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Antiviral prevention of sepsis induced cytomegalovirus reactivation in immunocompetent mice

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ABSTRACT

Introduction: Immunocompetent patients can reactivate latent cytomegalovirus (CMV) during critical illness and reactivation is associated with significantly worse outcomes. Prior to clinical trials in humans to prove causality, we sought to determine an optimal antiviral treatment strategy.
Methods: Mice latently infected with murine CMV (MCMV) received a septic reactivation trigger and were randomized to receive one of four ganciclovir regimens or saline. Lungs were evaluated for viral transcriptional reactivation and fibrosis after each regimen. Influences of ganciclovir on early sepsis-induced pulmonary inflammation and T-cell activation were studied after sepsis induction.
Results: All ganciclovir regimens reduced measurable MCMV transcriptional reactivation, and 10 mg/day for 7 or 21 days was most effective. Lower dose (5 mg/kg/day) or delayed therapy was associated with significant breakthrough reactivation. Higher doses of ganciclovir given early were associated with the lowest incidence of pulmonary fibrosis, and delay of therapy for 1 week was associated with significantly worse pulmonary fibrosis. Although bacterial sepsis induced activation of MCMV-specific pulmonary T-cells, this activation was not influenced by ganciclovir.
Conclusion: These results suggest that antiviral treatment trials in humans should use 10 mg/kg/day ganciclovir administered as early as possible in at-risk patients to minimize reactivation events and associated pulmonary injury.

1. Introduction

Cytomegaloviruses (CMVs) for all species are endemic and display classic characteristics of the *Betaherpesvirinae*. Following immune control of the primary lytic infection, CMV establishes life-long infection in its host. CMV becomes dormant in multiple end organs, a state also referred to as latency, and can later be reactivated by a variety of stimuli, including immunosuppression and inflammation (reviewed in Hummel and Abecassis, 2002). We first became interested in cytomegalovirus (CMV) reactivation in critically ill patients in the late-nineties (Cook et al., 1998), and since then it has become increasingly clear that up to 30–35% of latently infected immunocompetent individuals experience CMV reactivation during critical illness. This finding has now been reproduced independently by 7 different groups (Chiche et al., 2009; Heininger et al., 2001; Jaber et al., 2005; Kutza et al., 1998; Limaye et al., 2008; von Muller et al., 2006; Ziemann et al., 2008). Roughly 60% of people older than age 6 in this country have been infected with human CMV (HCMV) (Staras et al., 2006), and this percentage increases with age (Musiani et al., 1988). Thus most patients harbor latent virus when they develop critical illness, making them "at-risk" for reactivation.

Although the occurrence of viral reactivation during critical illness is now indisputable, the real question remains: is HCMV a pathogen in immunocompetent patients during critical illness, or simply an innocent bystander identifying patients with severe disease? HCMV is a well described pathogen in those without fully functional immune systems, such as neonates, patients with HIV, and transplant recipients receiving concurrent immunosuppression (Gaytant et al., 2002; Gor et al., 1998; Simmons et al., 1977; Steininger, 2007). Interestingly, the preponderance of recent clinical data supports the hypothesis that HCMV is also a pathogen in immunocompetent patients that develop critical illness. Studies to date have demonstrated surprisingly consistent morbidity in these patients, including increased durations of mechanical ventilation, prolonged hospitalizations, and worsened survival (Chiche et al., 2009; Cook et al., 1998, 2003; Heininger et al., 2001; Jaber et

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al., 2005; Kutza et al., 1998; Limaye et al., 2008; von Muller et al., 2006; Ziemann et al., 2008). It is intriguing that HCMV reactivation is associated with increased durations of mechanical ventilation, particularly because lungs are a primary site of latent virus (Toorkey and Carrigan, 1989), and a consistent site of reactivation (Cook et al., 1998, 2003; Heininger et al., 2001; Jaber et al., 2005; Kutza et al., 1998; Limaye et al., 2008; von Muller et al., 2006; Ziemann et al., 2008). Even more importantly have been recent associations of CMV reactivation with worsened mortality (Limaye et al., 2008; Ziemann et al., 2008). Perhaps the strongest support for this association comes from a recent meta-analysis that suggests a doubled mortality risk in patients with reactivation during critical illness (Kalil and Florescu, 2009). Thus available clinical data are consistent with the hypothesis that pulmonary HCMV reactivation during critical illness is pathogenic.

Because of ethical limitations associated with CMV research in very sick humans, we have developed a murine model to study reactivation and pathogenesis. This model was modified from the first described murine CMV (MCMV) model (Gonczol et al., 1985), and utilizes a septic challenge as a trigger for viral reactivation (Cook et al., 2002). Fortunately, HCMV and MCMV share many similarities (Collins et al., 1993; Henson et al., 1966; Rawlinson et al., 1996): both establish clinical latency following acute primary infection (Ho, 1982), reactivation has been associated with sepsis in both (Cook et al., 2002, 2003; Cook et al., 2006a; Heininger et al., 2001; Kutza et al., 1998; Limaye et al., 2008), and importantly MCMV has the same proclivity as HCMV for several organs, including lungs (Balthesen et al., 1993; Koffron et al., 1998; Kurz et al., 1997). These characteristics make MCMV an ideal model to study the pulmonary effects of reactivation, and using this model we have recently demonstrated that MCMV reactivation by sepsis in immunocompetent mice causes lung injury (Cook et al., 2006b). These studies also suggest that antiviral treatment with ganciclovir can prevent both sepsis-induced CMV reactivation and CMV-associated lung injury in immunocompetent mice.

