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Acute virus control mediated by licensed NK cells sets primary CD8+ T cell dependence on CD27 costimulation[1],[2],[3]

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Abstract

Natural killer (NK) cells represent a critical first-line of immune defense against a bevy of viral pathogens, and infection can provoke them to mediate both supportive and suppressive effects on virus-specific adaptive immunity. In mice expressing MHC I D^k, a major MCMV resistance factor and self-ligand of the inhibitory Ly49G2 (G2) receptor, licensed G2+ NK cells provide essential host resistance against murine (M)CMV infection. Additionally G2+ NK cell responses to MCMV increase the rate and extent of dendritic cell (DC) recovery, as well as early priming of CD8+ T-cell effectors in response to MCMV. However, relatively little is known about the NK-cell effect on co-stimulatory ligand patterns displayed by DCs, or ensuing effector and memory T-cell responses. Here we found that CD27-dependent CD8+ T-cell priming and differentiation is shaped by the efficiency of NK responses to virus infection. Surprisingly, differences in specific NK responses to MCMV in D^k-disparate mice failed to distinguish early DC co-stimulatory patterns.

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[3]Abbreviations: G2, Ly49G2; D^k, MHC-I D^k; MCMV, murine cytomegalovirus; DC, dendritic cell; SLEC, short-lived effector cell; MPEC, memory precursor effector cell

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Experimental conception and design: JJT, TNJB and MGB. Performed the experiments: JJT, AEG and MDS. Generated mice and reagents: JJT, AEG, ALG and HY. Analysis and interpretations of the data: JJT and MGB. Wrote the paper: JT, TNJB and MGB.

Conflict of Interest Disclosure

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Nonetheless, while CD27 deficiency did not impede licensed NK-mediated resistance, both CD70 and CD27 were required to efficiently prime and regulate effector CD8+ T-cell differentiation in response to MCMV, which eventually resulted in biased memory T-cell precursor formation in D^k mice. In contrast, CD8+ T-cells accrued more slowly in non-D^k mice, and eventually differentiated into terminal effector cells regardless of CD27 stimulation. Disparity in this requirement for CD27 signaling indicates that specific virus control mediated by NK cells can shape DC co-stimulatory signals needed to prime CD8+ T cells and eventual T-cell fate decisions.

Keywords

MHC; MCMV; CD70; costimulation; T cell differentiation

Introduction

NK cells are vital to protect against a variety of viral infections and cancer cells (1). Classical and functional deficiencies in human NK cells are associated with increased susceptibility to recurrent infections, particularly by members of the Herpesviridae family, including cytomegalovirus (CMV) (2, 3). While their role as direct antiviral mediators has been well studied (4, 5), recent investigations have revealed broader immunoregulatory roles for NK cells, including regulation of adaptive immune responses (6–8). As potent cytokine producers and highly cytotoxic effector cells, NK cells can bridge innate and adaptive immunity by aiding dendritic cell (DC) maturation for better T cell priming (9–12) and increasing the availability of antigen for cross presentation (13). Efficient NK cell resistance to infection can also limit antigen availability and decrease inflammation, which may decrease the overall magnitude of CD8+ T cell responses (14, 15). Furthermore, NK cell lysis of activated CD4+ and CD8+ T cells, and even other NK cells, which could prevent immune-related pathologies, was also shown to interfere with virus clearance during chronic infections (16–20). NK cells therefore serve in a variety of different roles during viral infection, and both the type of viral infection and the host-genetic factors regulating NK cell responses likely guide their involvement in antiviral defenses and host immunity.

MHC I D^k is a dominant genetic resistance factor that enables NK cells expressing Ly49G2 (G2) inhibitory receptors, for which D^k is a cognate ligand, to efficiently restrain MCMV infection (21, 22). While D^k expression on healthy cells prevents G2+ NK cell responses, altered expression of D^k during MCMV infection is thought to release G2-mediated inhibition of stimulatory NK receptors, thereby triggering efficient target cell lysis (23). Alternately, D^k-dependent licensing could serve to increase G2+ NK cell functionality and specific responsiveness to MCMV targets (22–25). In either case, licensed G2+ NK cells were found to selectively and specifically expand and limit MCMV spread only in mice with self-MHC D^k ligands expressed (21, 22, 25–27).

In response to virus infection, NK cells have also been shown to help protect, recover, and license DCs, which resulted in enhancements to cytotoxic T-cell immunity (28–30). In particular, we found that D^k-licensed G2+ NK cells can accelerate the recovery of CD8α+ and CD11b+ DCs, which then corresponds to enhanced virus-specific CD8+ T cell

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accumulation (29). Cross-presenting CD8α+ DCs have been shown to be critical for priming non-inflationary MCMV-specific CD8+ T-cell responses (31), and DC interactions with early responding NK cells may indirectly impact the quality of subsequent T-cell priming (9).

Given the differences in CD8+ T-cell kinetics in D^k-disparate mice (29), we predicted that D^k-licensed G2+ NK cells regulate co-stimulatory ligands displayed on DC early during MCMV infection. CD28-CD80/86 (32) and TNF superfamily members CD27-CD70 (33), OX40/40L (34), and 4-1BB/4-1BBL (35) were previously shown to provide critical co-stimulatory signals to virus-specific CD8+ T cells, though their spatiotemporal importance varied over the course of MCMV infection (36). Whereas CD28 is necessary for T-cell expansion during initial priming events (32, 37), OX40 and 4-1BB pathways have been demonstrated to drive latent and inflationary CD8+ T-cell accumulation during persistent MCMV infection (34, 35). CD27 plays an interesting role in MCMV infection since it has been shown to be critical for both acute non-inflationary and persistent inflationary accumulation of MCMV-specific CD8+ T cells (33). Moreover, immature NK cells express CD27, and several studies have correlated CD27 stimulation with the functional responsiveness of NK cells (38–40). Although these co-stimulatory pathways have been extensively studied, whether NK cells also specifically license DC for co-stimulatory ligand expression is unknown. Moreover, as host- and virus-specific effects influence how CD8+ T cells are primed via specifically licensed DC, understanding the NK cell role in shaping DC co-stimulatory ligand display is highly relevant (41, 42).

This study investigated how D^k-licensed G2+ NK cells accelerate and shape virus-specific CD8+ T cell accumulation during acute infection. We predicted that licensed NK-mediated virus resistance would enhance DC licensing by regulating co-stimulatory ligands required in CD8+ T-cell priming. We found that CD70-CD27 co-stimulatory interactions were required in licensed NK cell enhancement of CD8+ T-cell responses. Although CD70 ligand up-regulation in DCs was found to be a common response to MCMV, irrespective of the extent of virus resistance, we discovered that the efficiency of NK-mediated resistance determined the requirement for specific co-stimulatory cues to prime CD8+ T cells. Thus, in mice without licensed NK-mediated MCMV resistance, CD8+ T-cell priming was delayed and occurred independent of CD27 signaling. Our results indicate that specific virus control via licensed NK cells influences the set of cues that prime and shape primary effector CD8+ T-cell responses.

Materials and Methods

Ethics Statement

All mouse experiments conducted in this investigation were carried out in accordance with the Animal Welfare Act and the recommendations in the Guide for Care and Use of Laboratory Animals of the National Institutes of Health. Experimental procedures and *in vivo* treatments were approved by the University of Virginia Animal Care and Use Committee (Protocol Number: #3050).

