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Interferon-γ production by CMV-specific CD8+ T lymphocytes provides protection against cytomegalovirus reactivation in critically ill patients

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Take-home message: Cytomegalovirus reactivation is frequent in critically ill patients and is associated with poor outcome. The study of clinical risk factors such as underlying disease and its severity has not shown conclusive results for the prediction of CMV reactivation; therefore, we need immunological markers to determine which patients could receive prophylaxis with antiviral drugs. The results of our study show that the determination of interferon-γ by CMV-specific CD8+ T cells can be useful for predicting the risk of CMV reactivation.

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Abstract *Purpose:* To evaluate the usefulness of the secretion of interferon-γ (IFNγ) by cytomegalovirus (CMV)-specific CD8+ T cells to determine the risk of CMV reactivation in critically ill non-immunosuppressed patients. *Methods:* Two-center prospective cohort study including critically ill non-immunosuppressed CMV-seropositive patients admitted between December 2012 and March 2013. The incidence of CMV reactivation by polymerase chain reaction (real-time PCR) in plasma was investigated. IFNγ secretion by CMV-specific CD8+ T lymphocytes was determined at the time of admission to the intensive care unit (ICU) by means of the QuantiFERON®-CMV (QF-CMV) test. Cox regression analyses were performed to investigate CMV reactivation risk factors. *Results:* Fifty-three patients were included, of whom 13 (24.5 %) presented CMV reactivation. Twenty-six patients (49.1 %) were QF-CMV "reactive" ($QF\text{-}CMV_R$). Of the 26 $QF\text{-}CMV_R$ patients, 11.5 % (3/26) had CMV reactivation, whereas 37 % (10/27) of QF-CMV "non reactive" patients ($QF\text{-}CMV_{NR}$) presented reactivation ($p = 0.03$). By Cox regression, the presence of $QF\text{-}CMV_R$ at ICU admission (HR 0.09, 95 % CI 0.02–0.44; $p = 0.003$) was associated with a decreased risk of CMV reactivation. The sensitivity, specificity, positive predictive value, and negative predictive value of QF-CMV were 77, 57, 37, and 88 %,

respectively. Eleven of the 53 patients (20.7 %) died during the follow-up period. Mortality was more frequent in patients with CMV reactivation (6/13, 46.1 vs. 5/40, 12.5 %; $p = 0.015$). *Conclusions:* In critically ill non-immunosuppressed patients, the presence of functional CMV-specific CD8+ T lymphocyte response at intensive care unit admission provides protection against CMV reactivation.

Keywords Cytomegalovirus · Critical care · Interferon-γ · Mechanical ventilation · Replication · Mortality

Introduction

Cytomegalovirus (CMV) has a high seroprevalence in adults. Once primary infection has occurred, the virus remains dormant in tissues and can be reactivated during periods of immunosuppression, as frequently occurs in solid organ transplant patients or hematopoietic progenitors [1–3].

In addition to immunosuppressed patients, in recent years it has been shown that up to 40 % of critically ill CMV-seropositive patients present reactivation of the virus despite not presenting immunosuppression previously [4–7]. Some studies have associated these reactivations with higher rates of nosocomial infection, prolonged hospitalization, longer duration of mechanical ventilation, and increased mortality [4, 8, 9]. Therefore the performance of randomized placebo-controlled trials has been proposed to determine the benefit of prophylaxis against CMV in the prognosis of these patients [10]. However, these studies have significant limitations, such as the scarcity of data demonstrating a direct causal relationship between CMV reactivation and the worst prognosis, and primarily the lack of factors associated with the development of reactivation, which would allow higher-risk patients to be identified and avoid exposing lower-risk patients to antiviral drugs.

Until now, the study of clinical risk factors such as underlying disease and its severity have not shown conclusive results for the prediction of CMV reactivation [4, 9]. Added to this is the fact that the heterogeneity of the pathologies presented by patients admitted to intensive care units (ICU) represents a major constraint for drawing conclusions in this regard.

