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CD28/B7-Mediated Co-stimulation Is Critical for Early Control of Murine Cytomegalovirus Infection

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Abstract

Control of acute murine cytomegalovirus (MCMV) infection is dependent upon both innate and adaptive immune responses, relying primarily upon natural killer (NK) and T-cell responses for control. Although CD28/B7 plays a clear role in T-cell responses in many antigen systems including some viral infections, the importance of co-stimulation during MCMV infection is unconfirmed. In addition, recent data suggest that CD28/B7 co-stimulation might also be important to $Ly49H^+$ NK-cell expansion. We therefore hypothesized that CD28/B7 co-stimulation is critical to viral control after MCMV infection, and further that CD28/B7 co-stimulation plays a role in MCMV-specific T- and NK-cell responses. To test these hypotheses, we utilized C57BL/6 mice lacking the co-stimulatory molecules B7-1 and B7-2 or CD28. After primary infection with MCMV, viral titers are significantly elevated in mice lacking CD28 or B7 compared with wild-type mice. Impaired viral control is associated with significant defects in peripheral T-cell responses to MCMV, which appear to be dependent upon CD28/B7 co-stimulation. Abnormal hepatic T-cell responses in $CD28^{-/-}$ mice are preceded by impaired MCMV-specific $Ly49H^+$ NK-cell responses. Cytokine evaluations confirm that CD28/B7 co-stimulation is not required for non-specific antiviral responses. We conclude that CD28-mediated co-stimulation is critical for early viral control during acute MCMV infection.

Introduction

Control of acute cytomegalovirus (CMV) infection is dependent upon both innate and adaptive immune responses. It has been demonstrated that natural killer (NK) cell responses are critical to early viral control following acute CMV infection in some mouse strains (7,13,14,51,62). Viral clearance is further dependent upon intact T-cell responses, with CMV inducing specific cytotoxic T-cell (CTL) responses in infected hosts (16,48,52,54–56,66,67). Optimal CTL responses require professional antigen-presenting cells (APCs), and APC/T-cell interactions are thus critical to CTL differentiation in infected hosts (1,19,41). Although adoptive transfer of anti-CMV antibody is protective during acute infection (5,22,30,32,61), humoral responses to murine CMV (MCMV) are slower to develop than cellular responses (9), and both B cells and antibody appear dispensable during acute infection (29,74). Thus defects in either NK- or T-cell responses have significant implications for viral control, which is clinically most obvious in patients with impaired NK- or T-cell immunity (10,63,65).

T-cell responses to CMV occur through clonally restricted antigen receptors, resulting in proliferation and clonal expansion of CMV-specific cells (55). Generally, optimal activation of T cells requires co-stimulation in addition to antigen-specific signals (59). One such co-stimulatory mechanism is functionalized by activating receptor CD28 expressed on T-cell surfaces. Ligands for CD28, namely B7-1 and B7-2 (hereafter referred to as B7 molecules), are prototypic co-stimulatory molecules expressed primarily on antigen-presenting cells (6,23,24,34–36,59,76). Thus optimal activation of T cells relies upon antigen presentation to the T-cell receptor (TCR), and is enhanced by co-stimulation via CD28/B7 ligation.

Although numerous studies demonstrate the importance of co-stimulation in varying *in-vitro* antigen systems, there are few data published on co-stimulation during anti-viral responses, and even fewer utilizing *in-vivo* models. Of the few studies done to date, CD28/B7 co-stimulation has been shown to have varying importance for T-cell regulation of other viruses (18,44,49,70,71, and reviewed in [8]), and there are no studies evaluating the importance of co-stimulation in

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control of CMV infection. Despite this, there are circumstantial data suggesting that co-stimulation is important to the control of MCMV. Included in MCMV's immune evasion repertoire are genes that interfere with expression of B7 molecules on APCs. MCMV has been shown to downregulate surface expression of both B7-1 (CD80) and B7-2 (CD86) co-stimulatory molecules in monocyte/macrophage and dendritic cells during infection (2,37,42). Given the known importance of these co-stimulatory proteins in development of adaptive T-cell responses in other systems, we hypothesized that if MCMV has evolved specific immune evasion mechanisms that downmodulate B7 molecule expression, then CD28/B7 co-stimulation must play a critical role in anti-viral defense to infection with MCMV.

In addition to antigen-specific T-cell responses, NK-cell subsets have recently been shown to expand in response to specific antigenic stimuli (21,26,58). One example is $Ly49H^+$ NK-cell subset expansion in response to MCMV, which is triggered by activating receptor Ly49H binding to its recently described ligand MCMV protein m157 (4,12,21,25, 51,64,73). Because this NK subset expansion is similar to adaptive T-cell responses, it has been postulated that co-stimulation might also be important to NK-cell expansion and effector function (26,45). Murine NK cells have been shown to express CD28, and current data suggest that CD28 activation is important for optimal NK proliferation by enhancing cytokine production in these cells (26,45). Additionally, NK-cell cytotoxicity is enhanced by either type of B7 molecule (26,39,75), although B7-stimulated NK cytolysis does not absolutely require CD28 (17,40). Taken together, these data suggest that co-stimulation could indeed play an important role in NK-cell subset expansion in response to MCMV infection.

To test these hypotheses, we utilized C57/BL6 mice lacking the co-stimulatory molecules B7-1 and B7-2 or CD28. After infection with MCMV, these mice demonstrate significant defects in viral control associated with impaired T- and NK-cell responses. Impaired cellular responses to infection do not appear to be consequent to defective cytokine responses after infection in knockout mice. Thus our study for the first time confirms that CD28/B7-mediated co-stimulation is critical for early viral control during acute MCMV infection, and provides evidence that co-stimulation plays a role in adaptive cellular responses to MCMV.

Materials and Methods

Mice

Male C57BL/6 mice deficient of both B7-1 (CD80) and B7-2 (CD86) (referred to as $B7^{-/-}$) (11) or deficient of CD28 (referred to as $CD28^{-/-}$) (26,60) were obtained from our colony. Age-matched male C57BL/6 mice 8–10 wk of age (Charles River Laboratories, Boston, MA) were used as controls. All animals were housed in a pathogen-free environment, adhering to the "Guide for the Care and Use of Laboratory Animals," prepared by the National Research Council (National Institutes of Health Publication No. 86-23, revised 1985). After humane euthanasia, mice liver and spleen tissues were dissected aseptically, and underwent cellular isolation for flow cytometry analysis, viral titer analysis, or were frozen immediately in liquid nitrogen, then stored at −80°C for later analysis.

Viral infection

Primary CMV infection was achieved by IP injection of tissue culture passaged (once) through salivary gland–derived 5×10^4 PFU Smith strain MCMV (VR-1399/1981) obtained from the ATCC (Rockville, MD). Prior to infection (day 0) cohorts of 3 animals underwent analysis as controls. For infection experiments, 6 each of wild type, $B7^{-/-}$, and $CD28^{-/-}$ mice were infected, with cohorts of 3 animals each euthanized at 2 and 6 days post-infection (dpi).

Antibodies and flow cytometry

Fluorescent dye–conjugated antibodies specific for CD3 (PerCP-Cy5.5), NK1.1 (PE), interferon-γ, and bromodeoxyuridine (BrdU, PRB-1) were all purchased from BD PharMingen (San Diego, CA). Cells isolated from livers or spleens of mice were stained with fluorescent dye–conjugated mAbs and were analyzed by flow cytometry (FACScalibur, Becton Dickinson, Mountain View, CA) (26). Intracellular IFN-γ was analyzed in splenic NK cells (not further stimulated *ex vivo*) using FITC-conjugated mAb to IFN-γ from BD PharMingen as previously described (27). Ly49H (APC) antibody was a kind gift from Dr. Wayne Yokoyama.