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Mice

All mice used in this study were bred and maintained under specific pathogen-free conditions at the University of Virginia. C57L-derived MHC I D^k congenic (R7) and D^k transgenic (L.Tg1 and L.Tg3) mouse strains were described previously (21, 22). C57Bl/6 (B6).*Nkc*^{c57l} (NKC^l) and B6.*Cg-Nkc*^{c57l}*-D*^{k} (NKC^l-D^k) congenic mice with wild-type CD27 were generated by introgressing a C57L natural killer gene complex (NKC) into the B6 background using genome-wide marker-assisted selection as described previously (21, 43). These mice were compared to D^k-disparate B6.*Cd27*$^{-/-}$*-Nkc*$^{129/P2Ola}$ (CD27 KO) and B6.*Cg-Cd27*$^{-/-}$*-Nkc*$^{129/P2Ola}$*-D*^{k} (CD27 KO-D^k) mice. CD27 KO mice, which had been previously backcrossed to B6 from 129/P2Ola-founders, retain a CD27-linked NKC^{129} on chromosome 6 (33, 40) and were kindly provided by Jannie Borst (The Netherlands Cancer Institute, Amsterdam, Netherlands) via Ross Kedl (University of Colorado-Denver, CO, USA) (44). Importantly, *NKC-Ly49* haplotypes in 129 and C57L are highly related (45), *Ly49g* alleles in 129 and C57L mice are identical (21, 46), and both G2 receptors specifically bind D^k (47). CD27 KO mice were thus crossed to B6.D^k mice (a by-product of NKC^l-D^k production) to generate B6.*Cg-Cd27*$^{-/-}$*-Nkc*$^{129/P2Ola}$*-D*^{k} (CD27 KO-D^k) mice. Of note, both 129- and C57L-derived NKC haplotypes lack a *Ly49h* gene and, consequently, Ly49H+ NK-mediated MCMV resistance. All mice in this study were managed using a Colony Management System (Jackson Labs, JCMS Access, Version 6.1.9). All protocols were approved by the IACUC.

Virus infection and antibody treatments

Smith strain MCMV salivary gland stock virus (SGV) was prepared and titered on NIH-3T3 cell monolayers as described (26). SGV was administered via i.p. injection of 2×10^4 PFU. Where indicated, neutralizing mAbs specific for CD70 (mAb FR70; 250 μg/dose i.p. injected on 0, 2 and 4 d after infection), CD80 (mAb 16-10A1, BioXCell; 200 μg/dose i.p. injected on 0 and 3 d after infection), CD86 (mAb GL1, BioXCell; 200 μg/dose i.p. injected on 0 and 3 d after infection), and CD40L (mAb MR1, BioXCell; 250 μg/dose on 0, 2 and 4 d after infection) were administered. For *in vivo* G2+ NK cell depletions, 200 μg mAb AT8 or mAb 4D11 were i.p. injected 2 d prior and on the day of infection. For *in vivo* CD4+ T-cell depletions, 200 μg of mAb GK1.5 were i.p. injected on d 5, 4, and 0 before infection. Control IgG from rat serum (Sigma Life Sciences) or Syrian Hamster serum (Jackson ImmunoResearch Laboratories, Inc.) was administered in equivalent dose regimens, accordingly. Lymphocyte depletions exceeded 95–99% efficiency.

Flow cytometry and antibodies

Spleens were harvested from mice at the indicated time points postinfection and homogenized into single cell suspension through nylon cell strainers (Falcon Corning Brand; Life Sciences). Analyses of dendritic cells required additional processing with Collagenase D (0.5 mg/mL; Roche), as previously described (48). Single cell suspensions were pre-blocked with Fc receptor blocking antibody (24G2; UVA Lymphocyte Culture Center, Charlottesville, VA). All antibody incubations were performed on ice, and cells were washed with PBS or sorting buffer after each stain. Labeled cells were analyzed using the BD FACS Canto II (BD BioSciences) and the CytoFLEX (Beckman Coulter, Inc.). Data were collected

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using FACSDiva software (v8.0; BD BioSciences) or CytExpert software (v1.2.8.0; Beckman Coulter, Inc.) and analyzed with FlowJo (Versions 9.7.2 and 10.1; FlowJo LLC).

Fluorescently labeled and biotin-conjugated antibodies were purchased from BioLegend (San Diego, CA), BD Biosciences (San Diego, CA), and eBiosciences (San Diego, CA). Antibodies included anti-CD3 (145-2C11), CD19 (6D5), CD8 (53-6.7), CD4 (GK1.5; RM4-4), NKp46 (29A1.4), CD11b (M1/70), CD27 (LG.7F9), Ly49G2 (4D11), CD44 (IM7), CD11c (N418), KLRG1 (2F1), CD127 (A7R34), IFNγ (XMG1.2), TNFα (MP6-XT22), CD40L (MR1), CD49b (DX5), CD69 (H1.2F3), CD70 (FR70), CD86 (GL-1), and MHC II I-A/I-E (2G9). M45-D^b-tetramer was acquired from the NIH NIAID Tetramer Facility (Bethesda, MD). LIVE/DEAD Fixable Violet and Aqua Dead Cell staining kits were purchased from ThermoFisher Scientific (Waltham, MA).

Peptide restimulation assays

Single cell suspensions of mouse splenocytes from d 6 infected mice were incubated with either immunodominant M45 peptide (HGIRNASFI) or less immunodominant m139 peptide (TVYGFCLL) peptides as described previously (29). Briefly, splenocytes were resuspended in modified IMDM (ThermoFisher Scientific), Brefeldin A (5 μg/mL; BD Biosciences), and the indicated MCMV-derived peptide and subsequently incubated at 37°C, 5% CO_2 for 5 hours. Cytokine production was analyzed via intracellular cytokine staining. Surface proteins and fixable live/dead stains were performed first, as described above. Cells were then fixed and permeabilized (BD CytoFix/Cytoperm Kit) and subsequently stained on ice for IFNγ and TNFα.

Nucleic acid isolation and Quantitative PCR

Spleen DNA was prepared and analyzed for MCMV genomes using quantitative (q)PCR as previously described (49). For analysis of *Cd70* expression, splenic CD11c+ DCs were positively selected using CD11c+ MACS microbeads (Miltenyi Biotec, San Diego, CA). RNA was isolated using TRIzol (ThermoFisher Scientific) according to manufacturer guidelines, and converted to cDNA using Advantage RT for PCR Kit (Clontech, Mountain View, CA). *Cd70* cDNA was amplified using gene-specific primers: *Cd70*-Forward, 5′-TGC TGT TGG TTT CAT TGT AGC G-3′; *Cd70*-Reverse, 5′-ATC CTG GAG TTG TGG TCA AGG G-3′, as reported (50). *Hprt* was also amplified using gene-specific primers (22) and used to normalize and compare *Cd70* expression in infected and naïve DCs.

Statistical methods

All statistical measures were produced in Prism (GraphPad software, v6.0d). Graphs depict mean (± SD) for all data shown. Experimental phenotypes were tested for statistical significance using one-way ANOVA in conjunction with Tukey's post-hoc test, unless otherwise noted. Unpaired student t-tests were used to assess differences between viral titers where appropriate.

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Results

CD70 prompts accumulation of antiviral CD8+ T cells without impacting licensed NK cell responses to MCMV

We first examined DC co-stimulatory effects on virus-specific T cells acquired during MCMV infection in MHC I D^k congenic mice with licensed G2+ NK cells. Heterozygous ($D^{k/b}$) mice were used so that MCMV M45-specific (i.e. M45-D^b-restricted) CD8+ T cells could be analyzed. As expected, CD3+ T cells on the whole expanded significantly by d 6 in response to MCMV (Fig. 1A). However, CD70 neutralization effectively blocked accumulation of total CD8+ and antigen-specific CD8+ T cell subsets, including M45-specific CD8+ T cells, in comparison to isotype-treated control mice (Fig. 1A). It is unlikely this was due to a direct effect on NK cells since virus control in spleen was intact at 3.5 and 6 d post-infection, and G2+ NK cells accrued and matured normally in CD70-blocked mice (Fig. 1B–D, Supplemental Fig 1A). In contrast, sole blockade of either CD86 (Supplemental Fig 1B) or CD80 (Supplemental Fig 1C) failed to reduce T-cell accumulation, though in combination they did (Supplemental Fig. 1D). These results are consistent with previous work to indicate that both CD27 and CD28 signals contribute to non-inflationary T-cell outcomes (33, 51). Our results further demonstrated compensatory mechanisms for CD28 stimulation via CD80 or CD86, whereas CD27 was critically dependent on signals from CD70.

