the transplanted organ infected by the virus. Overall, the association between acute rejection, immune activation and local anti-CMV CD8⁺ T-cell immunity provides evidence supporting a role of CMV-specific CD8⁺ T-cells in acute rejection of transplanted lungs infected with CMV.

4. Discussion and conclusions

A clear understanding of the causes and mechanisms that underlie acute lung rejection post-transplant is necessary to optimize specific treatments and improve patient survival. In this study, we aimed to provide *in vivo* evidence to support the hypothesis that the CMV-specific T-cell response

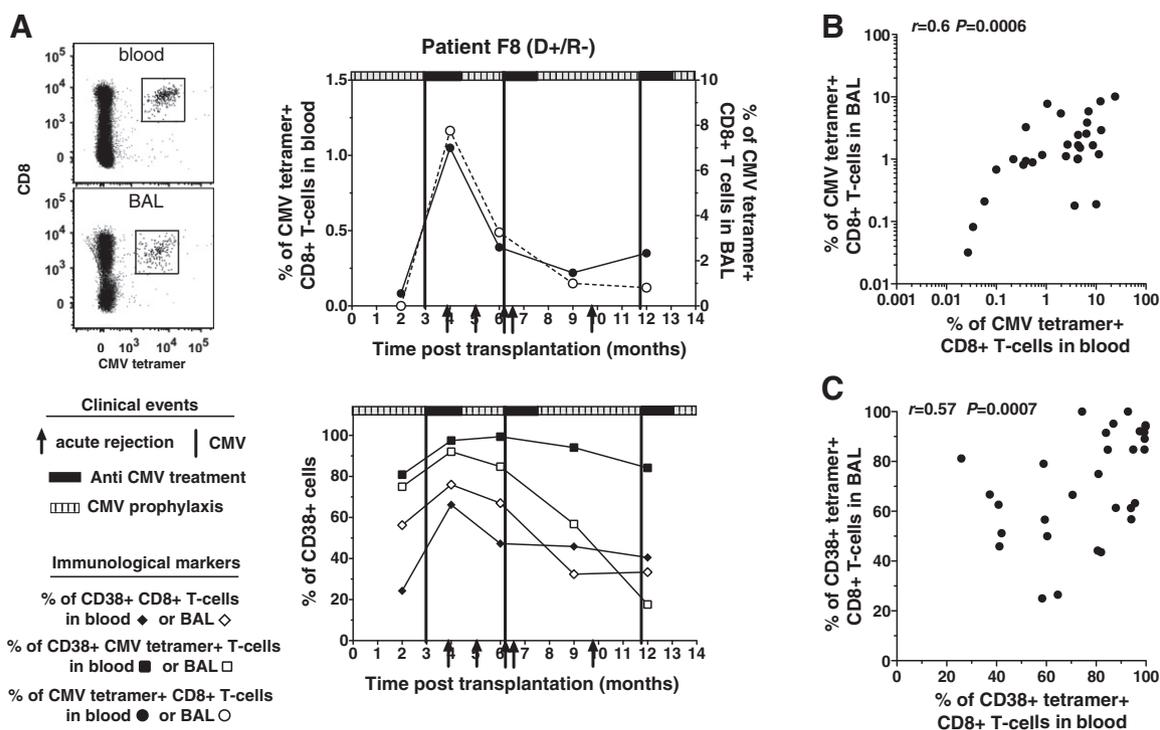


Figure 4 CMV-specific CD8⁺ T-cell responses in lung versus blood. (A) Representative examples of flow cytometry dot plots showing percentages of HLA-A*0201 pp65-NV9 tetramer⁺ CD8⁺ T-cells in blood and BAL from one D^+/R^- patient at month 6 post-transplant (upper left panel). Evolution of immunological markers in blood and BAL from one representative D^+/R^- patient over the post-transplant follow-up period. (B) Correlation between the frequency of CMV tetramer⁺ CD8⁺ T-cells in blood and in BAL from D^+/R^- patients. (C) Correlation between CD38 expression on CMV tetramer⁺ CD8⁺ T-cells in blood and in BAL from D^+/R^- patients. The Spearman's rank test was used to determine correlations.

