TRANSPLANT INTERNATIONAL

Transplant International ISSN 0934-0874

ORIGINAL ARTICLE

Cytomegalovirus exposure, immune exhaustion and cancer occurrence in renal transplant recipients

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Keywords
cancer, cytomegalovirus, kidney transplantation.

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Conflicts of Interest
The authors have declared no conflicts of interests.

Received: 13 December 2011
Revised requested: 15 January 2012
Accepted: 4 June 2012
Published online: 11 July 2012

doi:10.1111/j.1432-2277.2012.01521.x

Summary

The role of Cytomegalovirus (CMV) in carcinogenesis is controversial. We studied whether CMV may contribute to cancer occurrence in renal transplant recipients. We studied a prospective cohort of 455 consecutive patients who received a kidney transplant between January 1995 and December 2006. All cancers and types of cancers were assessed. Lymphocyte phenotype and cytokines production were analysed according to CMV status in a subset population of this cohort. Mean follow-up was 84 ± 29 months. One hundred and nineteen cancers (26.2%) occurred during the study follow-up. There was a higher cumulated incidence of cancers in CMV-exposed patients (30.4% vs. 20%; $P = 0.018$). Mean time to cancer occurrence was shorter in CMV-exposed patients than in CMV-naïve patients (4.7 ± 2.6 vs. 6.7 ± 2.8; $P = 0.001$). Cox regression analysis revealed that both pretransplant CMV exposure (HR, 1.83; 95% CI, 1.17–2.88; $P = 0.009$) and post-transplant CMV replication (HR, 2.17; 95% CI, 1.02–4.59; $P = 0.044$) were risk factors for cancer. Among CD8+ T cells, exhausted T cells assessed as CD57+CD28- were expanded in CMV-exposed patients (26 ± 20 vs. 9 ± 8%; $P < 0.0001$), whereas CD8+CD57+IL2- cells were more frequent in CMV-exposed patients. Our results highly suggest that CMV increases the risk of cancer after transplantation.

Introduction

A wide-ranging excess risk of cancer after solid organ transplantation has been increasingly recognized over recent decades as advances in medicine have extended the life of transplant recipients [1–4]. As a consequence, malignancy is now a leading cause of patient's death with functional graft, an outcome that can predominantly be attributed to the iatrogenic immunosuppression required to avoid rejection of the transplanted organ [1–4]. Several viruses with oncogenic properties, such as papilloma virus, EBV and HHV-8, have been implicated in this increased incidence of cancer after transplantation [5]. Nevertheless, few data are available concerning the potential role of Cytomegalovirus (CMV) in post-transplant cancer. However, recent evidences indicate the frequent presence of genome and antigens of HCMV in certain malignant tumours, such as colon cancer [6], malignant glioma [7], EBV-negative Hodgkin's lymphoma [8], cervix cancer [8], prostatic intraepithelial neoplasia and prostatic carcinoma [9]. This information suggests that a persistently active HCMV infection may be present in

certain tumours. Moreover, CMV is the main cause of immune exhaustion, which could prevent the activation and maintenance of an efficient immune response against tumours [10].

These observations have been recently challenging in transplant patients. Indeed, Couzi *et al.* [11] reported that CMV-naive recipients had an approximately fivefold higher risk of cancer compared with CMV-exposed patients. Moreover, in a randomized clinical trial comparing pre-emptive therapy with prophylaxis in renal transplant recipients, Spinner *et al.* [12] showed that although the incidence of death censored graft loss was not different, deaths with a functioning graft (2/4 of which were because of post- transplant malignancies) were more likely to occur in patients who received prophylaxis [12]. These two results suggest that immune response against CMV may enhance antitumour immunity and could challenge the relevance of CMV prophylaxis in transplant patients [13].

To better answer this important question, we analysed the role of CMV in a large cohort of consecutive renal transplant recipients. Moreover, we studied lymphocyte phenotype and cytokines production according to CMV status in a subset population of this cohort.