Ultimately, proof of pathologic causality will require antiviral treatment trials in critically ill patients at-risk for reactivation (Cook, 2007). These patients often tolerate drug side effects poorly, and therefore prior to embarking on such trials, we felt it critical to investigate the ideal dosing of antiviral medication. Although ganciclovir treatment was very effective in our previous studies, we chose several alternative strategies to reduce drug exposure. Using our murine model, we confirm that antiviral treatment with ganciclovir prevents sepsis-induced CMV reactivation and its attendant pulmonary injury. In addition, we report the influence of several antiviral dosing strategies on this reactivation induced injury mechanism. Finally, we present preliminary evidence that suggests that lung resident T-cells may contribute to CMV induced pulmonary injury during reactivation.

2. Methods

2.1. Animals, viral infection, and confirmation of latency

Female BALB/c mice (Harlan, Indianapolis, IN) 6–8 weeks of age were used in this study. Purified Smith strain (VR-194/1981) MCMV was obtained from ATCC (Rockville, MD). Primary CMV infection was achieved by intra-peritoneal (i.p.) injection of 2×10^5 PFU Smith-MCMV and latency was confirmed as previously described (Cook et al., 2006a,b). Mice were euthanized by cervical dislocation under inhalation anesthesia. Mouse tissues were dissected aseptically and snap frozen in liquid nitrogen, then stored at −80 °C. Primary infection and latency/reactivation were confirmed as previously published (Cook et al., 2002, 2006a). As previously published, we define latency as viral DNA present in host tissues, without transcription of viral genes (Cook et al., 2002, 2006a,b). All mice were housed adhering to the *Guide for the Care and Use of Laboratory Animals* prepared by the National Research Council (NIH Publication No. 86-23, revised 1985) following protocol approval by our Institutional Review Board.

2.2. Sepsis and CMV reactivation

We have previously shown that an LD_{50} model of polymicrobial sepsis induced by cecal ligation and puncture (CLP) will stimulate pulmonary transcriptional reactivation of latent MCMV in 100% of surviving mice (Cook et al., 2002). We defined transcriptional reactivation from latency as mRNA transcription of MCMV glycoprotein-B (GB) known to be expressed at early/late temporal phases (reviewed in Reddehase et al., 2002). In our model, transcriptional activity of MCMV-GB becomes detectable between 7 and 14 days following CLP, with peak transcription occurring 21 days after CLP (Cook et al., 2002).

Mice underwent CLP as previously described (Cook et al., 2002, 2006b) and were randomly divided into cohorts receiving saline (no treatment), ganciclovir 10 mg/kg/day × 3 weeks, ganciclovir 10 mg/kg × 1 week, ganciclovir 5 mg/kg/day × 3 weeks, or ganciclovir 10 mg/kg/day × 2 weeks, beginning 1 week after CLP. Three weeks after CLP, surviving mice were euthanized and lungs evaluated for viral reactivation and inflammatory mediator expression using PCR and RT-PCR. Tissue samples fixed in formalin and paraffin embedded underwent histologic analyses.

2.3. Antiviral therapy

Ganciclovir dosing of 10 mg/kg/day (subcutaneous in 0.2 cm^3 saline vehicle) was chosen because this has been previously shown to be efficacious in mice (Cook et al., 2006b; Duan et al., 1998; Lenzo, 2001) and is a standard dose in adults for CMV disease. Steady state plasma level comparisons were made between mice receiving subcutaneous and intravenous administration of ganciclovir and these were not significantly different after 5 days of treatment (data not shown). For reactivation experiments, we define 4 ganciclovir treatment groups: (a) 10 mg/kg/day for 21 days, (b) 5 mg/kg/day for 21 days, (c) 10 mg/kg/day for 7 days, or (d) delayed therapy, 10 mg/kg/day started 7 days after CLP (total of 2 weeks before evaluation). Groups a–c are considered prophylactic treatment, because therapy is being initiated on post-sepsis day 1, well before transcriptional activity of early/late genes can be detected. Group d could be considered pre-emptive therapy because it is started 1 week after sepsis onset, and mimics delayed treatment until viral activity is detected in humans. For T-cell experiments, mice received ganciclovir pretreatment (10 mg/kg/day) for 1 week prior to sepsis induction. This duration was chosen to allow development of steady state tissue concentrations (>5 doses) in an attempt to ensure treatment effect.

