Dual function of CD70 in viral infection: modulator of early cytokine responses and activator of adaptive responses[1]

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Abstract

The role of the tumor necrosis factor family member CD70 in adaptive T cell responses has been intensively studied but its function in innate responses is still under investigation. Here we show that CD70 inhibits the early innate response to murine cytomegalovirus (MCMV) but is essential for the optimal generation of virus-specific CD8 T cells. CD70^{-/-} mice reacted to MCMV infection with a robust type I interferon and proinflammatory cytokine response. This response was sufficient for initial control of MCMV, although at later time points, CD70^{-/-} mice became more susceptible to MCMV infection. The heightened cytokine response during the early phase of MCMV infection in CD70^{-/-} mice was paralleled by a reduction in regulatory T cells (Treg). Treg from naïve CD70^{-/-} mice were not as efficient at suppressing T cell proliferation compared to Treg from naïve WT mice and depletion of Treg during MCMV infection in Foxp3-DTR mice or in WT mice recapitulated the phenotype observed in CD70^{-/-} mice. Our study demonstrates that while CD70 is required for the activation of the antiviral adaptive response, it has a regulatory role in early cytokine responses to viruses such as MCMV, possibly through maintenance of Treg survival and function.

Keywords

CD70; dendritic cell; NK cell; regulatory T cell; interferon; virus

Introduction

Members of the tumor necrosis factor (TNF) family and the TNF receptor (TNFR) family provide costimulatory signals which facilitate optimal adaptive antiviral responses (1, 2).

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They promote the expansion of virus-specific T cells in primary responses and the generation of a memory T cell pool for secondary responses, mostly through induction of T cell survival pathways. Interactions between TNF and TNFR family members also induce signals that influence the functional polarization of CD4 T helper cells (1, 2).

CD70 is a TNF family member that is constitutively expressed on certain antigen-presenting cells in the thymic medulla, possibly mTEC (3), and the intestinal mucosa (4, 5). Additionally, CD70 is transiently expressed on DC, B and T cells after activation (3, 6-8). CD70 binds and activates CD27, a TNFR family member constitutively expressed on the surface of naïve CD8 T cells, CD4 T cells, and subsets of NK cells and B cells (6). Many studies have demonstrated that the CD70-CD27 costimulatory interaction supports clonal expansion of CD8 T cells during primary responses and/or the long-term survival of memory CD8 T cells, depending on the nature of the antigen (7-12). By interacting with DC, CD4 T helper (Th) cells induce expression of CD70 on DC, which engages CD27 on naïve CD8 T cells, providing the help that CD8 T cells require to generate memory responses (13). The CD70-CD27 interaction gives CD8 T cells survival signals (14, 15), which prevent TRAIL-mediated antigen-induced cell death (13).

CD70-CD27 interaction also facilitates CD4 T cell responses and directs CD4 Th cell functional polarization, promoting Th1 cell differentiation (16-18) and inhibiting Th17 effector functions (19). A recent study has shown that the CD70-CD27 interaction enables epithelial cells and DC in the thymic medulla to induce the development of CD4^{+}Foxp3^{+} regulatory T cells (Treg) (20). Finally, CD27 is highly expressed on a subset of mature NK cells endowed with great capacity to release lytic granules and IFN-γ, as well as high homeostatic proliferation compared to NK cells with low amount of CD27 (21). Whether CD70-CD27 interaction has a substantial impact on the expansion and effector functions of these NK cells is not known.

The function of CD70-CD27 interaction in viral infections has been extensively investigated in the context of adaptive responses, leading to the conclusion that this interaction impacts primary and/or memory antiviral adaptive responses, depending on the type of virus (9-11, 22-25). However, the impact of CD70-CD27 interactions on antiviral innate responses remains largely unknown. To address this issue we studied murine cytomegalovirus (MCMV) infection in CD70^{-/-} mice. We chose MCMV infection because the response to this virus in the C57BL/6 background is largely dependent on NK cells and cytokines, i.e. IFN-α and IL-12, which are produced by antigen presenting cells such as plasmacytoid dendritic cells (pDC) and conventional DC (cDC) (26, 27). We found that CD70^{-/-} mice responded to MCMV infection with an early burst of IFN-αIL-12 and other proinflammatory cytokines. Although this response was sufficient to control MCMV in the first 36 h, at later time points, CD70^{-/-} mice became more susceptible to MCMV infection compared to their wildtype (WT) counterparts because of a defect in the adaptive response. Furthermore, we found that the augmented cytokine response during the initial phase of MCMV infection in CD70^{-/-} mice was a consequence of reduced numbers and impaired function of Treg. Depletion of Treg during MCMV infection in Foxp3-DTR mice or in WT mice recapitulated the phenotype observed in CD70^{-/-} mice. Thus, our study demonstrates

that while CD70 is required for the activation of the antiviral adaptive response, it has a regulatory role in early cytokine responses to viruses such as MCMV.

Materials and Methods

Mice, Viral Infections and Treatments

All animal studies were approved by the Washington University Animal Studies Committee. Male and female C57BL/6 and CD70$^{-/-}$ mice on the C57BL/6 background (25) were bred in house and used between 8–12 weeks of age. Foxp3-diphtheria toxin receptor (DTR) mice were generated by Alexander Rudensky (Memorial Sloan-Kettering Cancer Center, New York, NY) (28) and kindly provided by Chyi-Song Hsieh (Washington University, St. Louis, MO). Foxp3-DTR mice were bred in house. Smith strains MCMV and AT1.5 (m157) were provided by W. Yokoyama and A. French (Washington University, St. Louis, MO). Mice were infected with salivary gland MCMV or AT1.5 stocks prepared from BALB/c mice (doses specified in figure legends). To deplete Treg in Foxp3-DTR mice, diphtheria toxin (DT) (1 μg/mouse) was injected i.p. on days -3 and -1 of infection; control mice received phosphate buffered saline (PBS). To deplete Treg with antibody, C57BL/6 and CD70$^{-/-}$ mice were injected i.p. with 300-500 μg of anti-CD25 Ab (clone PC-61.5.3, Bio X Cell) or a rat IgG isotype control Ab on days -3 and -1 of infection. To block CD70-CD27 interactions *in vivo*, we injected C57BL/6 mice i.p. with 150 μg of anti-CD70 Ab (clone FR-70, Bio X Cell) or a rat IgG isotype control Ab on days -1 and 0 of infection (29). The following primers were used to quantify MCMV in tissues using TaqMan PCR: For MCMV IE1: Probe 5'-/56-FAM/TCT CTT GCC/ZEN/CCGTCCTGAAAACC/3IABkFQ/-3', Primer 1 5'- CCC TCT CCT AAC TCT CCC TTT-3', Primer 2 5'-TGG TGC TCT TTT CCC GTG; For Actin: Probe 5'-/56-FAM/TTC AGG GTC/ZEN/AGGATACCTCT CTT GCT/ 3IABKFQ/-3', Primer 1 5'-AGC TCA TTG TAG AAG GTG TGG-3', Primer 2 5'-GGT GGG AAT GGG TCA GAA G-3'.