Isolation of hepatic mononuclear leukocytes

Hepatic mononuclear cells (MNCs) were isolated as previously described (26). Briefly, livers were perfused through the portal vein with 10 mL phosphate-buffered saline (PBS) to remove blood cells. Individual livers were ground with a 3-mL syringe insert and suspended in 10 mL of medium. Single-cell suspensions were collected and centrifuged, and tissue debris was discarded. Cells were re-suspended in 2 mL 40% Percoll (Pharmacia, Uppsala, Sweden), and layered on 2.5 mL 70% Percoll in 15-mL conical tubes. MNCs were recovered at the interface between 40% and 70% Percoll after centrifugation at 1600 rpm at room temperature for 25 min. Remaining red blood cells in MNCs were lysed by ammonium chloride solution. Hepatic MNCs were then subjected to flow cytometry analysis.

BrdU incorporation assay in vivo

To determine cell proliferation *in vivo*, BrdU (1 mg/0.1 mL/mouse; Sigma, St Louis, MO) was injected IP into mice 3 h prior to acquiring liver and spleen tissues. Splenocytes and hepatic MNCs were isolated, stained with fluorescent-conjugated mAbs to CD3, NK1.1, and Ly49H, followed by intracellular staining with FITC-labeled mAb to BrdU, and were analyzed by four-color flow cytometry as previously described (26). Subsets of $CD3^+NK1.1^-$, $CD3^-NK1.1^+Ly49H^+$, and $CD3^-NK1.1^+Ly49H^-$ cells were respectively gated to analyze BrdU incorporation. Ly49H monoclonal antibody was a gift from Dr. Wayne Yokoyama and biotinylated in our laboratory.

In vitro *plaque assay*

Spleen and liver tissues from 0, 2, and 6 dpi underwent *in-vitro* plaque assay (IVPA) analysis to determine viral titer, as well as real-time PCR to determine viral load. For IVPA, mouse embryo fibroblasts were grown to confluence in 6-well plates in DMEM (Gibco BRL, Carlsbad, CA). Following

centrifugation (1000×g for 10 min) of 5 mL of homogenized tissue, 1 mL of supernatant was placed in each well. These plates were centrifuged at 1000 g, and incubated at 37°C in 5% CO_2 for 3–4 h. The plates were washed three times with PBS, then covered with 3 mL of 1% agar in DMEM. Following 6–7 days of incubation (37°C in 5% CO_2), the plates were fixed in 10% formalin, stained with 1% crystal violet, and analyzed by low-power phase contrast microscopy for plaque formation. All IVPA experiments were performed in duplicate, and viral titers are reported as mean ± standard error of mean of both experiments.

IVPA techniques are time consuming and laborious, so we performed side-by-side comparisons of IVPA to real-time PCR to quantitate viral load in liver and spleen tissues. Linear regression analysis showed good correlation between IVPA and real-time PCR results (R^2 0.89; $p < 0.001$, data not shown). A drawback to real-time PCR is that occasionally it was not sensitive enough to detect low viral copies, requiring the use of IVPA. Therefore when copy numbers are adequate enough to allow detection, real-time quantitative PCR is an accurate surrogate for IVPA to quantify viral load in tissues.

DNA isolation

DNA was extracted from tissues using QIAamp Tissue Kit (Qiagen GmbH, Hilden, Germany). DNA extracted from tissue homogenates was eluted in 100 μL of distilled water and stored at −20°C until analysis. DNA were amplified in a total volume of 25 μL with 200 nm of each primer and 1.0 U of Taq DNA polymerase (Gibco BRL) added in 2.5 μL of a PCR buffer (50 mM KCl, 20 mM Tris-HCl [pH 8.4], and 1.5 mM $MgCl_2$).

Quantitative real-time PCR

Primer sets and fluorescence resonance energy transfer type probes were designed and optimized for MCMV GB and β-actin genes. Plasmids homologous to the sequences amplified by our PCR primers were cloned for these genes, and using serial dilutions of known plasmid concentrations, standard curves were constructed for quantitation. Regression equations had very high R^2 values (.95 β-actin and .99 gB), and were used for subsequent copy number calculations. Real-time PCR results are expressed as copies of DNA/mRNA per copies of β-actin. All samples were analyzed in duplicate, and results are expressed as mean copies± standard error of mean. Sequences for the real-time primers/probes designed were as follows: β-actin forward: attgtgatggactccggtga, reverse: agctcatagctcttctccag, probe: cacccacactgtgcccatctac, CMV GB; forward: tgtactcgaagggagagct, reverse: cgttcaccaccgaagacac, and probe: cgcctcgaacgtgttcagcctg. For cytokine analyses, primers for IL-1, TNF-α and IFN-γ were obtained from Super Array (Biosciences). Sequences of other primers are as follows from 5′ to 3′: IL-2, forward: tcactcctcacagtgacctcaagt; reverse, agcg cttactttgtgctgtcct; IL-12α, forward: tgtcttagccagtcccgaaacct, reverse: gtgaagcaggatgcagagcttcatt; IL-15, forward: ccatctc gtgctacttgtgtttcct, reverse: caggacgtgttgatgaacatttggac; IL-7α, forward: atccacctcacacgaggcacaagt, reverse: caaacacgaagca gtttgggacc; IFN-α, forward: tgtctgatgcagcaggtgg, reverse: aag acagggctctccagac; GAPDH, forward: aactttggcattgtggaagg, and reverse: acacattgggggtaggaaca.

PCR reactions were carried out using a Smart-Cycler (Cepheid, Sunnyvale, CA), using the following program: initial denaturation 4 min at 94°C, 35 cycles of denaturation for 30 sec at 94°C, annealing for 30 sec at 53°C, elongation for 30 sec at 72°C, followed by final elongation for 7 min at 72°C, then hold at 4°C.

Statistics

Statistical analyses utilized one-way ANOVA or one- or two-tailed Student's t-test. p Values ≤0.05 were considered significant for all testing. Means are expressed as mean ± standard error. Statistical software used was GraphPad Prism (Version 4.03; GraphPad Software, San Diego, CA).

Results

CD28/B7 co-stimulation is critical for early viral control

To evaluate influences of the co-stimulatory molecules B7 and CD28 on early viral control, wild-type, $B7^{-/-}$, and $CD28^{-/-}$C57BL/6 mice (H-2^b) were infected with MCMV. Viral titers were measured 2 and 6 dpi using both IVPA and quantitative real-time PCR for CMV DNA of tissue homogenates from spleen and liver.

In both liver and spleen, there were significantly elevated MCMV titers (threefold) by day 2 in $CD28^{-/-}$ mice, while viral titers in $B7^{-/-}$ mice were not significantly different than wild-type mice (Fig. 1A and B). Previous studies have shown that 6 d after MCMV infection, MCMV-specific NK subset expansion peaks, and anti-viral T-cell expansion is beginning (20,21,58). In the current study viral titers in both $B7^{-/-}$ and $CD28^{-/-}$ mice were significantly elevated (~2 logs) compared to wild-type mice in both liver and spleen 6 dpi (Fig. 1). Interestingly, $CD28^{-/-}$ mice had significantly higher viral titers (double) than $B7^{-/-}$ mice in both tissues by 6 dpi (Fig. 1A and B). In addition, hepatic titers were slightly but significantly higher than splenic titers at both time points (comparison not shown). Viral DNA loads from all tissues correlated very well with IVPA titers, further confirming IVPA results ($R^2 = 0.87$; data not shown). We therefore conclude that both B7 and CD28 molecules play essential roles in early control of primary MCMV infection.

CD28 and B7 are required for T-cell expansion in response to MCMV

CD8 T-cells are known to be vitally important to viral control following MCMV infection (16,53,55,56). Because CD28/B7 co-stimulation is known to be variably important to T-cell responses in other models of viral infection (8,18,44,49), one possible explanation for the observed differences in viral titers was that T cells in these knockout mice do not respond normally to MCMV without co-stimulation. To test this hypothesis, we evaluated $CD3^+$ $NK1.1^-$ T-cell expansion in liver and splenic tissue following acute MCMV infection using flow cytometry to measure total numbers and cell division.