CD70-CD27 and CD80/86-CD28 interactions were previously shown to serve complementary and non-redundant roles during influenza infection (37). Whereas CD28 stimulated CD8+ T cells to begin cycling, CD27 promoted survival and accumulation of activated CD8+ T cells. Herein we observed that transient CD70-blockade had little impact on the frequency of total CD3+ T cells, but significantly reduced the relative proportion of CD8+ T cells (Supplemental Fig. 1E). In contrast, dual blockade of CD80/86 significantly reduced the frequency of total CD3+ T cells, but failed to similarly affect the proportion of CD8+ T cells (Supplemental Fig. 1F). These observations may implicate a similar complimentary co-stimulation cascade, as CD28 stimulation drives total T cell accumulation, and CD27 specifically supports the proportional accumulation of activated CD8+ T cells. Interestingly, TLR agonism alone, such as that induced during viral infection, is sufficient to drive CD80 and CD86 expression on DCs, whereas CD70 typically requires additional co-stimulatory input (e.g. CD40/40L) (48, 52, 53). We therefore further investigated CD70-CD27 signaling and its accessibility to licensed NK-cell regulation.

CD70 controls effector CD8+ T cell differentiation during acute infection in mice with licensed NK cell control of MCMV

During viral infection, CD8+ T cells are exposed to many antigenic, co-stimulatory and cytokine signals that can affect expression of key transcription factors such as T-box expressed in T cells (Tbet) and eomesodermin (Eomes), and ultimately cell survival and effector functionality (54–56). While the duration of antigenic stimulation and exposure to specific cytokine milieus can affect differentiation of CD8+ T effector cells (54, 55, 57), CD70-CD27 co-stimulation can also impact the quality of effector and memory T cell differentiation via the regulation of Eomes (33, 58–60).

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To determine its role in T-cell priming, we first examined how CD70 blockade affects CD8+ T-cell differentiation. We assessed CD44 and the β2-integrin CD11c to broadly examine total antigen-experienced T cells, rather than solely focusing on M45-specific CD8+ T cells (61, 62). Importantly, CD44+ CD11c+ T cells were specifically detected after infection, but not in naïve mice (Fig. 2A). As with M45-specific T cells, accumulation of CD44+ CD11c+ CD8+ T cells in response to MCMV was CD70 dependent (Fig. 2A–C). We thus further examined CD70's effect on the differentiation of antigen-experienced CD8+ T cells into either short-lived effector cells (SLEC; KLRG1+ CD127−) or memory precursor effector cells (MPEC; CD127+ KLRG1−) as defined by Kaech and colleagues for T cells responding to lymphocytic choriomeningitis virus (LCMV) (54, 63). Similar to LCMV infection, antigen-experienced T cells were skewed towards KLRG1+ SLECs in MCMV-infected (d 6) mice, with a near 10-fold accrual of SLECs over naïve controls (Fig. 2A, 2D). CD70 neutralization decreased the accumulation of SLECs to less than half of what was observed in control mice. Even within the population of antigen-experienced CD8+ T cells, CD70 blockade reduced the proportion of CD8+ T cells that were KLRG1+ (Fig. 2D). We also observed significant differences in the numbers of CD127+ antigen-experienced MPECs when we compared control and CD70-blocked mice. However, this could have been due to disparities in the total number of antigen-experienced cells since CD127+ cell frequencies were equivalent (Fig. 2E). These results suggested that CD70 stimulation promotes MPEC accumulation during acute MCMV infection, consistent with previous reports (33).

To determine whether the differences in SLEC differentiation also affected functionality, we next assessed CD8+ T-cell cytokines produced in response to *ex vivo* restimulation with two different MCMV peptides, both of which are expressed during acute infection. Similar to its effect on T-cell differentiation, CD70 blockade dramatically decreased the numbers of M45- and m139-specific IFNγ+ (Fig. 2F) and TNFα+ (Fig. 2G) CD8+ T cells in MCMV-infected mice. Even without additional peptide restimulation, CD8+ T cells from isotype-treated mice exhibited a trend toward higher cytokine production. Taken together, these results suggested that CD70-CD27 signals prime CD8+ T cells to efficiently produce effector cytokines in mice with licensed NK cell control of MCMV.

CD8+ T cell dependence on CD70 signaling is intact in CD4+ T cell-depleted mice with licensed NK control of MCMV

We previously found that licensed NK cells can augment virus-specific CD8+ T-cell immunity without CD4+ T-cell help (29), consistent with prior work that demonstrated CD4+ T cell-independent CD8+ T-cell responses during MCMV infection (64). However, DC licensing for CD70 expression was thought to require co-stimulatory support from CD4+ T cells or helper subsets (e.g. NKT) that support CD40/40L interactions (48, 52, 53, 65–68). To pursue this, we analyzed the effect of CD70 neutralization on CD8+ T cells responding to MCMV in CD4+ T cell depleted mice (Fig. 3A, 3B). The mere absence of CD4+ T cells had little impact on CD8+ T cells responding to MCMV, whereas additional blockade of CD70 reduced total CD8+ T-cell numbers somewhat (Fig. 3C). We suppose that increased homeostatic T cell expansion after CD4+ T cell depletion may have accounted for the reduced efficacy of CD70 blockade on overall CD8+ T cells (see Figs. 1, 2). Nonetheless, CD70 blockade significantly muted M45-specific CD8+ T-cells in CD4 T cell-

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depleted mice (Fig. 3D). Still, we observed some variability in the extent of its effect in CD4+ T cell replete and depleted settings, which resulted in 4-fold or 2-fold declines in virus-specific CD8+ T cells, respectively (Fig 1, 3D). The results suggested that licensed NK cell control of MCMV may impart CD70-mediated enhancement of CD8+ T cell immunity without CD4+ T cell help.

We next addressed this question by repeating the CD70 neutralization treatment in experimental groups of mice, with or without CD4+ T cells, that could be directly compared (Supplemental Fig. 2). As expected, CD4+ T cell depletion *per se* had no effect on CD8+ T cells responding to MCMV (Supplemental Fig. 2A, 2C, 2D). Importantly, direct comparisons showed that CD70 signaling was equally important to expand (4-fold) overall and virus-specific CD8+ T cells, irrespective of CD4+ T cells. Similar patterns were also detected for M45-specific SLECs and MPECs (Supplementary Fig. 2E, 2F). Thus, these results indicated that enhanced accumulation of MCMV-specific CD8+ T cells in the presence of licensed NK cells is CD70-dependent and proceeds even in the absence of CD4+ T cells.

We next sought to determine whether interactions between CD40L and CD40 were necessary to prime efficient virus-specific CD8+ T-cell responses. While helper CD4+ T cells are perhaps best known for CD40L expression, the ligand can also be displayed by other leukocytes including CD8+ T cells, γδ-T cells, NK cells, NKT cells, monocytes, basophils, and mast cells (65). In fact, NK cells were found to drive *in vitro* maturation of monocytes via CD40/40L interactions, which then primed *M. tuberculosis* antigen-specific CD8+ T cells (69). Thus, we assessed if licensed NK cells express CD40L in response to viral infection. We found only limited intracellular stores of CD40L, however, and there were no differences observed among licensed and unlicensed NK cells (Supplemental Fig. 2G). To determine if CD40L has a role in helping licensed NK cells to prime CD8+ T cells, we gave repeated doses of anti-CD40L blocking mAb to D^k mice during infection. CD40L blockade, however, had little impact on total CD8+ T-cell accumulation in comparison to control mice (Fig. 3E). Additionally, CD40L blockade yielded only modest and insignificant reductions in M45-specific CD8+ T cells (Fig. 3F). In aggregate, these data demonstrated that CD70 effectively costimulates CD8+ T cell immunity in a CD4+ T cell- and CD40/40L-independent manner in mice with licensed NK cell control of MCMV.