Patients and methods

Study design and populations

We analysed a prospective cohort of 455 consecutive RTR. All the patients received a deceased kidney transplant at the transplant unit of the University hospital of Besançon between January 1995 and December 2006. The ethic committee of Franche-Comté has approved the study.

All the patients received a quadruple sequential immunosuppression. Induction consisted of either ATG (n = 278, 61%) [ATG Fresenius® (day 0: 9 mg/kg; days 1–4: 3 mg/kg/d, n = 177, 64%) or Thymoglobulin® (Genzyme) (day 0: 2 mg/kg; days 1–4: 1 mg/kg/d, n = 101, 36%)] or monoclonal anti-CD25 antibody (n = 177, 39%) [Simulect® (Novartis) (day 0: 20 mg, day 4: 20 mg)]. The same maintenance immunosuppressive treatments were used including Cyclosporine (January 1995–July 2001) or Tacrolimus (August 2001–December 2006), Azathioprine (January 1995–October 2000) or Mycophenolate Mofetil (November 2000–December 2006) and steroids.

All the patients except CMV seronegative recipients of a CMV seronegative donor received CMV prophylaxis with Valaciclovir in the first 3 months following transplantation.

Characteristics of the study population are described in Tables 1 and 2.

Table 1. Description of variables.

Variable	Category	Frequency (%)
Gender	Male	296 (65%)
	Female	159 (35%)
Past history of cancer	Yes	29 (6.4%)
	No	426 (94%)
Tobacco consumption	Yes	109 (24%)
	No	346 (76%)
Diabetes	Yes	54 (12%)
	No	401 (88%)
CMV serology	Negative	223 (49%)
	Positive	232 (51%)

CMV, cytomegalovirus.

Table 2. Characteristics of patients according to CMV exposure.

Variable	CMV-exposed (n = 270)	CMV-naïve (n = 185)	P
Age (years)	48 ± 14	47 ± 14	0.678
Gender (male %)	176 (65%)	120 (65%)	0.929
Past history of cancer	19 (7%)	10 (5.5%)	0.614
Tobacco consumption	63 (23%)	47 (25%)	0.813
Diabetes	35 (13%)	21 (11.5%)	0.712

CM infection and disease

CMV serology (ELISA) was performed before transplantation. Donor CMV serology was assessed through medical records.

CMV PCR were performed weekly until 3 months post-transplant, monthly until 6 months post-transplant and each year during follow-up. Patients were considered to have CMV infection in any case of positive PCR.

CMV disease was defined by the need of treatment in a patient with viral replication.

CMV exposure was defined by a positive pretransplant CMV serology and/or post-transplant CMV infection or disease.

Confounding factors

Age, gender, weight, size, haemodialysis duration before transplantation, diabetes mellitus, smoking status, pretransplant history of cancer, HLA matching, Panel Reactive Antibody, rank of transplantation (first vs. iterative), donor type and immunosuppressive treatment (type of induction, cyclosporine vs. tacrolimus, azathioprine vs. mycophenolate mofetil) were assessed.

Immune exhaustion

Absolute numbers of circulating B and T cells, $CD4^+$ and $CD8^+$ T cells were determined as previously described

[14] in 135 consecutive patients from the main cohort and in 98 patients of a prospective independent multicentre study. These patients have been included in the ORLY-EST study (Influence de l'**O**rientation de la **R**éponse **LY**mphocytaire dans l'athérosclérose post-transplantation). Briefly, ORLY-EST, started in November 2008, is an observational study including all incident renal transplant recipients in seven French transplant centres (Strasbourg, Nancy, Reims, Dijon, Clermont-Ferrand, Kremlin-Bicêtre, Besançon). Blood samples were collected at transplant and 1 year after transplantation, and sent with written consent to the Biomonitoring Plateform (CIC-BT506, EFS Besançon, France) for processing and storage. To date, 355 patients have been included in this study. Ninety-eight patients were extracted from the main cohort to explore thymic function. The ethic committee of Franche-Comté approved the study (2008).