2.4. PCR and RT-PCR

PCR and RT-PCR were performed as previously described (Cook et al., 2006a). If the first reaction yielded no visible product, a second (nested) PCR or RT-PCR reaction was performed using 1 μl of this first PCR product. Primers for MCMV-GB and GAPDH were as previously published (Cook et al., 2009b). Each RT-PCR experiment was performed in triplicate, and if any one of the three replicates was "positive", the mouse was considered to have transcriptional reactivation. Concomitant "no-RT" reactions were performed for each sample for each run to confirm lack of DNA contamination. For inflammatory mediator mRNA quantitative PCR, RNA were extracted from tissues as previously described (Cook et al., 2009a). Relative mediator mRNA was calculated using the $2^{-\Delta\Delta CT}$ method

(Livak and Schmittgen, 2001). Primers for tumor necrosis factor alpha (TNF-α) were obtained from SABiosciences (Frederick, MD).

2.5. Image analysis for fibrosis

Lung tissues from each treatment group were obtained 3 weeks after CLP. Lung tissues were fixed, sectioned, and stained with Gomori's trichrome to identify the presence of mature collagen and fibrosis. After image acquisition and digitization into our image analysis system, images were color segmented and analyzed for fibrosis as previously described (Cook et al., 2006b). All image acquisition and analyses were performed by a technician blinded to study groups.

2.6. Antibodies and flow cytometry

Fluorescent dye-conjugated antibodies specific for CD8 (PerCP) and CD43 (PE-Cy7) were used (BD PharMingen, San Diego, CA). MCMV-specific T-cells were identified using MHC-I tetramers specific for MCMV proteins pp89 ($H2L^d$-restricted ^{168}YPHFMPTNL^{176} (Del Val et al., 1988)) and m164 ($H2D^d$-restricted ^{257}AGPPRYSRI^{265} (Holtappels et al., 2002b)) as previously described (Sierro et al., 2005). Briefly, lungs were digested in RPMI with fetal calf serum containing collagenase, filtered, washed, and lymphocytes were isolated by Percoll gradient centrifugation. MHC class I peptide tetrameric complexes were produced and assembled as previously described (Altman et al., 1996). Lymphocytes were incubated with tetramers (37 °C) for 1 h followed by antibody surface staining (4 °C) for 1 h, fixed, and analyzed by flow cytometry (FACScalibur, Becton Dickinson, Mountain View, CA) and results analyzed using FlowJo software (Tree Star Inc., Ashland, OR).

2.7. Statistical analyses

Statistical analyses using two-tailed Student's *t*-test, Chi-square or Fisher's Exact tests were performed where appropriate. *p*-values < 0.05 were considered significant for all testing. Means are expressed as mean ± standard error. Statistical software used was Graphpad Prism (GraphPad Software Inc., La Jolla, CA).

3. Results

3.1. Influence of ganciclovir on MCMV transcription after sepsis

We have previously shown that bacterial sepsis induced by CLP causes systemic transcriptional reactivation of latent MCMV (Cook et al., 2002). More recently, we have shown that 3 weeks of ganciclovir (10 mg/kg/day) will prevent detectable transcriptional reactivation of CMV from latency (Cook et al., 2006b). In an attempt to minimize antiviral exposure, we were interested to test alternate ganciclovir regimens to prevent MCMV reactivation. We therefore performed CLP on MCMV-latent mice, randomly assigning mice to groups receiving ganciclovir: (a) 10 mg/kg/day for 21 days, (b) 5 mg/kg/day for 21 days, (c) 10 mg/kg/day for 7 days, or (d) delayed therapy, 10 mg/kg/day started 7 days after CLP. Because our previous experience has shown that maximal MCMV transcriptional activity occurs 3 weeks after a sepsis trigger (Cook et al., 2002), we evaluated mice for transcriptional activity at this time point.

Fig. 1 shows examples of RT-PCR results from representative cohorts from each treatment group, and Fig. 2A summarizes results from all experiments. Consistent with our previously published results, all saline treated mice show viral transcription after CLP. Mice receiving ganciclovir show significant reductions in viral transcription when compared to saline treatment (Chi-