CD27-deficiency dampens virus-specific CD8+ T cell immunity without impairing licensed NK cell control of MCMV

Next we examined the role of CD70-CD27 co-stimulation in distinct CD8+ T cell immune responses in mice with or without licensed NK cell control of MCMV by assessing the effect of CD27 deficiency in our D^k-disparate mouse strains (see Materials and Methods). To validate the model of licensed G2+ NK-cell virus control in CD27 KO mice, we examined spleen tissues on d 6 after infection. As expected, MCMV was below detection in mice with D^k-licensed NK cells, irrespective of CD27 expression (Fig. 4A). In fact, virus spread was fully restrained by 3.5 days p.i., which further indicated that licensed NK-mediated virus control in D^k mice is not delayed by CD27 deficiency (Supplemental Fig. 3A, 3B). In contrast, virus levels were generally 3-logs higher in both strains without D^k. Although

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larger numbers of total and G2+ NK cells responding to MCMV were generally observed in CD27 KO-D^k mice, they did not differ significantly from CD27 KO NK cells in non-D^k mice (Supplemental Fig. 3C, 3D). Significant elevations in activation (CD69+) and maturation (CD11b+) markers were also observed in the CD27 KO NK cells, which suggested that these cells were indeed responding and specifically controlling viral infection (Supplemental Fig. 3E, 3F). Non-D^k mice exhibited a 2-fold greater number of total CD69+ NK cells (d 3.5), which likely reflected the sustained elevation of splenic viral levels and the continued non-specific activation of NK cells, as these parameters have been previously correlated (21). Nonetheless, these results indicated that licensed NK cell-mediated resistance to MCMV is CD27-independent.

While the ability of D^k-licensed NK cells to efficiently respond to and restrain acute MCMV infection was unhindered by CD27 deficiency, previous work established that CD8+ T cell expression of CD27 is necessary to mediate CD70-dependent virus-specific T cell immunity (33). Thus, we further examined the role of CD70-CD27 signaling in driving distinct CD8+ T cell responses to MCMV in our D^k-disparate congenic strains. As expected (44), CD4+ and CD8+ T cell numbers in naïve mice were unaffected, indicating that CD27 had little impact on the homeostatic maintenance of naïve T cells (Fig. 4B–D). However, MCMV induced accumulation of total and M45-specific CD8+ T cells in mice with both D^k and CD27 (Fig. 4B, 4D, 4E). Total numbers of CD4+ T cells were unaffected by the presence of D^k or CD27 (Fig. 4C), however previous studies have identified deficiencies in antigen-specific CD4+ T cell responses to class II restricted MCMV peptides in the absence of CD27 (33). As with CD70 neutralization, CD27-deficiency severely limited M45-specific CD8+ T cell accumulation in mice with D^k-licensed NK cell control of MCMV. In fact, CD8+ T cells expanded similarly sans CD27 signaling regardless of whether D^k was expressed.

In addition to CD27-dependent accrual of virus-specific CD8+ T-cells, we found that CD27 was also critical for priming SLECs and IFNγ-competent CD8+ T cells in D^k mice. In comparison, the absence of either CD27 or D^k significantly interfered with CD8+ T SLEC differentiation and cytokine production at d 6 (Fig. 4F, 4H). Once again, these effects were not limited to a single T-cell epitope, as peptide re-stimulation with m139 recapitulated the difference in numbers of cytokine producing CD8+ T cells amongst different groups. In the M45-specific population, there was little evidence of CD8+ T MPEC differentiation at d 6 (Fig. 4G). In aggregate, these results indicated that although licensed NK cell activation, accumulation and control of MCMV proceeded independent of CD27, CD8+ T cell priming in the wake of efficient MCMV control still required CD70-CD27 signaling for early accumulation of KLRG1+ SLECs.

Licensed NK cell control of MCMV adjusts the duration of CD70 expression on DC

Intriguingly, CD27-deficiency did not further impede CD8+ T-cell accumulation in mice without D^k (Fig. 4D, 4E). This suggested one of two possibilities: (i) CD70 could be selectively regulated by efficient NK cell control of MCMV. If so, CD27 expression on CD8+ T cells should have little impact on virus-specific T-cell accumulation in non-D^k mice. (ii) Alternatively, poor virus control due to the absence of licensed NK cells might

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interfere with early CD8+ T-cell priming, regardless of access to co-stimulatory mediators. If so, analysis of d 6 T-cell features to test the role of CD70-CD27 signaling in non-D^k mice may be challenging since previous work showed that poor virus control corresponded with a delay in the acquisition of functionally competent effector T cells *in vivo* (29). Both of these possibilities were therefore pursued to investigate if licensed NK cells regulate CD27-dependent T-cell accumulation.

To investigate whether licensed NK cells influence CD70 expression on DCs, we examined splenic DCs in D^k-disparate mice after infection. MCMV levels were similar at 36 h, but began diverging as early as 48 h for the two mouse strains (Fig. 5A). Surprisingly, *Cd70* gene expression increased with similar kinetics and magnitude in D^k and non-D^k mice early after infection (Fig. 5B). While *Cd70* expression had increased slightly in DC relative to naïve controls by 36 h, it increased substantially in both mouse groups by 48 h postinfection. By 72 h, *Cd70* expression in D^k mice had returned to baseline, whereas it remained elevated in non-D^k mice 72–96 h. These data indicated that licensed NK-cell resistance coincided with restrained *Cd70* expression in DCs. We next examined MCMV-induced DC cell surface expression of costimulatory ligands and MHC class II (I-A/I-E) (Fig. 5C). Consistent with transcriptional kinetics, both D^k and non-D^k mice had increased expression of CD70 and CD86 at 48 h postinfection. For D^k mice, maximal CD70 and CD86 expression occurred at 48 and 72 h, respectively, and then decreased afterward. In contrast, non-D^k mice had increased expression (MFI) for both CD86 and CD70 through 96 h postinfection. These results suggested that co-stimulatory ligands in DCs were negatively regulated in D^k mice in comparison to prolonged CD70 and CD86 expression in non-D^k mice. In addition to higher expression of these maturational markers, DC frequencies and total numbers of DCs expressing CD70 and CD86 were also significantly elevated in non-D^k mice at 96 h (Fig. 5D, 5E). Whether this was due to downregulation of co-stimulatory ligands in mature DCs, or expansion of immature DCs from pre-DCs in D^k mice remains to be determined. Thus, counter to expectations, MCMV infection resulted in increased costimulatory ligand gene and protein expression, with slightly greater and extended up-regulation in non-D^k mice lacking highly efficient licensed NK cell virus control.

Inefficient NK-mediated control of MCMV results in delayed and CD27-independent accumulation of KLRG1+ SLECs

Given CD70’s protracted expression in DC due to inefficient virus control, we pursued its effect on CD8+ T cells later during infection. As in Figure 4, total and antigen-experienced CD8+ T cells were again less abundant in infected (d 6) non-D^k mice (Fig. 6A–D). As a result, deficits in SLECS and MPECs were also observed, regardless of CD27 expression (Fig. 6E, 6F). However, by d 8 CD3+ and CD8+ T cells in both WT and KO non-D^k mice were on par with T cells in D^k mice (Fig. 6B, 6C). Moreover, as the antigen-experienced CD8+ T cells in D^k mice declined at d 8, in non-D^k mice they significantly increased (Fig. 6A, 6D). Even more striking, KLRG1+ SLEC differentiation increased more than 2-fold in both WT and KO non-D^k mice (Fig. 6A, 6E). These data suggested that a lack of efficient MCMV control, rather than CD70-CD27 signaling, led to significantly altered CD8+ T-cell differentiation. On the other hand, CD127+ KLRG1− CD8+ T cells decreased as the population of KLRG1+ CD127− cells increased, but this had little effect on the total number

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of CD127+ CD8+ T cells (Fig. 6A, 6F). Significant contractions of the SLEC population in D^k mice and compensatory growth in the number of differentiated CD127+ MPECs at d 8 were also observed. Moreover, this bias toward SLEC contraction and MPEC accrual was reversed in CD27 WT-D^k mice through specific depletion of G2+ NK cells prior to MCMV infection (Fig. 6G), which confirms a specific role of D^k-licensed G2+ NK cells in shaping CD8+ T-cell immunity. Together these data therefore suggested that the balance of licensed NK cell-mediated virus resistance distinguished the patterns of CD8+ T cell differentiation in response to MCMV infection. Whereas more efficient NK-mediated virus control coincided with earlier CD8+ T-cell priming and SLEC formation, higher viral burden corresponded to later CD27-independent accumulation of KLRG1+ SLECs and fewer MPECs.

