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Sepsis Erodes CD8+ Memory T Cell Protective Immunity Against an EBV Homolog in a 2B4-Dependent Manner

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Abstract

Epstein–Barr virus (EBV) reactivation commonly occurs following sepsis but the mechanisms underlying this are unknown. We utilized a murine EBV homolog (gHV) and the cecal ligation and puncture model of polymicrobial sepsis to study the impact of sepsis on gHV reactivation and $CD8^+$ T cell immune surveillance following a septic insult. We observed a significant increase in the frequency of gHV-infected germinal center B cells on day 7 following sepsis. This increase in viral load was associated with a concomitant significant decrease in the frequencies of gHV-specific $CD8^+$ T cells, as measured by class I MHC tetramers corresponding to the immunodominant viral epitopes. Phenotypic analysis revealed an increased frequency of gHV-specific $CD8^+$ T cells expressing the 2B4 coinhibitory receptor in septic animals compared to sham controls. We sought to interrogate the role of 2B4 in modulating the gHV-specific $CD8^+$ T cell response during sepsis. Results indicated that in the absence of 2B4, gHV-specific $CD8^+$ T cell populations were maintained during sepsis, and gHV viral load was unchanged in $2B4^{-/-}$ septic animals relative to $2B4^{-/-}$ sham controls. WT $CD8^+$ T cells upregulated PD-1 during sepsis, while $2B4^{-/-}$ $CD8^+$ T cells did not. Finally, adoptive transfer studies revealed a T cell intrinsic effect of 2B4 coinhibition on virus-specific $CD8^+$ T cells and gHV viral load during sepsis. These data demonstrate that sepsis-induced immune dysregulation erodes antigen-specific $CD8^+$ responses against a latent viral infection, and suggest that blockade of 2B4 may better maintain protective immunity against EBV in the context of sepsis.

Keywords

sepsis; virus-specific T cell responses; $CD8^+$ T cell; immune memory

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Introduction

Sepsis, which is defined as life-threatening organ dysfunction caused by a dysregulated host response to infection, is a leading cause of morbidity and mortality in intensive care units (ICU) (1). Outside of antibiotics, treatment of sepsis is non-specific, aimed at early cardiopulmonary resuscitation to minimize the adverse systemic effects of infection (2). While this approach is frequently effective (3), there are no approved therapeutics available for sepsis once antibiotics and supportive therapy fail (4). Importantly, recent studies have shown that a major cause of death in septic patients is the development of secondary infections (5). These secondary infections manifest as a result of post-septic functional immunosuppression, as has been well described during sepsis in both animal and clinical studies (6, 7). Thus, identifying targets that could restore immune competence and prevent secondary infections during sepsis remains an important goal.

Herpesviruses such as cytomegalovirus (CMV) and Epstein–Barr virus (EBV) have a high seroprevalence in adults (8, 9). A defining feature of this virus family is the ability to establish latency within host cells, leading to lifelong infection. However, in the setting of immunosuppression such as is seen in HIV infection or following organ transplantation, viral reactivation can occur, leading to life-threatening complications (10, 11). In addition, herpesvirus reactivation has frequently been reported in septic patients (12–15), including a study by Walton and colleagues, which found that nearly 60% of septic patients showed signs of viral reactivation within 7 days of their septic event (13). EBV was the most frequently detectable virus, with 53% of septic patients testing positive for EBV viremia, as compared to only 11.8% of non-septic critically ill patients and 3.6% of healthy controls (13). These findings are of clinical relevance because studies also showed that viral reactivation is associated with longer hospital stays and increased mortality (12, 14, 16–18).

Taken together, these findings suggest that sepsis-induced immunosuppression may be causally responsible for the observed viral reactivation in human septic patients. However, the mechanisms underlying this process are unknown. It is well established that antigen-specific $CD8^+$ memory T cells are responsible for the immunosurveillance that protects against recrudescence of latent viral infections (19). One class of molecules that has been shown to inhibit $CD8^+$ T cell survival and functionality and contribute to post-septic immune incompetence in both mouse and humans is the family of T cell coinhibitory receptors (20). Importantly, expression of coinhibitory molecules including PD-1 and BTLA have been identified on the surface of T cells isolated from septic patients as opposed to those obtained from non-septic controls (21). Subsequent studies on both PD-1 and BTLA revealed that coinhibitory signals play an important role in the pathogenesis of sepsis, and that blockade of these pathways may represent a therapeutic strategy for the amelioration of morbidity and mortality in septic individuals (22–30). In addition, we recently showed that the 2B4 coinhibitory pathway plays a critical role in sepsis-induced pathology. Additional evidence exists to suggest that 2B4 may be playing an important role on memory T cells. For example, studies in models of chronic viral infection revealed that 2B4 functions predominantly to limit the expansion and functionality of memory T cells, as opposed to primary effectors (31).

We therefore aimed to develop a mouse model to assess the quantity and quality of the gHV-specific $CD8^+$ T cell response in the setting of sepsis, and to evaluate the role of 2B4 in this process. We utilized the mouse model of EBV (mouse gammaherpesvirus-68, referred to here as gHV) to assess the fate of $CD8^+$ T cell responses in the context of the cecal ligation and puncture (CLP) model of polymicrobial sepsis. gHV readily infects mice and establishes a latent infection that is harbored for life in B cells, macrophages, and splenic DC. Factors that support the applicability of gHV to EBV disease are: (*1*) gHV encodes several genes involved in latency, reactivation, and viral replication that are well conserved between human and murine γ-herpes viruses (32); (*2*) Functional studies on "unique" gHV gene products have demonstrated conservation of key functions of EBV proteins (33); and (*3*) Both gHV and EBV are lymphotropic and associated with lymphoproliferative disorders and lymphomas. These conserved genetic and functional aspects of the virus suggest that gHV is a good model for the study of the host response to EBV.

Using this model, we found that animals that were infected with gHV 30 days prior to undergoing CLP experienced a significant reduction in gHV-specific $CD8^+$ T cells and an elevated gHV viral load compared to non-septic controls. Mechanistically, our study also revealed that 2B4 was upregulated on gHV-specific T cells following sepsis, and that the genetic absence of 2B4 resulted in the preservation of gHV-specific $CD8^+$ memory T cells and reduction in viral load following sepsis. This study therefore demonstrates that sepsis erodes the viral-specific $CD8^+$ T cell responses against an EBV homolog in a 2B4-dependent manner.