Naive CD4 T cells were also assessed as $CD45RA^+$, $CD62L^+$, $CD45RO^-$ $CD4^+$ $CD3^+$ cells using the following antibodies: FITC-conjugated CD45RA (clone HI100), phycoerythrin-CD62L (Dreg56) (BD Biosciences, Le Pont de Claix, France), ECD-CD45RO (UCHL1), PC7-CD3 (13B8.2) and allophycocyanin-CD3 (UCHT1) (Beckman Coulter). Exhausted T cells were assessed as CD57+CD28- using the following antibodies: FTIC-conjugated CD57 (Beckman Coulter), CD28 perCP/Cy5.5 (BD Biosciences Pharmingen). Naïve T cells were defined as $CD45RA^+$ $CD28^+$.

Cytokines production was assessed as followed: PBMCs were isolated from blood samples using density gradient centrifugation (Ficoll-Hypaque; GE Healthcare) and cryopreserved in aliquots before being analysed. After thawing, PBMCs were washed twice in RPMI 1640+ GlutaMAX™-I (Invitrogen, France) containing 10% serum foetal calf (Invitrogen, France). 10^6 cells are distributed in a 96-well plate (Greiner bio-one, France). For each sample, cells were incubated during 1 h at 37 °C with 5% CO_2 with PMA (50 ng/ml) and Ionomycine (2 μg/ml) (Sigma, France), CMV peptides IE1, IE2 ou pp65 (1 μg/ml) (jpt Innovative Peptide Solutions, Germany) or RPMI 1640+ GlutaMAX™-I (Invitrogen, France) containing 10% serum foetal calf (Invitrogen, France) as a negative control. Brefeldin A (1 mg/ml) (Sigma, France) was then added and incubation time was pursued for a total time of 4.5 h. Cells were washed twice and first incubated with the following directly conjugated monoclonal antibodies for 20 min at 4 °C: anti-CD3-APC/Cy7 (clone HIT3a; Biolegend), anti-CD4-Pacific blue (clone RPA-T4; Biolegend), anti-CD8-PC7(clone RPA-T4; Biolegend), anti-CD57-FITC (clone NC1; Beckman Coulter). Cells were washed twice. To detect intracellular cytokines, surface staining PBMCs were processed using fixation buffer and permeabilization buffer (BD Biosciences) and incubated with the following conjugated monoclonal antibodies: anti-IL-2-PC7 (clone MQ1-17H12; BD Biosciences), anti-IL-10-PE (clone JES3-19F1; BD Biosciences), anti-IFN-γ-APC (clone 25723.11; BD Biosciences), anti-TNF-α-PerCP/Cy5.5 (clone MAb11; BD Biosciences). Cell debris and doublets were excluded on the basis of side versus forward scatter. All cells were analysed on a FACS CanthoII (BD Biosciences) using FACS Diva (BD Biosciences) software.

Cancer

All cancers and types of cancers are prospectively assessed in our centre since January 1995. Death was considered to be because of cancer if directly as a result of neoplasic disease or antineoplasic treatments.

Two physicians independent of the study were responsible for diagnostic ascertainment. This analysis was performed without knowledge of baseline characteristics.

Statistical analysis

Arithmetic mean was calculated and expressed as ± SD.

The patients were first divided in four groups according to CMV donor/recipient status (D–/R–, D–/R+, D+/R+, D+/R–). In a second analysis, we separated patients with any contact with CMV (R+ and R– with post-transplant CMV infection or disease) from those who had never been exposed to CMV (D–/R– and D+/R– without post-transplant CMV infection or disease).