Although CD27 stimulation has been implicated as a primary mediator of memory conversion in CD8+ T cells (33, 37, 44, 59), CD70-CD27 interactions have also been proposed to have opposing effects on CD8+ T cells, depending on the chronicity of infection (70, 71). Penaloza-MacMaster et al. (70) demonstrated that CD70-CD27 interactions are vital for priming CD8+ T cells during acute LCMV Armstrong infection, but that persistent CD70 signaling during chronic LCMV clone-13 infection actually decreases CD8+ T cell accumulation by d 21. Similarly, prolonged CD27 stimulation during chronic LCMV has also been demonstrated to increase CD4+ T cell production of IFN-γ and TNF-α, both of which disrupt splenic architecture and interfere with viral clearance by neutralizing antibodies (71). Given the prolonged exposure to active viral replication, it is possible that persistent CD27 signaling due to ineffective NK-mediated virus control is detrimental to CD8+ T cell survival and memory conversion.

To investigate if CD27 hinders the generation of memory CD8+ T cells in response to MCMV in non-D^k mice, we infected WT and CD27 KO D^k-disparate mice with MCMV and followed the infections to d 21. Surprisingly, two major phenotypes were noted: First, CD27 expression had little impact on frequencies of CD3+ or CD8+ T cells in peripheral blood from D^k or non-D^k mice (Supplemental Fig. 4A–C). However, the frequency of circulating M45-specific CD8+ T cells was decreased in CD27 KO mice relative to WT mice (Supplemental Fig. 4D). Hence, CD27 was required to maintain antigen-specific CD8+ T cells after virus clearance from spleen, which was independent of the extent of NK-mediated MCMV resistance or a robust CD8+ T cell response during acute infection. Second, the frequencies of SLEC and MPEC differentiation within the respective populations of virus-specific CD8+ T cells revealed that effector T cell populations expressing KLRG1 or CD127 trended within D^k and non-D^k mice (Supplemental Fig. 4A, 4E, 4F). Regardless of CD27 expression, mice with licensed NK-cell control tended to skew towards CD127+ memory CD8+ T cell responses, whereas mice without licensed NK cell control biased CD8+ T cells towards a KLRG1+ effector CD8+ T cell fate. Thus, these results indicated that while CD70-CD27 interactions improve the survival and accumulation of antigen-specific CD8+ T cells, differentiation skewing is substantially influenced by early viral control mediated by NK cells.

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Discussion

The ability to mount highly functional and specific NK cell responses to viral infection is integral to protecting hosts against repeated virus challenges (1, 2). In addition to the direct antiviral properties of NK cells, these innate lymphocytes also play critical roles in preventing excessive immunopathology (16, 20, 72), preserving lymphoid organ architecture (73), and licensing DCs for improved priming of T cells (10, 11, 74). Whether the impacts of NK cells on CD8+ T-cell immunity are ultimately beneficial or detrimental to protective host immunity is still debated, and likely depends on host- and virus-specific contexts (6, 7, 75). Particularly for NK cells, host genetic factors yield considerable influence over the cues to which the NK cells can respond (4, 23, 76), and likewise, the manner in which the virus manipulates infected target cells informs the type and quality of NK reactivity (5).

Here we investigated pathways through which licensed NK cells responding to MCMV infection improve adaptive CD8+ T cell immunity. We found that similar to other mouse models of efficient NK-mediated MCMV resistance (32, 33), CD27 and CD28 co-stimulatory interactions were necessary to promote accumulation of CD8+ T cells by d 6. Moreover, CD70 specifically impacted the proportional accumulation of CD8+ T cells and shaped effector cell differentiation. Given the critical role for CD27 in driving CD8+ T cell responses in mice with D^k-licensed NK cells and the minimal effect of CD27 deficiency in mice with much less efficient virus resistance, we expected licensed NK cells responding to MCMV to specifically induce of CD70 on DCs. However, we found that CD70 was equally upregulated on splenic DCs early after infection, even in the absence of licensed G2+ NK cells. Thus, rather than influencing the expression of the CD70 on splenic DCs, licensed G2+ NK cells determined the set of cues necessary for priming and differentiating acute CD8+ effector T cells.

CD8+ T cells are exposed to a variety of signals during viral infection that influence their priming and differentiation. These signals include (i) the strength and duration of antigen recognition by the TCR (signal 1), (ii) co-stimulatory interactions at the immunological synapse (signal 2), and (iii) cytokines in the inflammatory milieu (signal 3) [reviewed in (77)]. Tight regulation and coordination of signal 2 is key to driving a controlled cytotoxic T-cell response without inducing anergy or immunopathology (36, 78). While many of these co-stimulatory pathways are shared across diverse viral infections, several reports have acknowledged that distinct families of viruses as well as the relative persistence of viral infections can significantly impact T-cell reliance and host-benefits from certain co-stimulatory cues (41, 42, 70, 71, 79). Croft and colleagues (79) previously correlated the virulence of recombinant vaccinia strains with increased utilization of CD27 and OX40 co-stimulatory pathways. In that study, deficiencies in CD27 or OX40 receptors were only detrimental when the host was challenged with highly virulent vaccinia virus. In related work, CD70-CD27 interactions were shown to be either critical or dispensable for CD8+ T-cell immunity depending on whether mice were infected with acute LCMV Armstrong or chronic LCMV clone-13, respectively (70). It was hypothesized that while CD70-CD27 co-stimulation was vital for immune responses to acute LCMV with low antigenic exposure, chronic LCMV infection likely induced alternative co-stimulatory pathways that precluded dependence on CD70. Moreover, over-stimulation of CD27 has actually been shown to

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impede adaptive immune responses during chronic viral infection and prolong the time to viral clearance (70, 71).

Here, rather than comparing the impacts of different virus strains (e.g. Armstrong vs clone-13) on the outcomes of co-stimulatory signaling pathways, we varied the host models and their ability to efficiently control infection. We predicted that prolonged exposure to MCMV in mice without efficient licensed NK cell control would increase the number and diversity of co-stimulatory signals utilized by antigen-specific CD8+ T cells. Similar to the studies with LCMV Armstrong and clone-13, the efficiency of viral control was a chief determinant of acute CD8+ T cell dependence on CD27. Whereas D^k mice were critically dependent on CD27 for enhancement of CD8+ T cell accumulation, mice without D^k demonstrated a delayed but CD27-independent accrual of T cells. While conditions of prolonged viral antigen exposure likely induced additional co-stimulatory or inflammatory pathways that could drive acute CD8+ T cell accumulation and differentiation, CD27 was still integral for establishing CD8+ T memory cells in both D^k and non-D^k mice, and did not provoke the negative effects previously seen with LCMV clone-13 (70). Thus, regardless of the efficiency of viral control, CD27 plays a critical role in shaping memory differentiation and survival, consistent with previous studies (33, 58–60). However, when MCMV is efficiently dampened via licensed NK cells and putatively less antigen and inflammation is present, the potency of CD70-CD27 interactions is necessary to promote immunity over tolerance. Future studies are warranted to further explore the long-term impacts of licensed NK-mediated MCMV control on memory T cells. Given that additional stimuli may be guiding delayed CD8+ T cell priming and that differentiation skewing is informed by the presence of licensed G2+ NK cells, we speculate that the early influence of licensed NK cells during MCMV infection may have lasting impacts on host immunity.

As we begin to dissolve the complexities of NK regulation of adaptive immunity, it is clear that the '*one size fits all*' approach is insufficient to explain the diversity of NK cell influences that we observe across disease models. Particularly as we develop and implement immunotherapeutic strategies against infectious diseases and varieties of cancer, it will be important to evaluate the impact of NK cells in host-specific contexts. In genetically diverse inbred mouse strains, we observe a plethora of NK cell influences ranging from vital support for T cell immunity (13, 28, 29, 69) to cytolytic elimination of activated lymphocytes (16, 18–20, 72). From studies of human NK cell responses to viral infections – including HCMV, HIV, and HCV – it is understood that specific pairings of NK cell receptors and HLA alleles could influence the extent of viral resistance (4, 23). Moreover, such receptor-ligand interactions may directly influence selective DC survival or maturation early during infection (12). In the same vein, while the diversity of T cell co-stimulatory cues for various models of disease have been well studied (80), it is clear that different viral infections will influence the pathways utilized to promote protective immunity (41, 70, 79). Still, even with all of these cells and signals in place, host-genetic factors that instruct NK cell responses may consequently shape the impact and necessity of those signals, as demonstrated herein. Thus, in addition to studying the cell types and signals that can foster cell-mediated immunity, we will need to develop a more thorough understanding of early innate immunity and how it affects the immunogenic milieu.

