Primary Cytomegalovirus Phosphoprotein 65–Specific $CD8^+$ T-Cell Responses and T-bet Levels Predict Immune Control During Early Chronic Infection in Lung Transplant Recipients

Matthew R. Pipeling, Emily R. John, Jonathan B. Orens, Noah Lechtzin, and John F. McDyer

Division of Pulmonary and Critical Care Medicine, Johns Hopkins University, School of Medicine, Baltimore, Maryland

Background. Cytomegalovirus (CMV) remains an important pathogen in solid organ transplantation, particularly lung transplantation. Lung transplant recipients (LTRs) mismatched for CMV (donor positive/recipient negative [D^+R^-]) are at highest risk for active CMV infection and have increased mortality. However, the correlates of immune control during chronic CMV infection remain incompletely understood.

Methods. We prospectively studied 22 D^+R^- LTRs during primary CMV infection and into chronic infection. Immune responses during primary infection were analyzed for association with viral relapse during early chronic infection.

Results. Primary CMV infection was characterized by a striking induction of T-box transcription factor (T-bet) in $CD8^+$ T cells. CMV-specific effector $CD8^+$ T cells were found to be T-bet^+. After primary infection, 7 LTRs lacked immune control with relapsing viremia during early chronic infection. LTRs with relapsing viremia had poor induction of T-bet and low frequencies of phosphoprotein 65 (pp65)–specific $CD8^+$ effector T cells during primary infection. However, frequencies of IE1-specific $CD8^+$ effector T cells during primary infection were not associated with early relapsing viremia.

Conclusions. T-bet plays an important role in coordinating $CD8^+$ effector responses to CMV during primary infection. Moreover, $CD8^+$ T-bet induction and pp65-specific $CD8^+$ effector responses at the time of primary infection are important predictors of immune control of CMV during early chronic infection.

Cytomegalovirus (CMV) remains a significant opportunistic infection in solid organ transplant recipients, particularly lung transplant recipients (LTRs) [1–5]. LTRs have increased susceptibility to CMV infection, perhaps because the lung is a major reservoir for latent virus [6]. As a β-herpesvirus, CMV establishes a state of chronic infection after primary infection, with the risk of reactivation. LTRs mismatched for CMV (donor positive/recipient negative [D^+R^-]) have the highest risk of active CMV infection and demonstrate increased 1- and 5-year mortality [7]. Active CMV infection is a risk factor for the development of chronic allograft rejection (bronchiolitis obliterans syndrome), a major limiting factor for long-term survival in LTRs [8, 9]. Importantly, CMV viremia early after lung transplantation is strongly associated with the development of bronchiolitis obliterans syndrome in LTRs [10]. Moreover, repeated episodes of relapsing viremia in LTRs are associated with a decreased survival [11]. However, despite the impact of CMV on outcomes in LTRs, prophylaxis, monitoring, and treatment regimens vary widely among institutions and the correlates of protective immunity remain to be fully elucidated.

The adaptive immune system, particularly $CD8^+$ T cells, plays a key role in the control of acute viral infections, including CMV infection [12]. However, control of CMV during chronic infection requires ongoing effective immune surveillance to prevent viral relapse in its host [13]. Viral-specific effector $CD8^+$ T cells exert their antiviral activities through the production of type 1

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Correspondence: John F. McDyer, MD, Johns Hopkins University, Department of Medicine, 5501 Bayview Circle, Rm 4B51, Baltimore, MD 21224 (jmcdyer@jhmi.edu).

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cytokines (eg, interferon γ [IFN-γ] and tumor necrosis factor α [TNF-α]) and cytolytic activity. The differentiation of effector T cells, including $CD8^+$ T cells, is transcriptionally regulated—often by the same transcription factors that mediate their effector function [14]. The T-box transcription factor, T-bet, was first described as being critical for the development of $CD4^+$ T-helper 1 cells in mice [15] and subsequently shown to be important in $CD8^+$ T cells [16–18]. However, although T-bet is critical for the development of effective $CD8^+$ T cells in animal models, little is known about its functional importance in humans.

We elsewhere demonstrated the acquisition of de novo primary CMV-specific $CD8^+$ T cell effector responses in D^+R^- LTRs predominated by IFN-γ production toward the structural protein of phosphoprotein 65 (pp65) [19]. We have subsequently noted relapsing CMV viremia after completion of therapy for primary infection in a subset of these D^+R^- LTRs during early chronic infection. We therefore hypothesized that LTRs who developed adequate $CD8^+$ effector responses during primary infection would be protected from viral relapse during early chronic CMV infection. Herein, we report our findings of the importance of adequate induction of T-bet and its facilitation of pp65-specific effector responses in $CD8^+$ T cells during human primary CMV infection in viral control during early chronic infection.