Using log rank tests on Kaplan Meier nonparametric estimates of the survival without cancer distribution, we selected variables with a P value lower than, or equal to, 0.20. The selected variables were included into a Cox proportional hazards model, and a backward stepwise selection process was performed, this time at a classical $\alpha = 0.05$. Gender and age being potential confounding variables, they were also entered into the Cox model, no matter the significance of their relationships with death. Tobacco consumption was accounted for as currently smoking versus nonsmoking definition variables.

Results are expressed as hazard ratio (HR) and 95% confidence interval (CI), with a P value testing the null hypothesis: HR = 1. Therefore, when P value is less than 0.05, HR is significantly different from 1, either greater than 1 (i.e. risk of death is increased) or less than 1 (i.e. risk of death is decreased). Assumptions of Cox models (log-linearity, proportionality of risk in time) were met in this analysis.

T lymphocytes subset counts were compared between CMV-exposed and CMV-naïve patients using student t test.

Results

Study population

Patient's characteristics are depicted in Tables 1 and 2. Mean age was 47 ± 14 years. The patients were followed for a mean duration of 84 ± 29 months.

CMV exposure

Two hundred and twenty-three patients were CMV seronegative at transplant (49%). Of them, 116 (52%) received a CMV seropositive kidney. There was no CMV infection in D-/R- patients, but 38 D+/R– patients (32.7%) experienced CMV infection or disease.

Among 232 patients with positive CMV serology at transplant, 112 (48.3%) received a CMV-positive kidney. There was a similar rate of CMV disease or infection (28.3% vs. 27.7%) in D–/R+ and D+/R+ patients.

As a whole, 270 patients were considered as CMV-exposed patients (232 R+ and 38 R– with CMV infection or disease) and 185 as CMV-naïve patients (R– without any CMV infection).

As a whole, 103 patients developed either CMV infection or disease. The only risk factor for CMV infection/disease was older age ($P = 0.029$). There was a trends towards a higher incidence of CMV infection/disease in patients having received ATG (25.2% vs. 18.7%; $P = 0.131$). Therefore, ATG use was forced in the Cox model for further analyses.

Characteristics of CMV-exposed and CMV-naïve patients are depicted in Table 2.

Cancer

One hundred and nineteen cancers (26.2%) occurred during the study follow-up.

There was a trend towards a higher rate of cancer in CMV seropositive patients (30% vs. 22.4%; $P = 0.095$).

There was a higher cumulated incidence of cancers in CMV-exposed patients as compared with CMV-naïve patients (30.4% vs. 20%; $P = 0.018$). There was also a significant higher death rate because of cancer in CMV-exposed patients (8.9% vs. 3.2%, respectively; $P = 0.039$). Mean time to cancer occurrence was shorter in CMV-exposed patients than in CMV-naïve patients (4.7 ± 2.6 vs. 6.7 ± 2.8 years; $P = 0.001$) (Fig. 1).

Cancer sites are depicted in Table 3. The cancer ratio CMV-exposed/CMV-naïve was 1.83. For cancer with at least four cases, the ratio exceeds three for lung, prostate, oesophagus, uterus and colon carcinomas. Lung cancers were marginally more frequent in CMV-exposed patients (3.3% vs. 0.5%; $P = 0.092$).

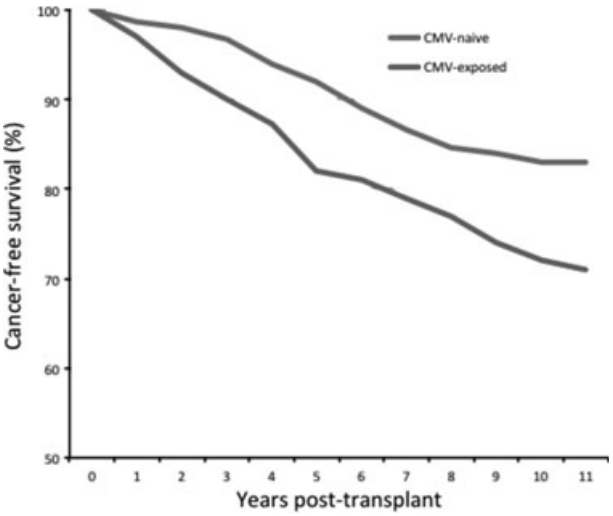

Figure 1 Cancer-free survival in CMV-exposed and CMV-naïve patients.