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Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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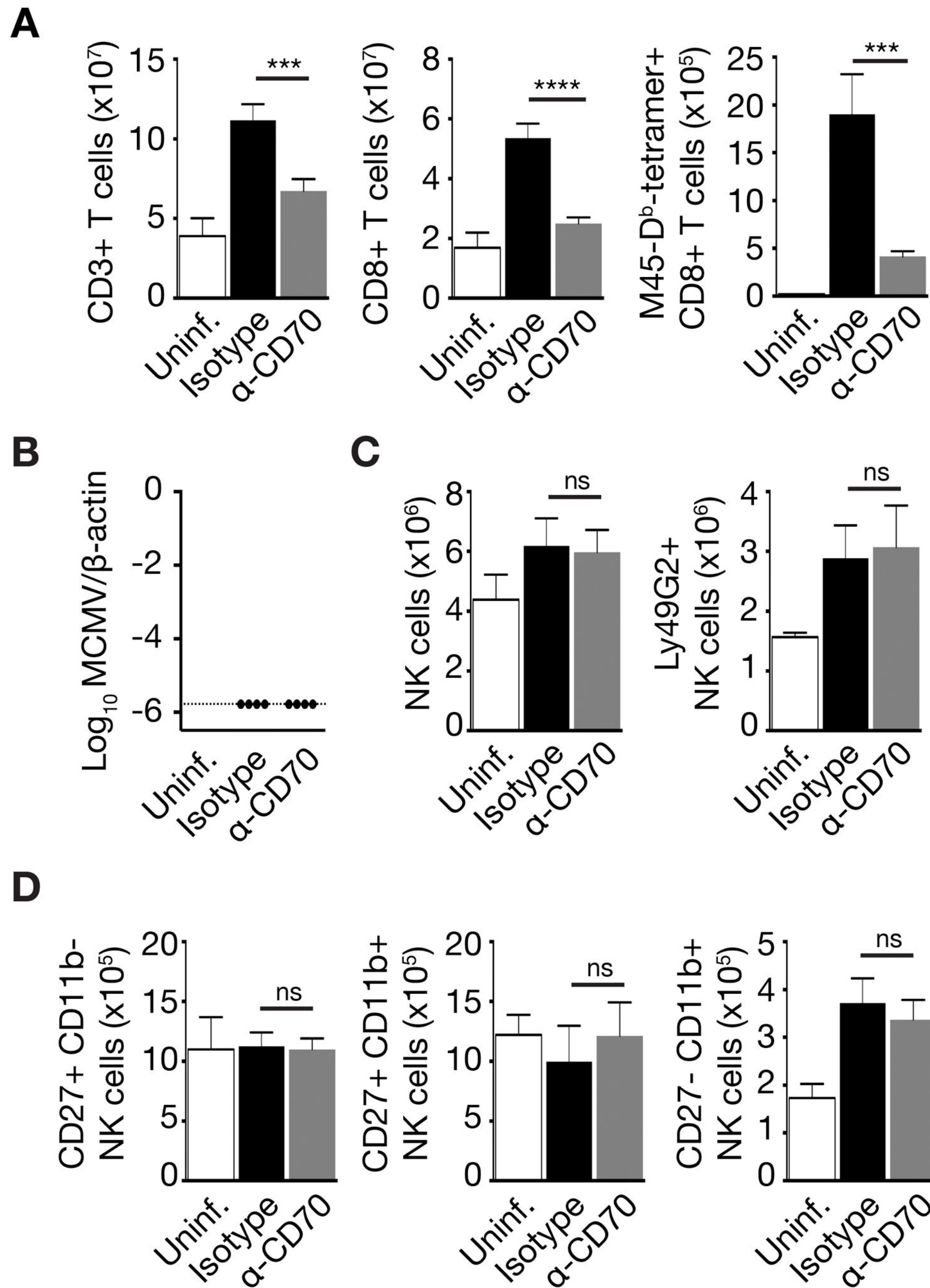

Figure 1. CD70 prompts accumulation of antiviral CD8+ T cells without impacting licensed NK cells responding to MCMV

MHC congenic R7 ($D^{k/b}$) mice were either PBS-injected (uninf), or treated with rat IgG (isotype) or mAb FR70 (α-CD70) prior to and concurrent with MCMV infection. MCMV levels (d 6) in spleen tissues were measured and fluorescent mAb-stained splenocytes were analyzed by flow cytometry. (A) Graphs show the means (± SD) for the number of viable single cell-gated CD3+ CD19− T cells, and total and M45-specific CD8+ T cells for the indicated groups of mice (n = 3–4 mice/group). (B) The graph shows individual spleen virus levels in the indicated mice. (C) The bar graphs show the mean (± SD) number of total

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viable single-cell gated CD3− CD19− NKp46+ NK cells (left) and G2+ NK cells (right). (D) Graphs show the mean (± SD) number of immature CD27+ CD11b− (left), maturing CD27+ CD11b+ (center), and mature CD27− CD11b+ (right) NK cells. Results are representative of at least 4 independent studies. Statistical comparisons were calculated using one-way ANOVA when appropriate (*P < .05, **P < .01, ***P < .001, ****P < .0001).

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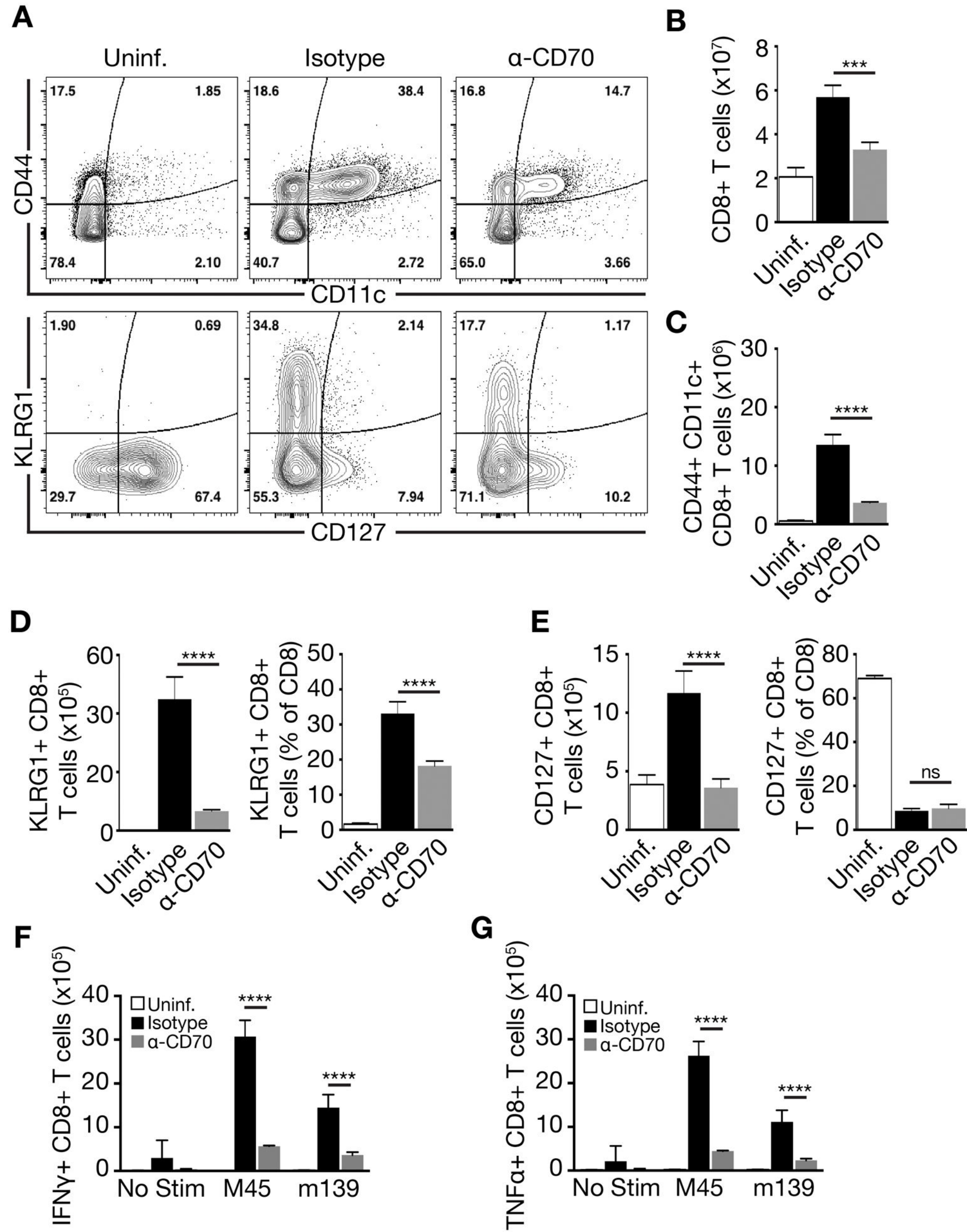