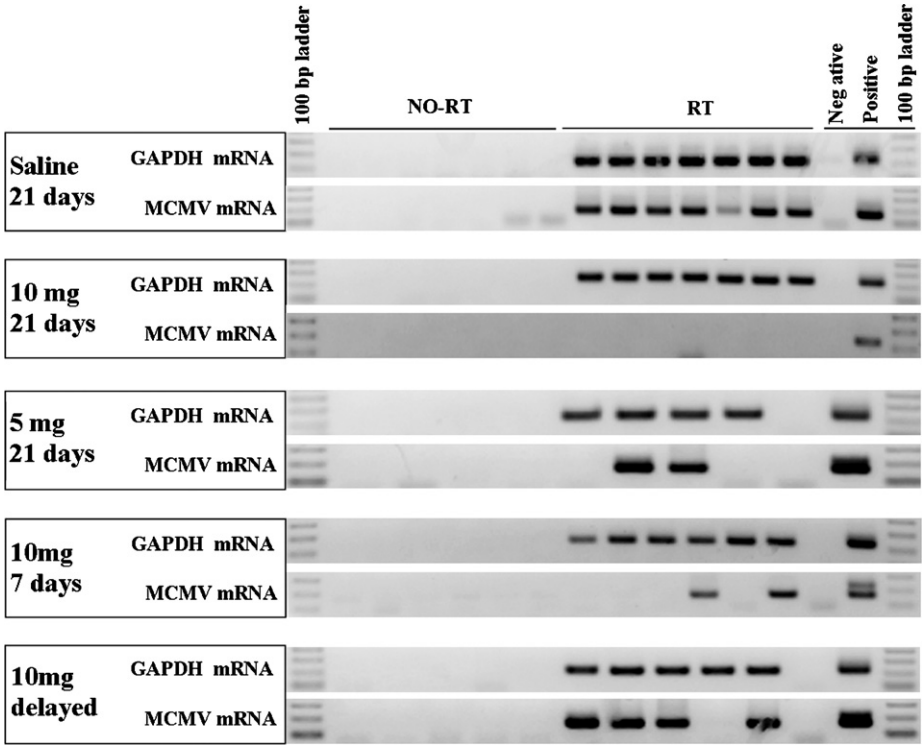

Fig. 1. Cytomegalovirus transcriptional reactivation during bacterial sepsis. Mice latently infected with murine cytomegalovirus (MCMV) had bacterial sepsis induced by cecal ligation and puncture (CLP). After CLP, mice received ganciclovir or saline treatments as indicated. Three weeks after CLP and subsequent antiviral treatment, mouse lung homogenates were evaluated for viral transcriptional reactivation. Representative gels from nested reverse transcription polymerase chain reactions (RT-PCR) from each group are shown. Each lane represents lung tissue results from individual mice. RT results for GAPDH confirm mRNA extraction, and murine cytomegalovirus (MCMV) mRNA represents MCMV gene glycoprotein-B transcription. Presence of DNA contamination was evaluated by NO-RT controls for each specimen. Negative and positive refer to technique controls. All RT-PCR evaluations were performed in triplicate.

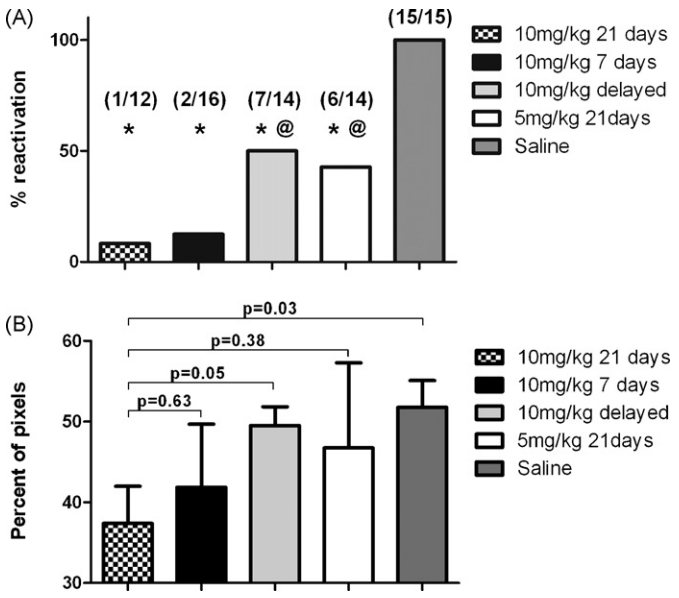

Fig. 2. Ganciclovir treatment influences reactivation and pulmonary injury. Mice latently infected with murine cytomegalovirus (MCMV) had bacterial sepsis induced and received one of the listed ganciclovir regimens or saline (control). (A) Three weeks later lungs from surviving mice were evaluated for MCMV transcriptional reactivation. Each bar represents the percentage reactivation (# reactivation/n) for each treatment group. *Significant difference from saline controls ($p < 0.05$), and @significant difference from 10 mg/kg 21 days group ($p < 0.05$). All evaluations were performed in triplicate. (B) Surviving mice were also evaluated for pulmonary fibrosis. Lungs were fixed, embedded, sectioned, and stained with Gomori's trichrome. Images were acquired, color segmented, and fibrosis quantitated as percent of pixels. Each bar represents the mean ± standard error for 5–7 mice.

square $p < 0.002$). Mice receiving 10 mg/kg/day for 21 days after CLP show the lowest incidence of detectable viral transcription (1/12, 8.3%). Limiting ganciclovir exposure by shortening treatment duration (10 mg/kg/day for 7 days) shows comparable transcription rates to those receiving 10 mg/kg/day for 21 days (12.5% versus 8.3%, Fisher's Exact $p = 1$). In contrast, reducing ganciclovir exposure by lowering the dose (5 mg/kg/day for 21 days) allows breakthrough transcription in ~40% of mice, which is significantly worse than 10 mg/kg/day for 21 days (Fisher's Exact $p = 0.04$), but still better than saline treatment. Finally, delaying therapy for 1 week after sepsis is associated with viral transcription rates significantly higher than either 7 or 21 days of early initiated therapy (Fisher's Exact $p < 0.05$). There was no MCMV mRNA detectable in MCMV-naïve healthy mice undergoing CLP (data not shown). Overall survival following CLP was not significantly different between treatment groups (not shown), which is consistent with our previous observations (Cook et al., 2006b).

Taken together these results suggest that early initiation of antiviral therapy improves control of sepsis-induced viral transcriptional reactivation, and reduction of antiviral exposure by delaying treatment or decreasing ganciclovir dosing to 5 mg/kg/day allows significant breakthrough reactivation.