Table 3. Cancer sites in CMV-exposed and CMV-naïve patients.

	CMV-exposed (cancer-related death)	CMV-naïve (cancer-related death)
Skin	30	17
Lymphoma	10 (70%)	6 (50%)
Lung	9 (78%)	1
Prostate	6 (17%)	2
Kidney	5	3
Oesophagus	3 (100%)	0
Uterus	6	2
Colon	3	1
Breast	2	2 (50%)
Larynx	0	1 (100%)
Sarcoma	1	0
Ovary	2 (100%)	0
Pancreas	2 (100%)	0
Bladder	2 (50%)	0
Liver	1 (100%)	1 (100%)
Stomach	0	1
Total	82	37

One hundred and three (22.6%) patients experienced CMV infection or disease. Cancer occurrence was more frequent in these patients as compared with those without neither CMV infection nor disease (34% vs. 23.9%; $P = 0.047$).

In univariate analysis, age ($P = 0.003$), smoking status ($P = 0.009$), male gender ($P = 0.017$), CMV exposure ($P = 0.006$) and diabetes mellitus ($P = 0.043$) levels were predictive of cancer occurrence.

Cox regression analysis revealed that age (HR, 1.04; 95% CI, 1.01–1.06; $P = 0.027$), smoking status (HR, 1.93;

Table 4. Cox model: hazard ratio (HR) of cancer and 95% confidence intervals (CI).

	HR	CI 95%	*P*
CMV exposure	1.83	1.17–2.88	0.009
Smoking status	1.93	1.07–3.48	0.020
Age	1.04	1.01–1.06	0.027

95% CI, 1.07–3.48; $P = 0.020$) and CMV exposure (HR, 1.83; 95% CI, 1.17–2.88; $P = 0.009$) were risk factors for cancer. Hazard risks and their 95% CI of cancer occurrence for each variable in the Cox model are displayed in Table 4, along with *P* values.

To clarify whether CMV exposure or CMV replication after transplantation is the risk factor for cancer, we then considered three groups of patients: CMV-negative patients for the whole period ($n = 185$), CMV-positive patients without replication after transplantation ($n = 167$) and those with CMV replication after transplantation ($n = 103$).

Cancer rates were 20%, 28% and 34% in the three groups respectively. In univariate analysis, both CMV-positive patients without replication ($P = 0.073$) and those with CMV replication ($P = 0.009$) had an increased risk of cancer. Cox regression analysis revealed that both CMV-positive patients without replication (HR, 1.71; 95% CI, 0.99–2.95; $P = 0.053$) and those with CMV replication (HR, 2.17; 95% CI, 1.02–4.59; $P = 0.044$) had an increased risk of cancer.

Immune exhaustion, CMV exposure and cancer occurrence

Retrospective cohort

Patients included in this subanalysis did not differ from the main cohort (data not shown). Eighty-four patients (62%) had had a previous exposure to CMV, whereas 51 were CMV-naïve. Age was similar in the two groups (50 ± 12 vs. 51 ± 13 years; $P = 0.931$). The same proportion of patients in the two groups had received ATG induction (64% vs. 68%; $P = 0.380$).

Immune exhaustion and CMV

The CD8+/CD3+ ratio was increased in CMV-exposed patients (30 ± 14 vs. 20 ± 11%; $P = 0.003$). Among CD8+ T cells, CD57+CD28- T cells were expanded in CMV-exposed patients (26 ± 20 vs. 9 ± 8%; $P < 0.0001$), whereas CD45RA+CD28+ T cells were more frequent in CMV-naïve patients (46 ± 19 vs. 26 ± 21%; $P = 0.007$).