MATERIALS AND METHODS

Study Participants

D^+R^- LTRs from the Johns Hopkins Lung Transplant Program were identified and provided informed written consent for participation in an institutional review board–approved protocol. All patients were treated with standard immunosuppression. Antiviral prophylaxis with ganciclovir and/or valganciclovir was used for the initial 3 months after transplantation. Patients were prospectively monitored at least weekly for the development of primary CMV infection (defined as de novo detection of viral replication by quantitative polymerase chain reaction [PCR]). CMV viral loads were determined using quantitative PCR of plasma samples by the Johns Hopkins Hospital Clinical Virology Laboratory. Patients developing primary CMV infection were treated with antiviral therapy until 2 consecutive weekly quantitative CMV PCR results revealed undetectable viremia. After completion of antiviral therapy for primary infection, patients were prospectively monitored with quantitative PCR at least biweekly for the development of relapsing viremia (defined as the detection of >300 copies/mL CMV by quantitative PCR on 2 consecutive samples after the completion of antiviral therapy for primary infection). Patients with relapsing viremia received antiviral therapy if clinically indicated.

Tissue Samples

Blood samples were obtained before the discontinuation of initial antiviral prophylaxis (pre-CMV time point) and within 5–14 days of detection of de novo viremia (primary CMV time point). Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood samples by density gradient centrifugation using Ficoll-Paque (GE Healthcare).

Antigen Stimulation

Single pools of overlapping 15-mer peptides for pp65 (SwissProt No. P06725; 138 peptides) and IE1 (SwissProt no. P13202; 120 peptides) were gifts from Dr David Stroncek of the National Institutes of Health. PBMCs were cultured in round-bottom tissue culture tubes (Sarstedt) in the presence or absence of pooled pp65 or IE1 peptides (1 μg/mL) or the positive control of staphylococcal enterotoxin B (SEB; 1 μg/mL). All stimulations for intracellular cytokine production were performed as described elsewhere [19].

Flow Cytometry

After stimulation, cells were surface-stained with the fluorochrome-labeled antibodies anti–CD3-AlexaFluor700, anti–CD8-AmCyan (BD Biosciences), and anti–CD4–phycoerythrin (PE)–Texas Red (Abcam). Live/Dead Fixable Blue Dead Cell Stain (Invitrogen) was used to allow gating on viable cells. Cytofix/Cytoperm (BD Biosciences) reagents were used to fix and permeabilize cells. Intracellular staining was performed with the fluorochrome-labeled antibodies anti–IFN-γ–allophycocyanin, anti–TNF-α–PE–cyanine 7 (BD Biosciences), and anti–T-bet–PE or anti–T-bet–AlexaFluor 647 (eBiosciences). Cell fluorescence was analyzed using a FACSAria cytometer, and data were analyzed using FlowJo software (Tree Star).

Statistical Analysis

Statistical analysis was performed using the GraphPad Prism 5.04 software package. No assumption was made regarding the Gaussian distribution of measured variables, and therefore the nonparametric tests of Wilcoxon signed-rank, Mann–Whitney–Wilcoxon, and Spearman rank correlation were used. A 2-tailed P value of $<.05$ was considered to be statistically significant.

RESULTS

Relapsing Viremia After Primary CMV Infection in D^+R^- LTRs

Since we reported the development of de novo CMV-specific $CD8^+$ effector responses during primary CMV infection in D^+R^- LTRs, we have expanded our prospective cohort of D^+R^- LTRs to study primary $CD8^+$ T cell effector responses and their role during early chronic infection. The details of the 22 D^+R^- LTRs studied during primary CMV infection and closely monitored for ≥6 months after primary infection are presented in Table 1. With close prospective monitoring (see the Materials and Methods), we were able to detect primary CMV infection at a median of 156 days after transplant (~60 days after discontinuation of initial CMV antiviral prophylaxis) in these 22 LTRs. Continued prospective monitoring after completion of antiviral therapy for

Table 1. Characteristics of Lung Transplant Recipients (LTRs), Primary Cytomegalovirus (CMV) Infection, and Relapse