Some studies have therefore analyzed immunological variables to identify patients at greater risk of CMV reactivation. In this respect, it was recently shown that the weakness in natural killer (NK) cells function, measured according to the decrease in the secretion of interferon-γ (IFNγ), preceded CMV reactivation in critically ill patients [11]. In addition to NK cells, the immunity produced by T cells plays a crucial role in the prevention of CMV reactivation [12]. In this sense, studies have shown that the assessment of T cell functionality has the potential to become a useful tool for managing CMV infection in transplant patients [13]. Indeed, decreased secretion of IFNγ by CMV-specific CD8+ T lymphocytes has been associated with increased risk of CMV reactivation in transplant recipients [14–17].

Therefore, the aim of this study was to prospectively analyze the impact of the functionality of CMV-specific CD8+ T lymphocytes on the risk of CMV reactivation in critically ill non-immunosuppressed patients. To do this, we determined whether the decreased secretion of IFNγ by CMV-specific CD8+ T cells at the time of ICU admission is associated with increased risk of reactivation in these patients.

Materials and methods

Study population and design

This prospective observational study was conducted in the ICUs of two third-level centers (Ciudad Real General University Hospital and Reina Sofia University Hospital in Córdoba) between December 2012 and March 2013. All adult patients who were newly admitted to both ICUs either without hospitalization or after a stay in hospital wards were assessed daily for inclusion in the study. The inclusion criteria were as follows: (1) age over 18 years; (2) seropositivity for CMV; (3) expected survival more than 72 h; (4) expected stay in the ICU more than 4 days; (5) no antiviral medication used in the previous month; (6) absence of congenital or acquired immunosuppression; (7) no steroid treatment in the previous month; (8) no hemoconcentrated blood transfusions in the week prior to admission.

At the time of ICU admission (within the first 24 h) and after verification of compliance with the inclusion criteria, blood samples were obtained to determine (1) CMV serology; (2) quantification of CMV-DNA; and (3) production of IFNγ by CMV-specific CD8+ T lymphocytes. Clinical variables were also collected at this time to determine risk factors for CMV reactivation. Patients with evidence of viral reactivation at admission were not included in the study. Patients were monitored for CMV reactivation by measuring viral load upon admission and at least once a week until discharge from ICU or death. Duration of viremia was considered as the number of days that elapsed between detection of the first positive viral load and the first negative viral load or until ICU

discharge. The attending physicians were unaware of the virological and immunological results gathered in an internal database designed for the study. No patient was given antiviral treatment against CMV during follow-up.

The research protocol was approved by the ethics committees of each center. The study was performed in accordance with the Declaration of Helsinki and its later amendments. All patients gave their informed consent prior to the inclusion in the study.

Determination of anti-CMV IgG antibodies and CMV viral load

Antibodies to CMV indicating prior CMV infection were assessed using a commercial chemiluminescence immunoassay kit (Architect CMV IgG 6C15, Abbott, Sligo, Ireland). The assay was performed and interpreted according to the manufacturer's recommendations. The serological study was processed independently in the microbiology laboratory at each center.

The quantification of CMV-DNA was tested by an Abbott RealTime CMV kit (Abbott Molecular, Illinois, USA). DNA extractions were performed from 500 µL of plasma using an Abbott mSample preparation system DNA kit on an m2000sp instrument (Abbott Molecular, Illinois, USA) and eluted in a volume of 70 µL. Amplification and real-time detection of CMV DNA was performed on an m2000rt instrument. Results were standardized to international units per milliliter (IU/mL) using the World Health Organization (WHO) International Standard for Human CMV for Nucleic Acid Amplification Technique (National Institute for Biological Standards and Controls, NIBSC 09/162).

CMV viral load was determined by real-time PCR at least once per week during the ICU stay. All viral load determinations were performed at Ciudad Real University General Hospital. According to the manufacturer, the detection limit of this method is 31.2 IU/mL (20 copies/mL). CMV reactivation was considered upon detection of CMV viral load by PCR.

The laboratory personnel who performed the analyses had no contact with patients or knowledge of their clinical data.