3.2. Pulmonary fibrosis following CMV prevention strategies

Transcriptional reactivation is associated with pulmonary fibrosis that can be prevented by ganciclovir treatment in MCMV-infected immunocompetent mice (Cook et al., 2006b). We therefore tested the hypothesis that our chosen ganciclovir dosing strategies have variable effects on pulmonary fibrosis after a sepsis trigger. To test this hypothesis, lung tissues from each group stained with Gomori's trichrome underwent color segmentation and quantitative analysis of fibrosis as previously described (Cook et al., 2006b). As shown in Fig. 2B, ganciclovir treatment (10 mg × 21 days) is associated with significantly less fibrosis than saline treatment, confirming our previous results (Cook et al., 2006b). Shortening therapy to only 7 days does not significantly increase fibrosis, but importantly, delay of therapy for 7 days is associated with significantly increased fibrosis. Finally, lower dose ganciclovir therapy (5 mg/day) shows fibrosis results not significantly worse than 10 mg/kg doses. These results suggest that early antiviral prophylaxis is critical to reduce pulmonary injury associated with CMV reactivation.

3.3. Influence of ganciclovir upon T-cell responses to MCMV

We were intrigued by the worse fibrosis in mice with delayed antiviral treatment, which suggested to us a possible link between early inflammatory events during sepsis in latently infected mice and development of eventual pulmonary fibrosis. We have previously shown that latently infected mice display exaggerated early TNF-α responses to bacterial sepsis (Cook et al., 2006b). This exaggerated response is consistent with a preconditioned innate immune response previously described by others (Barton et al., 2007) that is not seen in MCMV-naïve mice. In addition to these changes in innate immunity, it is well known that CMV infection induces increased resident pulmonary CD8+ T-cells in both humans and mice (Holtappels et al., 2000; Karrer et al., 2003; Pipeling et al., 2008). Lung-resident MCMV-specific T-cells maintain an effector memory (T_{EM}) phenotype (Karrer et al., 2003; Podlech et al., 2000; Sierro et al., 2005), and we have corroborated these findings in our model (not shown). MCMV-specific T_{EM}-cells can be activated in vitro with MHC-I presented MCMV peptides, and after long durations of latency develop an increasing propensity to secrete TNF-α (Babel et al., 2008; Munks et al., 2006; Simon et al., 2006). We therefore hypothesized that lung resident T_{EM}-cells may contribute to the enhanced TNF-α response observed in lungs early after sepsis in latently infected mice.

To test this we evaluated pulmonary lymphocytes from MCMV-naïve and latent mice by flow cytometry and confirmed that in our model MCMV-latent mice have significantly increased pulmonary resident T-cells (Fig. 3A and B). To determine if sepsis influences activation of lung resident T-cell activation in latently infected mice during early sepsis, lung lymphocytes were isolated 1 day after sepsis induction and evaluated by flow cytometry for CD8 T-cell activation. As shown in Fig. 3C, sepsis induces a fourfold increase in CD43 expression in pulmonary CD8+ T-cells in mice with MCMV.

To confirm that this early T-cell activation occurs in MCMV-specific T-cells, and is not a generic response to sepsis, we studied a second cohort of latently infected mice. Additionally, half of these mice received ganciclovir pretreatment before the septic challenge to determine if this T-cell activation might be prevented. As shown in Fig. 4A MCMV-specific T-cells identified by either pp89 or m164 tetramer binding had very high levels of activation marker CD43 expression when compared to tetramer negative T-cells. Ganciclovir pretreatment did not seem to influence this activation (Fig. 4A), and consistent with this ganciclovir did not influence early TNF-α mRNA after sepsis induction (Fig. 4B). Altogether, these results suggest that the previously observed early exaggerated pulmonary inflammatory response to sepsis in MCMV-latent mice may be in part a consequence of MCMV-specific T-cell activation.

4. Discussion

The main questions addressed in the current report are (1) determining the optimal daily dose and duration of ganciclovir therapy, and (2) determining when to initiate therapy to best prevent reactivation of CMV in non-immunosuppressed hosts. Our data clearly demonstrate that higher doses of ganciclovir (10 mg/kg/day)