LTR	Age, y	Sex	Primary diagnosis	Immunosuppressive agent at primary CMV onset, dose (doses per day)	Interval from transplantation to onset of primary CMV, d	Relapsing viremia after primary infection (Interval, d)
22	60	Female	Idiopathic pulmonary fibrosis	TAC, 3.5 mg (2); MMF, 1 g (2); Pred, 10 mg (1)	157	No
24	31	Female	Cystic fibrosis	TAC, 1.5 mg (2); MMF, 0.5 g (3); Pred, 10 mg (1)	96	Yes (105)
25	34	Female	Primary pulmonary hypertension	TAC, 4 mg (2); MMF, 0.5 g (2); Pred, 10 mg (1)	129	No
28	33	Female	Cystic fibrosis	TAC, 6 mg (2); MMF, 0.5 g (2); Pred, 5 mg (1)	210	No
29	62	Female	COPD	TAC, 2.5 mg (2); MMF, 0.5 g (3); Pred, 10 mg (1)	168	Yes (75)
30	61	Female	Idiopathic pulmonary fibrosis	CSA, 175 and 100 mg; RAPA, 1 mg (1); Pred, 5 mg (1)	94	No
31	55	Male	Cystic fibrosis	TAC, 3 mg (2); AZA, 50 mg (1); Pred, 5 mg (1)	248	No
33	51	Female	Idiopathic pulmonary fibrosis	TAC, 2 mg (2); MMF, 0.5 g (3); Pred, 7.5 mg (1)	186	Yes (74)
34	59	Female	COPD	TAC, 4 mg (2); MMF, 0.25 g (2); Pred, 10 mg (1)	174	No
35	27	Male	Cystic fibrosis	TAC, 1 mg (2); MMF, 0.5 g (2); Pred, 7.5 mg (1)	219	No
36	49	Female	Idiopathic pulmonary fibrosis	TAC, 4 mg (2); MMF, 0.25 g (2); Pred, 10 mg (1)	167	Yes (84)
37	56	Female	Obliterative bronchiolitis	TAC, 5 mg (2); MMF, 0.5 g (4); Pred, 10 mg (1)	133	No
38	56	Male	COPD	TAC, 1 mg (2); MMF, 1 g (2); Pred, 10 mg (1)	155	No
40	54	Female	COPD	TAC, 1.5 mg (2); MMF, 0.5 g (4); Pred, 15 mg (1)	122	No
41	64	Female	Bronchiectasis	TAC, 4 mg (2); MMF, 0.5 g (3); Pred, 5 mg (1)	184	No
42	37	Female	Sarcoidosis	TAC, 5.5 mg (2); MMF, 0.5 g (4); Pred, 12.5 mg (1)	138	No
43	51	Male	Sarcoidosis	TAC, 4 mg (2); MMF, 0.5 g (2); Pred, 15 mg (1)	83	Yes (83)
45	21	Male	Cystic fibrosis	TAC, 2.5 mg (2); MMF, 0.5 g (3); Pred, 15 mg (1)	92	No
46	59	Male	COPD	TAC, 1.5 mg (2); MMF, 1 g (2); Pred, 20 mg (1)	37	Yes (103)
48	47	Male	Idiopathic pulmonary fibrosis	TAC, 2 mg (2); MMF, 0.25 g (2); Pred, 10 mg (1)	162	Yes (77)
50	64	Male	Idiopathic pulmonary fibrosis	TAC, 0.5 mg (2); MMF, 0.5 g (3); Pred, 10 mg (1)	127	No
51	41	Female	Pulmonary hypertension	TAC, 2 mg (2); MMF, 0.5 g (2); Pred, 7.5 mg (1)	214	No
Summary	53[a]	Female in 64%	…	…	156[a]	Yes in 32% (83[a])

Abbreviations: AZA, azathioprine; COPD, chronic obstructive pulmonary disease; CSA, cyclosporine; MMF, mycophenolate mofetil; Pred, prednisone; RAPA, sirolimus; TAC, tacrolimus.

[a] Values represent medians.

primary infection revealed relapsing viremia within 6 months of primary infection in a subset of these D^+R^- LTRs during chronic infection (7 [32%] of 22 patients) with relapsing viremia (median viral load, 2538 copies/mL) occurring at a median of 83 days (range, 74–105 days) after initial detection of primary infection. Six of the 7 LTRs with relapsing viremia required retreatment with antiviral therapy. All episodes of relapsing viremia were independent of episodes of acute rejection, augmented immunosuppression, or other infectious insults. Additionally, there was no clinical evidence of ganciclovir resistance as all LTRs with relapsing viremia responded to ganciclovir.

Relapsing Viremia Is Associated With Low pp65-Specific $CD8^+$ T-Cell Effector Responses

Because the detected $CD8^+$ T cells during primary infection were predominantly pp65-specific, we assessed whether there were differences in pp65-specific $CD8^+$ effector responses between LTRs who demonstrated durable viral control and those with relapsing viremia after primary infection. As shown in Figure 1*A*, 2 representative LTRs (LTR 37, who had viral control, and LTR 48, who had relapsing viremia) demonstrated distinct patterns of pp65-specific effector responses on evaluation during primary infection. LTR 37 demonstrated robust pp65-specific $CD8^+$ T-cell IFN-γ production during primary infection, whereas LTR 48 had minimal pp65-specific responses during primary infection.

We then investigated the development of pp65-specific $CD8^+$IFN-γ^+ effector responses during primary CMV infection in the entire cohort of 22 LTRs who had been monitored for relapsing viremia. Figure 1*B* shows significant differences in primary effector responses between LTRs with viral control after antiviral therapy and those with relapsing viremia (median frequency of pp65-specific $CD8^+$IFN-γ^+ T cells, 1.79% vs 0.07%, respectively; $P = .0006$ by Mann–Whitney–Wilcoxon test). In contrast, $CD8^+$ T-cell effector responses to IE1 were not significantly associated with the development of relapsing viremia during early chronic infection. Likewise, there were no significant differences in effector responses to the positive control of SEB between controllers and relapsers (data not shown). In addition, as shown in Figure 1*C*, we found that frequencies of pp65-specific $CD4^+$IFN-γ^+ T cells differed between these groups, but with absolute frequencies of pp65-specific $CD4^+$IFN-γ^+ T cells being significantly lower than $CD8^+$ responses and near the limit of detection in this assay (median frequency of pp65-specific $CD4^+$IFN-γ^+ T cells, 0.08% vs 0.01%, respectively; $P = .046$ by Mann–Whitney–Wilcoxon test).