As previously published, wild-type mice showed significant expansion of hepatic T-cell counts by 6 dpi, which corresponded with increased BrdU incorporation (Fig. 2A and B). In contrast, $B7^{-/-}$ and $CD28^{-/-}$ mice had significantly lower hepatic T-cell counts at 6 dpi ($p = 0.04$, $p = 0.03$; Fig. 2A and B). Although this corresponded to impaired hepatic T-cell division, as suggested by impaired BrdU incorporation, which was significantly lower in $B7^{-/-}$ and $CD28^{-/-}$ mice

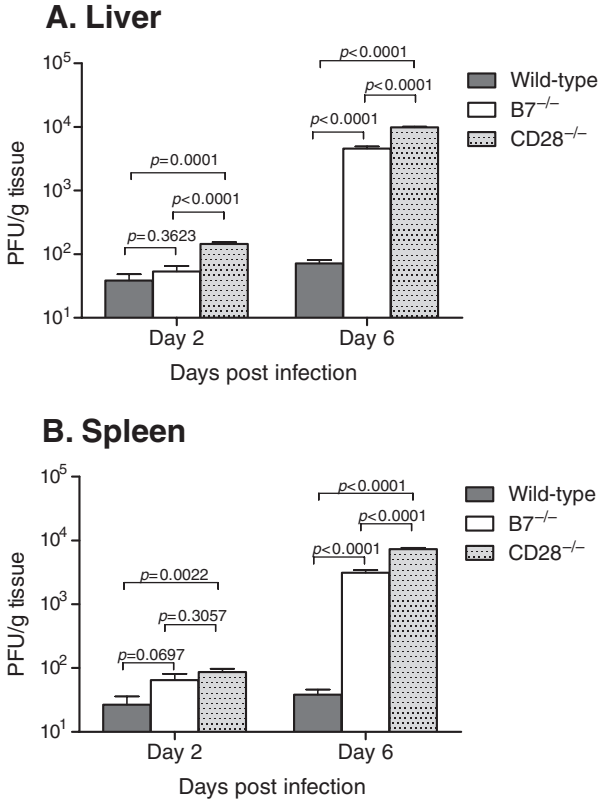


FIG. 1. Effect of co-stimulation upon viral titers during acute MCMV infection. Wild-type C57BL/6, $B7^{-/-}$, or $CD28^{-/-}$ knockout mice were infected with Smith MCMV (5×10^4 PFU) or receiving vehicle (PBS), and liver (**A**) and spleen (**B**) tissues were acquired 2 or 6 dpi. Tissues were analyzed by plaque assay to determine viral titers, and results are reported in plaque-forming units (PFU) per gram of tissue. Each data bar represents 3 mice. The data shown are representative of three comparable experiments.

than in wild-type mice ($p = 0.01$, $p = 0.002$; Fig. 2B), it is also possible that low hepatic T-cell counts could be consequent to impaired hepatic T-cell migration or T-cell death.

In contrast to liver, overall splenic T-cell counts in $B7^{-/-}$ and $CD28^{-/-}$ mice were comparable to those of wild-type mice ($p = 0.88$, $p = 0.36$; Fig. 2A and B). Curiously, this was observed despite significant impairment of T-cell division in $B7^{-/-}$ and $CD28^{-/-}$ mice as demonstrated by decreased BrdU incorporation ($p = 0.0007$, $p = 0.05$; Fig. 2A and B). Despite maintenance of normal numeric T-cell responses, knockout mice have dramatically elevated splenic viral titers compared to wild-type mice 6 dpi (Fig. 1B). One possible explanation for these observations is that small changes in T-cell numbers driven by antigen-specific T-cell proliferation could be obscured in the spleen, as this is a primary lymphoid organ. Similar analyses of $CD3^+NK1.1^+$ cells (NKT) showed no significant expansion, and no significant differences between wild-type and knockout mice (data not shown). From these studies we conclude that CD28/B7 co-stimulation is critical to T-cell division and subsequent peripheral expansion after infection with MCMV, and additionally that both B7 and CD28 influence accumulation of T cells in the liver.

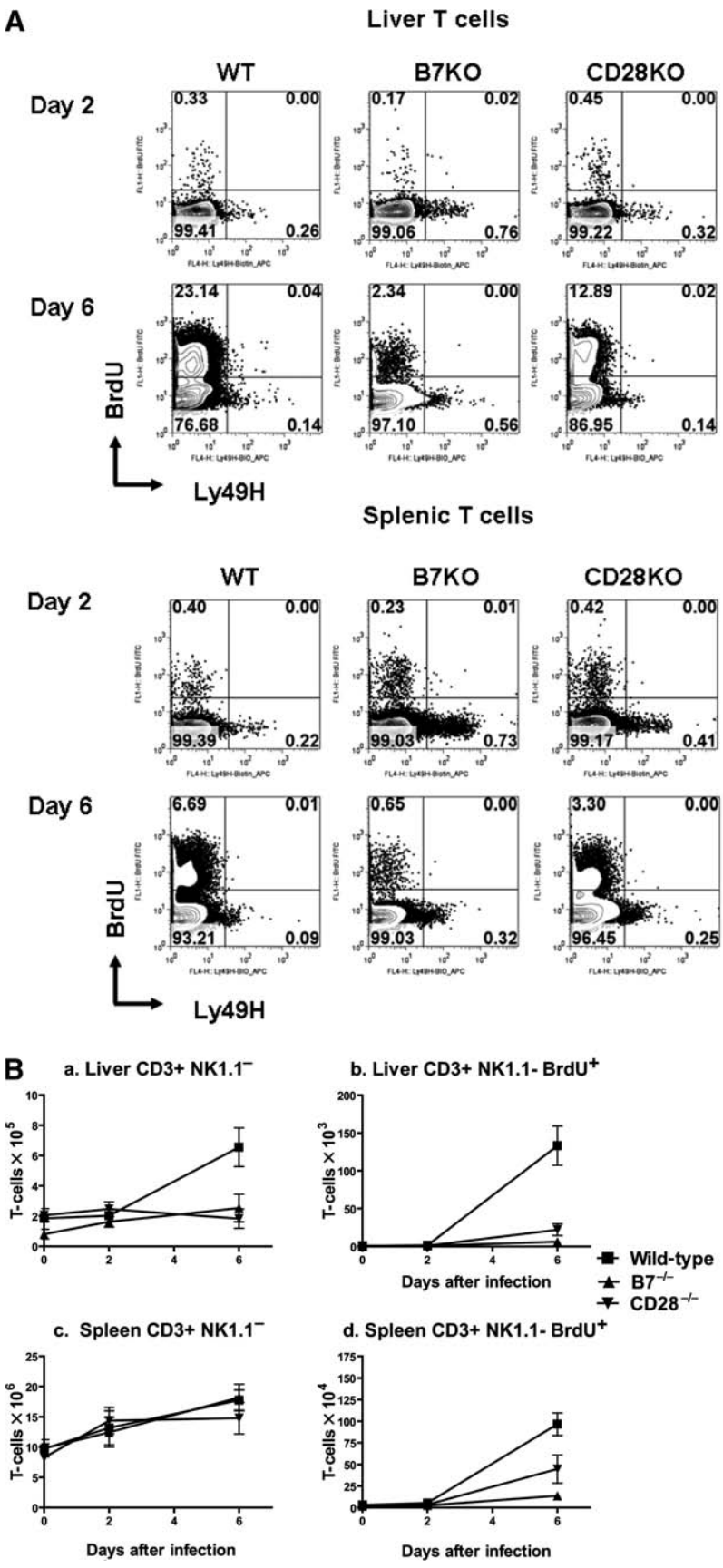
CD28, but not B7, is required for expansion of Ly49H$^+$ NK cells in liver

Robbins *et al.* have recently shown that early T-cell responses and viral control of MCMV are dependent upon preceding NK-cell responses (57). Therefore we considered the possibility that diminished T-cell proliferative responses, and elevated viral titers in spleen and liver, could also be additionally consequent to abnormal NK-cell responses in mutant mice. NK-cell responses to MCMV infection in normal mice begin at 2 dpi as non-specific responses, and peak at 6 dpi, with subset expansion of $Ly49H^+$ cells (20,21,58). We therefore hypothesized that the impairment of early viral control observed in $CD28^{-/-}$ and $B7^{-/-}$ mice could be consequent to inadequate NK-cell expansion, and compared expansion between $Ly49H^+$ and $Ly49H^-$ NK cells in livers after MCMV infection.