Figure 2. CD70 controls effector CD8+ T cell differentiation during acute infection in mice with licensed NK cell control of MCMV

Mice were treated with rat isotype IgG or anti-CD70 and infected with MCMV as in Figure 1. (A) Shown are representative contour flow plots for the frequencies of antigen-experienced (CD44+ CD11c+) T cells (top) and SLEC (KLRG1+ CD127−) and MPEC (CD127+ KLRG1−) differentiated CD8+ T cells (bottom). (B–C) Graphs represent the mean (± SD) number of total CD8+ (B) and antigen experienced CD8+ T cells (C). (D–E) Histograms show the mean (± SD) numbers and frequencies of antigen experienced SLEC (D) and MPEC (E) CD8+ T cell populations. (F–G) Bar graphs show the intracellular expression of INFγ (F) and TNFα (G) by CD8+ T cells for each peptide restimulation. (n =

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3–4 mice/group). Data is representative of at least 4 independent studies. Statistical comparisons were calculated using one-way ANOVA when appropriate (*P < .05, **P < .01, ***P < .001, ****P < .0001).

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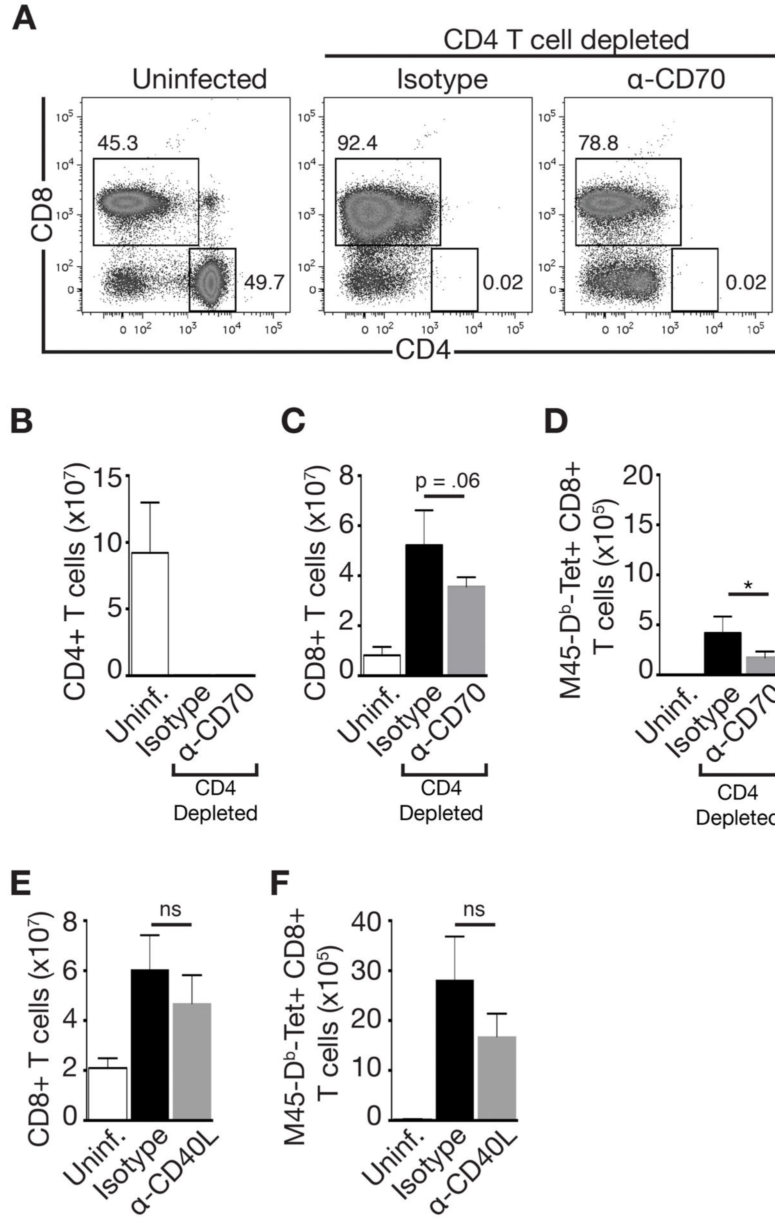

Figure 3. CD8+ T cell dependence on CD70 signaling is intact in CD4+ T cell-depleted mice with licensed NK control of MCMV

Anti-CD4 (GK1.5) depleting antibodies were administered to MHC congenic R7 ($D^{k/b}$) mice to effectively deplete CD4+ T cells. CD4-depleted mice were additionally treated with rat isotype IgG or anti-CD70 blocking antibodies during MCMV infection. (A) The dot plots represent frequencies of splenic CD8+ and CD4+ T cells from the indicated cohorts (d 6). (B–D) Histograms show the mean (± SD) of total CD4+ (B), CD8+ (C) and tetramer+ CD8+ T cells (D). Data are representative of 2 experiments with 3–4 mice per group. (E–F) Congenic R7 ($D^{k/b}$) mice were treated with neutralizing anti-CD40L (MR1) monoclonal antibody during MCMV infection. Bar graphs show the mean (± SD) number of total (E)

and M45-D^b-tetramer+ CD8+ T cells at d 6. Data are representative of 3 experiments (n = 3–4 mice/group). Statistical analyses were performed using one-way ANOVA (*$P < .05$).

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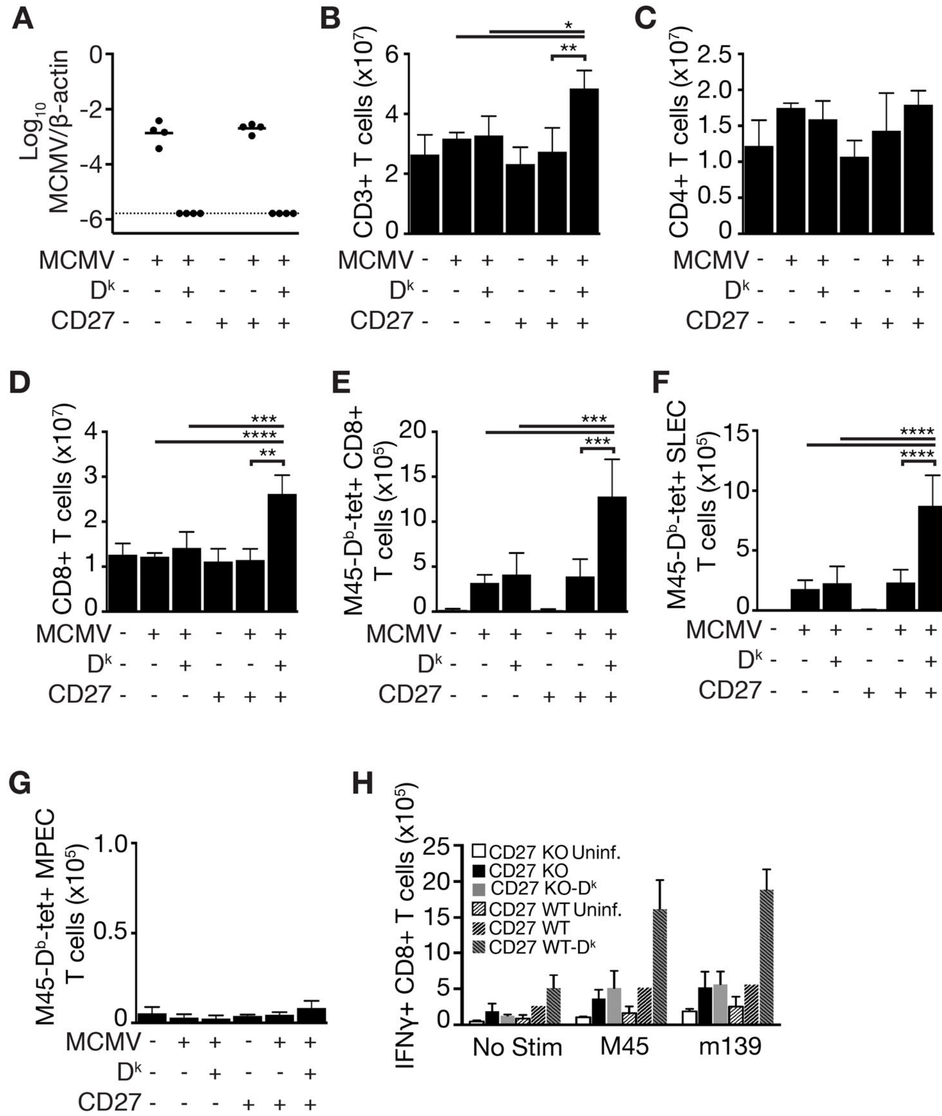