		TRBV	CDR3 sequence (aa)	TRBJ	Freq (%)		
Patient F8 - month 4	CMV pp65-NV9 specific CD8 ⁺ T-cells	Blood	27	CASTDVSGLYNEQFF	2-1	36	
			7-6	CASSLAPGTTNEKLFF	1-4	26	
			30	CAWSVSDLAKNIQYF	2-4	12	
			2	CASIDRVRLLETQYF	2-5	6	
			7-6	CASSLAPGATNEKLFF	1-4	6	
			27	CASSLSAGTVLETQYF	2-5	5	
			15	CATQTHSYEQFF	2-1	5	
			7-6	CASSLAPGVTNEKLFF	1-4	2	
			6-5	CASSSQTGTGSYGYTF	1-2	2	
	BAL	27	CASTDVSGLYNEQFF	2-1	95		
		7-6	CASSLAPGTTNEKLFF	1-4	3		
		30	CAWSVSDLAKNIQYF	2-4	1		
		12-4	CASSPGSLLYGYTF	1-2	1		
		27	CASTDMSGALYNEQFF	2-1	1		
		27	CASTDVSGLYNERFF	2-1	1		
		27	CASTNVSGALYNEQFF	2-1	1		
		Patient F22 - month 4	CMV pp65-NV9 specific CD8 ⁺ T-cells	Blood	12-4	CASSSAHYGYTF	1-2
29-1	CSVDLGGQNTAEFF				1-1	30	
12-4	CASSSAHYGYTF				1-2	1	
12-4	CASSSARYGYTF				1-2	1	
29-1	CSVDLGGQNAEAF				1-1	1	
12-4	CVSSSAHYGYTF				1-2	1	
BAL	29-1				CSVDLGGQNTAEFF	1-1	52
	12-4				CASSSAHYGYTF	1-2	32
	12-4				CASSSAHYGCTF	1-2	4
	29-1		CNVDLGGQNTAEFF	1-1	2		
	29-1		CSVAGTVNEQYF	2-7	2		
	12-4		CASSSAHYDYTF	1-2	1		
	28		CASSVGPSSGELFF	2-2	1		
	9		CASSYLGGAGTGELFF	2-2	1		
	29-1		CSVETRDYEQYF	2-7	1		
Patient F11 - month 9	CMV pp65-NV9 specific CD8 ⁺ T-cells		Blood	2	CASSEGLLGGAEGLSPLHF	1-6	66
				5-1	CASSFEGGVNGYGYTF	1-2	23
		12-4		CASSSSTPGFPFSEQYF	2-7	5	
		12-4		CASSSVSTQYF	2-3	5	
		9		CASSVGGGRDEQFF	2-1	1	
		7-6		CASSLAPGTTNEKLFF	1-4	1	
		BAL		2	CASSEGLLGGAEGLSPLHF	1-6	73
				5-1	CASSFEGGVNGYGYTF	1-2	22
				2	CASSEGLLGGAEGLSSLHF	1-6	1
	2		CASSERLLGGAEGLSPLHF	1-6	1		
	5-1		CTSSFEGGVNGYGYTF	1-2	1		
	5-1		CVSSFEGGVNGYGYTF	1-2	1		

Figure 5 Clonotypic analysis of CMV-specific CD8⁺ T-cells in lung *versus* blood. TRBV and TRBJ usage, CDR3 β amino acid sequence, and relative frequency are shown for CMV pp65-NV9 tetramer⁺ CD8⁺ T-cell populations isolated from the blood and BAL of three patients (F8, F22 and F11) at time of acute rejection. Colored lines highlight dominant clonotypes common to both compartments. Common clonotypes were identical at the nucleotide level.

may play a role in acute lung rejection. Initially, we focused our investigation on D⁺/R⁻ patients, in whom primary CMV infection occurs post-transplant due to the presence of the virus in the transplanted lung. We found that CMV replication and the antiviral CD8⁺ T-cell response were major drivers of immune activation, both in the periphery and in the lungs; this, in turn, was associated with acute lung rejection. Our study does not provide an absolute demonstration of the causal relationship between anti-CMV immunity and acute rejection. On the one hand, acute rejection may promote CMV replication, thus resulting in enhanced anti-CMV T-cell responses. However, on the other hand, our observations support the possibility that CMV specific immune response and mediated inflammation may actually promote acute rejection.

On the basis of our results and the literature, the following model may thus be proposed. Despite prophylaxis, primary CMV infection with extensive viral replication occurs in D⁺/R⁻ patients, inciting a robust CMV-specific T-cell immune response, especially within the CD8⁺ T-cell compartment. The magnitude of this primary response can be immense. Even in chronically infected healthy individuals, up to 40% of the entire CD8⁺ T-cell memory pool can be specific for CMV [36]. These features are sufficient to explain the elevated levels of immune activation observed in D⁺/R⁻ patients post-transplant. In these patients, the transplanted lungs are not only the source of CMV, they are also important targets of the newly established CMV-specific peripheral CD8⁺ T-cells, which are characterized by a strong pro-inflammatory functional profile [22,36,41], and can migrate readily to the lungs to exert their anti-viral function. However, collateral damage may ensue with the generation of local conditions that favor acute rejection. CMV-specific CD8⁺ T-cells may cause damage locally, upon activation with CMV or allo-antigens [25–30]. These different factors, either alone or in combination, are likely to alter the fragile balance between the graft and its host. Further studies will be necessary to decipher the precise mechanisms that underlie the potentially damaging effects of CMV-specific CD8⁺ T-cell activity. In particular, it will be important to elucidate how such responses may affect the outcome of transplantation in the context of HLA mismatch. Of note, along with CD8⁺ T-cells, other pro-inflammatory members of the cellular immune arm may play a role in promoting acute rejection in CMV infected lung transplant patients. For instance, CMV infection is known to shape the compartment of the non-conventional $\gamma\delta$ T-lymphocytes [42], and studies in organs or allogeneic stem cells transplanted patients indicate that $\gamma\delta$ T-cells are important players of the cellular immune response against this virus [43–46]. The ligand recognized by a $\gamma\delta$ TCR on CMV infected stressed human cells has in fact recently been discovered [47]. $\gamma\delta$ T-cells are often found in peripheral tissues and organs, where they can engage potent effector functions both in terms of cytokine and chemokine production capacity and direct cytotoxicity [48]. Their potential role in linking further the relationship between CMV infection and acute lung rejection will therefore require some attention.