MNCs isolated from livers after MCMV infection were evaluated for $Ly49H^+$ and $Ly49H^-$ NK-cell expansion by flow cytometry as shown in Fig. 3A, and summarized in Fig. 3B. Confirming previously published findings, Fig. 3B-a shows significant expansion from baseline of $Ly49H^+$ NK cells in wild-type mice at day 2 of MCMV infection ($p = 0.01$) that rise more than fivefold by day 6 of infection ($p < 0.01$) (20,21,58). This expansion is due to $Ly49H^+$ NK-cell division, as confirmed by significant increases of BrdU incorporation in this subset at 2 and 6 dpi in wild-type mice (Fig. 3B-b; $p < 0.01$ for both time points, compared to baseline). In contrast, non-specific $Ly49H^-$ NK-cell expansion and division were modest (Fig. 3B-c and d), which has also been consistently described by others (20,21,58).

As shown in Fig. 3B, CD28 appears to be important for MCMV-specific NK subset expansion/accumulation in liver tissue during MCMV infection. Unlike wild-type mice, $CD28^{-/-}$ mice do not develop $Ly49H^+$ NK-cell expansion/accumulation, showing no significant increase in $Ly49H^+$ NK counts above baseline ($p > 0.05$ for either time point;

FIG. 2. Co-stimulation in T-cell responses during acute MCMV infection. Wild-type C57BL/6 (WT), $B7^{-/-}$ (B7KO), or $CD28^{-/-}$ knockout (CD28KO) mice infected with Smith MCMV (5×10^4 PFU) or vehicle had liver and spleen tissues removed 2 or 6 dpi. Three hours prior to organ removal, each mouse received IP 1.0 mg of BrdU. Single splenocyte preparations or hepatic MNCs were prepared, stained with mAb to CD3 (PerCP-Cy5.5), NK1.1 (APC), and Ly49H (PE), followed by intracellular staining with mAb to BrdU (FITC) as described in materials and methods, and analyzed by flow cytometry. $CD3^+NK1.1^-$ T cells were gated and analyzed for BrdU and Ly49H expression. Note that a small portion of $CD3^+NK1.1^-BrdU^-$ T cells expressed Ly49H, consistent with our previous observation (unpublished data). (**A**) Representative flow cytometric plots of liver and spleen from mice at 2 or 6 dpi. (**B**) T-cell numbers for each group at various time points post infection from a representative experiment. Each data point represents mean ± standard error for 3 mice. The experiment was repeated twice with similar results.


A
Liver T cells
WT
B7KO
CD28KO
Day 2
0.33
0.00
99.41
0.26
0.17
0.02
99.06
0.76
0.45
0.00
99.22
0.32
Day 6
23.14
0.04
76.68
0.14
2.34
0.00
97.10
0.56
12.89
0.02
86.95
0.14
BrdU
Ly49H
Splenic T cells
WT
B7KO
CD28KO
Day 2
0.40
0.00
99.39
0.22
0.23
0.01
99.03
0.73
0.42
0.00
99.17
0.41
Day 6
6.69
0.01
93.21
0.09
0.65
0.00
99.03
0.32
3.30
0.00
96.45
0.25
BrdU
Ly49H
B
a. Liver CD3+ NK1.1−
T-cells × 10^5
b. Liver CD3+ NK1.1- BrdU+
T-cells × 10^3
Days after infection
Wild-type
B7−/−
CD28−/−
c. Spleen CD3+ NK1.1−
T-cells × 10^6
Days after infection
d. Spleen CD3+ NK1.1- BrdU+
T-cells × 10^4
Days after infection

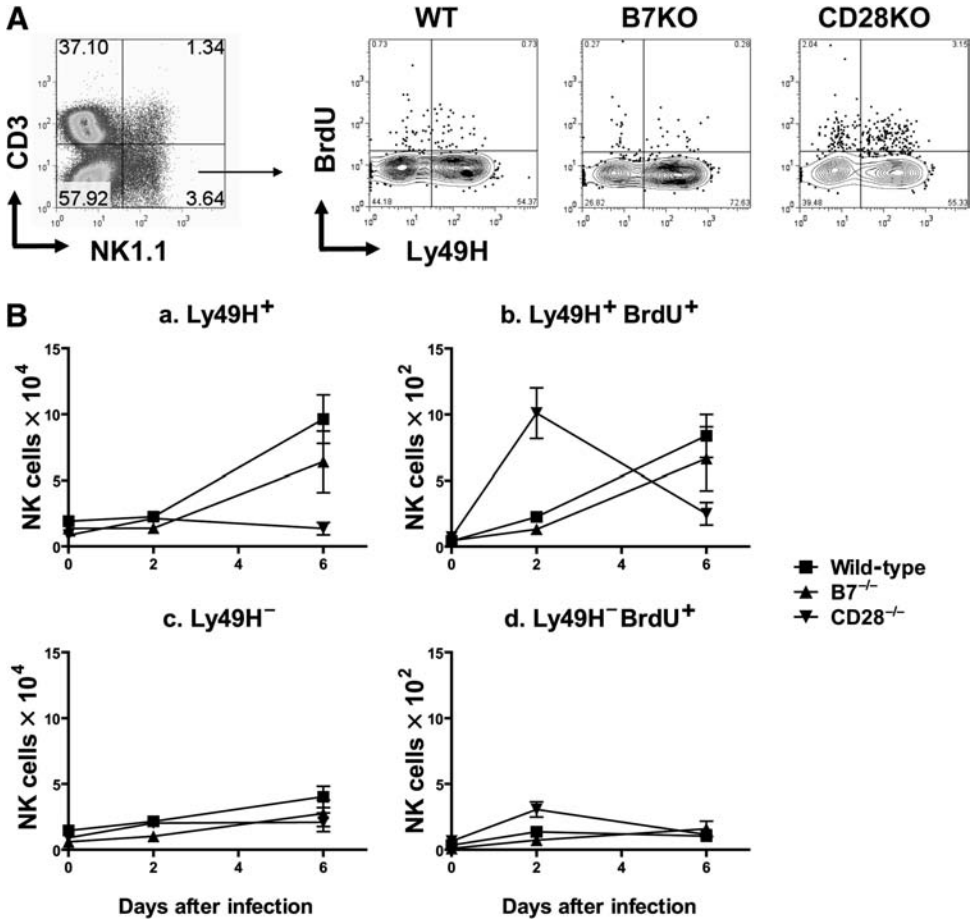


FIG. 3. Effect of co-stimulation on LY49H$^+$ NK-cell expansion in liver during acute MCMV infection. Wild-type C57BL/6, B7$^{-/-}$, or CD28$^{-/-}$ knockout mice infected with Smith MCMV (5×10^4 PFU) had liver tissues removed 2 or 6 dpi. Three hours prior to organ removal, each mouse received BrdU. Hepatic MNCs were prepared and NK cells were enumerated and had division estimated by four-color flow cytometry. The MNCs were stained with fluorescent dye-conjugated mAbs to CD3 (PerCP-Cy5.5), NK1.1 (PE), and Ly49H (APC) for cell surface markers, and intracellular staining with mAb to BrdU (FITC) as described in materials and methods. Percentages of each subset (CD3$^-$NK1.1$^+$Ly49H$^+$, CD3$^-$NK1.1$^+$Ly49H$^-$, CD3$^-$NK1.1$^+$Ly49H$^+$BrdU$^+$, or CD3$^-$NK1.1$^+$Ly49H$^-$BrdU$^+$) were determined and then multiplied by numbers of bulk MNCs. (**A**) Representative flow cytometric plots at 2 dpi from CD3$^-$NK1.1$^+$ gated cells. (**B**) Mean cell numbers of CD3$^-$NK1.1$^+$ gated Ly49H$^+$ and Ly49H$^-$ NK cells and BrdU incorporation in hepatic MNC over time. Each data point represents mean ± standard error of mean for 3 mice. This experiment was performed twice with comparable results.