QuantiFERON-CMV assay

The QuantiFERON-CMV® test was performed in accordance with the manufacturer's instructions (Cellestis, a Qiagen company, Melbourne, Australia) (Fig. 1). In brief, 1 mL of heparinized whole blood was collected in three QuantiFERON-CMV blood collection tubes. Tubes contained either (1) a mix of 22 CMV peptides from a variety of proteins; (2) no antigens (negative control); or (3) phytohemagglutinin (positive mitogen control). All

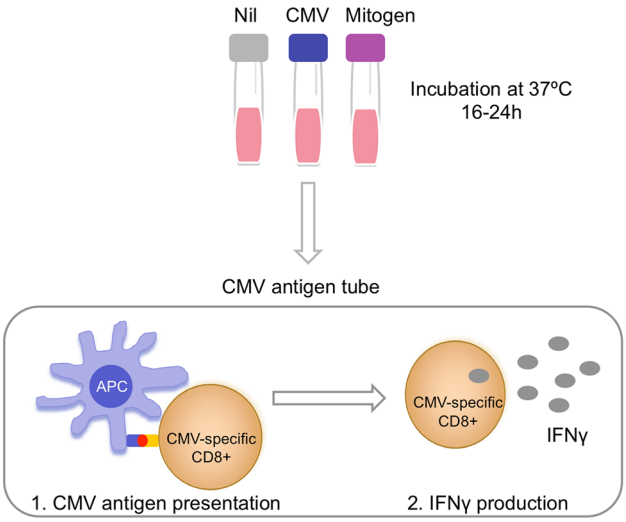

Fig. 1 Schematic representation of QuantiFERON-CMV assay. In the cytomegalovirus antigen tube, CMV-specific CD8+ T cells of patients who have been previously exposed to the virus recognize cytomegalovirus antigen and respond by secreting interferon-γ

candidates enrolled in the study had HLA class I alleles capable of binding CMV peptides. After collection, the tubes were shaken vigorously and incubated for 16–24 h at 37 °C. Subsequently, supernatants were recovered and analyzed for IFNγ (IU/mL) by standard ELISA. According to the manufacturer's instructions a result for the CMV antigen was considered "reactive" when the CMV antigen response minus the negative control response was greater than 0.2 IU/mL of IFNγ. The result of QuantiFERON-CMV was considered "indeterminate" when the IFNγ level in the CMV antigen tube minus the negative control was less than 0.2 IU/mL and the IFNγ level in the mitogen tube (once the negative controls had been subtracted) was less than 0.5 IU/mL. For the purpose of statistical analysis, "indeterminate" results were considered as "non-reactive".

Statistical analysis

Data were analyzed using the SPSS statistical package (version 15.0). The baseline characteristics of patients with and without CMV reactivation were compared using the Chi-square test or Fisher's exact test for categorical variables and the Mann–Whitney *U* test for continuous variables. To study the factors associated with CMV reactivation, a Cox regression analysis was performed that included clinically relevant variables taken upon completion of the QuantiFERON-CMV in spite of not being significant in the univariate analysis. The multivariate model was limited to four factors present at admission to the ICU because of the limited number of events or patients in each case. The following variables

were analyzed: age (years), SAPS II at inclusion (score points), diabetes mellitus (yes/no), and QuantiFERON-CMV (reactive/non-reactive). The model included testing for co-linearity, interactions, and proportional hazard assumption for the risk factors. The sensitivity, specificity, positive predictive values (QuantiFERON-CMV non-reactive patients presenting reactivation), and negative predictive values (QuantiFERON-CMV reactive patients not presenting reactivation) of QuantiFERON-CMV were calculated. p values below 0.05 were considered statistically significant for all tests.

Results

A total of 98 patients were initially assessed for inclusion in the study. Of them, 24 were excluded because of high probability of death or discharge within the first 4 days of admission ($n = 13$), negative serology for CMV ($n = 4$), need for hemoconcentrated blood transfusion ($n = 4$), and immunosuppression ($n = 3$). A total of 74 patients signed informed consent (Fig. 2). Twenty-one of these patients were excluded because of presence of CMV reactivation at ICU admission ($n = 10$), unexpected early discharge ($n = 6$), and lost to follow-up ($n = 5$). Thus a total of 53 patients completed the study. The median time of ICU stay was 23 days (interquartile range 13–36 days). The demographic and clinical characteristics of these patients are shown in Table 1.