We also analyzed whether pp65-specific production of TNF-α by $CD8^+$ T cells was predictive of viral relapse and found no significant association (median frequency of pp65-specific $CD8^+$TNF-α^+ T cells, 0.09% vs 0.00%, respectively; $P = .13$ by Mann–Whitney–Wilcoxon test). We found that pp65-specific $CD8^+$IFN-γ^+ T cells have a $CD45RA^{low}$ phenotype consistent with our previous findings during acute primary CMV infection (data not shown) [19]. We also detected both $CD8^{high}$ and $CD8^{low}$ effector populations in a significant proportion of our participants, as shown in our representative LTR plots in Figure 1*A* and as has been observed during active human immunodeficiency virus (HIV) replication [20]. However, we did not detect significant differences in cytokine production between these $CD8^+$ populations. Taken together, these data suggest that acquisition of adequate pp65-specific $CD8^+$ T-cell immunity during primary infection results in durable viral control during early chronic CMV infection.

Striking Induction of T-bet Expression in $CD8^+$ T Cells During Primary Infection

T-bet has been proposed to be a master regulator of type 1 effector responses (eg, IFN-γ). To further characterize the development of CMV-specific $CD8^+$ effector responses during primary infection in D^+R^- LTRs, we evaluated the intracellular expression of T-bet protein in $CD8^+$ T cells during a CMV-naive period (pre- CMV); and 3 months after transplantation, before discontinuation of prophylaxis and at the time of primary infection. As shown for a representative LTR in Figure 2*A*, there is a marked increase in the percentage of $CD8^+$ T cells expressing T-bet during primary infection compared with the pre-CMV time point. Within the entire cohort of D^+R^- LTRs, there was a significant increase in the frequency of T-bet^+CD8^+ T cells during primary infection, as demonstrated in Figure 2*B* (median frequency of T-bet^+CD8^+ T cells before CMV vs with primary infection, 16.79% vs 68.52%, respectively; $P = .0002$ by Wilcoxon signed-rank test). Of note, we did not detect any differences in T-bet expression between $CD8^{high}$ and $CD8^{low}$ cells (data not shown). We also assessed the relationship between the production IFN-γ and T-bet expression in CMV-specific $CD8^+$ T cells during primary viral infection. As shown for a representative LTR in Figure 2*C*, CMV-specific IFN-γ production is derived from $CD8^+$ T cells that express T-bet.

Low Frequencies of T-bet^+CD8^+ T Cells Are Associated With Relapsing Viremia and Correlate With pp65-Specific $CD8^+$IFN-γ^+ Effector Responses

Because we noted individual variability in T-bet induction, we next sought to assess whether the level of induced T-bet expression in $CD8^+$ T cells during primary CMV infection was predictive of relapsing viremia during chronic infection. As shown in Figure 3*A*, relapsing viremia occurs in D^+R^- LTRs in whom there is failure to induce a significant frequency of T-bet^+CD8^+ T cells during primary CMV infection (median frequency of T-bet^+CD8^+ T cells with relapsing viremia vs those with viral control, 33.79% vs 72.27%, respectively; $P = .003$ by Mann–Whitney–Wilcoxon test). Additionally, because both pp65-specific $CD8^+$ T-cell effector responses and T-bet expression were predictive of relapsing viremia during chronic CMV infection, we assessed whether these parameters were