Fig. 3B-a). In fact, CD28$^{-/-}$ mice showed no significant difference between Ly49H$^+$ (specific) and Ly49H$^-$ (non-specific) NK-cell counts at 6 dpi ($p > 0.05$; compare Fig. 3B-a and c). Curiously, this deficit in Ly49H$^+$ NK cells occurred despite significantly higher division rates in Ly49H$^+$ NK cells at 2 dpi in CD28$^{-/-}$ mice ($p < 0.01$; Fig. 3B-b). It is possible that this enhanced division was in part non-specific, as there was a concomitant similar, albeit smaller, elevation in Ly49H$^-$ NK-cell division at this same time ($p < 0.05$; Fig. 3B-d). Despite this, by 6 dpi, Ly49H$^+$ NK counts were significantly lower than wild-type controls, and Ly49H$^-$ NK counts were no different than wild-type controls in liver tissues. Thus CD28 appears to be required for specific Ly49H$^+$ NK-cell accumulation in the liver of infected mice, but does not appear to influence non-specific NK-cell responses to MCMV infection. It is unclear whether failed NK-cell expansion in CD28$^{-/-}$ mice livers reflects increased apoptosis in this population, and/or simply failure of normal recruitment of antigen-specific NK cells to the liver.

In contrast to CD28, B7 expression plays little or no role in expansion/accumulation of hepatic NK cells after infection. Fig. 3B-a shows that Ly49H$^+$ NK cells in B7$^{-/-}$ mice significantly expand by 6 dpi compared to baseline. Thus wild-type and B7$^{-/-}$ mice showed no significant difference in Ly49H$^+$ NK-cell counts at 6 dpi ($p = 0.32$), and BrdU incorporation in Ly49H$^+$ NK cells was also similar between wild-type and B7$^{-/-}$ mice (Fig. 3B-b).

Neither CD28 nor B7 are required for expansion of splenic Ly49H$^+$ NK cells

To explore whether co-stimulation is required for splenic NK-cell expansion, we evaluated Ly49H$^+$ and Ly49H$^-$ NK cells after infection by flow cytometry. In contrast to liver, and similar to our T-cell findings, there was no significant difference in splenic Ly49H$^+$ NK-cell numbers between wild-type, B7$^{-/-}$, or CD28$^{-/-}$ mice at either 2 or 6 dpi ($p = 0.56$ and $p = 0.95$, respectively; Fig. 4A and B), although splenic expansion

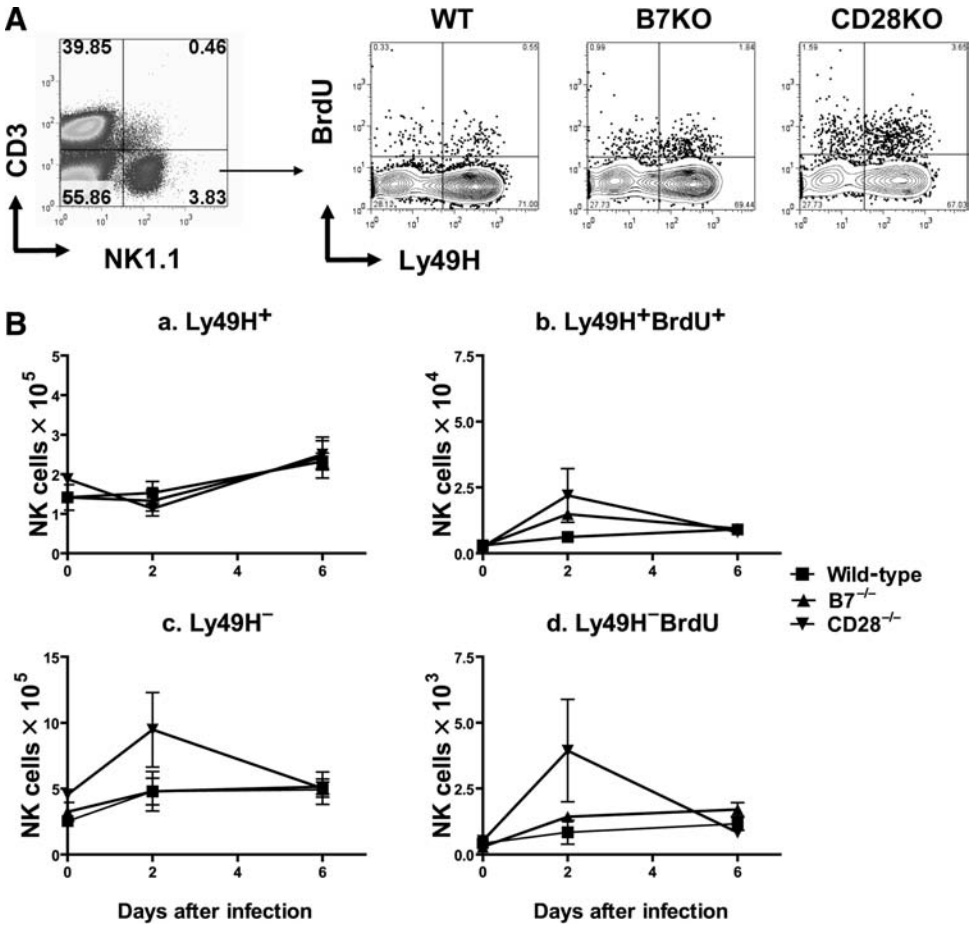


FIG. 4. Effect of co-stimulation on splenic NK-cell expansion during acute MCMV infection. Wild-type C57BL/6 (WT), $B7^{-/-}$ (B7KO), or $CD28^{-/-}$ knockout (CD28KO) mice infected with Smith MCMV (5×10^4 PFU) or vehicle had splenic tissues removed 2 or 6 dpi. Three hours prior to organ removal, each mouse received IP 1.0 mg BrdU. $CD3^{-}NK1.1^{+}$ gated splenocytes were enumerated and had division estimated by flow cytometry, as described in Fig. 3. (**A**) Representative flow cytometric plots 2 dpi. (**B**) $Ly49H^{+}$ and $Ly49H^{-}$ NK-cell numbers and BrdU incorporation in spleens over time. Each data point represents mean ± standard error for 3 mice. This experiment was performed twice with comparable results.

of $Ly49H^{+}$ NK cells was much more modest (~twofold) than that seen in livers (>fivefold). In wild-type mice, MCMV-specific $Ly49H^{+}$ NK cells showed a non-linear increase by day 6 (Fig. 4B), a result that has been previously published (20,21). The initial decline in splenic NK counts at 2 dpi has been previously postulated to be secondary to either redistribution of splenic NK cells to more peripheral sites of infection, or to apoptosis or consumption of these cells in the spleen (20).