QuantiFERON-CMV assay results

Because all QuantiFERON-CMV tests were performed at ICU admission we have the results of the 74 patients initially included in the study. Forty-seven of them (63.5 %) were "reactive". Of the 53 patients who completed the study, 26 (49.1 %) were QuantiFERON-CMV reactive. Of the remaining patients, 25 (47.2 %) were QuantiFERON-CMV non-reactive and two patients (3.7 %) were indeterminate. The median level of IFNγ in QuantiFERON-CMV reactive patients was 2.02 IU/mL (interquartile range 0.8–15.6 IU/mL).

Characteristics of CMV reactivation

CMV reactivation was detected in 13 of the 53 patients (24.5 %). The characteristics of these patients are shown in Table 1. In these 13 patients, the median time of onset of viremia was 14 days (interquartile range 12–27 days). The cumulative incidence of reactivation greater than 1000 IU/mL was 23.1 % (3/13 patients), occurring at a median of 10 days (interquartile range 7–20 days). The median duration of viremia was 18 days (interquartile range 8–33 days). The frequency of reactivation was higher in the QuantiFERON-CMV non-reactive individuals than in the QuantiFERON-CMV reactive individuals (37 vs. 11.5 %; $p = 0.03$). The median onset of viremia was 20 days (interquartile range 14–63 days) in QuantiFERON-CMV reactive patients and 14 days (interquartile range 9–27 days) in QuantiFERON-CMV non-reactive patients ($p = 0.28$). Median peak viral load in QuantiFERON-CMV reactive patients was 3521 IU/mL (interquartile range 40–6376 UI/mL) and in QuantiFERON-CMV non-reactive patients 377 IU/mL (interquartile range 58–520 IU/mL) ($p = 0.39$). No significant differences in the duration of viremia were detected in QuantiFERON-CMV reactive patients (median 35 days, interquartile range 12–57 days) compared to QuantiFERON-CMV non-reactive patients (median 15 days, interquartile range 10–24 days), ($p = 0.63$). The percentage of patients without CMV reactivation according to the result of the QuantiFERON-CMV test is shown in Fig. 3. The sensitivity and specificity of QuantiFERON-CMV were 77 and 57 %, respectively. The positive predictive and negative predictive values were 37 and 88 %, respectively.

Eleven of the 53 patients (20.7 %) died during hospitalization (median 52 days, interquartile range 32–105 days). In-hospital mortality was significantly more frequent in patients with CMV reactivation than those without CMV reactivation (6/13, 46.1 % vs. 5/40, 12.5 %; $p = 0.015$). Unfortunately, as a result of the small number of patients who died, the confidence interval was very wide in the Cox regression and we could not analyze the joint association of these variables.

Factors associated with CMV reactivation

To evaluate factors associated with CMV reactivation in critically ill non-immunosuppressed patients a Cox regression was performed. This analysis included the following variables: age, SAPS II, diabetes mellitus, and reactive QuantiFERON-CMV. In Cox regression, QuantiFERON-CMV test result was the only variable with statistically significant association with CMV reactivation (HR 0.09, 95 % CI 0.02–0.44; $p = 0.003$). In particular, display CD8+ T cell response to CMV (i.e., being QuantiFERON-CMV reactive) at ICU admission was associated with a protection against CMV reactivation. Other factors included in the analysis such as the severity of the underlying disease at ICU admission determined by the SAPS II (HR 1.02, 95 % CI 0.96–1.07; $p = 0.47$), diabetes mellitus (HR 4.73, 95 % CI 0.57–39.21; $p = 0.15$), and age (HR 0.97, 95 % CI 0.92–1.03; $p = 0.40$) were not associated with CMV reactivation.