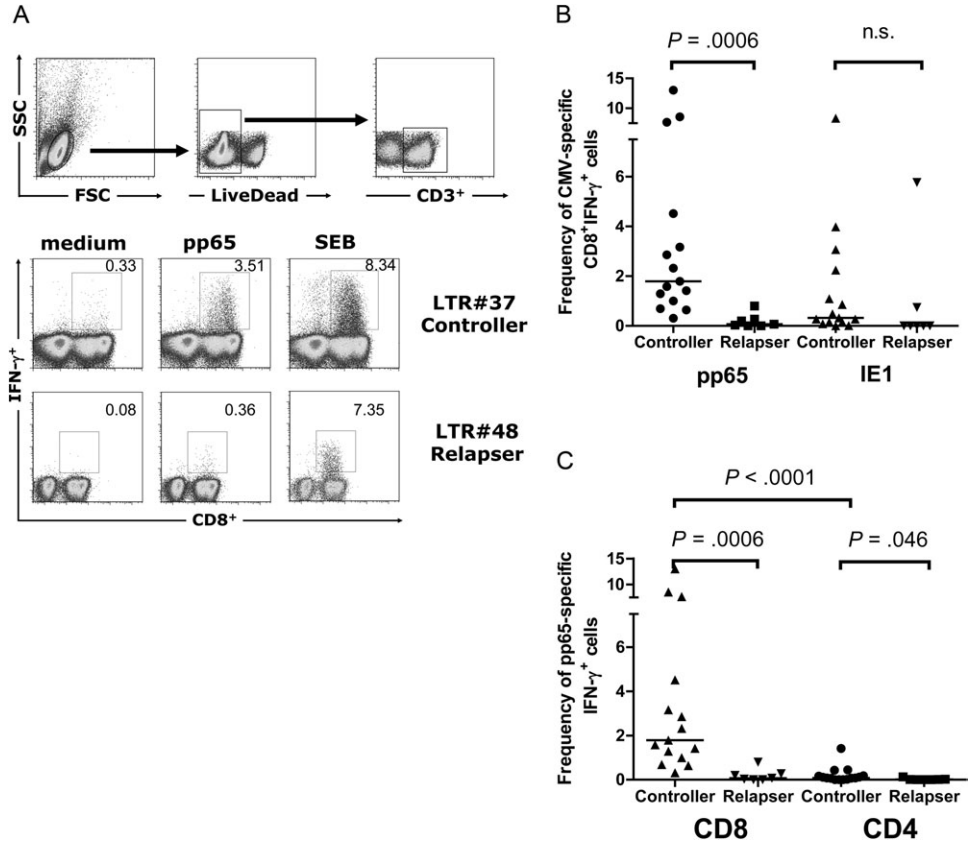

Figure 1. Inadequate phosphoprotein 65 (pp65)–specific $CD8^+$ effector responses during primary cytomegalovirus (CMV) infection are associated with relapsing viremia. *A,* Representative gating strategy for analysis of side scatter (SSC), forward scatter (FSC), LiveDead, CD3+ and CD8+ for peripheral blood mononuclear cells (PBMCs) and staining for interferon γ (IFN-γ) from 2 representative lung transplant recipients (LTRs). PBMCs were restimulated with either medium, pooled pp65 peptides, or staphylococcal enterotoxin B (SEB) and evaluated for $CD3^+CD8^+$ T cell production of IFN-γ with values indicating the frequency of $CD3^+CD8^+$ T cells producing IFN-γ. *B,* Comparison of pp65-specific and IE1-specific $CD8^+$IFN-γ^+ responses during primary CMV infection between 15 LTRs experiencing durable viral control and 7 LTRs experiencing relapsing viremia during early chronic infection. Bars represent median values, and *P* values were calculated by the Mann–Whitney–Wilcoxon test. *C,* Comparison of pp65-specific $CD8^+$ and $CD4^+$ production of IFN-γ between 15 controller and 7 relapser LTRs. Bars represent median values, and *P* values were calculated by the Mann–Whitney–Wilcoxon or Wilcoxon signed-rank test, as appropriate. Abbreviation: n.s., not significant.

related during primary infection. Figure 3*B* shows the relationship between the frequency of pp65-specific $CD8^+$IFN-γ^+ T cells and the frequency of T-bet$^+CD8^+$ T cells during primary infection. We found a strong nonlinear association (1-phase exponential association due to the rapid approach to the asymptote of 100% T-bet$^+$ cells; $r_s = 0.674$; $P = .006$ by Spearman rank correlation test). Together, these data suggest that D^+R^- LTRs with appropriate induction of $CD8^+$ T-bet and pp65-specific effector responses during primary infection establish durable immune control during early chronic CMV infection.

Frequency of pp65-Specific $CD8^+$IFN-γ^+ and T-bet$^+CD8^+$ T Cells During Primary Infection Have High Sensitivity and Specificity for Predicting Viral Control During Early Chronic CMV Infection

Finally, we used receiver operating characteristic (ROC) curves to assess the diagnostic power of both $CD8^+$pp65-specific IFN-γ production and $CD8^+$ T-bet levels during primary infection in predicting viral control versus relapsing viremia during early chronic CMV infection. Figure 4*A* shows the ROC curve analysis for the frequency of pp65-specific $CD8^+$ T-cell IFN-γ production during primary CMV infection and demonstrates high levels of sensitivity and specificity (85.7% and 86.7%, respectively) at a threshold frequency of 0.475% pp65-specific $CD8^+$ T cells producing IFN-γ at primary infection for predicting relapsing viremia during early chronic infection. Likewise, given the association between T-bet and pp65-specific IFN-γ responses, the ROC curve in Figure 4*B* shows a threshold frequency of 46.2% of $CD8^+$ T cells expressing T-bet during primary CMV infection and allows a sensitivity of 71.4% and a specificity of 93.3% for the prediction of adequate viral control during early chronic infection. Taken together, these data demonstrate the potential clinical utility of these $CD8^+$ immune parameters in predicting viral control or relapse during early chronic CMV infection.