Fig. 4B-b and c show the effects of B7 and CD28 on splenic NK-cell division following MCMV infection. Both $B7^{-/-}$ and $CD28^{-/-}$ mice show small but statistically significant increases in BrdU incorporation in $Ly49H^{+}$ NK cells 2 dpi when compared to wild-type mice ($p < 0.05$). Nonetheless, this increased division corresponds to either no change or slight decreases in splenic $Ly49H^{+}$ NK-cell numbers on day 2 (Fig. 4B-a). Although the hypothesis that these cells might be redistributing to other sites of infection is sensible, there is no evidence that this occurs on day 2 post infection in the liver, when liver counts of $Ly49H^{+}$ NK cells are not significantly increased in either $B7^{-/-}$ or $CD28^{-/-}$ mice (Fig. 3B). Six days after infection, wild-type, $B7^{-/-}$, and $CD28^{-/-}$ mice show comparable BrdU incorporation that is significantly elevated from baseline. This cell division correlates with modest increases in $Ly49H^{+}$ NK-cell numbers in splenic tissues by 6 dpi (Fig. 4B-a and c). Importantly, when cell division rates for splenic $Ly49H^{+}$ and $Ly49H^{-}$ NK cells are compared, there is identical BrdU incorporation in $Ly49H^{-}$ NK cells for wild-type and knockout mice (compare Fig. 4B-b and d). Thus it seems that non-specific NK-cell expansion is intact in knockout mice, and that splenic NK expansion in response to MCMV occurs by a non-specific mechanism that does not require CD28 or B7 co-stimulation.

Effects of co-stimulatory molecules on early cytokine production

As an alternative hypothesis, it is possible that $B7^{-/-}$ or $CD28^{-/-}$ mice have some fundamental defect in early cytokine responses that could explain the observed defects in T- and NK-cell expansion independent of co-stimulation. To test this hypothesis, we examined cytokine mRNA in liver and splenic tissues from wild-type and $CD28^{-/-}$ mice, including IFN-α, IFN-γ, IL-1, IL-2, IL-12, IL-15, IL-17, and

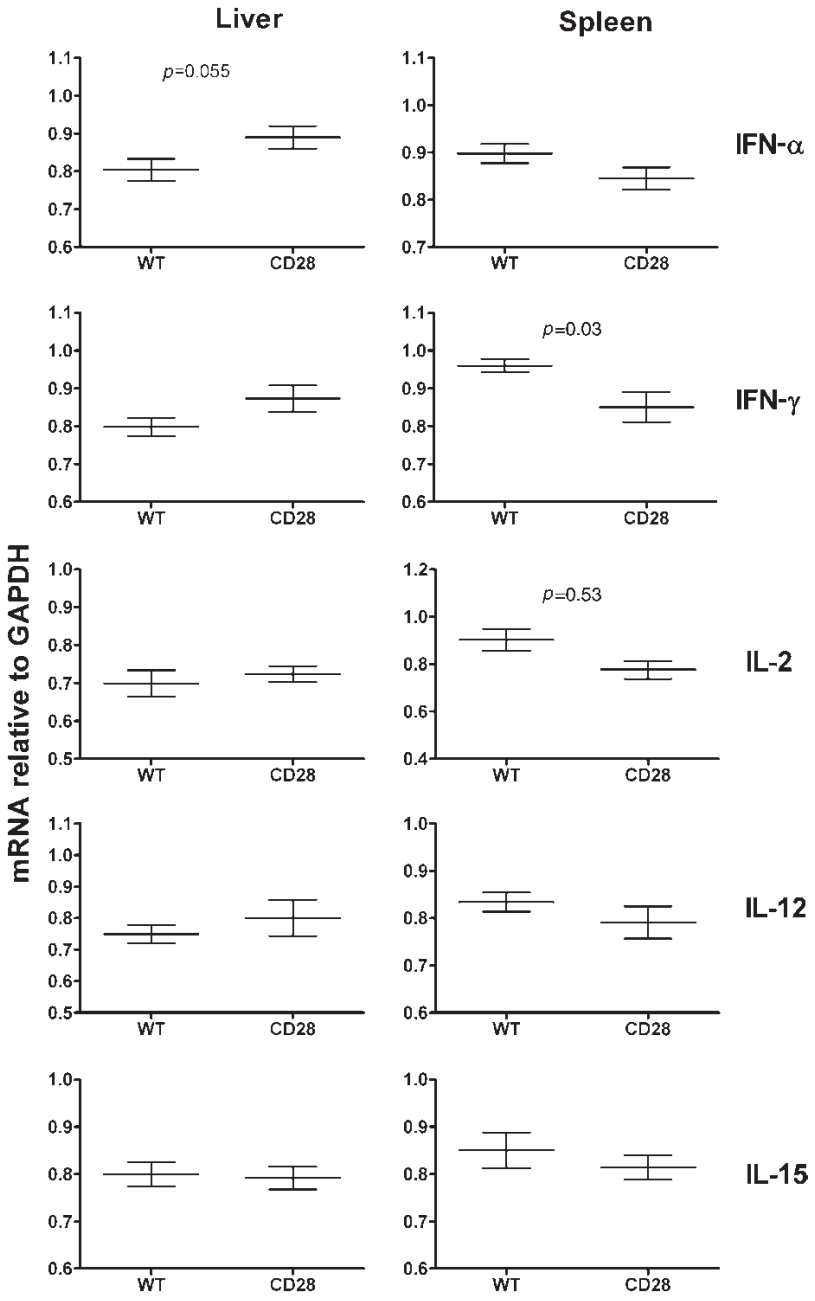


FIG. 5. Effect of co-stimulation of early cytokine production upon MCMV infection. Wild-type C57BL/6 or $CD28^{-/-}$ knockout mice infected with Smith MCMV (5×10^4 PFU) had liver and splenic tissues evaluated at 2 dpi. Total RNA was prepared and subjected to real-time PCR analysis for IL-1, IL-2, IL-12, IL-15, IL-17, IFN-γ, TNF-α, and IFN-α. There were no significant differences in IL-1, IL-17, or TNF-α transcripts between wild-type and $CD28^{-/-}$ mice in the liver or the spleen (not shown). Data shown are arbitrary transcript units (relative to GAPDH) for IFN-α, IFN-γ, IL-2, IL-12, and IL-15, in liver or spleen at 2 dpi. Significant differences are noted by *p* values in this figure. Each data point represents mean ± standard error of mean for 3–4 mice.

TNF-α, at both 2 and 6 dpi. There were no significant differences between $CD28^{-/-}$ and wild-type mice for any cytokine transcripts at 6 dpi in either tissue (data not shown). There were several differences noted at 2 dpi, which are illustrated in Fig. 5 and discussed below.

Several cytokines are known to be critical to T-cell responses to MCMV, including IFN-α and γ, IL-2, and IL-12 (43,57). There were no significant differences in IL-2 transcription in hepatic tissues at 2 or 6 dpi that might explain abnormal T-cell numbers and division in $CD28^{-/-}$ mice (Fig. 5). There were, however, elevated hepatic IFN-α transcripts observed in $CD28^{-/-}$ mice at 2 dpi that became comparable to wild-type by 6 dpi (Fig. 5). It has been previously shown that a lack of early viral control by NK cells leads to overexpression of IFN-α after MCMV infection, and that elevated IFN-α impairs T-cell responses to MCMV (57). Although there were decreased levels of IL-2 and IFN-γ transcripts observed in spleens of $CD28^{-/-}$ mice at 2 dpi