Cell Preparations

Single-cell suspensions of spleens were prepared by collagenase D digestion and passage through nylon mesh cell strainers (BD Biosciences). Red blood cells (RBC) were lysed with RBC lysis buffer (Sigma-Aldrich). Whole blood was collected by cardiac puncture and serum was stored at -20°C until analysis.

Antibodies and Flow Cytometry

The following reagents were from BD Biosciences, eBioscience or BioLegend: fluorochrome labeled anti-CD69 (H1.2F3), anti-CD11c (HL3), anti-CD8α (53-6.7), anti-CD4 (GK1.5), anti-I-Ab (AF6-120.1), anti-CD3 (145-2C11), anti-NK1.1 (PK136), anti-Ly49H (3D10), anti-IFN-γ (XMG1.2), anti-Granzyme B (GB11), anti-CD11b (M1/70), anti-Ly6C (AL-21), anti-Ly6G (1A8), anti-CD25 (PK61.5), anti-Foxp3 (150D/E4), anti-CD262 (DR5, MD5-1), anti-TRAIL (N2B2), and Streptavidin. The m45 tetramer used to visualize virus-specific CD8 T cells was a generous gift from M. Brown (University of Virginia, Charlottesville, VA). Fc receptors were blocked before surface staining with supernatant from HB-197 cells (ATCC). Dead cells were excluded with propidium iodide (PI). Intracellular staining for IFN-γ and Granzyme B was performed on freshly isolated spleen

cells or after 4 h incubation with Brefeldin A using the BD Cytofix-Cytoperm kit (BD Biosciences) according to the manufacturer instructions. All flow cytometry was conducted on a dual laser FACSCalibur or LSR II (BD Biosciences) and analyzed with FlowJo software (Tree Star, Inc.). For NK cell purification, DX5^{+} cells were purified from spleens with anti–DX5 microbeads as recommended by the manufacturer (Miltenyi Biotec). Purities were always >85%.

ELISA and Cytometric Bead Array

Serum samples from infected mice were collected at various time points p.i. IFN-α concentrations were measured by ELISA (PBL Assay Science). IL-12p70, IL-10, IL-6, MCP-1, TNF-α and IFN-γ were measured by flow cytometry with the Mouse Inflammation CBA kit (BD Biosciences).

Cell Lines and Tissue Culture

YAC-1 and primary cells were cultured in RPMI 1640 with 10% bovine calf serum (BCS), 1% glutamax, 1% HEPES, 1% nonessential amino acids, 1% sodium pyruvate, 1% penicillin/streptomycin and 1% kanamycin sulfate (Gibco-Invitrogen).

Cytotoxicity Assays

For NK cell cytotoxicity assays, splenic NK cells purified from MCMV-infected mice were resuspended in complete RPMI and serially diluted in 96-well round bottom plates. YAC-1 cells were labeled with ^{51}Cr for 2 h then incubated with effector cells at 37°C for 4 hr. ^{51}Cr release in supernatants was measured with a γ-counter.

Treg Suppression Assays

CD4^{+}CD25^{+}Treg were isolated from naïve C57BL/6 and CD70^{-/-} mice with the mouse CD4^{+}CD25^{+} Regulatory T Cell Isolation Kit (Miltenyi Biotec). CD4^{+}CD25[-]cells from C57BL/6 mice were used as T conventional cells (Tconv). Tconv were labeled with 5 uM CFSE and cultured with Treg according to Collison and Vignali (30) with modifications. Briefly, 96-well flat bottom bottoms were coated overnight at 4°C with anti-CD3 and anti-CD28 Abs in PBS (2.5 μg/ml). Two-fold serially diluted Treg from C57BL/6 or CD70^{-/-} mice were plated per well then CFSE-labeled Tconv cells were added to each well at a concentration of 1×10^5 cells/well. Co-cultures were incubated at 37°C for 72 h then analyzed by flow cytometry. Cells were stained with PI and anti-CD4 to visualize live, proliferating CFSE^{+} Tconv cells.

Tissue Samples and Immunohistochemistry

Formalin-fixed, paraffin-embedded tissue sections were used for immunohistochemical staining to visualize T cells and B cells. Sections were stained with anti-CD3 and anti-B220. Digital images were captured and processed using Analysis Image Processing software (Olympus).

Statistical Analysis

The statistical significance of differences in mean values was analyzed with unpaired, two-tailed Student's t-test. p values less than 0.05 were considered statistically significant.

Results

CD70 deficiency has opposing consequences on MCMV clearance at early and late time points of infection

We infected CD70$^{-/-}$ mice and WT C57BL/6 controls intraperitoneally (i.p.) with MCMV and measured viral burden in tissues. At 1.5 d post-infection (p.i.) CD70$^{-/-}$ mice showed lower MCMV titers than WT mice. However, at 3 d p.i., MCMV titers were significantly higher in spleens and livers of CD70$^{-/-}$ mice compared to WT controls (Fig. 1A). Since MCMV has a tropism for salivary glands where it remains detectable for a longer period of time after infection (31), we also measured viral titers in salivary glands on day 8 p.i. Again, viral loads were higher in CD70$^{-/-}$ mice than in WT controls (Fig. 1A). To determine whether CD70$^{-/-}$ mice were more susceptible to infection we injected a higher dose of MCMV (2×10^5 pfu). Both CD70$^{-/-}$ and WT mice lost a significant amount of body weight; however, while 90% of WT mice recovered 5 days p.i. and survived infection, only 30% of CD70$^{-/-}$ mice recovered and survived (Fig. 1B). Together, these results indicate that lack of CD70 delays viral replication during the early stage of infection but, ultimately, affects MCMV clearance and survival at later time points once the infection is established.