The proportion of CD4 T cells was similar in the two groups. By contrast, among CD4 T cells, CD57+CD28- T cells were markedly increased in CMV-exposed patients (8 ± 6 vs. 0.6 ± 0.2%; $P < 0.0001$).

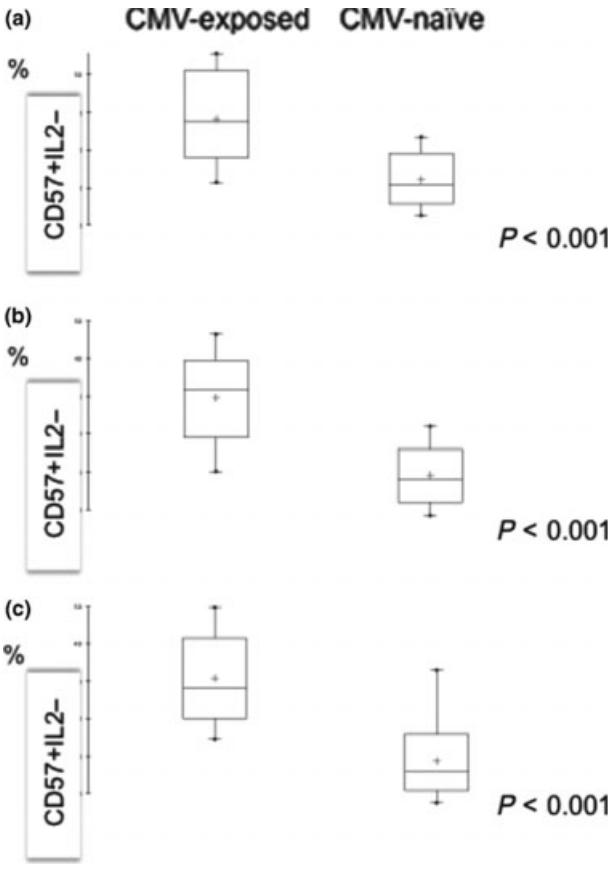

Figure 2 Proportion of CD57+IL2- T cells among CD8+ T cells in CMV-exposed and CMV-naïve patients at basal state (a), after incubation with PMA-Ionomycine (b) and after incubation with pp65 antigen (c).

PD1+ expression on both CD4+ (2.1 ± 1.4 vs. 0.9 ± 0.5%; $P = 0.03$) and CD8+ (4.9 ± 3.1 vs. 2.3 ± 1.4%; $P = 0.008$) T cells was more frequent in CMV-exposed patients.

Among CD8+ T cells, CD8+CD57+IL2- cells were more frequent in CMV-exposed patients at basal state, after stimulation by PMA or CMV peptides (Fig. 2).

We then considered three groups of patients: CMV-negative patients for the whole period ($n = 44$), CMV-positive patients without replication after transplantation ($n = 53$) and those with CMV replication after transplantation ($n = 38$). CD8+CD57+CD28- T cells frequency gradually increased from CMV-naïve patients to those with post-transplant replication (6.2% (range: 0.1–46.5), 13.7% (range: 2.7–60.4) and 23.6% (3.6–70.5); $P < 0.001$) (Fig. 3).

Immune exhaustion and cancer

Among these 135 patients, 20 developed a cancer during follow-up (46 ± 21 months). CD8+CD28- T cells were