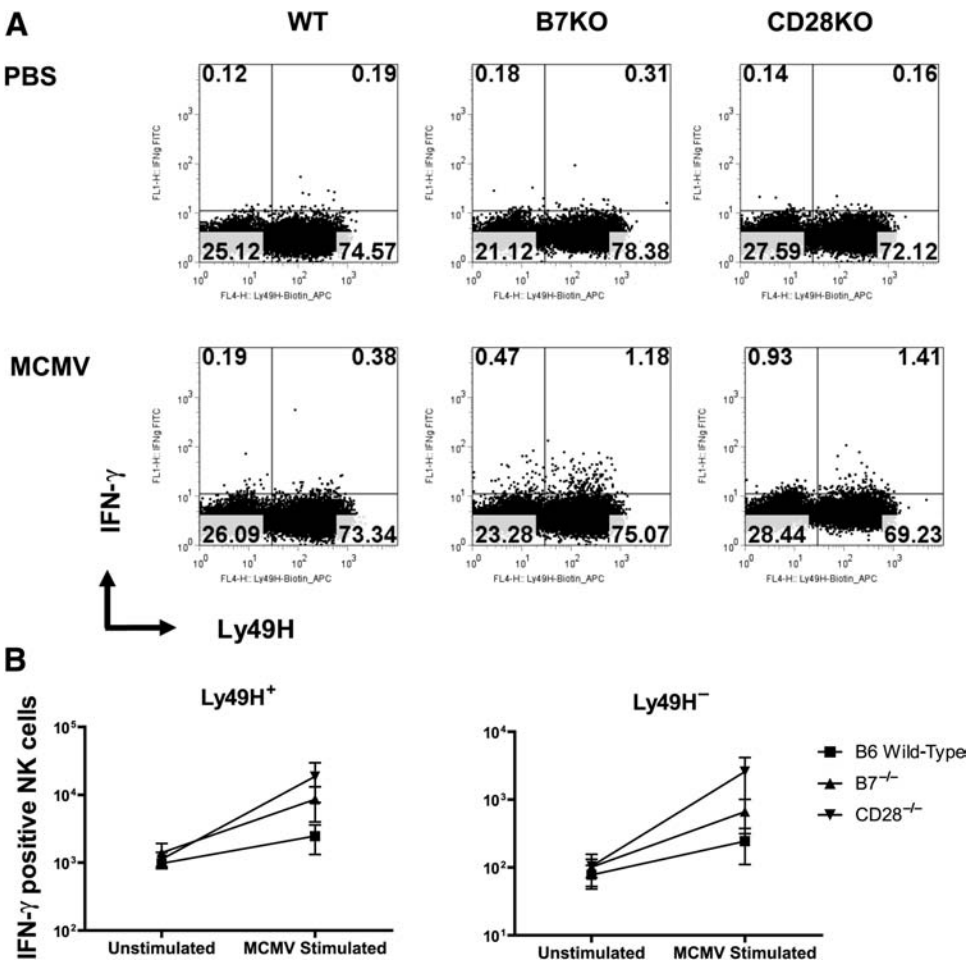


FIG. 6. Co-stimulation and Ly49H$^+$ NK-cell IFN-γ responses during acute MCMV infection. Wild-type C57BL/6 (WT), B7$^{-/-}$ (B7KO), or CD28$^{-/-}$ (CD28KO) knockout mice infected with Smith MCMV (5×10^4 PFU) or receiving vehicle (PBS) had splenic tissues removed at 2 dpi. Single splenocytes were prepared, stained with mAb to CD3 (PerCP-Cy5.5), NK1.1 (PE), and Ly49H (APC), followed by intracellular staining with FITC-conjugated mAb to IFN-γ and analysis by four-color flow cytometry. CD3$^-$NK1.1$^+$ cells were gated as described in Fig. 3, and further analyzed for Ly49H$^+$ and IFN-γ expression. Ly49H$^+$ NK cells expressing IFN-γ were enumerated. (**A**) Representative of flow cytometric plots for Ly49H$^+$IFN-γ^+ NK cells from each group. (**B**) Numbers of IFN-γ-producing Ly49H$^+$ and Ly49H$^-$ NK cells in each group. Each data point represents mean $\pm$ standard error of mean for 3 mice.

compared to wild-type mice, these returned to normal by 6 dpi (not shown), and these deficits did not appear to influence overall splenic T-cell numbers after infection (Fig. 2).

Furthermore, there are several cytokines known to be critical in early NK responses to MCMV, including IFN-α, IL-2, IL-12, and IL-15 (45,46). Importantly, there were no significant differences in these cytokine transcripts in liver between wild-type and CD28$^{-/-}$ mice to explain defective hepatic Ly49H$^+$ NK-cell expansion observed in CD28$^{-/-}$ mice (Fig. 5). IL-2, IL-12, and IL-15 levels were comparable in wild-type and CD28$^{-/-}$ mice, and as previously mentioned, day 2 liver IFN-α mRNA levels were actually higher in CD28$^{-/-}$ mice. Although IL-2 levels were lower in spleens at 2 dpi, this did not appear to influence splenic NK responses (Fig. 4). Finally, there were no defects in non-specific Ly49H$^-$ NK-cell expansion after infection in knockout mice when compared to wild-type mice in either liver (Fig. 3) or spleen (Fig. 4), which functionally confirms that non-specific responses to MCMV are intact in these knockout mice.

Because IFN-γ is a dominant cytokine produced by NK cells during MCMV infection (21), we examined whether co-stimulation influences early IFN-γ production by NK cells in response to MCMV infection. As shown in Fig. 6, numbers of Ly49H$^+$ IFN-γ-producing NK-cells in spleens 2 days after infection are not impaired in knockout mice. One might anticipate early NK IFN-γ responses to be consequent to m157-Ly49H interaction, but this does not appear to be the case at 2 dpi, because Ly49H$^-$ NK cells showed the same fold increase in IFN-γ expression at 2 dpi (Fig. 6B). These findings also suggest that the decreased transcription of IFN-γ observed in whole spleen lysates (Fig. 5) is occurring outside the NK-cell compartment. Although wild-type splenic Ly49H$^+$ NK cells expressing IFN-γ are numerically somewhat lower than those previously published at this time point, we did not stimulate our cells *ex vivo* to elicit this response. Unfortunately, hepatic NK cells were too few to allow similar analysis of IFN-γ-producing cells by flow cytometry (all were used in Ly49H enumeration and BrdU experiments), but transcripts

of IFN-γ at 2 and 6 dpi were comparable in liver between wild-type and CD28$^{-/-}$ mice (Fig. 5).

Interpreting these cytokine results is somewhat complicated by differences in viral titers in knockout mice, as well as a lack of cellular fraction–specific analyses. Differences in IFN-γ expression in splenocyte lysates that were not observed in NK cells highlight the possibility that splenocyte and hepatic lysates could mask subtle differences in cytokine production in individual cell subsets. Nonetheless, there appear to be no glaring defects in cytokine responses to infection in CD28 knockout mice, and taken together, these observations suggest that non-specific cytokine responses to MCMV are intact in CD28$^{-/-}$ mice.

Discussion

This study confirms that the co-stimulatory molecules B7 and CD28 are critical to host viral control during acute MCMV infection. Our viral titer data clearly demonstrate that both molecules are required for host immune control of virus replication following acute MCMV infection in both hepatic and splenic tissues. This influence in viral titers began as early as 2 dpi, becoming more dramatic at 6 dpi in mice lacking B7$^{-/-}$ or CD28$^{-/-}$. Early responses to MCMV (days 1–2) are known to be largely a consequence of APC release of cytokines, resulting in non-specific NK-cell expansion (3,19,47), while later viral control (day 6) is dependent upon antecedent non-specific and MCMV specific NK-cell responses, as well as subsequently developing T-cell responses (14,16,33,48,50,55,57). Because of the known importance of co-stimulation in adaptive immune responses, we further explored influences of co-stimulation on T- and NK-cell responses in these knockout mice.

It is not entirely surprising that co-stimulation is critical to MCMV control, given recent data demonstrating the importance of B7 during herpes simplex virus and Epstein-Barr virus infection (44,70,71). Further data supporting this hypothesis include MCMV targeting of DC responsiveness and co-stimulation as part of its immune evasion armamentarium (2,37,41,42). B7 proteins enhance NK-mediated killing, and conversely, B7-1–deficient APCs display significant reductions in their ability to promote T-cell activation (23,39,75). Thus circumstantially, it seemed likely that co-stimulation via B7 and CD28 would be critical to MCMV control after acute infection. Despite these circumstantial data, there are other viruses, such as lymphocytic choriomeningitis virus, that actually develop vigorous CD8 responses without co-stimulation (49). Thus ours are the first data of which we are aware to definitively prove that co-stimulation is required for early control of CMV infection.