Lack of CD70 causes an early and exuberant cytokine response to MCMV infection

We next assessed the impact of CD70-deficiency on cytokine responses to MCMV. Remarkably, serum IFN-α levels were higher in CD70$^{-/-}$ mice than in WT mice at 36 and 48 h p.i. (Fig. 2A); at 72 h p.i., systemic IFN-α was undetectable in both strains (data not shown). Similarly, serum IL-12p70 was ~3-fold higher in CD70$^{-/-}$ mice than WT controls at 36 h p.i., but then sharply declined (Fig. 2B). Serum TNF-α (Fig. 2C) and IL-6 (Fig. 2D) also peaked at 36 h p.i. and higher levels were detected in CD70$^{-/-}$ mice. Taken together these data indicate that loss of CD70 impacts both the magnitude and timing of IFN-α and pro-inflammatory cytokine production, resulting in an early burst of cytokine secretion in response to MCMV. Most likely, the early wave of IFN-α accounts for the transient reduction of MCMV viral load during the first 36 h of infection in CD70$^{-/-}$ mice.

In parallel with the enhanced inflammatory cytokine response, serum levels of CCL2 (MCP-1) were also elevated in CD70$^{-/-}$ mice compared to WT mice at 24 and 36 h p.i. (Fig. 2E). CCL2 promotes the recruitment of myeloid cells; accordingly, about 3-fold more CD11b$^+$Ly6C^{hi}Ly6G$^-$ monocytes were observed in the spleens of CD70$^{-/-}$ mice at 24 and 36 h p.i., but these numbers declined rapidly (Fig. 2F). Thus, lack of CD70 also causes the rapid influx of monocytes into the spleen in response to MCMV infection.

The robust cytokine response to MCMV in CD70$^{-/-}$ mice results in accelerated activation of NK cells

Since NK cells play an essential role in controlling MCMV infection through lysis of infected cells and IFN-γ production (26, 27), and CD70$^{-/-}$ mice have defective control of

MCMV infection, we wanted to determine the impact of CD70-deficiency on NK cells. Prior to infection, NK cell numbers were similar in naïve CD70^{-/-} and WT mice (**data not shown**). CD70^{-/-} mice had more NK cells in the spleen than WT mice 24 h p.i. but these numbers declined more rapidly in CD70^{-/-} mice than in WT controls, such that at 48 h p.i. CD70^{-/-} mice had fewer NK cells than WT mice (Fig. 3A, B). To evaluate NK cell activation we monitored CD69 expression. Prior to infection, NK cells from both CD70^{-/-} and WT mice expressed low and similar levels of CD69 (**data not shown**). A higher frequency of CD69^{+} NK cells was noted in CD70^{-/-} mice at 24 and 36 h p.i., but by 48 h p.i., the majority of NK cells in both strains of mice expressed CD69 (Fig. 3C, D). Considering the changes in the absolute numbers of NK cells, more CD69^{+} NK cells were present in the spleens of CD70^{-/-} mice at early time points p.i. but these numbers declined very rapidly in comparison to WT mice (Fig. 3C, D). These results indicate that CD70-deficiency results in accelerated activation of NK cells followed by a sharp decline in NK cell numbers during MCMV infection.

We next analyzed the effector functions of NK cells. Serum levels of IFN-γ were 3-fold higher in CD70^{-/-} mice than in WT mice 36 h p.i. (Fig. 3E). At this time point after infection serum IFN-γ most likely reflects NK cell secretion. Consistent with this, the frequency and mean fluorescent intensity (MFI) of IFN-γ-producing NK cells was higher in CD70^{-/-} than in control mice at 36 h p.i. (Fig. 3F). However, at 48 h p.i., NK cells from WT mice appeared to be making more IFN-γ on a per cell basis even though the frequencies of IFN-γ-producing NK cells were similar in both groups of mice (Fig. 3F). At higher effector:target ratios, NK cells from CD70^{-/-} mice also showed a slight increase in cytolytic capacity (Fig. 3G) and expressed more Granzyme B (**data not shown**) at 36 h p.i. than NK cells from WT mice. At 48 h p.i., specific lysis and Granzyme B expression were similar in WT and CD70^{-/-} mice (Fig. 3G and **data not shown**), however, we did observe a higher frequency of NK cells that expressed TRAIL in CD70^{-/-} mice (**data not shown**). Taken together, these results indicate that CD70-deficiency elicits activation and function early on during MCMV infection followed by a reduction in NK cell numbers. Most likely, this initial activation of NK cells is due to the early boost of cytokines, such as IFN-α and IL-12p70, which activate NK cells (27).

The failure of CD70^{-/-} mice to effectively control MCMV is independent of NK cell numbers

NK cells in C57BL/6 mice recognize MCMV-infected cells through the receptor Ly49H, which detects the MCMV-encoded MHC class-I like molecule m157 (26). To determine whether the increased MCMV burden in CD70^{-/-} mice is due to defective NK cell numbers, we infected CD70^{-/-} and WT mice with Δm157 MCMV. This virus eludes recognition by Ly49H, therefore its clearance is NK cell-independent (26). Similar to WT MCMV, viral loads were higher in CD70^{-/-} mice than in WT mice on day 3 p.i. (Fig. 3H), indicating that the impaired control of MCMV infection in CD70^{-/-} mice is not directly related to a defect in NK cell numbers. Likewise, the IFN-α response to Δm157 MCMV was about 2.5-fold higher in CD70^{-/-} mice compared to WT mice (Fig. 3I), suggesting that the enhanced cytokine response of CD70^{-/-} mice to MCMV is independent of NK cell recognition of MCMV.

CD70-deficiency affects CD8α DC numbers and the MCMV-specific CD8 T cell response

Since a NK cell defect may not be the culprit of impaired MCMV control in CD70^{-/-} mice, we investigated whether CD70-deficiency affected DC-T cell interaction and adaptive responses. We first examined whether DC express CD70 during MCMV infection. At 36 h p.i. approximately 30% of splenic CD8α DC expressed CD70 and by 48 h p.i. CD70 was present on 60% of splenic CD8α DC (data not shown and Fig. 4A). When we evaluated DC numbers in CD70^{-/-} and WT mice infected with MCMV, WT mice showed a progressive decline in total DC numbers and CD8α DC in the first 72 h p.i. and CD70^{-/-} mice exhibited a more marked decrease (Fig. 4B, C). At 48 h p.i., CD8α DC in CD70^{-/-} mice were greatly reduced compared to WT controls and were almost undetectable at 72 h p.i. We also observed that during MCMV infection higher frequencies of CD8α DC in CD70^{-/-} mice expressed the TRAIL receptor DR5 compared to WT mice (Fig. 4D). Thus, it is plausible that the marked reduction of CD8α DC in CD70^{-/-} mice is due at least in part to activation-induced cell death.