Results

gHV-specific $CD8^+$ memory T cells are lost following sepsis

To establish the kinetics of the $CD8^+$ T cell response following gHV infection, WT mice were infected with gHV intraperitoneally and the gHV-specific $CD8^+$ T cell response was assessed through serial bleeds at day 7, 14 and 28 post-infection (Fig. 1A). The gHV-specific T cell response for both p56 and p79 epitopes peaked at day 14 post-infection (Fig. 1B and C), as has been previously described (34). To assess the gHV-specific immune response in the setting of sepsis, septic peritonitis was established by CLP at 30 days post-infection. Three days later the impact of sepsis on $CD8^+$ gHV-specific immunity and viral load was assessed (Fig.1A). To measure viral load in this system we utilized a transgenic virus harboring a YFP-$H2^b$ fusion protein that allows for the detection of cells that are latently infected with MHV-68 via flow cytometry (35). It is well recognized that the murine gammaherpesviruses preferentially infect the germinal center B cell compartment in order to establish latency and gain access to the long-lived memory B cell pool (36, 37). We thus sought to determine the proportion of cells harboring latent virus on a per-cell basis within the GC B cell compartment ($CD19^+$ $GL7^+$ $CD95^+$) of septic mice at 30 days post infection. Compared to animals receiving sham surgery, septic animals exhibited significantly higher frequencies of virally infected germinal center B cells (Figure 1D and E). Uninfected negative controls are shown in Supplemental Fig. 1A. Correspondingly, septic animals also exhibited a significant reduction in the frequencies and number of $CD44^{hi}$ activated $CD8^+$ T cells (Fig. 1F and G). In addition, gHV-specific, p56- and p79-$tetramer^+$ $CD8^+$ T cells were

also significantly lower in septic mice relative to non-septic sham controls (Fig. 1H and I). Similar results were observed in enumerating the absolute number of gHV-specific, p56- and p79-tetramer$^+$ CD8$^+$ T cells per spleen in WT CLP and sham controls (Fig. 1I right panels).

2B4 expression is upregulated on a subset of antigen-experienced and gHV-specific CD8$^+$ T cells during sepsis

2B4 (CD244) has recently been shown to be inducibly expressed on both CD4$^+$ and CD8$^+$ T cells and to exert coinhibitory function on these cell populations (31, 38, 39). Additionally, previous work from our lab has uncovered a novel role for 2B4 coinhibitory signaling on CD4$^+$ T cells in mediating immune dysregulation following sepsis (40). Given these data and the clinical descriptions of significant rates of viral recrudescence following sepsis, we sought to interrogate the role of 2B4 in mediating the gHV-specific CD8$^+$ T cells response in the setting of sepsis. We first assessed the frequency of 2B4 expression on antigen-experienced CD44^{hi} activated CD8$^+$ T cells following sepsis, where a higher proportion of cells expressed 2B4 compared to the non-antigen-experienced CD44^{lo} population. Importantly, we observed an increased frequency of 2B4-expressing cells within the CD44^{hi} compartment of CD8$^+$ T cells isolated from septic animals as compared to sham controls (Fig. 2A and B). Similar results were observed in the CD4$^+$ T cell compartment, in that the frequencies of 2B4-expressing cells increased following CLP in the CD44^{hi} but not the CD44^{lo} subset (Supplemental Fig. 2A, B). Next, the proportion of gHV-specific tetramer-positive CD8$^+$ T cells expressing 2B4 following the induction of sepsis was assessed. Again, we observed that higher proportions of tetramer-positive T cells expressed 2B4 compared to the non-tetramer positive population. Importantly, a greater frequency of 2B4- expressing cells was observed within the gHV-specific p56- and p79-tetramer$^+$ CD8$^+$ T cell populations isolated from septic animals as compared to sham non-septic controls (Fig. 2C-F).

Expression of 2B4 on gHV-specific CD8$^+$ T cells modulates the anti-viral T cell response during sepsis

Because 2B4 was upregulated on gHV-specific CD8$^+$ T cells following CLP, we next tested whether 2B4 co-inhibitory signals were modulating the antigen-specific T cell response to gHV during sepsis. 2B4$^{-/-}$ mice were infected with gHV, and gHV-specific CD8$^+$ T cell responses were measured in the blood on D7, D14 and D28 post-infection (Fig. 3A). Similar to what was observed in WT mice (Fig. 1B and C), the gHV-specific T cell response peaked at day 14 post-infection (Fig. 3B and C), indicating that gHV-specific responses could be elicited in these mice and that the lack of 2B4 did not alter the kinetics of the viral response. We next assessed the role of 2B4 in the CD8$^+$ gHV-specific immune response and subsequent viral load in the setting of sepsis, as described above, using 2B4 deficient animals (Fig. 3A). In contrast to the significant increase in the frequency of virally infected gHV-YFP$^+$ GC B cells observed in WT animals following sepsis, both sham and septic 2B4$^{-/-}$ animals exhibited equivalent, low frequencies of YFP$^+$ GC B cells (Fig. 3D and E). WT and 2B4$^{-/-}$ animals exhibited similarly low frequencies of YFP$^+$ GC B cells in the absence of CLP (Supplemental Fig 1B). These data demonstrate that while sepsis resulted in an increase in gHV viral load in WT animals, viral load was not increased in 2B4$^{-/-}$ hosts during sepsis. Moreover, in contrast to the decrease in antigen-experienced T cells that was observed in WT animals following sepsis (Fig. 1F-I), the frequencies of CD8$^+$ CD44^{hi}

activated T cells was not decreased following sepsis in the absence of 2B4 (Fig. 3F and G). No decrease in CD44^{hi} CD4^{+} T cells was observed following CLP, either in WT or 2B4$^{-/-}$ animals (Supplemental Fig. 2A-B). Importantly, both the frequency and number of gHV-specific, p56- and p79-tetramer^{+} CD8^{+} T cells were maintained in 2B4$^{-/-}$ septic mice relative to 2B4$^{-/-}$ non-septic sham controls (Fig. 3H and I). These data suggest that in the absence of 2B4, virus-specific CD8^{+} T cells are better able to perform immune surveillance and prevent latent viral re-activation.

2B4 deficiency did not improve antigen-specific CD8^{+} T cell activation following sepsis

To determine the potential mechanism by which 2B4 signaling modulated the CD8^{+} T cell response to gHV during sepsis, we next assessed the immune phenotype of antigen-experienced CD8^{+} CD44^{hi}, as well as antigen-specific p56- and p79-tetramer^{+} T cells. Within total CD44^{hi} cells, the proportion of cells expressing the activation markers CD25 (IL-2Ra) and CD69 were significantly increased in 2B4$^{-/-}$ animals relative to WT mice during sepsis (Supplemental Fig. 3A and B). Interestingly, however, this was not the case within the p56- and p79-tetramer^{+} populations, wherein lack of 2B4 had no effect on the proportion of cells expressing either marker during sepsis (Supplemental Fig. 3C-F).

2B4 signaling modulates IFN-γ cytokine secretion and viral load during sepsis in a cell-intrinsic manner

Given these results that gHV-specific CD8^{+} T cells were preserved during sepsis in the absence of 2B4, we next queried the functionality of these CD8^{+} T cells in WT vs. 2B4$^{-/-}$ septic animals. WT or 2B4$^{-/-}$ mice were infected with gHV and 30 days later subjected to CLP. Three days later, spleens were harvested and cells were restimulated ex vivo with PMA and ionomycin to detect IFN-γ production. Results indicated that 2B4$^{-/-}$ mice exhibited increased frequencies of IFN-γ^{+} CD8^{+} T cells as compared to WT mice following CLP (Figure 4A, 4B). These data indicate that there are more functional effectors in the 2B4$^{-/-}$ gHV-infected septic mice as compared to the WT gHV-infected septic mice.