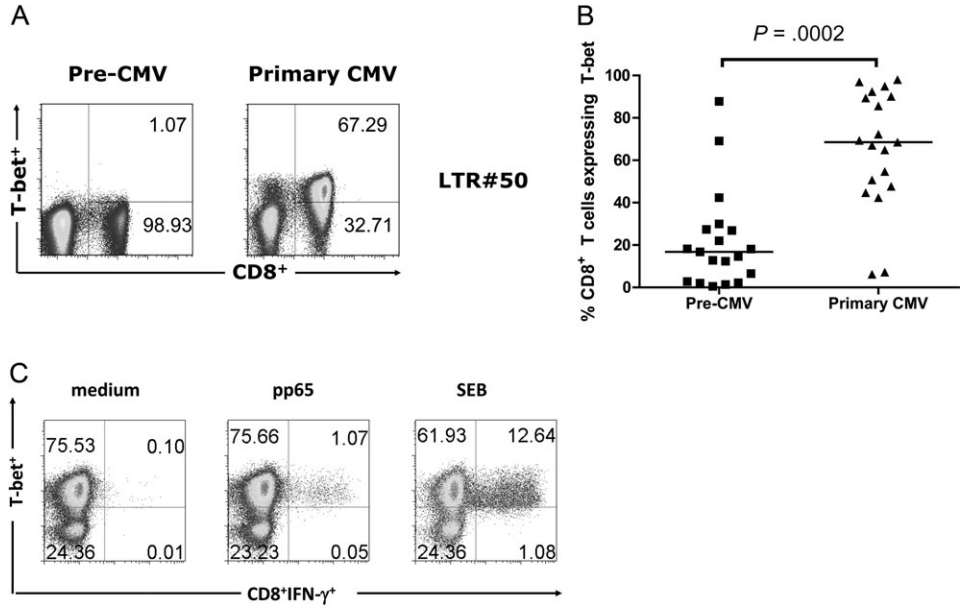

Figure 2. Massive induction of T-box transcription factor (T-bet) expression in $CD8^+$ T cells with type 1 effector function caused by primary cytomegalovirus (CMV) infection. *A,* $CD3^+CD8^+$ T cells from a representative donor-positive/recipient-negative lung transplant recipient (LTR 50) stained for intracellular T-bet expression at a time point after lung transplantation but before primary CMV infection (pre-CMV) and then during primary CMV infection, with values indicating the frequency of $CD3^+CD8^+$ T cells expressing T-bet. *B,* Comparison of intracellular expression of T-bet between pre-CMV and primary CMV infection time points among 19 LTRs. Bars represent median values, and *P* value was calculated by the Wilcoxon signed-rank test. *C,* Peripheral blood mononuclear cells from a representative LTR (31) restimulated with medium, pooled phosphoprotein 65 (pp65) peptides, or staphylococcal enterotoxin B (SEB) during primary CMV infection and $CD3^+CD8^+$ T cells were evaluated for coexpression of intracellular T-bet and interferon γ (IFN-γ).

DISCUSSION

CMV infection remains a major factor on outcomes in lung transplantation, with D^+R^- LTRs having increased mortality despite improved antiviral therapy options [2, 7]. Therefore, understanding whether adequate immune viral control occurs in these high-risk LTRs is critical given the impact of relapsing viremia on survival in LTRs [11]. In this study, we provide evidence for the importance of pp65-specific $CD8^+$ T-cell effector responses during primary infection in predicting control of relapsing viremia during early chronic infection and the important role of T-bet in directing these responses in a prospective cohort of 22 D^+R^- LTRs. Our findings are consistent with findings of prior studies showing an important role for cell-mediated immunity, particularly $CD8^+$ T cells, in CMV host defense in either bone marrow or hematopoietic stem cell transplant recipients or solid organ transplant recipients [13, 21, 22]. Moreover, studies in the murine CMV (MCMV) model have