Since CD8α DC prime naïve CD8 T cells against MCMV through cross presentation, we next investigated the generation of MCMV-specific CD8 T cells by m45 tetramer staining. We found a significant reduction in total and MCMV-specific CD8 T cells in MCMV-infected CD70^{-/-} mice compared to WT mice at day 7 p.i., suggesting that the CD8 T cell response was indeed affected by the loss of CD8α DC (data not shown and Fig. 4E). Immunohistochemical analysis of spleens from CD70^{-/-} and WT mice at day 7 p.i. confirmed a reduction of CD3^{+} cells in CD70^{-/-} mice (**data not shown**). We envision that the defect of the adaptive T cell response, and particularly of specific CD8 T cells, is a factor contributing to the impaired MCMV control in CD70^{-/-} mice. Interestingly, CD70^{-/-} mice showed a modest increase in Ly49H^{+} NK cells compared to WT mice on day 7 p.i. (Fig. 4F), suggesting that after the initial phase of infection, MCMV-specific NK cells expand to greater numbers in CD70^{-/-} mice as a consequence of increased viral replication and an impaired adaptive response.

Regulatory T cells modulate innate responses to MCMV infection

We sought to investigate the mechanism behind the exuberant cytokine response that occurs in CD70^{-/-} mice very early after MCMV infection. We hypothesized that lack of CD70 may release the innate immune system, particularly antigen-presenting cells (APC), from an inhibitory signal such that APC become hyper-responsive to microbial stimuli that engage Toll-like receptors (TLR). It has been shown that CD70^{-/-} mice have reduced numbers of Treg (20) and that Treg regulate DC responses *in vivo*, such that in the absence of Treg, DC exhibit trafficking defects to sites of infection and can be more responsive to stimulation (32-36). Therefore, we examined numbers of Treg in WT and CD70^{-/-} mice during MCMV infection. In agreement with a previous study, naïve CD70^{-/-} mice had a slight reduction of Treg compared to WT mice (Fig. 5A) (20). Following MCMV infection, Treg were consistently ~2-3-fold less in CD70^{-/-} mice at 24, 48 and 72 h p.i. (Fig. 5B). To determine whether Treg influenced the innate response to MCMV infection we injected Foxp-DTR mice with DT or PBS to deplete Treg or not prior to infection with MCMV. We found that DT eliminated almost all CD3^{+}CD4^{+}Foxp3^{+} cells (Fig. 5C) and that depletion of Treg resulted in elevated serum IFN-α and TNF-α but reduced IL-12p70 levels (Fig. 5D). With

respect to NK cell activation and function, frequencies of NK cells from Treg-depleted mice were similar to non-depleted mice but they expressed more CD69 (Fig. 5E), consistent with the elevated levels of IFN-α. In accordance with the lower levels of IL-12p70 in Treg-depleted mice, fewer NK cells produced IFN-γ by intracellular staining (Fig. 5F); however, systemic levels of IFN-γ were relatively similar to non-depleted mice (Fig. 5F). Thus, Treg appear to regulate innate responses during MCMV infection.

Regulatory T cells are functionally impaired in CD70^{-/-} mice

We next asked whether depletion of Treg in CD70$^{-/-}$ mice would have an impact on innate responses to MCMV infection. To test this, we depleted Treg in WT and CD70$^{-/-}$ mice using antibody-mediated depletion. Injection of anti-CD25 Ab eliminates the majority of CD25$^+$Treg. Anti-CD25 Ab treatment in WT mice resulted in a 50% reduction of CD3$^+$CD4$^+$Foxp3$^+$ cells (Fig. 6A) and consistent with data obtained using Foxp3-DTR mice, WT mice treated with anti-CD25 Ab had elevated levels of IFN-α and proinflammatory cytokines after infection with MCMV (Fig. 6B). WT mice depleted of Treg with anti-CD25 Ab also had increased NK cell activation as measured by CD69 and IFN-γ production (Fig. 6C-6E). In contrast to Treg depletion in WT mice, cytokine responses and NK cell activation in CD70$^{-/-}$mice treated with anti-CD25 Ab were not altered in the absence of Treg (Fig. 6F-6J). These data suggested that Treg might already be functionally impaired in CD70$^{-/-}$ mice. To address this, we performed *in vitro* Treg suppression assays (30). We found that Treg isolated from naïve CD70$^{-/-}$ mice were not able to suppress proliferation of CD4$^+$CD25$^-$T cells (Tconv) as efficiently as Treg from naïve WT mice (Fig. 6K). Also supporting the idea that Treg from CD70$^{-/-}$ might have a moderate, intrinsic defect in their suppressive capacity, transient blockade of CD70-CD27 interactions in WT mice had no impact on Treg numbers (Fig. 7A) or on cytokine responses and NK cell activation during MCMV infection (Fig. 7B-7C). Taken together, our findings indicate that Treg control innate responses to MCMV infection in WT mice and that reduced numbers and impaired function of Treg in CD70$^{-/-}$ mice contribute to hyper-activation of the innate response during MCMV infection.

Discussion

Our study shows that CD70 has two major functions in the antiviral immune response. On one hand CD70 is required for an optimal CD8 T cell response and control of MCMV load. On the other hand we found that CD70 is essential for regulating the innate inflammatory response during the initial phase of infection. The impairment of the adaptive T cell response was expected because activation of CD8 T cells through CD27 has been shown to provide survival signals that counter TRAIL-induced apoptosis (13-15). However, we found that lack of CD70 also resulted in reduced DC numbers early after MCMV infection, which may contribute to the reduction in the CD8 T cell response. CD70-deficient DC expressed more DR5 than their WT counterparts, which may increase their susceptibility to TRAIL-induced apoptosis. The remarkable finding of this study is that CD70 is required for the control of innate inflammatory response in the initial phase of infection. Accordingly, CD70$^{-/-}$ mice exhibited an early robust cytokine response to MCMV infection. The increased IFN-α response in CD70$^{-/-}$ mice facilitated the control of MCMV in the first 36 h

of infection, and together with the burst of IL-12, probably promoted the nonspecific activation of NK cells and the enhanced secretion of IFN-γ. This elevated cytokine response appeared to be a consequence of a defect in Treg numbers and function. We found that CD70^{-/-} mice have a modest reduction of Treg in steady state, as recently reported (20), which was intensified during viral infection and that Treg from CD70^{-/-} mice were not as efficient at suppressing responses by other cell types. Because Treg inhibit the activation and promote the trafficking of APC, it is likely that impaired survival and function of Treg in CD70^{-/-} mice results in exuberant responsiveness of these cells to inflammatory stimuli and lessens their numbers at sites of infection (32-36). Corroborating this, WT but not CD70^{-/-} mice depleted of CD25^{+}Treg displayed greater cytokine production after infection with MCMV. However, transient blockade of CD70-CD27 interactions was not sufficient to cause changes in Treg numbers or the innate response, which is in agreement with a recent study (29). Since CD70 mediates reverse signaling (37) and translocates together with the invariant chain to the endosomal/lysosomal compartments (38), CD70 may also act by modulating TLR signaling and/or translocation of TLR into endosomal compartment where they interact with microbial ligands.