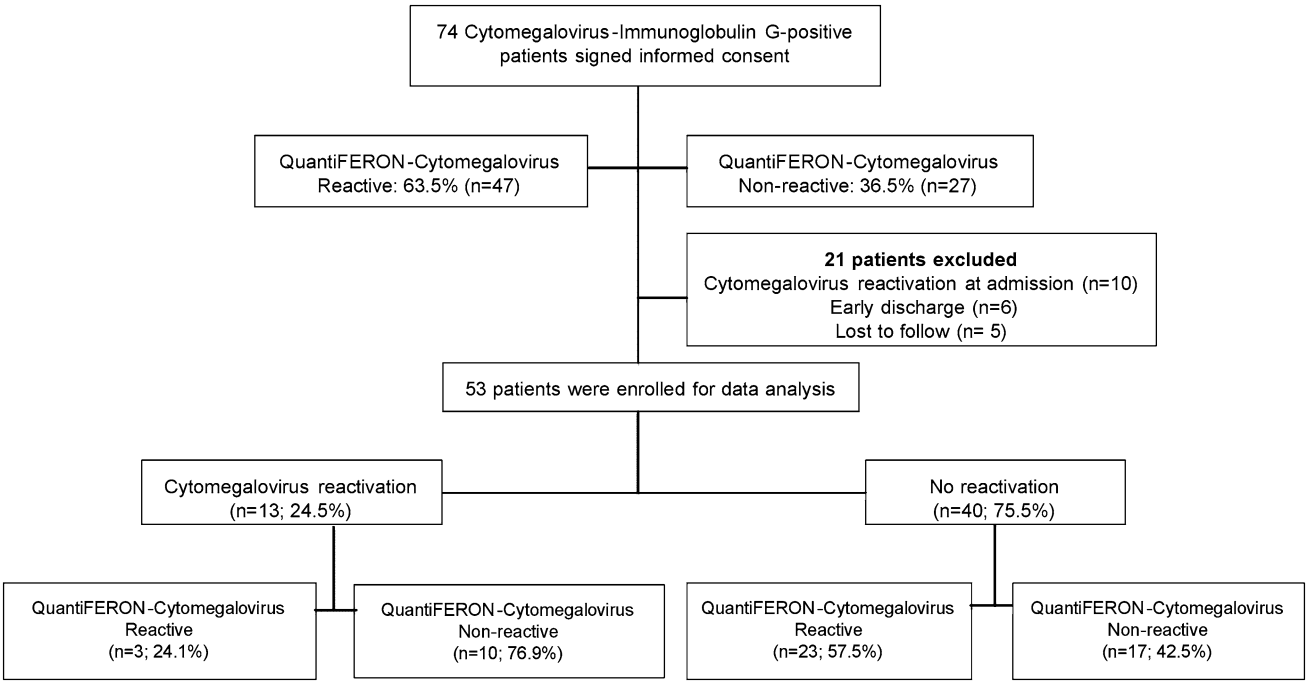

Fig. 2 Patient selection

Table 1 Patient characteristics according to CMV reactivation

Variable	All patients $n = 53$ (100 %)	CMV reactivation		p
		Yes ($n = 13$)	No ($n = 40$)	
Sex: male	40 (75.5 %)	8 (61.5 %)	32 (80 %)	0.2[b]
Age (years)[a]	59.28 (46–75)	58.54 (43–76)	59.53 (46–64)	0.8[c]
Type of admission				
Medical	18 (34 %)	6 (46.2 %)	12 (30 %)	0.3[b]
Surgical	35 (66 %)	7 (53.8 %)	28 (70 %)	
APACHE II[a]	16 (6–26)	16.5 (8–26)	15.8 (6–24)	0.6[c]
SAPS II[a]	34.9 (13–68)	37.2 (26.5–46)	34.1 (28–37.5)	0.4[c]
Septic shock	28 (52.8 %)	8 (61.5 %)	20 (50 %)	0.4[b]
Diabetes mellitus	10 (18.9 %)	6 (46.2 %)	4 (10 %)	0.009[d]
Mechanical ventilation	47 (88.7 %)	13 (100 %)	34 (85 %)	0.3
Duration of mechanical ventilation (days)[a]	21.6 (10–27.5)	33.5 (21–45.5)	17.2 (6–22)	0.007[c]
Acute kidney failure	25 (47.2 %)	8 (61.5 %)	17 (42.5 %)	0.2[b]
Bacteremia	25 (47.2 %)	7 (53.8 %)	18 (45 %)	0.5[b]
Candidemia	7 (13.2 %)	2 (15.4 %)	5 (12.5 %)	1[d]
Ventilator-associated pneumonia	21 (39.6 %)	8 (61.5 %)	13 (32.5 %)	0.1[b]
QuantiFERON®-CMV reactive	26 (49.1 %)	3 (23.1 %)	23 (57.5 %)	0.03[b]
Hospitalization time (days)[a]	45.3 (29–61)	52 (37–76)	34 (23–54)	0.9[c]
ICU stay (days)[a]	28.1 (13.5–36)	43 (31–51)	17 (12–26.5)	0.01[c]
Mortality	11 (20.7 %)	6 (46.2 %)	5 (12.5 %)	0.01[d]

CMV cytomegalovirus, *SAPS* simplified acute physiology score, *ICU* intensive care unit

[a] Median (interquartile range)

[b] Chi-square test

[c] Mann–Whitney *U* test

[d] Fisher's exact test

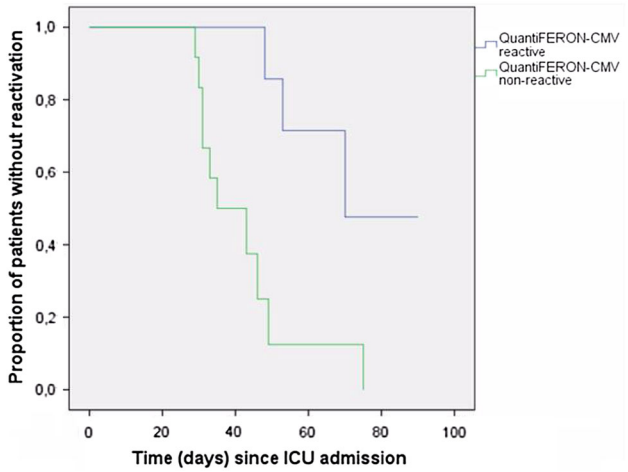

Fig. 3 Comparative analysis using Kaplan–Meier curves of the estimated survival free of cytomegalovirus reactivation in QuantiFERON-CMV reactive patients (*blue line*) and in QuantiFERON-CMV non-reactive patients (*green line*) (Log rank test $p = 0.004$)