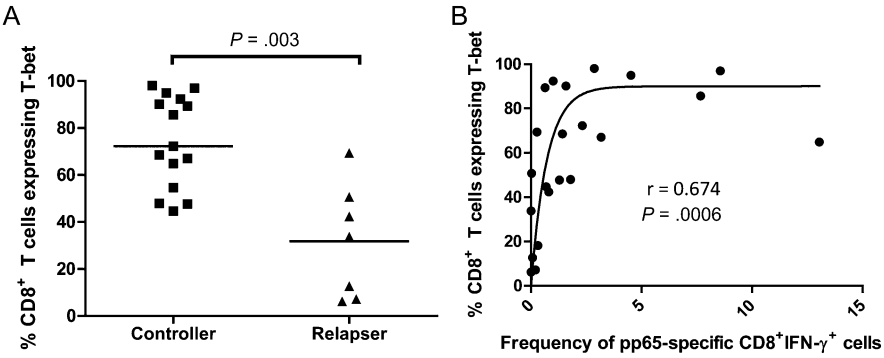

Figure 3. Association of inadequate induction of T-box transcription factor $(T\text{-bet})^+CD8^+$ T cells during primary cytomegalovirus (CMV) infection with relapsing viremia and correlation with phosphoprotein 65 (pp65)–specific $CD8^+$interferon γ $(IFN\text{-}\gamma)^+$ effector responses. *A,* Comparison of expression of T-bet in $CD3^+CD8^+$ T cells during primary infection between 15 lung transplant recipients (LTRs) with durable viral control and 7 LTRs with relapsing viremia. Bars represent median values, and *P* value was calculated with the Mann–Whitney–Wilcoxon test. *B,* Evaluation of the relationship between pp65-specific production of IFN-γ and T-bet expression in $CD3^+CD8^+$ T cells during primary CMV infection by scatterplot analysis; a best-fit nonlinear correlation (1-phase exponential association) was used. Correlation coefficients (r_s) and *P* value were calculated using the Spearman rank correlation test.

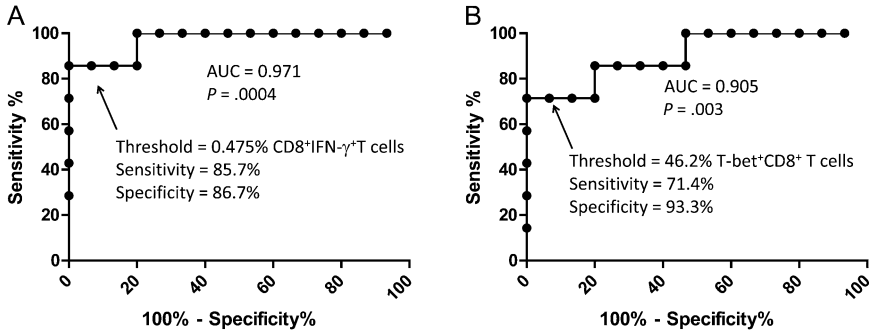

Figure 4. Receiver operator characteristic (ROC) curves of T-box transcription factor (T-bet)$^+$CD8$^+$ and phosphoprotein 65 (pp65)–specific CD8$^+$ interferon γ (IFN-γ)$^+$ effector T cells for prediction of relapsing viremia during early chronic cytomegalovirus (CMV) infection. ROC curve (1 − specificity value vs sensitivity value) analyses, with calculation of the area under the curve (AUC), of the ability of the frequency of pp65-specific CD8$^+$IFN-γ$^+$ T cells (*A*) or T-bet$^+$CD8$^+$ T cells (*B*) during primary CMV infection to predict relapsing viremia during early chronic infection were performed. Indicated threshold values for each characteristic demonstrate optimal balance between sensitivity and specificity.

demonstrated the importance of CD8$^+$ T cells in maintaining immune control [23]. However, other studies have failed to demonstrate an ability to predict immune control of CMV [24, 25]. This may be due to differences in groups of solid organ transplant recipients studied [24] or differences in CMV immune status before transplantation [25]. Furthermore, our results differ from those reported by Bunde et al [26], who found that IE1-specific, but not pp65-specific, CD8$^+$ T-cell responses correlated with symptomatic CMV reactivation. However, there are key distinctions between these studies, because the participants in that study were all recipient seropositive with preexisting immunity, and the majority were heart transplant recipients, whereas we investigated primary CMV-specific CD8$^+$ effector responses in LTRs and their subsequent capacity for viral control during chronic infection. Indeed, the discordance between this study and our results might be due to the evolution of the CMV-specific CD8$^+$ memory response from acute into chronic infection. For example, a study of CMV infection in neonates by Gibson et al [27] showed that IE1-specific responses were increased at 1 year after primary infection. Moreover, significant changes in the immunodominance of CD8$^+$ T-cell memory from acute into chronic infection have been demonstrated in MCMV infection [28–30]. Thus, the hierarchical antigen-specific determinants for CD8$^+$ T-cell-mediated immune control of CMV seem dynamic and may differ according to time from primary infection.