Surprisingly, although a substantial NK cell subset expresses CD27 (21, 39) and previous studies confirmed a role for DC-NK interaction in promoting control of viral infections (40, 41), NK cell effector functions were not reduced in CD70^{-/-} mice. In fact, NK cell activation was transiently increased in CD70^{-/-} mice at early time points after MCMV infection, which may reflect an increase of IFN-α and IL-12 as well as a decrease in Treg, which have been recently shown to modulate NK cell function and homeostasis (42-44). This early NK cell activation was followed by a reduction in NK cell numbers, but this minor change is unlikely to affect the control of MCMV infection, as CD70^{-/-} mice showed defective control of m157 MCMV, which evades NK cell surveillance in C57BL/6 mice. Altogether our results indicate the dual function of CD70 in viral infection as a modulator of early cytokine responses via maintenance of Treg function and survival and activator of adaptive responses through induction of virus-specific CD8 T cells.

Acknowledgments

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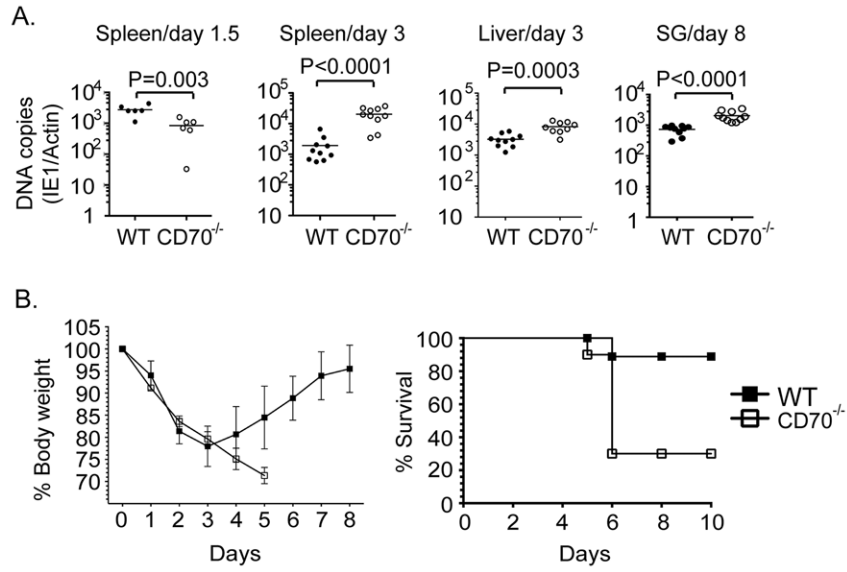
Figure 1. Effect of CD70-deficiency on viral load and surival during MCMV infection
(A) WT and CD70$^{-/-}$ mice were infected i.p. with 5×10^4pfu of MCMV. Viral loads were determined in spleen on day 1.5, in spleen and liver on day 3, or in salivary gland (SG) on day 8. (B) Body weight and survival were monitored in WT and CD70$^{-/-}$ mice infected i.p. with 2×10^5pfu of MCMV (n=10). Data are from two-four independent experiments. Statistical significance is indicated by p values.
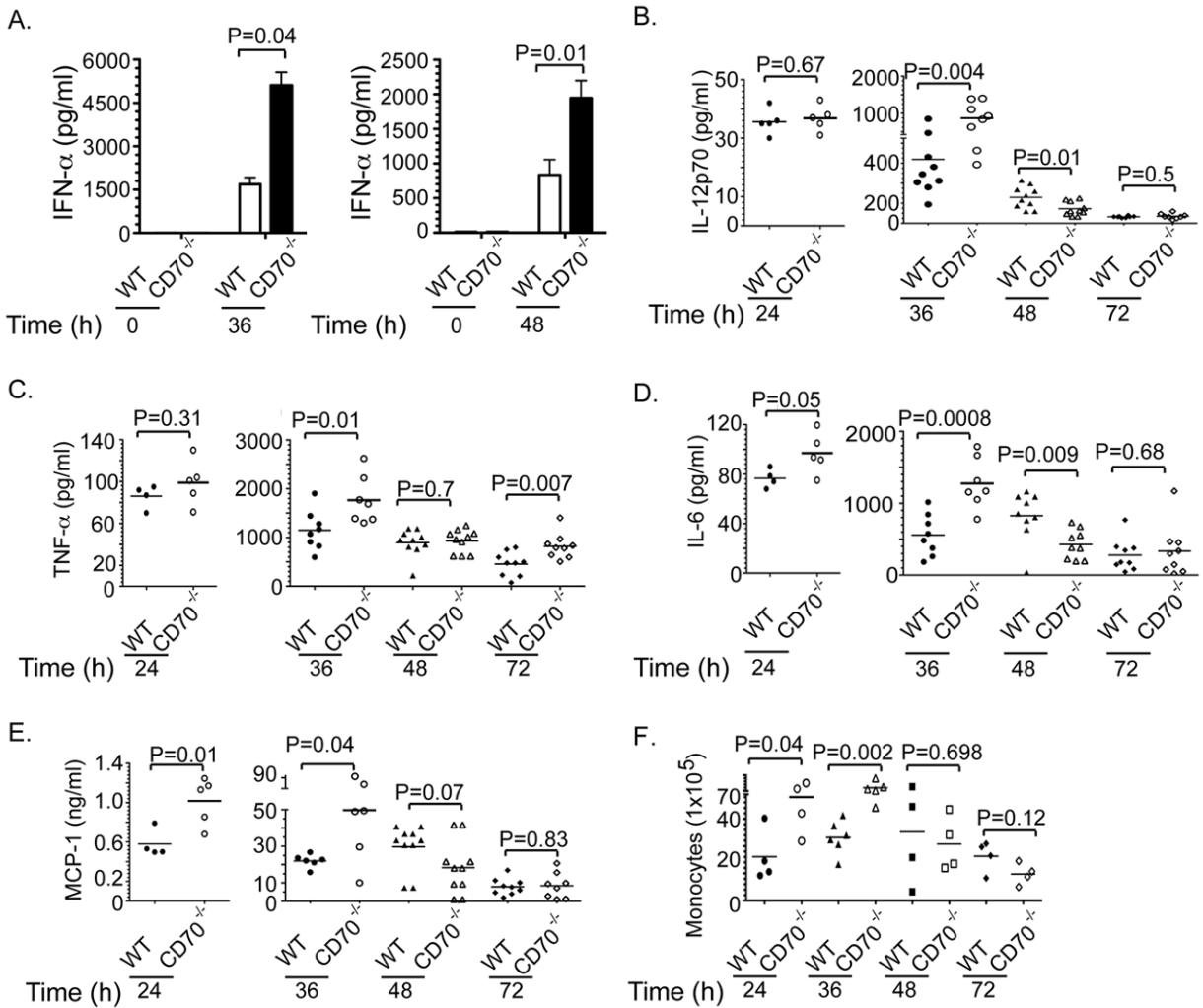
Figure 2. CD70 modulates the inflammatory response during MCMV infection