We next asked if the increased IFN-γ secretion was a result of T cell intrinsic 2B4 deficiency. To address this, either WT or 2B4$^{-/-}$ transgenic OT-I T cells specific for the chicken-ovalbumin protein and expressing the congenic marker Thy1.1 were transferred into naïve C57BL/6 recipients one day prior to infection with a genetically modified gHV expressing the OVA$_{257\text{-}264}$ epitope (gHV-OVA). Thirty days later, infected mice were subjected to CLP and the antigen-specific immune response was measured three days later (Fig. 4C). The use of this transgenic system allowed us to eliminate expression of 2B4 exclusively on the transferred, antigen-specific CD8^{+} T cells in order to determine whether 2B4 is acting in a T cell intrinsic manner. At three days post-CLP, results indicated that both the frequency and number of IFN-γ^{+} cells was increased in the population of 2B4-deficient OT-I T cells as compared to the WT OT-I T cells (Fig. 4D and E).

To determine whether T cell intrinsic 2B4 deficiency impacted gHV viral load, WT or 2B4$^{-/-}$ transgenic OT-I T cells were transferred into B6 recipients one day prior to infection with gHV-OVA, as described above (Fig. 4C). Thirty days later, infected mice were subjected to CLP and gHV viral load was measured in the spleen three days later via q-PCR.

Results indicated that WT animals contained significantly higher viral load as compared to $2B4^{-/-}$ septic animals (Fig. 4F). Taken together, these results therefore indicate that 2B4 functions in a cell intrinsic manner to modulate IFN-γ secretion in gHV-specific $CD8^+$ T cells and impact gHV viral load during sepsis.

2B4 signaling modulates PD-1 expression during sepsis in a cell-intrinsic manner

We next explored the hypothesis that improved functionality of the gHV-specific $CD8^+$ T cell responses following sepsis in the absence of 2B4 could be the result of altered expression of other coinhibitory receptors, which have been shown to contribute to functional T cell exhaustion and deletion, including PD-1, BTLA and LAG-3. We found that PD-1 was significantly increased, both proportionally and on a per-cell basis, on $CD8^+$ $CD44^{hi}$ antigen-experienced T cells isolated from WT septic animals compared to WT sham controls (Fig. 5A and B). There were no differences in BTLA or LAG-3 expression on $CD8^+$ $CD44^{hi}$ antigen-experienced T cells isolated from WT septic animals vs. WT sham controls (data not shown). Of note, following CLP, cells isolated from $2B4^{-/-}$ mice exhibited significantly lower levels of PD-1, indicating that the presence of 2B4 on WT cells may modulate the expression of this coinhibitory molecule (Fig. 5B, left). PD-1 expression in WT vs. $2B4^{-/-}$ sham animals were not different (Fig. 5B)

These findings demonstrated that 2B4 impacts the phenotype of gHV-specific $CD8^+$ T cell responses during sepsis, but this could be a direct effect of 2B4 signaling on those T cells or a secondary effect of 2B4 to impact APC or some other cell population. To determine whether 2B4 functions to modulate PD-1 expression on gHV-specific $CD8^+$ T cells during sepsis in a cell intrinsic manner, we conducted adoptive transfer studies similar to those shown in Figure 4, in which either WT or $2B4^{-/-}$ transgenic OT-I T cells specific for the chicken-ovalbumin protein and expressing the congenic marker Thy1.1 were transferred into naïve C57BL/6 recipients one day prior to infection gHV-OVA. Thirty days later, infected mice were subjected to CLP and the antigen-specific immune response was measured three days later (Fig. 5C). At three days post-CLP, results indicated that both the frequency and MFI of PD-1 expression was reduced on 2B4 deficient OT-I T cells as compared to the WT OT-I T cells (Fig. 5D and E). These results therefore indicate that 2B4 functions in a cell intrinsic manner to modulate PD-1 expression in gHV-specific $CD8^+$ T cells during sepsis.

Discussion

Recently, viral reactivation in septic patients has frequently been reported in clinical trials and viral recrudescence has been associated with secondary infection and worse outcome (12–14). We hypothesized that the loss of antigen-specific $CD8^+$ T cells, which are generally thought to be responsible for immunosurveillance against latent viral infections, may be an important cause of gHV reactivation in sepsis. In the present study, we establish a mouse model in which we observed increased gHV viral load following septic insult, and demonstrate that septic gHV-infected animals exhibited a concomitant reduction in the frequencies and absolute numbers of $CD44^{hi}$ $CD8^+$ antigen-experienced and gHV-specific p56- and p79-$tetramer^+$ $CD8^+$ T cells following sepsis (Fig. 1). Mechanistically, we found that expression of the coinhibitory receptor 2B4 contributed to the attrition of virus-specific

$CD8^+$ T cells during sepsis, because in the absence of 2B4, virus-specific T cells were maintained and viral load was not increased.

The finding that gHV-specific memory T cells are eroded during sepsis is consistent with previously published reports. Specifically, Badovinac and colleagues working in an LCMV model observed rapid apoptosis of pre-existing memory CD8 T cells after sepsis induction that led to a loss in CD8 T cell-mediated protection (41). In line with these studies, we previously reported that sepsis results in the preferential depletion of a subset of memory-phenotype CD8 T cells that remain “unactivated” (i.e., fail to up-regulate activation markers) (42). These data suggested that perhaps memory T cells that re-encounter cognate antigen during the septic event might be spared from sepsis-induced apoptosis. However, in our study we found that antigen-specific memory T cells specific for a latent virus (and thus which may receive persistent antigen exposure) were still susceptible to sepsis-induced attrition. Together, these data illuminate the impact of sepsis in shaping the quantity and quality of memory CD8 T cell-mediated protective immunity to a latent viral infection.