Discussion

The results of this prospective observational study show that the determination of IFNγ production by CMV-specific CD8+ T lymphocytes using the QuantiFERON-CMV test at admission in ICU is a good marker for identifying the risk of CMV reactivation in critically ill non-immunosuppressed patients.

The main utility of QuantiFERON-CMV® in these patients is its high negative predictive value as patients with QuantiFERON-CMV® reactive show protection against CMV reactivation. The administration of prophylaxis is therefore not recommended in these patients, thus preventing the effects of exposure to antiviral drugs (adverse reactions, resistance) [18].

In our study, only 10 of the 27 (37 %) QuantiFERON-CMV non-reactive patients presented CMV reactivation. Given this low positive predictive value, the QuantiFERON-CMV non-reactive result would not be adequate for identifying patients who may develop subsequent reactivation as 63 % of the QuantiFERON-CMV non-reactive patients would receive prophylaxis unnecessarily. Therefore, the QuantiFERON-CMV non-reactive result would be useful to select a population in which monitoring for early detection and treatment of CMV reactivation (pre-emptive therapy) during their stay in the ICU would be a better strategy than universal prophylaxis.

The potential usefulness of evaluating the function of CMV-specific T cells using the QuantiFERON-CMV test has been demonstrated in both hematopoietic progenitor and solid organ transplant recipients [13, 15, 19]. Cantisán et al. reported that the decreased production of IFNγ by CMV-specific CD8+ T lymphocytes has been shown to predict CMV reactivation in both the pre-transplant and post-transplant periods [13].