WT and CD70^{-/-} mice were infected i.p. with 5×10^4 pfu of MCMV. (A) Serum IFN-α levels were measured by ELISA at 36 and 48 h p.i. Proinflammatory cytokine production in mice at various time points p.i. Serum levels of IL-12p70 (B), TNF-α (C), IL-6 (D) and MCP-1 (E). (F) Monocyte recruitment to spleens of WT and CD70^{-/-} mice after MCMV infection. Data shows numbers of CD11b^{+}Ly6C^{hi}Ly6G^{-} cells in spleens at various time points p.i. Data are from two-ten independent experiments. Error bars represent the mean ± SEM. Statistical significance is indicated by p values.
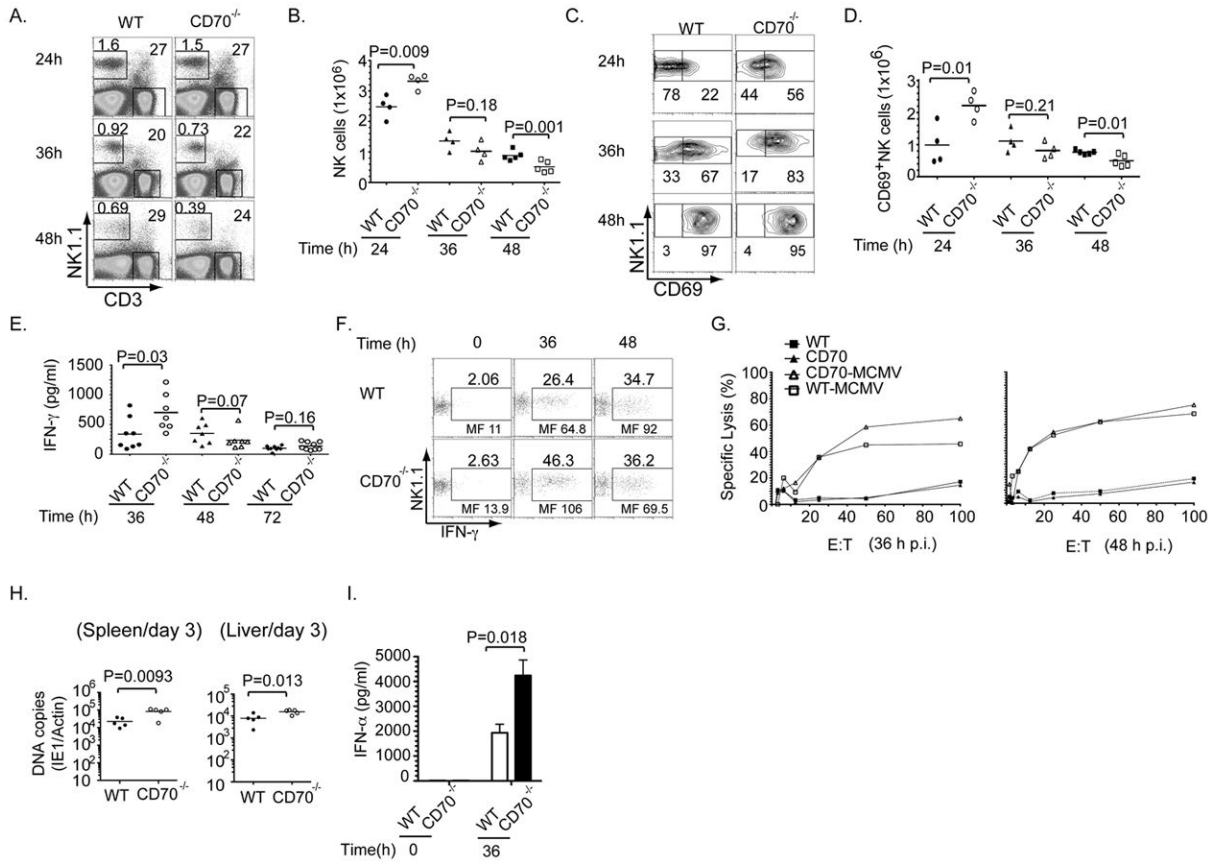
Figure 3. CD70-deficiency augments NK cell activation early during infection