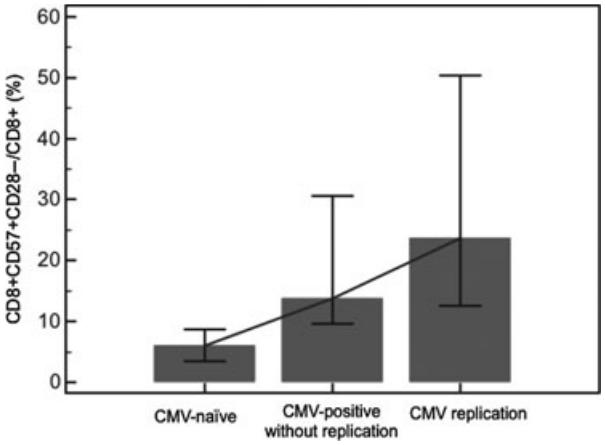

Figure 3 Proportion of CD57+CD28- T cells among CD8+ T cells in CMV-negative patients for the whole period (n = 44), CMV-positive patients without replication after transplantation (n = 53) and those with CMV replication after transplantation (n = 38).

significantly more frequent in these patients (48 ± 28 vs. 30 ± 22%; P = 0.031).

Prospective cohort

Before transplantation, CMV-positive patients had an increase in late stage differentiated CD8+T cells compared with CMV-negative (CMV-) patients (CD8+CD57+CD28- T cells: 23.3 vs. 14.2%, respectively, P = 0.035). Similar differences were observed for CD4+T cells. At 1 year after transplantation, late stage differentiated CD8+ T cells increased only in CMV-positive patients (Fig. 4).

Discussion

Our study demonstrates that both pretransplant CMV exposure and post-transplant CMV replication increased the risk of cancer after transplantation. Cancer-related death rate was also higher in CMV-exposed patients. As CMV is not considered to be directly oncogenic, other mechanisms may be involved to explain the association between viral exposure and cancer. Exhausted CD8+ T cells accumulated in CMV-exposed patients and seem to be associated with the subsequent occurrence of cancer. Collectively, all these results suggest that CMV contributes to the increased risk of cancer in transplant patients.

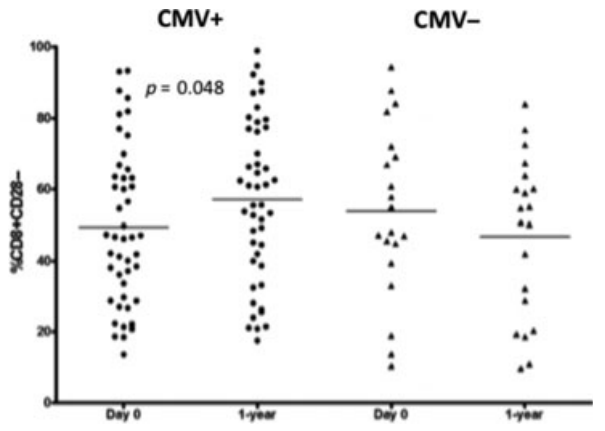

Figure 4 % of CD28- T cells among CD8+ T cells at transplant and 1 year post-transplant in CMV-positive patients and in CMV-negative patients.

Chronic CMV infection has been suggested as the main stimulus driving the *in vivo* process of immune exhaustion, which in many studies is associated with clonal expansion of CD8+ T cells, an inverted CD4:CD8 ratio (i.e. <1), and increased numbers of CD8+CD28- T cells [15]. Loss of CD28 and gain of CD57 are well described markers of immune exhaustion [15]. Exhaustion is characterized by the progressive loss of T cell function. IL-2 production is one of the first functions to be lost, whereas TNF production is lost later. At a severe stage of exhaustion, IFNγ production is eventually compromised [16]. Functional exhaustion develops when there is a high antigenic load and is considered to be a way of limiting the magnitude of effector T cell responses. Immune exhaustion has been associated with inflammatory diseases and recent data suggest that, in CMV-positive patients suffering rheumatoid arthritis, terminally differentiated CD4+T cells secreting IFNγ contributes to RA progression [17]. Although this may be relevant against autoimmune responses, it may also compromise effective immunity against infectious agents and tumours [18,19]. We showed that CMV-exposed patients exhibit features of immune exhaustion such as expansion of CD8+CD57+CD28- T cells, higher expression of PD1 in both CD4+ and CD8+ T cells and an increased proportion of CD8+CD57+ not producing IL-2. We suggest that CMV induces immune exhaustion in transplant patients and could, at least partially through this mechanism, favour post-transplant tumour occurrence. CMV reactivation may further stimulate immune exhaustion and increase the risk of cancer. Indeed, we observed both a higher rate of CD8+CD57+CD28- T cells and an increased incidence of cancer in patients with post-transplant CMV replication. This suggests that repeated immune challenges drive immune exhaustion, which could reduce immunity against cancer.