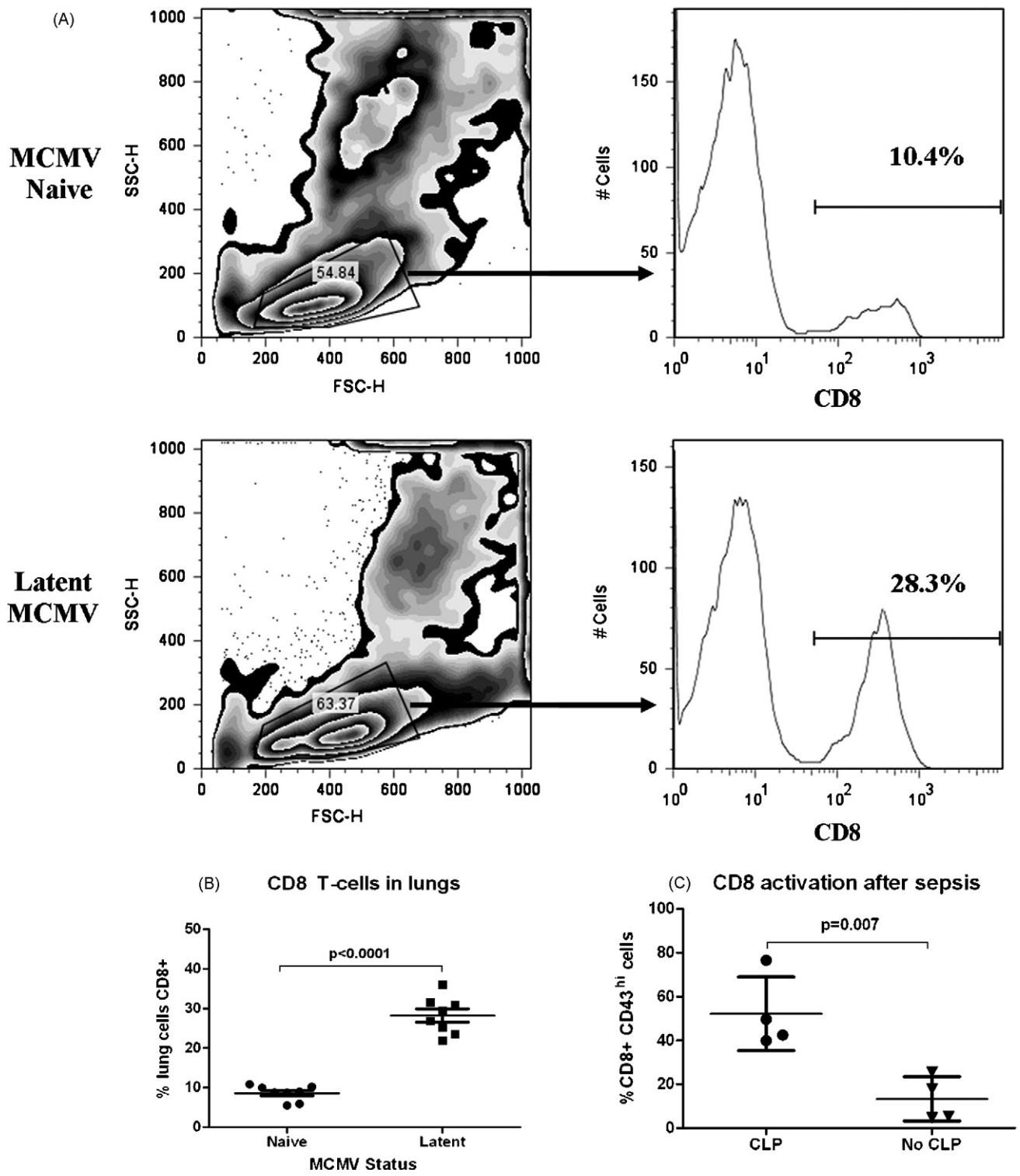

Fig. 3. Bacterial sepsis induces pulmonary T-cell activation. Flow cytometry for CD8+ T-cells was performed on pulmonary lymphocytes isolated from mice. (A) Representative lymphocyte gating and CD8 histograms for murine cytomegalovirus (MCMV) naïve or latently infected mice (non-septic). (B) Graphical summary of CD8 percentages in lungs of non-septic mice. (C) Pulmonary lymphocytes from MCMV-latent mice isolated 1 day after bacterial sepsis (CLP) or no sepsis (no CLP) were evaluated for CD8+ activation by colocalizing with activation marker CD43 using flow cytometry. For graphs each data point represents result from one mouse, and bars represent mean ± standard error.

are more efficacious at preventing MCMV transcriptional reactivation than the lower dose of 5 mg/kg/day. Perhaps even more importantly, only higher doses of ganciclovir appear to prevent MCMV associated pulmonary injury. Durations of either 1 or 3 weeks (10 mg/kg/day) appear to control transcriptional reactivation equally, but the best results for reducing pulmonary injury were achieved with 3 weeks of treatment. That said, there was still some reduction in viral transcription for the lower dose group (5 mg/kg/day), so if lower dosing is required for renal or marrow insufficiency we would still expect some benefit. Together these data suggest that to optimally prevent sepsis-induced CMV reactivation and resultant pulmonary injury in non-immunosuppressed