(A-G) WT and CD70$^{-/-}$ mice were infected i.p. with 5×10^4pfu of MCMV. (A) Frequencies of NK cells (NK1.1$^+$CD3$^-$) and CD3$^+$ T cells in spleens at various time points p.i. (B) Total numbers of splenic NK cells at 24, 36 and 48 h p.i. Frequencies (C) and numbers (D) of splenic CD69-expressing NK cells. Serum levels of IFN-γ (E) and frequencies and MFI (MF) of IFN-γ-producing NK cells (F) in spleens after MCMV infection. (G) Cytolytic activity of NK cells was assessed in standard 4 h ^{51}Cr release assays. Mice were infected for 36 or 48 h with 5×10^4pfu MCMV. NK cells were purified from spleens and incubated with YAC-1 target cells. (H, I) Mice were infected i.p. with 1×10^4pfu of m157 MCMV. Viral loads in spleen and liver were determined on day 3 p.i. (H) and serum IFN-α levels were measured at 0 and 36 h p.i. (I). Data are from two-three independent experiments. Error bars represent the mean ± SEM. Statistical significance is indicated by p values.
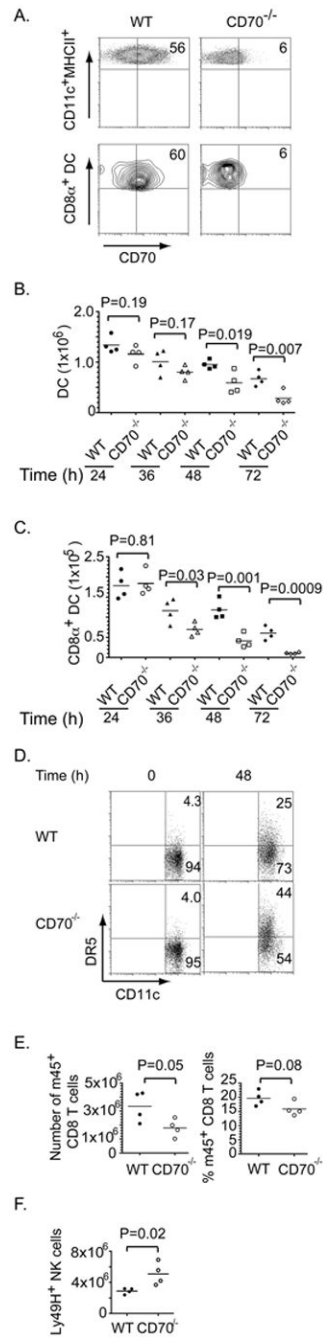
Figure 4. DC survival and the CD8 T cell response are impaired in CD70^{-/-} mice following MCMV infection

WT and CD70^{-/-} mice were infected i.p. with 5×10^4 pfu of MCMV. (A) Expression of CD70 on splenic CD11c^{hi}MHCII^{+} DC and CD8α DC 48 h p.i. Numbers of splenic CD11c^{hi}MHCII^{+} DC (B) and CD8α DC (C) at various time points p.i. (D) DR5 expression on splenic CD11c^{hi}MHCII^{+} DC 48 h p.i. (E) Numbers and frequencies of m45-specific CD8 T cells in spleens on day 7 p.i. as measured by tetramer staining. (F) Numbers of Ly49H^{+} NK cells in spleens on day 7 p.i. Data are representative of two independent experiments. Statistical significance is indicated by p values.
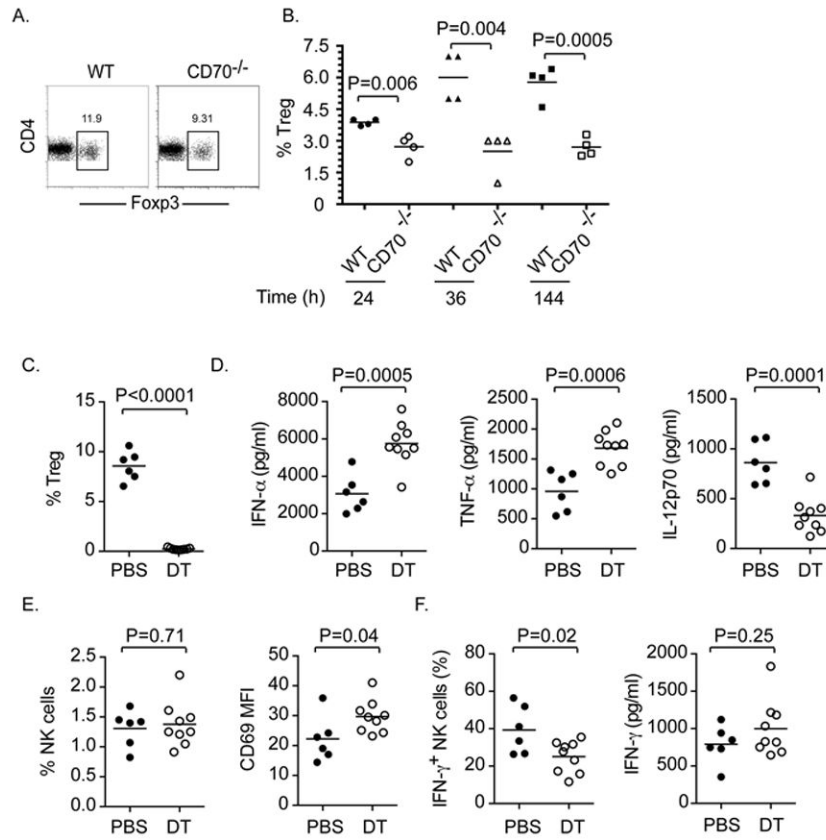
Figure 5. Regulatory T cells are reduced in CD70^{-/-} mice and control the innate response to MCMV