Antigen presentation and co-stimulation are known to be critical in development of normal T-cell responses in many antigen systems. Interestingly, MCMV has been shown to cause "immune paralysis" in DCs, which manifests as reduced release of IL-2 and IL-12, and impairment of T-cell activation (2,28). This impairment has recently been attributed to viral genes *m138* and *m147.5*, which cause down-regulation of the co-stimulatory molecules B7-1 (CD80) and B7-2 (CD86) (37,42) on DCs. Our studies confirm the importance of CD28/B7 co-stimulation in the development of T-cell responses to MCMV *in vivo*, as knockout mice showed impaired hepatic T-cell expansion and accumulation. This may be a direct consequence of diminished T-cell division, as there was significantly lower T-cell BrdU incorporation following MCMV infection in both B7$^{-/-}$ and CD28$^{-/-}$ mice compared to wild-type mice. It is possible that poor expansion of T cells in both B7$^{-/-}$ and CD28$^{-/-}$ mice is a consequence of early abnormalities in splenic IL-2 or IFN-γ transcription, but this will require further study. Overall, it is likely that this impaired T-cell response at least contributes to the elevated viral titers seen at 6 dpi.

Much like APC/T-cell interactions, APC/NK interactions have also been shown to be critical in development of NK-cell responses to MCMV. Early during MCMV infection, APCs produce IFN-α/β and IL-12, causing IFN-γ-dependent non-specific NK expansion *in vivo* (3,19,47). In addition to these non-specific NK responses, MCMV-specific Ly49H$^+$ NK subset expansion occurs 4–8 days after infection, consequent to stimulatory receptor Ly49H$^+$ activation (20), presumably activated by binding ligand m157 (12,64,73). This subset expansion has been reported to peak at 6 dpi in both liver and spleen tissue (3,20,21,58), and we reproduced these results in wild-type mice. Recent work has shown that interaction between CD8α^+ DC and NK cells is important for expansion of this MCMV-specific Ly49H$^+$ NK subset in response to acute infection (3). Altogether these observations by others are quite novel, demonstrating for the first time that NK cells, long considered to be central to "innate" immunity, actually have "adaptive" characteristics.

Because of these adaptive characteristics, and the obvious importance of APC/NK interactions, we postulated that similar to T cells, co-stimulation might be important to expansion of MCMV-specific NK-cell subsets. Indeed, CD28$^{-/-}$ mice had significantly reduced hepatic Ly49H$^+$ NK subset expansion, which correlated with significant impairment in Ly49H$^+$ NK-cell division at 6 dpi. In contrast, B7 appeared dispensable to MCMV-specific NK-subset expansion, as B7$^{-/-}$ mice developed normal hepatic Ly9H$^+$ NK counts and BrdU incorporation. Nonetheless it was conceivable that deficits in Ly49H$^+$ NK-subset expansion in CD28$^{-/-}$ mice could be explained by some fundamental defect in innate responses in these mice. Importantly, there were no significant differences in IFN-α, IFN-γ, IL-12, or IL-15 transcripts after infection that might explain impaired NK-subset expansion in CD28$^{-/-}$ mice. Thus, although not definitively proven, our data suggest that CD28 co-stimulation enhances subset expansion of peripheral NK cells. This provocative observation will require further study.

The mechanism by which CD28 might participate in hepatic NK-cell expansion is currently not known. MCMV-specific NK expansion is somewhat unique in that it does not depend upon MHC-I antigen presentation, but instead relies upon direct recognition of m157 (an MHC-I homologue) expressed on the surface of infected cells by activating receptor Ly49H$^+$ (72). We speculate that just as MHC/TCR signaling and antigen-specific T-cell expansion is enhanced by CD28/B7 co-stimulation, m157/Ly49H signaling may be similarly enhanced, and therefore lack of CD28 stimulation leads to the depressed NK responses seen in CD28$^{-/-}$ mice. Unfortunately, this hypothesis doesn't fit neatly with our results, as B7$^{-/-}$ mice do not show the same effect. This is in contrast to the utter dependence of peripheral T-cell responses upon both co-stimulatory molecules. At best, we can consider that peripheral NK-cell subset expansion is influenced by co-stimulatory molecule

CD28, but appears independent of co-stimulatory ligand B7. If true, this also suggests that CD28 might recognize a different B7 family ligand in $B7^{-/-}$ mice that facilitates MCMV-specific NK-subset expansion.

Although co-stimulation appears to be important to splenic T-cell division in response to MCMV infection, the decreased T-cell division observed in knockout mice did not negatively impact overall splenic T-cell counts. This could simply be because splenocytes responsive to MCMV comprise a small percentage of the splenocyte population, or that day 6 is too early to detect significant differences in T-cell counts. Alternatively, splenic expansion of T cells after MCMV infection might not be solely reliant on increased cell division co-stimulated by B7 or CD28 molecules, but may occur by some other mechanism, such as resistance to activation-induced T-cell death. In contrast, splenic NK-cell expansion appears to be altogether independent of co-stimulatory influence, occurring primarily by non-specific expansion, as shown by equal expansion and division of both $Ly49H^+$ and $Ly49H^-$ NK cells in wild-type and knockout mice. This is consistent with previously published hypotheses that splenic NK-cell expansion occurs by a mechanism different than that seen in the liver (68,69). Although this issue of splenic NK expansion has become somewhat controversial (38,68,69), it would appear that whatever the mechanism, splenic NK- and T-cell expansion occurs independent of co-stimulation by B7 or CD28. It is interesting to note that development of relatively "normal" MCMV-specific NK-cell and T-cell numbers in splenic tissues was associated with better viral control in spleen tissues compared to liver (comparison not shown). Nonetheless, splenic viral titers were still much higher in both $B7^{-/-}$ and $CD28^{-/-}$ than wild-type mice, suggesting that there is some important immune interaction missing in these knockout mice.

Interestingly, at almost every time point, $CD28^{-/-}$ mice had significantly higher viral titers than $B7^{-/-}$ mice. While this could be explained simply by CD28 being relatively more important to viral control, it is also possible that a lack of CD28 molecules could have additional consequences. $CD28^{-/-}$ mice still express B7 molecules, which can also bind CTLA-4 and PD-L1, known inhibitory receptors (15,23,31,40). It is therefore possible that absence of CD28 could cause unopposed B7/CTLA-4 or PD-L1 binding and signaling, downregulating adaptive responses to MCMV. This could explain the differences in viral titers between $CD28^{-/-}$ and $B7^{-/-}$ mice observed in livers as early as 2 dpi, and in both liver and spleen by 6 dpi. Alternatively, because T-cell responses are in part dependent upon preceding NK-cell responses (57), it is also possible that $B7^{-/-}$ mice, which develop normal NK but abnormal T-cell responses, have slightly better viral control than $CD28^{-/-}$ mice, which have sequential impairment of both NK- and T-cell responses. Robbins *et al.* have shown that poor viral control by NK cells leads to higher IFN-α levels after infection (57), which we observed in $CD28^{-/-}$ mice at 2 dpi. These hypotheses are not mutually exclusive, are both consistent with previously published data, and likely explain the differences in viral titers observed in our knockout mice.

Conclusion

In conclusion, the co-stimulatory molecules B7 and CD28 are critical to viral control in mice during acute CMV infection, as absence of either molecule leads to significant elevations in viral titers. We feel that the differences in viral titers observed in knockout mice are likely a direct consequence of impaired T-cell expansion, with more subtle contributions from impaired MCMV-specific NK-cell expansion in $CD28^{-/-}$ mice. Whether CD28/B7 co-stimulation is critical for NK-subset expansion remains unproven, but this provocative observation certainly deserves further study. These data thus support the hypothesis that downregulation of co-stimulatory molecules by MCMV is an important immune evasion strategy during acute infection.

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Disclosure Statement

No competing financial interests exist.

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