Our data revealed a functional role for 2B4 signaling in preserving gHV-specific $CD8^+$ T cell responses and preventing an increase in the frequency of gHV-infected B cells during sepsis. However, this observation could have been due to a direct effect of 2B4 signaling on $CD8^+$ T cells, or an indirect effect of 2B4 signaling on some other cell type that then secondarily impacted virus-specific $CD8^+$ T cell responses. Our adoptive transfer studies illuminated a T cell-intrinsic role for 2B4 in modulating the quality of virus-specific $CD8^+$ T cell responses during sepsis. Specifically, T cell intrinsic deficiency of 2B4 resulted in reduced expression of PD-1 on antigen-specific $CD8^+$ T cells. PD-1 has previously been shown to mark $CD8^+$ T cell responses that are more short-lived and exhibit diminished cytokine producing effector function in the context of gHV infection (43). However, these results do not rule out an additional, T cell extrinsic role for 2B4 on $CD8^+$ T cell responses during sepsis. For example, a previous study revealed that mice lacking 2B4 exhibited diminished LCMV-specific $CD8^+$ T cell responses and prolonged viral persistence relative to wild-type mice (44). Interestingly, these findings were not due to 2B4 deficiency in T cells; instead, the authors showed that in the absence of 2B4-mediated coinhibition, NK cells became hyper-activated and lysed activated $CD8^+$ viral-specific T cells, leading to loss of viral control (44). Indeed, our data showed that antigen-specific $CD8^+$ T cell numbers were only partially restored when 2B4 was deleted exclusively in T cells, thus pointing towards an additional, T cell-extrinsic effect for 2B4 during sepsis.

The observed maintenance of virus-specific $CD8^+$ T cell populations and reduced viral load in the setting of 2B4 deficiency suggests a critical role for 2B4 signaling in the loss of these antigen-specific populations and the erosion of protective immunity following sepsis. Thus, our data suggest that blocking the 2B4 signaling pathway may hold promise as a therapeutic target to modulate the immune system and maintain protective immunity in the setting of sepsis. There is precedent for this as therapeutic blockade of PD-1 and PD-L1 have been tested in clinical trials to improve immune dysregulation following sepsis (45, 46). Indeed, we recently showed that pharmacologic blockade of 2B4 reduced sepsis-induced mortality in a model of CLP (40). Critical questions that will arise in considering therapeutic 2B4 blockade in septic patients include the timing of anti-2B4 administration, because

administration of a coinhibitory receptor blocker too early during the hyper-inflammatory phase may exacerbate immune dysregulation and result in unwanted toxicities. It should be noted, however, that in our genetic knockout studies, animals lack 2B4 during the entire septic event and the outcome is beneficial. Future studies examining the impact of pharmacologic blockade on memory T cell survival, functionality, and overall mortality during sepsis are warranted.

Finally, studying the septic response in latently infected animals may represent a more physiologically relevant model in which to study other aspects of sepsis pathophysiology. This is due to the fact that in normal adult human subjects, the peripheral T cell compartment is comprised of ~50% memory T cells, a number that is driven in large part to exposure to viruses such as CMV and EBV. In contrast, laboratory mice housed under SPF conditions possess only ~10-20% memory T cells (47, 48). A recent seminal study by Beura and colleagues showed that exposure of laboratory animals to pet store or feral mice resulted in the rapid generation of $CD8^+$ memory T cell populations in these animals, at levels that approximate those observed in adult humans (49). Thus, the use of latently infected murine hosts may represent a way to better model the antigen-experienced, latently infected immune system of human septic patients in order to study the T cell response to sepsis in a more physiologically relevant context.

In sum, our results demonstrate that sepsis-induced immune dysregulation erodes $CD8^+$ memory T cell immunosurveillance against a latent viral infection. 2B4-mediated co-inhibitory signals play a functional role in this loss of protective immunity. These results indicate that the disruption of 2B4-mediated co-inhibitory signaling may better maintain protective immunity against EBV in the context of sepsis.

Methods and Materials

Mice and gHV infection

6- to 8-week-old male C57BL/6 mice were purchased from the Jackson Laboratory (Bar Harbor, ME, stock # 000664). Age-matched 2B4 knockout mice (denoted $2B4^{-/-}$) on a C57BL/6 background were a gift from C. Terhorst (Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA). OT-I transgenic mice were obtained from Taconic Farms. $2B4^{-/-}$ mice were bred onto OT-I xThy1.1 background at Emory University (Atlanta, GA). All animals were maintained in accordance with Emory University Institutional Animal Care and Use Committee guidelines (PROTO201700378, PI: Mandy Ford). Virus stocks were prepared as previously described (50). Mice were infected with 2×10^3 pfu intraperitoneally of a recombinant, transgenic murine gammaherpesvirus (gHV), MHV68-$H2^b$YFP, which expresses the enhanced yellow fluorescent protein (EYFP) (35). This allows for the monitoring of viral load via flow cytometry. Mice were allowed to establish a latent viral infection for 30 days before the establishment of sepsis.

Cecal ligation and puncture

Sepsis was induced using CLP, a murine model of polymicrobial sepsis, as previously described (51). Injury was titrated to achieve a ~50% 14-day mortality to mimic the clinical

scenario of sepsis. In brief, C57BL/6 mice were anesthetized using isoflurane and underwent laparotomy, the cecum was exteriorized, ligated distal to the ileocecal valve, and punctured twice with a 25-gauge needle. Sham-operated animals underwent laparotomy and exteriorization of the cecum only. Prior to incision, mice received buprenorphine, given subcutaneously at a dose of 0.1 mg/kg (McKesson Medical, San Francisco, CA, USA), to alleviate postoperative pain. Mice also received subcutaneous injections of normal saline (1 ml), ceftriaxone (50 mg/kg) (Sigma-Aldrich, St. Louis, MO, USA), and metronidazole (35 mg/kg) (Apotex, Weston, FL, USA) postoperatively to mimic the human condition where fluid resuscitation and antibiotics are a mainstay of sepsis management. Ceftriaxone and metronidazole were re-dosed at 12, 24, and 36 h post-CLP. All animals were killed at 3 days post CLP for tissue collection and immunophenotyping.

Cell transfers

Spleen and mesenteric lymph node cells were isolated from Thy1.1^{+} OT-1 C57BL/6 and 2B4$^{-/-}$ mice for adoptive T cell transfer. The number of OT-1 cells transferred was calculated using flow cytometric analysis, by staining for CD8, Thy1.1, Vα2 and Vβ5. The OT-I cells were diluted to a concentration of 2.0×10^4/ml and a volume of 500ul was transferred to mice via tail vein injection 24 hours prior to gHV infection.

Flow cytometry

Three days after CLP, mice were sacrificed and spleens were harvested. Splenocytes were stained for CD3 (Biolegend), CD4 (BD), CD8 (Biolegend), CD44 (Biolegend), Thy1.1 (BD), anti-CD25, anti-69, anti-CD62L (all from Biolegend), and anti-BTLA, anti-2B4, anti-PD-1 and anti-Lag-3 (all from eBioscience) for phenotypic analysis. Accucheck Counting beads (Thermo Fisher Scientific) were added before data collecting to calculate the absolute number per spleen. Samples were analyzed on a LSRII flow cytometer (BD) and data were analyzed using FlowJo software (Tree Star, Ashland, OR, USA).

Tetramer staining

We used class I MHC tetramers corresponding to the gHV-immunodominant epitopes (ORF61/p79/ K^{b} and ORF6/p56/D^{b}) to assess the gHV-specific CD8^{+} T cell responses (NIH MHC Tetramer Core, Atlanta, GA). Blood or splenocytes were collected at times indicated and were lysed with fixative-free lysing solution (Life Technologies). Prior to surface staining, samples were incubated with APC-conjugated ORF6/p56 and PE-conjugated ORF61/p79 tetramers at room temperature for 30 min.