Although our data support the possibility that the primary CMV-specific T-cell response disturbs the graft-host balance, the presence of CMV in the transplanted lung *per se* is also likely to have an effect. Indeed, this possibility is supported by our findings in patients (either R⁺ or R⁻) transplanted with an infected organ (D⁺). Unlike D⁻/R⁺ patients, the D⁺/R⁻ and D⁺/R⁺ patients in our cohort presented very similar characteristics with regards to the association between acute rejection and CMV-related cellular activation. Although D⁺/R⁺ patients do not experience primary infection after transplantation, they are exposed to superinfection (*i.e.* infection with a different CMV strain). As shown in non-human primates, this results in increased systemic CMV-specific T-cell activity and immune activation [49,50]. This, in turn, could influence graft rejection locally as pro-inflammatory CMV-specific T-cells move into the infected organ. The similarities between D⁺/R⁻ and D⁺/R⁺ patients support the homogenization of D⁺ patient treatment with strong and prolonged CMV prophylaxis (*i.e.* valgancyclovir), in line with recent recommendations [51], to dampen down the risk of acute rejection related to

enhanced CMV-specific T-cell responses during primary or superinfection. Moreover, since the level of CD8⁺ T-cell activation in the blood represents a good surrogate marker of lung CD8⁺ T-cell activity, which is associated with acute rejection, it is possible that this parameter could be usefully monitored during clinical follow-up. For example, straightforward assessment of CD38 expression on CD8⁺ T-cells from PBMCs could help to predict the risk of acute rejection events during the months following lung transplantation, and may thus help tailor immunosuppressive and anti-CMV regimens. Analyses of our data set using a parametric survival model yielded a significant association between circulating CD8⁺ T cell activation levels (*i.e.* CD38 expression) as a continuous variable and lung transplant rejection among D⁺ (but not D⁻) donors, and this was strongest soon after transplantation while slowly waning during follow-up ($P = 0.004$). Moreover, we observed that the change in circulating CD8⁺ T cell activation levels from the preceding visit was significantly associated with lung transplant rejection in the D⁺/R⁺ patient group ($P = 0.02$, HR = 1.04/1%). This did not reach significance in the D⁺/R⁻ patient group ($P = 0.16$). Using the same models, a significant association (strongest after transplantation and waning during follow-up) was also found between the magnitude of the CMV specific CD8⁺ T cell response (*i.e.* percentage of pp65 specific IFN γ ⁺ cells as a continuous variable) and lung transplant rejection among D⁺ donors ($P = 0.002$). Although they do not stand as proof for the predictive value of peripheral cellular activation markers for acute rejection, these encouraging observations should promote further studies that specifically address this point.

On the one hand, CMV-specific CD8⁺ T-cells may have a detrimental impact on the stability of the transplanted lung and therefore patient survival. However, on the other hand, CMV disease is an important determinant of mortality post-transplant, and control of CMV replication by the host immune system (in addition to prophylaxis) is necessary for long-term survival [52,53]. Thus, CMV-specific T-cell immunity likely represents a double-edged sword in the context of lung transplantation. In support of this notion, pp65 and IE1 specific CD8⁺ T-cell responses (measured using IFN γ intracellular staining upon stimulation with pp65 and IE1 overlapping peptides) appeared to be differentially associated with acute rejection and control of CMV in lung transplant patients. Strong IFN γ ⁺ CD8⁺ T-cells specific for pp65 were usually detectable in patients with evidence of CMV replication (Figs. S2A and B). Moreover, this response (*i.e.* mean frequency overtime) was associated with acute rejection (Fig. S2C). In contrast, IE1-specific IFN γ ⁺ CD8⁺ T-cells were only detected when CMV replication appeared to be controlled (either soon after transplantation or at later time points) (Figs. S2A and D), and was not associated with acute rejection (Fig. S2E). Although the administration of CMV prophylaxis complicates interpretation, these observations are consistent with previous reports of a protective advantage of IE1-specific over pp65-specific CD8⁺ T-cells in heart, lung and allogeneic stem cell transplant recipients [54,55]. Thus, differences in the antigenic repertoire of the CMV-specific T-cell response may impact viral control, with some targets providing protection and others potentially acting as “decoys” that act to expand T-cell populations and enhance graft injury.

In conclusion, the present clinical study provides further support that CMV-specific CD8⁺ T-cell immunity links the

observed relationship between CMV infection and the occurrence of acute lung rejection. Our findings can potentially be extrapolated to solid organ transplantation in general, and also help to inform the pathology of CMV-mediated disease in immunocompetent patients.

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Conflict of interest statement

The authors of this manuscript have no conflicts of interest to disclose.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.clim.2013.03.012>.

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