We reported elsewhere on primary CMV-specific T-cell responses in D$^+$R$^-$ LTRs, with pp65-specific CD8$^+$ effector responses found to be immunodominant compared with IE1-specific responses during acute primary infection, followed by a contraction of these responses 3 months into chronic infection [19]. Our current results indicate that the initial magnitude of these CD8$^+$ T-cell responses during acute infection can reliably predict the capacity for immune viral control during early chronic infection within 6 months after primary infection. These results are consistent with findings in murine Sendai virus infection showing that the magnitude of the contracted CD8$^+$ memory T-cell population is dependent on the initial effector burst of CD8$^+$ T cells [31]. In this regard, the quality and fitness of the CD8$^+$ effector memory response seem to be determined by the appropriate development of de novo responses during primary exposure to antigen [32, 33]. However, it remains incompletely understood what factor or factors govern optimal quality and fitness of the primary CD8$^+$ effector burst. Others have investigated the role of CD4$^+$ T-cell responses to CMV and viral control, which may suggest that appropriate CD4$^+$ T-cell help is important for the development of potent antiviral CD8$^+$ T cells to protect against relapsing viremia [34, 35]. We did find increased pp65-specific CD4$^+$ cells in LTRs with viral control, albeit at much lower frequencies than pp65-specific CD8$^+$ cells and near the limit of detection by flow cytometric analysis. Thus, pp65-specific CD8$^+$ T cells may have an increased hierarchical role in systemic immune viral control in this susceptible population. With respect to antigen levels, we did consider whether the duration of initial viremia during primary infection differed between controllers and relapsers, and we did not observe significant differences, which argues against suboptimal antigen exposure or poor strength of signal in the setting of antiviral therapy leading to impaired primary effector responses. In addition, although our studies focused on the production of the IFN-γ by CD8$^+$ T cells in the primary response to pp65 during CMV infection and its role in immune control, it is plausible that other CD8$^+$ effector functions (eg, cytolytic activity) are also important determinants of the capacity for immune viral control of CMV, as has been demonstrated in HIV infection by long-term nonprogressors [36].

Our results also show that induction of the transcription factor, T-bet, plays an important role in the establishment of CMV-specific immune viral control. Studies in the murine lymphocytic

choriomeningitis virus model have demonstrated the importance of T-bet in the generation of protective $CD8^+$ effector cells [16, 37]. Our results provide evidence that primary CMV infection leads to a massive induction of T-bet expression in the $CD8^+$ T-cell pool and are consistent with a recent report by Hertoghs et al [38], who found increased expression of T-bet with primary CMV infection in renal transplant recipients. Our study expands on this, as we show pp65-specific IFN-γ-producing cells that predict immune viral control are derived from this expanded population of T-bet^+CD8^+ T cells. Moreover, we also found variability in T-bet levels during acute CMV infection among individuals in a larger LTR cohort, with lower levels being predictive of relapsing viremia and a strong relationship between the frequencies of T-bet^+CD8^+ T cells and pp65-specific $CD8^+$IFN-γ^+ T cells during primary infection. Taken together, our data suggest that if adequate levels of T-bet expression in effector $CD8^+$ T cells are not induced during primary CMV infection, insufficient frequencies of protective pp65-specific $CD8^+$IFN-γ^+ effector cells are produced, resulting in relapsing viremia during early chronic infection. However, the factors leading to adequate T-bet induction and pp65-specific effector function remain incompletely understood and deserve further study. It is plausible that the environment created by antigen-presenting cells, such as dendritic cells, plays a key role in the induction of adequate T-bet expression and effector function in the primary $CD8^+$ effector pool necessary for immune control.

We should point out several limitations of our studies. We acknowledge the possibility that within our cohort of D^+R^- LTRs, there are confounding factors on the development of immune control during early chronic CMV infection. However, we did not find significant differences in our cohort between viral controllers and relapsers in regard to levels of immunosuppression, duration of primary viremia, or other monitored parameters after transplantation. We also recognize that even with close prospective monitoring for the development of relapsing viremia within our cohort of D^+R^- LTRs, it is possible that our finding of approximately one-third of LTRs developing relapsing viremia after primary infection underestimates the actual incidence of relapsing viremia. Likewise, although our studies focused on responses to pp65 and IE1, because these major CMV antigens have been demonstrated to induce substantial T-cell immunity among healthy individuals and transplant recipients [26, 39, 40], we acknowledge that the total effector response to CMV may be considerably larger given that additional antigens contribute to a broader CMV-specific $CD8^+$ T-cell effector response, as has been reported elsewhere [41, 42]. Thus, our findings of pp65-specific $CD8^+$ immunodominance during primary infection is relative to IE1-specific responses, as we cannot exclude other potentially dominant CMV-specific $CD8^+$ responses in our study participants. In this regard, future studies with additional CMV antigens may reveal an even stronger correlation between T-bet levels in the $CD8^+$ pool and CMV-specific IFN-γ responses. However, despite these potential limitations in our study, we have provided important evidence that systemic immune control during early chronic CMV infection without continued antiviral prophylaxis is possible in high-risk D^+R^- LTRs after primary infection.

Overall, we report the induction of T-bet in $CD8^+$ T cells during human primary CMV infection and its association with pp65-specific $CD8^+$ effectors. Our data show that a subset of D^+R^- LTRs do not develop sufficient frequencies of T-bet^+CD8^+ or pp65-specific $CD8^+$ effectors during primary infection and develop relapsing viremia during early chronic infection. Conversely, our data indicate that adequate induction of T-bet and pp65-specific $CD8^+$ effectors during primary CMV infection can reliably predict which D^+R^- LTRs will experience durable immune systemic viral control. With this better understanding of the factors during primary infection that predict subsequent viral control, it may become possible to risk-stratify D^+R^- LTRs for viral relapse and perform individualized antiviral immune monitoring and prophylaxis.

Notes

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Potential conflicts of interest. All authors: No reported conflicts.

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