Quantitative PCR (q-PCR) for viral load

Latent viral DNA was quantified by quantitative PCR (q-PCR) as previously described (52) . Briefly, DNA was extracted from whole splenocytes using a Qiagen DNeasy kit (Qiagen, Valencia, CA) and then quantified using a UV spectrophotometer. DNA (100 ng) was subjected to q-PCR using PrimeTime Gene Expression Master Mix (IDT, Coralville, IA), 1.0 nM primers complementary to the ORF50 gene, and 0.5 nM labeled probe complementary to the ORF50 gene. The samples were subjected to 40 cycles of 15 s at 95°C and 1 min at 60°C. q-PCR was performed using a QuantStudio Flex System (Thermo Fisher

Scientific, Waltham MA). To construct a standard curve, a graded number of copies of the pBluescript SK(+) plasmid, containing the 70bp fragment of the ORF50 gene generated from the following primers (3′ primer, CCCTGAGGCTCAACAATTGG; 5′ primer, GGATACGCCTGTCCAGCATATT), was mixed with 100 ng of naive splenocyte DNA and subjected to q-PCR. No-template controls containing 100 ng of uninfected splenocyte DNA were negative for all q-PCR assays. The assay was able to detect fewer than 10 viral genomes per sample.

Statistical analysis

All the data are expressed as mean ± SEM. Comparison of continuous variables between the groups was conducted using a Student's t-test or Mann-Whitney U-test depending on Gaussian distribution. All statistical analyses were performed using the statistical software Prism V. Two-tailed P-values < 0.05 were considered statistically significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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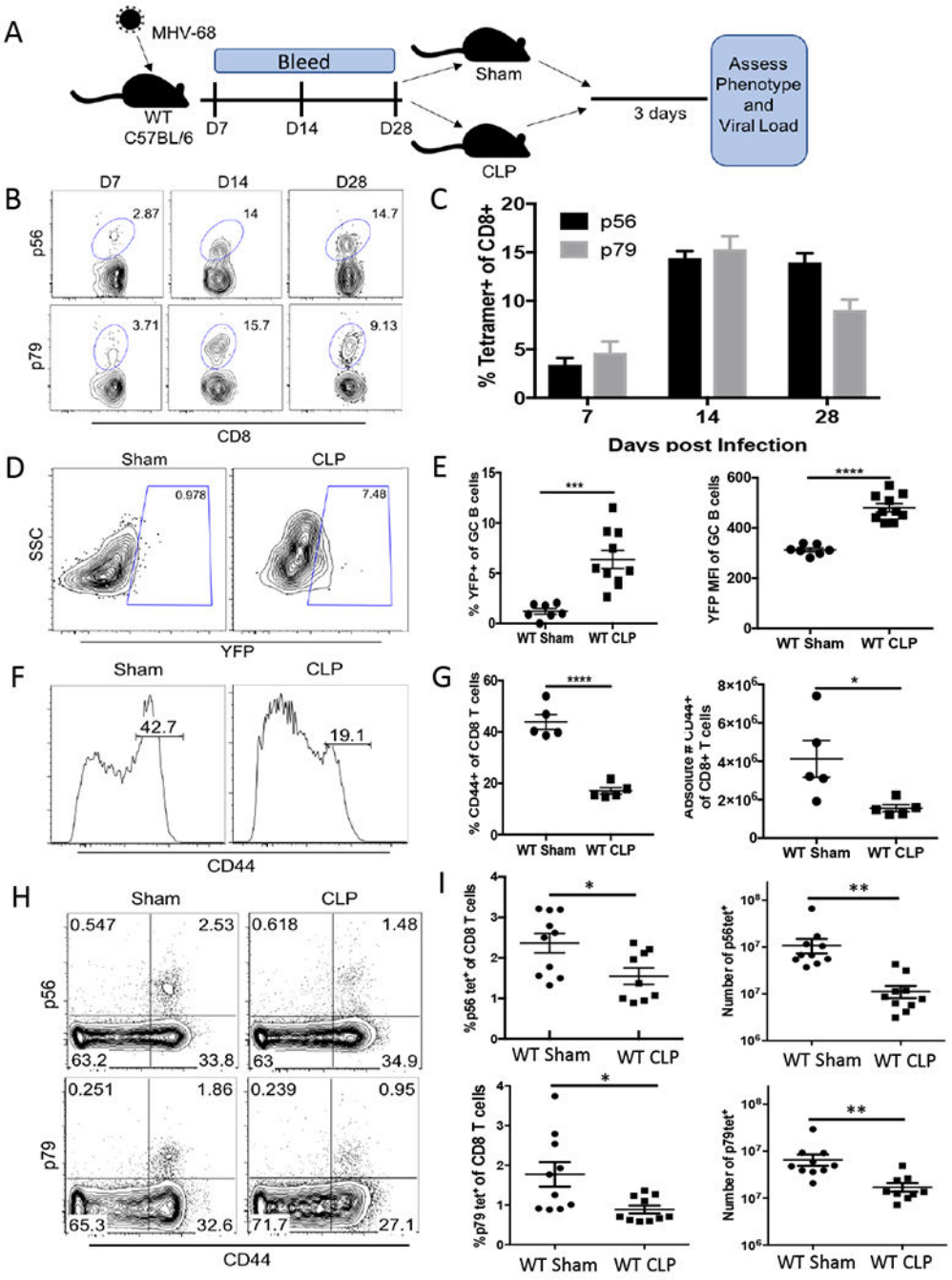

Figure 1. Sepsis results in a significant reduction in the frequencies and numbers of gHV-specific CD8$^+$ T cells.

Naïve mice were infected with 2×10^3 PFU intraperitoneally of a recombinant, transgenic murine gammaherpesvirus (gHV), MHV68-H2^b-YFP, which expresses the enhanced yellow fluorescent protein (EYFP) (A). Mice were serially bled on days 7, 14 and 28 post-infection. Representative flow plots (B) and summary data (C) of the kinetics of p56 and p79 tetramer staining in the blood following infection. At d 30 post-infection animals were subjected to CLP to induce sepsis. Three days post-CLP mice were sacrificed and splenocytes were harvested for assessment of viral load and immune phenotype. Representative flow plots (D)

and summary data (E) of viral load as measured by flow cytometry as the percent (left) and mean florescence intensity (right) of YFP on splenic $CD19^+$ $GL7^+$ $CD95^+$ GC B cells. Representative flow plots (F) and summary data (G) of percent (left) and absolute number (right) of $CD44^{hi}$ $CD8^+$ T cell populations in spleens of WT CLP animals and sham controls. Representative flow plots (H) of p56- and p79- specific $CD8^+$ T cell responses in the spleen. Summary data (I) of percent (left) and absolute number (right) of p56- and p79-specific $CD8^+$ T cell responses in WT CLP vs. sham controls. Data are representative of 2-3 independent experiments with 5-10 mice per group. Statistical analysis was conducted using a Student's t-test. *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$.