At present, data on the functionality of CMV-specific T lymphocytes in critically ill patients are scarce and present contradictory results. Two previous studies reported no differences in terms of the functionality of CMV-specific T cells in patients with and without CMV reactivation [20, 21]. Subsequently, the results of one study of cases and controls, which included 31 patients subjected to mechanical ventilation, showed how decreased levels of IFNγ-producing CMV-specific CD4+ and CD8+ T lymphocytes were associated with the presence of active CMV infection or increased risk of subsequent viral reactivation, although the absence of multivariate analysis does not rule out the possible existence of confounding factors [22].

Studies conducted so far show that although it may be variable, the mean incidence of CMV reactivation in critically ill patients is 25 % [9]. This variability in the incidence of reactivation may be due to differences in the type of population and the technique and frequency of viral monitoring employed. In our series, an incidence of CMV reactivation of 24.5 % was found, which is similar to the level reported previously [6, 11, 23]. Similarly, the median time of onset of viremia in our study was 14 days, which is similar to that reported in previous studies using PCR in viral monitoring [6]. Our results show that although reactivation is more frequent in QuantiFERON-CMV non-reactive, no differences were found in the timing of onset, duration, and magnitude of viremia. A study with a larger sample would probably reveal differences in these aspects.

As a result of the limited number of patients in the study, the multivariate analysis included only three risk factors (age, diabetes mellitus, and SAPS II score) in addition to the QuantiFERON-CMV. Numerous studies have shown the influence of age on immune response to CMV [24, 25], although our study did not find age to be associated with increased risk of CMV reactivation. In a similar manner, although diabetes mellitus has been associated with increased risk of infections and sepsis [26, 27], it was not associated with increased risk of CMV reactivation in our study. Moreover, in line with previously published data [6], this study found no association between severity of illness (as assessed by the SAPS II score) and CMV reactivation, thus decreasing the likelihood of CMV reactivation being an intermediate marker of disease severity.

In our study, the mortality rate was significantly higher in patients who developed CMV reactivation compared to those who did not. However, because of the small sample size (only 6 of the 13 patients with replication died), we did not perform a multivariate analysis to verify the joint influence of other potential risk factors. Studies evaluating the influence of CMV reactivation on the mortality of critically ill patients have reported contradictory results

[5, 7]. This may be because these studies include different populations with different degrees of severity and different strategies for monitoring CMV reactivation. A placebo-controlled clinical trial of antiviral prophylaxis would have to be conducted in order to determine the real influence of CMV reactivation on mortality in this population.

Our study has some strengths, among them the prospective design, the use of a robust PCR-based method to detect CMV reactivation, the evaluation of immune function using a standardized method, and the inclusion of representative patients in a general ICU setting.

Our study also has certain limitations. The main limitation is the small size of the sample studied, which does not allow definitive conclusions to be drawn regarding the existence of differences in the kinetics of viral reactivation based on the results of the QuantiFERON-CMV test. Another limitation is that the sample size does not allow one to perform an adequate assessment of the possible role of clinical variables on CMV reactivation. On the other hand, we do not know the value of QuantiFERON-CMV immediately before CMV reactivation because the QuantiFERON-CMV test was performed at admission. However, our objective is to identify patients according to their risk of reactivation at the time of admission to the ICU, and on the other hand, we cannot know the moment at which viral reactivation will occur.

Conclusions

This study shows that patients with functional CD8+ T lymphocyte response to CMV at ICU admission are protected against CMV reactivation, thus demonstrating that the use of prevention strategies (universal prophylaxis or pre-emptive therapy) should not be indicated in this group of patients. In patients lacking this specific response the monitoring of CMV viral load for the detection and early treatment of the virus can be an alternative to prophylaxis.

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Compliance with ethical standards

Conflicts of interest The authors do not have any financial relationship with the organization that sponsored the research. The authors declare that they have no conflict of interest.

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