Other mechanisms could be involved to explain the link between CMV and cancer. A number of investigations on infected tumour cell lines has implied that CMV infection may interfere with several key cellular signalling pathways, leading to enhanced survival and angiogenesis, as well as alterations in cell motility and adhesion [8]. For instance, US28, a viral G protein, activates signalling pathways linked to cell proliferation [20]. CMV-activated

gene transcription may thus promote malignant transformation by dysregulating various cellular normal physiological processes that control the cell cycle. This virus may also be involved in mutagenesis, inhibition of apoptosis, angiogenesis, cell invasion and modulation of the host immune system [21–23].

However, other observations have suggested that CMV may help in antitumour immunity. During CMV infection, γδ cells, a subset of T cells, undergo expansion and serve to contain the infection. γδ cells exert major histocompatibility complex unrestricted natural cytotoxicity against solid tumours and some leukaemias and lymphomas [24]. In a mouse xenograft tumour model, γδ lymphocytes isolated from renal transplant recipients with CMV infection killed both CMV infected cells and HT29 colon cancer cells [25]. A case-controlled study documented that renal transplant recipients who developed post-transplant malignancies had significantly lower γδ cells before the onset of malignancy compared to patients without malignancies [10]. The same authors reported a lower incidence of post-transplant cancer in CMV-exposed patients [10]. Nevertheless, the very small number of patients seriously hampers the results of this study. However, the net effect of CMV on oncogenesis may depend from different parameters. A robust expansion of γδ T cells could eventually counterbalance other deleterious effects of CMV.

We principally observed an increased incidence of lung cancer in CMV-exposed patients. CMV has been described as a possible risk factor for lung cancer in the general population. Indeed, CMV has been detected in lung carcinoma [26]. Of note, the proportions of current smokers were similar in CMV-naïve and CMV-exposed patients. Interestingly, colon cancer, cervix cancer and prostatic carcinoma were found to be modestly more frequent in CMV-exposed patients. CMV has been also identified in these tumours in immunocompetent patients [6,8,9].

Our study has some limitations. Bias inherent to retrospective studies cannot be excluded. Moreover, confounding factors associated with CMV exposure cannot be ruled out even when most of those implicated in cancer occurrence after transplantation have been taken into account. We considered all types of cancer. Mechanisms involved in carcinogenesis may vary from one cancer to another, but a defect in immune surveillance may have an impact on different types of cancer.

Our results highly suggest that both pretransplant CMV exposure and post-transplant CMV replication increase the risk of cancer after transplantation. Universal CMV prophylaxis could be required to avoid CMV replication. Whether markers of immune exhaustion could help to define the individual risk of cancer should be assessed in future studies.

Authorship

CC, JB, PS, DD and PT: designed the study concept and draft the manuscript. CC, JB, BG, CR, PS and DD: performed research/study. JB, BG, CR, and PS: contributed to acquisition of biological data. CC, JB, CR, CA, JMC, CB, MCWL and DD: participated in patients' follow-up and collected data. CC, PS, DD and PT: analyzed data. CC and DD: wrote the paper. PT: reviewed the manuscript.

Funding

The authors have declared no funding.

Acknowledgements

This study is supported by grants from the Fondation de Transplantation. CC received financial support from the Fondation de transplantation (#ET-031211 and #ET-050320, respectively).

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