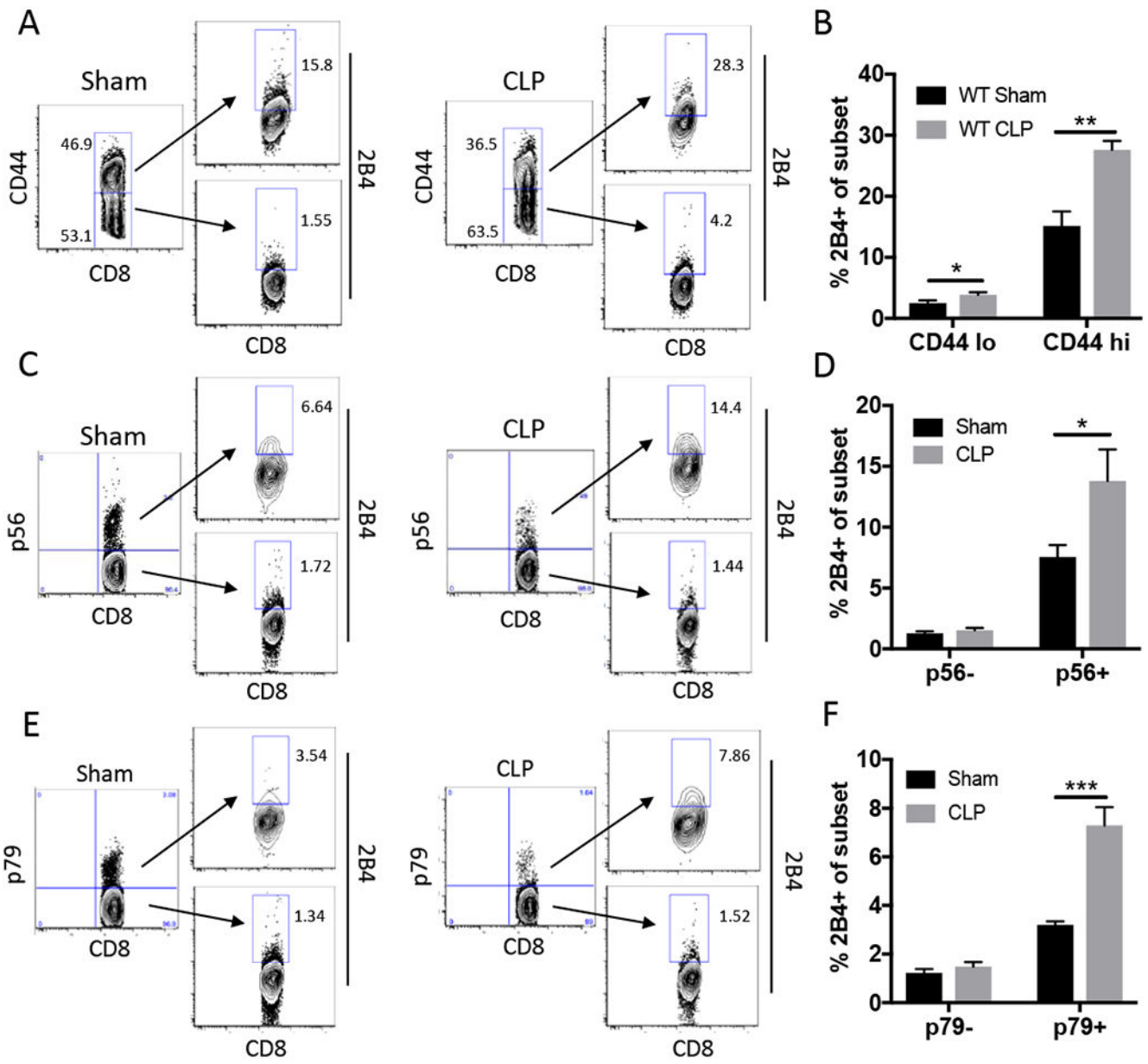

Figure 2. 2B4 expression is upregulated on a subset of antigen-experienced and gHV-specific CD8$^+$ T cells during sepsis.

Three days post CLP and 33 days post gHV infection, splenocytes from WT sham or CLP animals were assessed for the proportion of cells expressing the coinhibitory molecule 2B4. Representative plots of 2B4 expression (A) on CD8$^+$ CD44^{hi} antigen-experienced T cells taken from sham (left) and CLP (right) animals. Increased proportions of CD44^{hi} (A, top) T cells expressed 2B4 compared to CD44^{lo} T cells (A, bottom). These data are summarized in (B). Representative flow plots (C and E) and summary data (D and F) of 2B4 expression on gHV-specific p56 (C-D) and p79 (E-F) tetramer$^+$ CD8$^+$T cells isolated from WT sham or CLP animals. Data are representative of 2-3 independent experiments with 5-10 mice per

group. Statistical analysis was conducted using a Student's t-test. *p<0.05, **p<0.01, ***p<0.001.