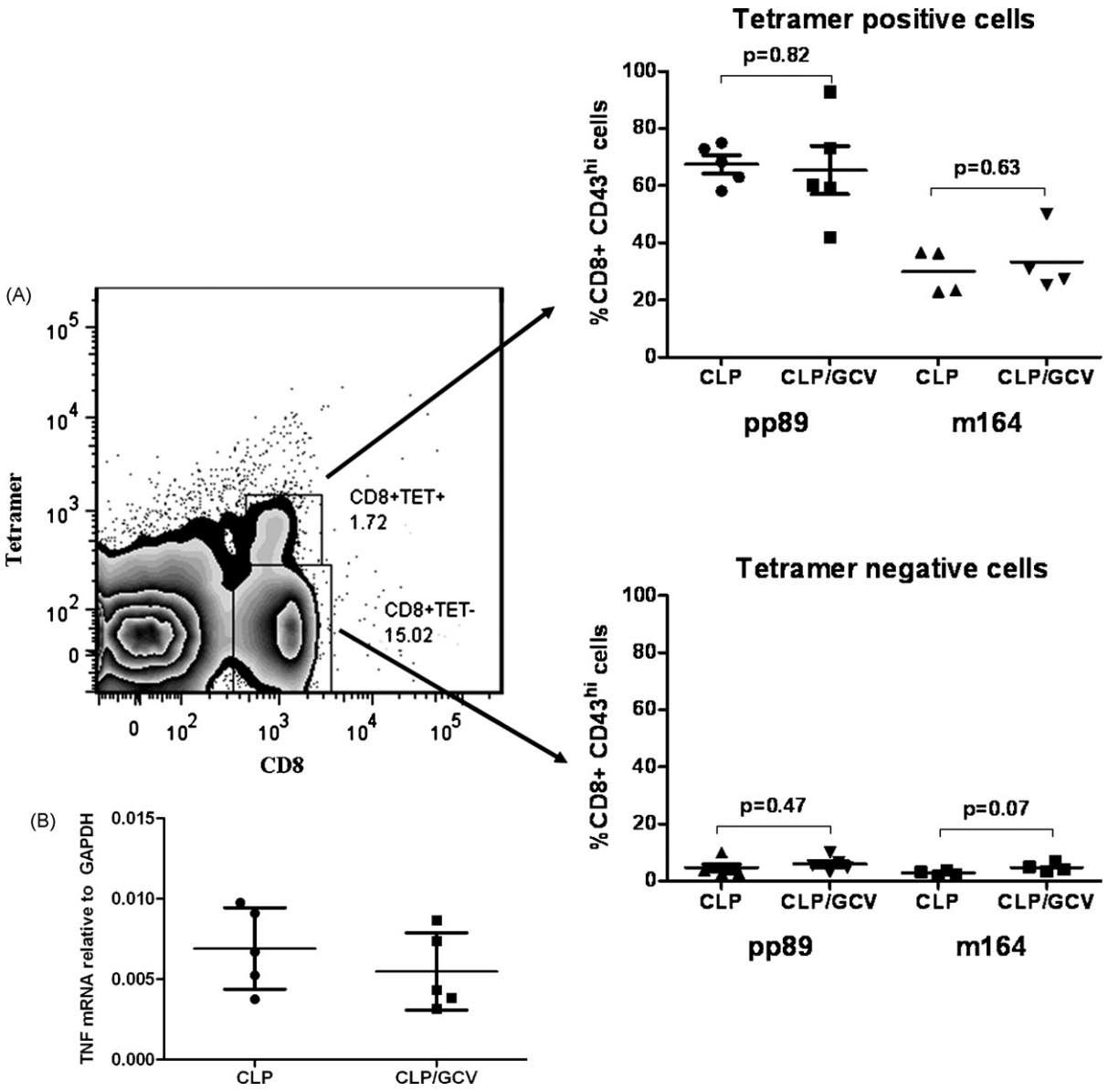

Fig. 4. Ganciclovir does not prevent sepsis induced T-cell activation in lungs. (A) Flow cytometry was performed on pulmonary lymphocytes isolated from mice previously infected with murine cytomegalovirus (MCMV) 1 day after induction of sepsis (CLP) or sepsis after ganciclovir pretreatment (CLP/GCV). Cells were incubated with fluorophore labeled anti-CD8 and CD43 antibodies as well as MCMV specific peptide loaded MHC-I tetramers specific for MCMV proteins pp89 and m164. Representative flow scatter plot shows gating for CD8 + tetramer-positive (CD8 + TET+) and CD8 + tetramer negative (CD8 + TET−) cells that were subsequently evaluated for activation ($CD43^{hi}$). Activation results are summarized graphically for tetramer-positive cells (above) and tetramer negative cells (below). Ganciclovir had no significant impact upon MCMV-specific T-cell activation. Significantly more MCMV-specific T-cells were activated ($CD43^{hi}$) than non-MCMV-specific T-cells after sepsis (analysis not shown). For graphs each data point represents result from one mouse, and bars represent mean ± standard error. Student's *t*-test was used for comparisons and *p*-values are shown.

ICU patients, ganciclovir 10 mg/kg/day should be given for 1–3 weeks.

Secondly, the current study addresses optimal timing of ganciclovir treatment. Although there are significant literature comparing prophylactic versus pre-emptive therapy in transplant patients, no such data exist in non-immunosuppressed critically ill patients. Our data clearly show that delay of therapy in septic mice for 1 week significantly reduces control of viral activity, and more importantly that delay in therapy allows significantly worse pulmonary injury. This is consistent with scant clinical data currently available in non-immunosuppressed critically ill patients that suggest no therapeutic benefit for antiviral treatment initiated after identification of reactivation (Chiche et al., 2009; Cook et al., 1998; Heininger et al., 2001; Jaber et al., 2005). It is important to emphasize that in both mice and humans, there is a time lag between the insult that triggers reactivation and detection of viral reactivation. In humans, Limaye et al. (2008) have shown that median time to detect DNAemia was 12 days, and in our murine model viral transcription is similarly not detectable for 7–14 days (Cook et al., 2002). It therefore seems that in non-immunosuppressed hosts, to achieve maximal reduction of both pulmonary viral activity and injury, that an antiviral prophylaxis strategy and not a pre-emptive approach should be employed. We therefore suggest that if CMV prevention trials are to be done in non-immunosuppressed ICU patients, prophylactic treatment should be initiated in "at-risk" patients as soon as this risk is identified, because waiting until CMV testing becomes "positive" may obviate benefits of antiviral therapy.