(A) Frequencies of Treg (CD3$^+$CD4$^+$Foxp3$^+$) in spleens of naïve WT and CD70$^{-/-}$ mice. (B) WT and CD70$^{-/-}$ mice were infected i.p. with 5×10^4pfu of MCMV and Treg frequencies in spleens were determined at various time points p.i. (C-F) Foxp3-DTR mice were injected with DT or PBS to deplete Treg or not, then infected with 5×10^4pfu of MCMV. Mice were analyzed 36 h p.i. (C) Frequencies of CD3$^+$CD4$^+$Foxp3$^+$ cells in spleens of infected mice. (D) Serum concentrations of IFN-α, TNF-α and IL-12p70 36 h p.i. (E) Frequencies of NK cells in spleens from infected mice and NK cell expression of CD69. (F) Frequencies of IFN-γ-producing splenic NK cells and serum IFN-γ levels in control and Treg-depleted mice. Data are representative of (A, B) or combined from (C-F) two independent experiments. Statistical significance is indicated by p values.
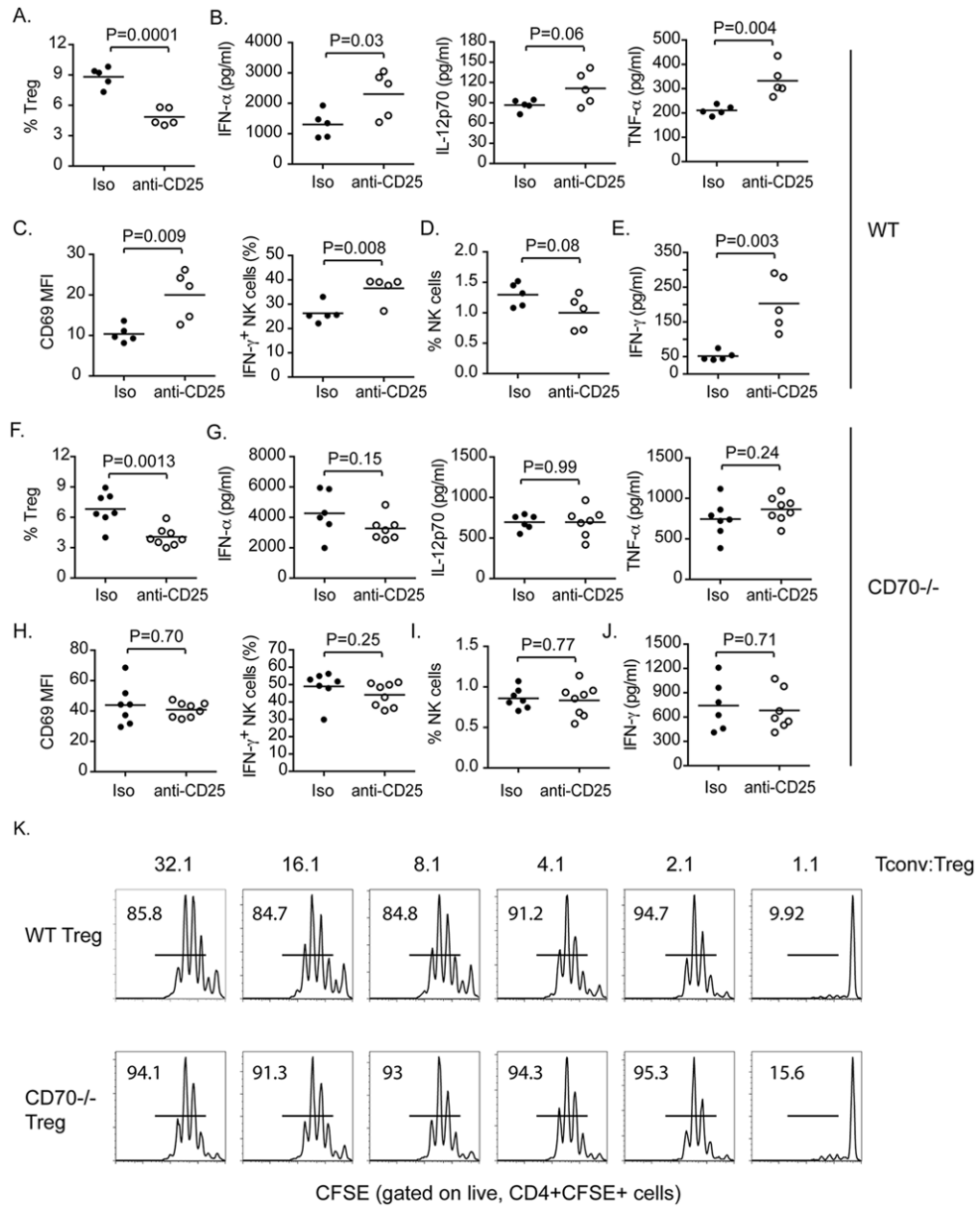
Figure 6. Treg are functionally impaired in CD70$^{-/-}$ mice

(A-J) WT and CD70$^{-/-}$ mice were injected with an isotype control Ab (Iso) or depleted of CD4$^+$CD25$^+$Foxp3$^+$ cells with anti-CD25 Ab then infected with MCMV. Mice were analyzed 36 h p.i. (A, F) Percentages of Treg in spleens. (B, G) Serum levels of IFN-α, IL-12p70 and TNF-α. (C, H) CD69 expression on NK cells and frequencies of IFN-γ-producing NK cells in spleens. (D, I) Frequencies of splenic NK cells and (E, J) serum IFN-γ levels. Data are combined from two independent experiments. Statistical significance is indicated by p values. (K) Treg were isolated from naïve WT and CD70$^{-/-}$ mice and co-cultured with CFSE-labeled naïve CD4$^+$CD25$^-$ T cells (Tconv) from WT mice for 3 d. Proliferation was measured by flow cytometry. Numbers indicate percentages of Tconv that divided two times or more.
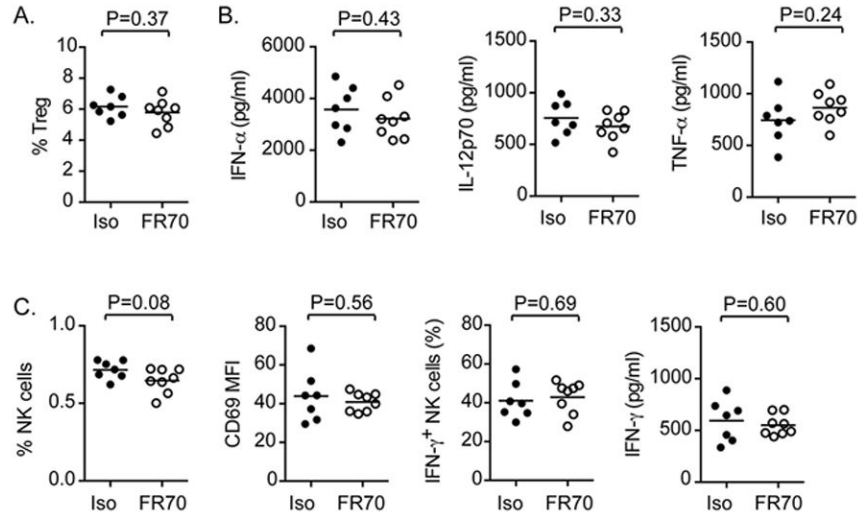
Figure 7. Transient blockade of CD70-CD27 interactions does not impact innate responses to MCMV

(A-C) WT mice were injected with an isotype control Ab(Iso) or anti-CD70 Ab(FR70) prior to MCMV infection. Mice were analyzed at 36 h p.i. (A) Treg frequencies in spleens of infected mice. (B) Serum levels of IFN-α, IL-12p70 and TNF-α. (C) Frequencies of splenic NK cells, CD69 expression on splenic NK cells, frequencies of splenic IFN-γ-producing NK cells and serum levels of IFN-γ. Data are combined from two independent experiments. Statistical significance is indicated by p values.