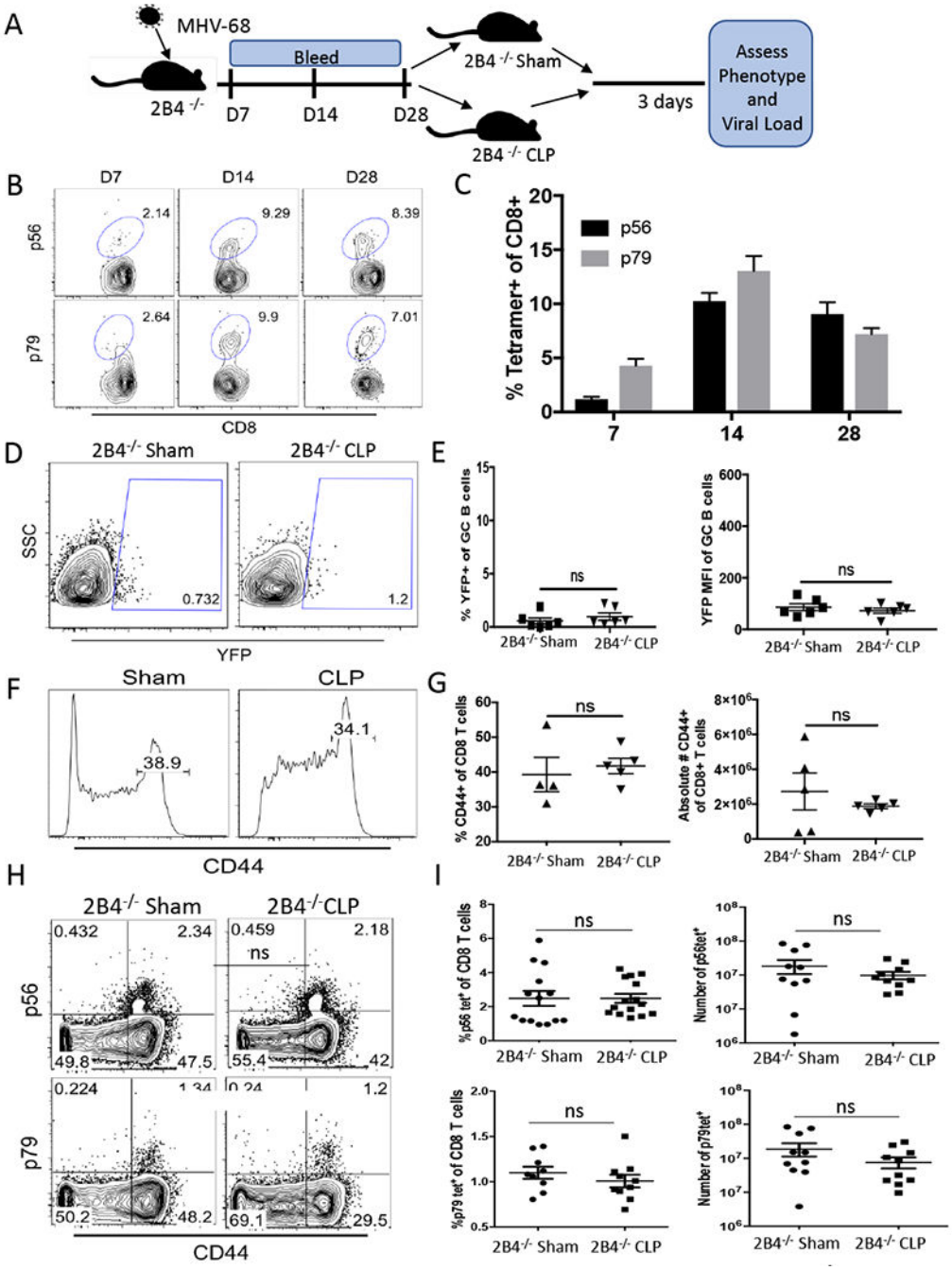

Figure 3: Expression of 2B4 on gHV-specific CD8$^+$ T cells modulates the anti-viral T cell response.

Naïve 2B4$^{-/-}$ mice were infected with 2×10^3 PFU intraperitoneally of a recombinant, transgenic murine gammaherpesvirus (gHV), MHV68-H2^b-YFP, which expresses the enhanced yellow fluorescent protein (EYFP) (A). Mice were serially bled on days 7, 14 and 28 post-infection. Representative flow plots (B) and summary data (C) of the kinetics of p56 and p79 tetramer staining in the blood of 2B4$^{-/-}$ mice following infection. At d 30 post-infection animals were subjected to CLP to induce sepsis. Three days post-CLP mice were sacrificed and splenocytes were harvested for assessment of viral load and immune

phenotype. Representative flow plots (D) and summary data (E) of viral load as measured by flow cytometry as the percent (left) and mean florescence intensity (right) of YFP $CD19^+$ $GL7^+$ $CD95^+$ GC B cells. Representative flow plots (F) and summary data (G) of percent (left) and absolute number (right) of $CD44^{hi}$ $CD8^+$ T cell populations in $2B4^{-/-}$ CLP animals and $2B4^{-/-}$ sham controls. Representative flow plots of p56- and p79-specific $CD8^+$ T cell responses measured in spleens of $2B4^{-/-}$ mice (H). Summary data of percent (left) and absolute number (right) of p56- and p79-specific $CD8^+$ T cell responses in spleens of $2B4^{-/-}$ CLP vs. $2B4^{-/-}$ sham controls (I). Data are representative of 2-3 independent experiments with 5-10 mice per group. Statistical analysis was conducted using a Student's *t*-test. "ns" signifies no significant difference between groups.

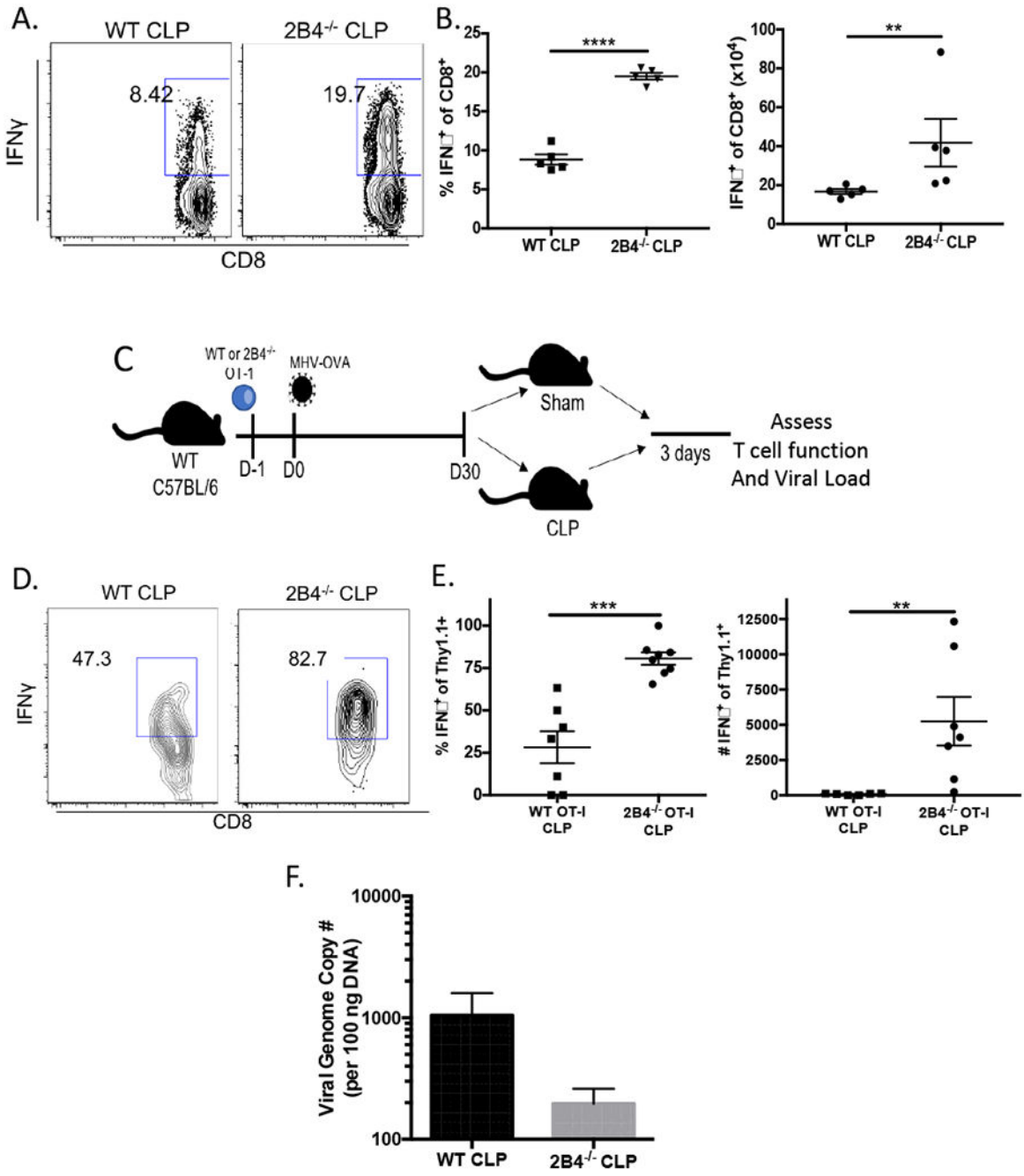

Figure 4. 2B4 signaling modulates IFNγ production on gHV-specific CD8$^+$ T cells and viral load in a cell-intrinsic manner.