CMV infection, both in mice and humans, is associated with localization of sizable populations of CMV-specific T_{EM}-cells in the lungs of latently infected hosts (Holtappels et al., 2000; Pipeling et

al., 2008). The propensity of these CMV-specific T_{EM}-cells to secrete TNF-α when stimulated (Babel et al., 2008; Munks et al., 2006; Simon et al., 2006) suggested the possibility that exaggerated early TNF-α responses previously observed in MCMV-latent mice might be consequent to viral stimulation of these lung resident memory T-cells. Importantly, previous work has shown that non-selective activation of these lung resident T-cells causes lethal pneumonitis associated with exaggerated inflammatory responses (Tanaka et al., 1994, 1997). In the current report bacterial sepsis was associated with fourfold activation of lung-resident CD8 T-cells during early sepsis in latently infected mice, and interestingly this activation was unique to MCMV-specific T-cells. An important question raised by these findings is whether activation of MCMV-specific T-cells in this model is driven by presentation of viral antigens. This is entirely possible, because neither pp89 nor m164 expression require viral replication (Holtappels et al., 2002a; Keil et al., 1985) and therefore should not be influenced by ganciclovir pretreatment. Despite this we were unable to detect pp89 or m164 mRNA using RT-PCR (data not shown), but it is possible that quantities were insufficient for detection at this early time point. We therefore conclude that lung-resident MCMV-specific T-cells are activated somehow by bacterial sepsis, possibly by expression of viral antigens at levels undetectable by the methods employed here. Regardless of the activation trigger, our results suggest that lung resident memory T-cells might contribute to sepsis-induced pulmonary injury in latently infected immunocompetent hosts, a hypothesis that merits future attention.

We chose to study ganciclovir in this study for several reasons. First, ganciclovir or its derivatives are the least toxic of the FDA approved anti-CMV drugs (Mercorelli et al., 2008). This is particularly important because critically ill patients often develop renal insufficiency and might be particularly susceptible to nephrotoxic drugs. Second, of its available derivatives, ganciclovir is available in intravenous formulation. Many patients develop ileus for the first several days of their critical illness and might have poor absorption and bio-availability of oral formulations precluding clinical efficacy. Finally, although there is some controversy regarding the role of the MCMV kinase M97 in the phosphorylation of ganciclovir (Okleberry et al., 1997; Wagner et al., 2000), ganciclovir inhibits both HCMV and MCMV replication in vivo (Lenzo, 2001; Mercorelli et al., 2008; Scott et al., 2005). Because prophylaxis trials will treat many patients that will not ultimately reactivate virus, such trials will require the least toxic clinically efficacious intravenous drug available, which for now is ganciclovir.

Detection of viral reactivation is problematic both in humans and mice because growing virus in culture is far less efficient than detecting by-products of full reactivation (DNA, RNA, CMV antigenemia). Indeed, in mice we can barely detect live virus after a reactivation trigger without any antiviral treatment (Cook et al., 2006a), making recovery of live virus a poor indicator of success or failure of an antiviral therapy. Similarly, in immunocompetent humans, transcriptional indicators such as PCR or antigenemia appear to be far more sensitive clinical methods for detecting reactivation episodes (Kalil and Florescu, 2009). We therefore chose to use transcriptional reactivation—defined as mRNA transcription of glycoprotein-B as our indicator of viral activity. Although one could argue that MCMV-GB transcription can occur without formation of infectious virus particles because of a checkpoint in replication (Reddehase et al., 2002), the converse is not true. Thus if antiviral treatment prevents detectable GB transcription, it undoubtedly prevents downstream viral replication and propagation. Moreover, during clinical trials one of the major endpoints will be prevention of reactivation/replication, which will no doubt be monitored by the most sensitive methods—either DNAemia or antigenemia, making our model relevant.

In conclusion, our data suggest that for CMV prevention trials in non-immunosuppressed critically ill patients, prophylactic antiviral treatment should be initiated as early as possible in patients "at-risk" for reactivation. Waiting until demonstrable reactivation, so-called "pre-emptive therapy", may significantly reduce the beneficial effects of antiviral therapy in these patients. We recommend that treatment with ganciclovir should ideally begin with 10 mg/kg/day dosing, and during initial trials that patients receive treatment for at least 7–21 days. Lower dosing may still provide some benefit in patients who might not tolerate therapeutic doses, but it is unclear if lower doses will obviate CMV induced pulmonary injury. Although ganciclovir therapy does not appear to influence the early exaggerated inflammatory response to sepsis seen in latently infected hosts, the possible pathogenic role of CMV-specific T-cell activation during sepsis merits further study.

Conflict of interest

CHC has served as a one time advisor to Roche pharmaceuticals. The other authors have no other conflicts to declare.

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