Three days post CLP and 33 days post gHV infection, splenocytes from WT or 2B4$^{-/-}$ sham or CLP animals were assessed for the proportion of cells expressing the cytokine IFNγ. Representative flow plots of IFNγ expression on CD8$^+$ T cells (A). Frequency (left) and absolute number (right) is summarized in (B). Schematic of adoptive transfer studies, in which either WT or 2B4$^{-/-}$ transgenic OT-I T cells specific for the chicken-ovalbumin protein and expressing the congenic marker Thy1.1, were transferred into naïve C57BL/6 recipients one day prior to infection with a genetically modified gHV expressing the

$OVA_{257-264}$ epitope (gHV-OVA). Thirty days later, infected mice were subjected to CLP and splenocytes were harvested three days post-CLP (C). Representative flow plots of OVA-specific $CD8^+$ $Thy1.1^+$ cells expressing IFNγ (D). Frequency (left) and absolute number (right) of IFNγ^+ $Thy1.1^+$ $CD8^+$ OVA-specific T cells is summarized in (E). DNA from splenocytes of mice receiving either WT or $2B4^{-/-}$ OT-I T cells was subjected to Q-PCR analysis with primers and probe specific to viral ORF50 (F). Data are representative of 2-3 independent experiments with 5-7 mice per group. Statistical analysis was conducted using a Student's t-test. **p<0.01, ***p<0.001, ****p<0.0001.

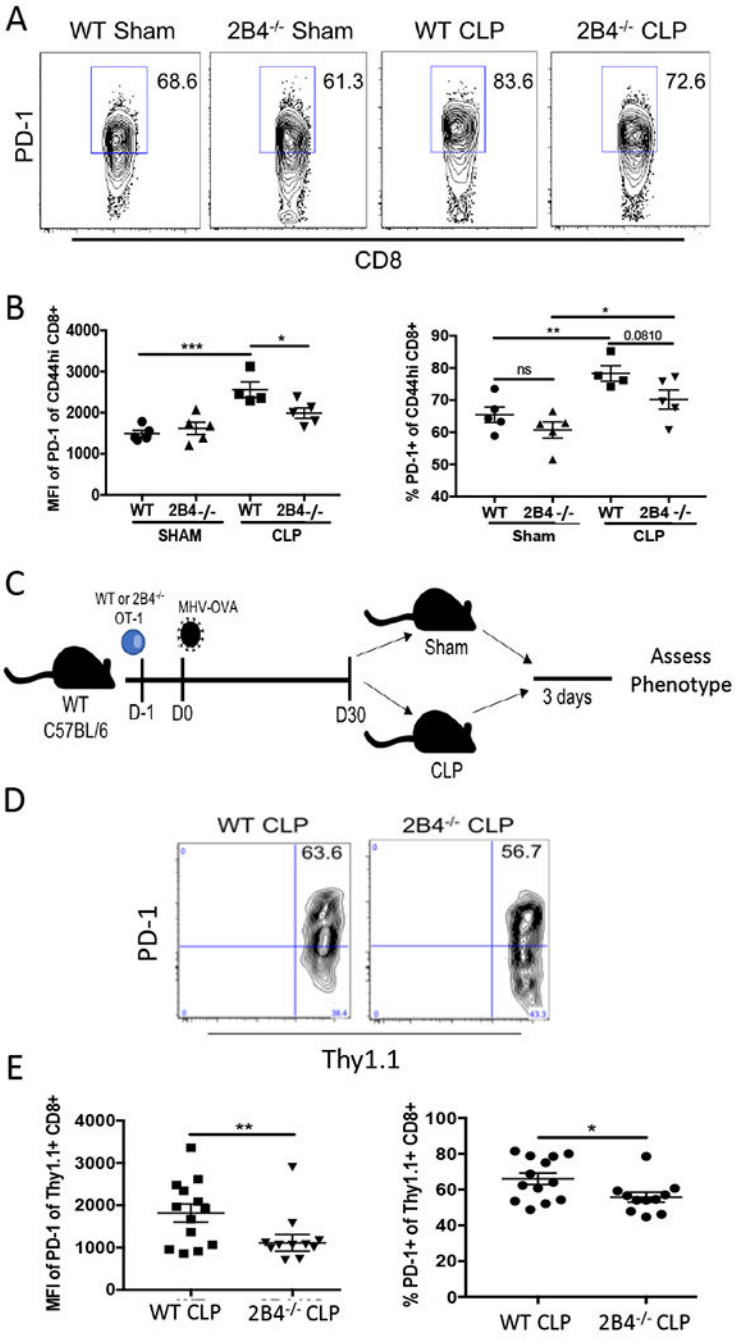

Figure 5. 2B4 signaling modulates PD-1 expression on gHV-specific CD8$^+$ T cells in a cell-intrinsic manner.

Three days post CLP and 33 days post gHV infection, splenocytes from WT or 2B4$^{-/-}$ sham or CLP animals were assessed for the proportion of cells expressing the coinhibitory marker PD-1. Representative flow plots of PD-1 expression on CD8$^+$ CD44^{hi} antigen-experienced T cells (A). Mean florescence (left) and percent (right) is summarized in (B). Schematic of adoptive transfer studies, in which either WT or 2B4$^{-/-}$ transgenic OT-I T cells specific for the chicken-ovalbumin protein and expressing the congenic marker Thy1.1, were transferred into naïve C57BL/6 recipients one day prior to infection with a genetically modified gHV

expressing the $OVA_{257-264}$ epitope (gHV-OVA). Thirty days later, infected mice were subjected to CLP and splenocytes were harvested three days post-CLP (C). Representative flow plots of OVA-specific $Thy1.1^+$ cells expressing PD-1 (D). Mean florescence (left) and percent positive (right) of $Thy1.1^+$ $CD8^+$ OVA-specific T cells is summarized in (E). Data are representative of 2-3 independent experiments with 5 mice per group. Statistical analysis was conducted using a Student's t-test. *p<0.05, **p<0.01, ***p<0.001, "ns" indicates no significant difference between groups.