Figure 4. CD27-deficiency dampens virus-specific CD8+ T cell immunity without impairing licensed NK cell control of MCMV

CD27 KO, CD27 KO-D^k, CD27 WT and CD27 WT-D^k mice were infected with MCMV for 6 d. (A) The graph shows individual spleen virus levels in the indicated mice. (B–E) Histograms show the mean (± SD) number of total CD3+ (B), CD4+ (C), CD8+ (D), and M45-D^b-tetramer+ T cells (E). (F–G) Bar graphs show the mean (± SD) number of tetramer + SLECs (F) and MPECs (G) per spleen at d 6. (H) Histograms represent the mean (± SD) number of total IFNγ+ CD8+ T cells detected for each peptide stimulation (No stimulation, M45, and m139). Data are representative of 3 independent experiments (n = 3–4 mice/group). Statistical comparisons were calculated using one-way ANOVA (*P < .05, **P < .01, ***P < .001, ****P < .0001).

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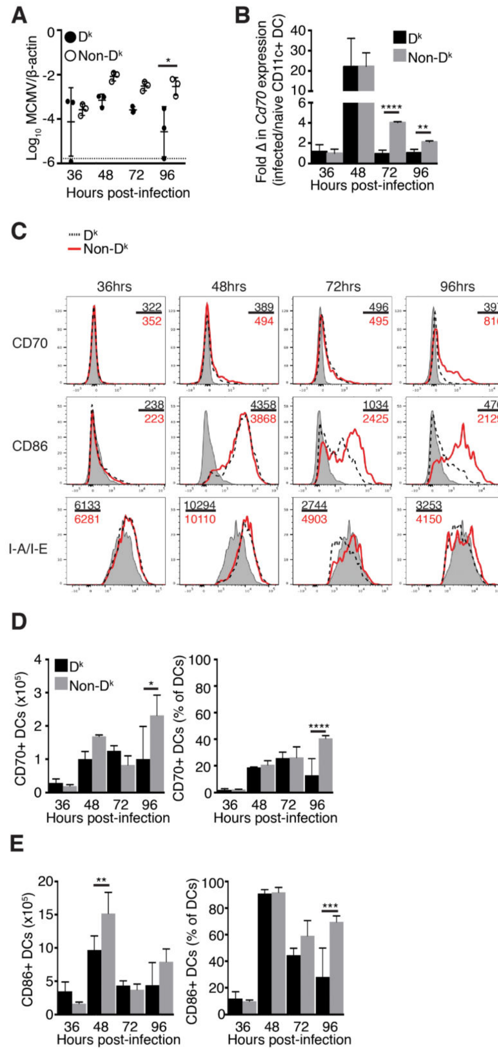

Figure 5. Licensed NK cell control of MCMV adjusts the duration of CD70 expression on DC

(A) MCMV genome levels in individual D^k-disparate mice at the indicated times postinfection are shown. (B) Spleen CD11c+ DCs were positively selected at the indicated times postinfection. The graph displays the mean (± SD) fold-change in normalized *Cd70* expression relative to naïve DCs. (C) The histogram overlays of viable (single-cell) CD3– CD19– MHC II^{hi} gated DCs demonstrate representative expression of co-stimulatory ligands CD70 (top) and CD86 (middle), as well as MHC II (I-A/I-E) (bottom) for the indicated time points (black dashed lines = D^k; red solid lines = non-D^k; shaded graphs = CD70 FMO or d 0 CD86 and MHCII). Median fluorescent intensities for each of the ligands from D^k (top black) and non-D^k (bottom red) mice are also displayed in each histogram. (D, E) Graphs represent the total number and frequency (± SD) of DCs expressing CD70 (D) or CD86 (E). Data are representative of 2-independent experiments (n = 3–4 mice/group) (*P < .05, **P < .01, ****P<.0001 by one-way ANOVA).

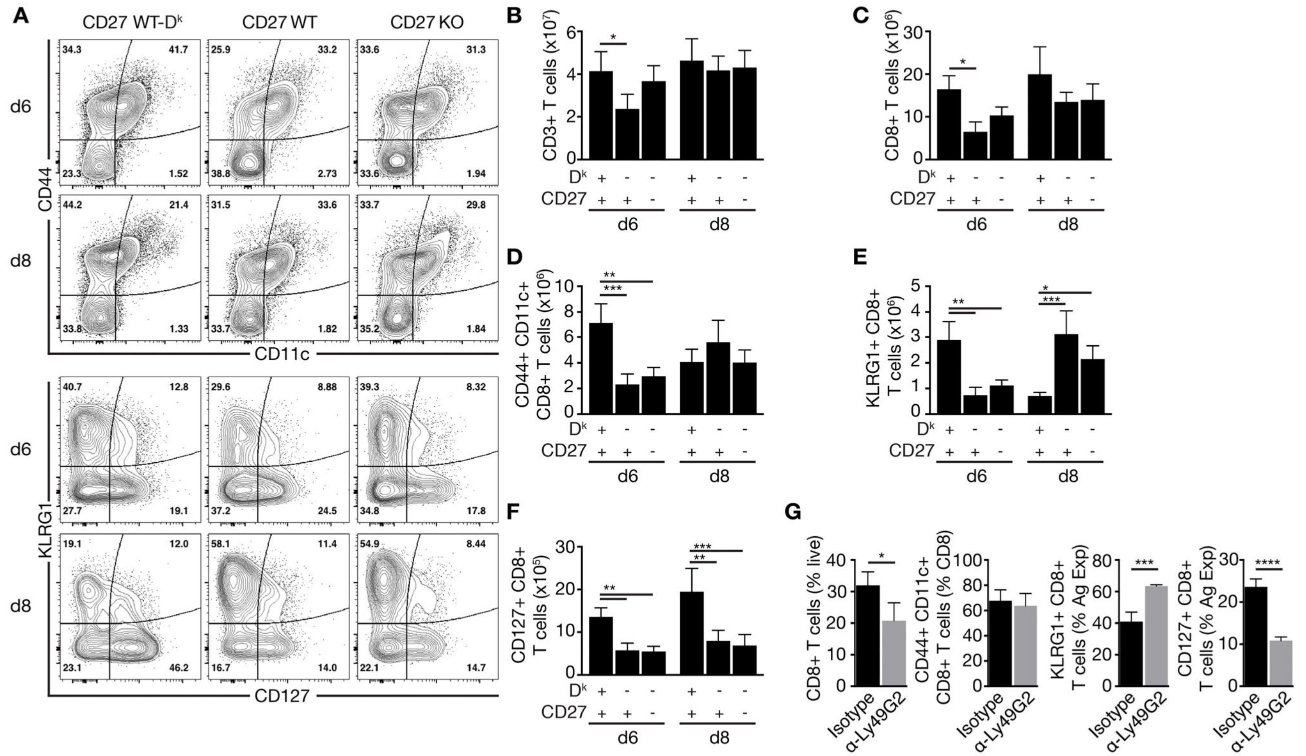

Figure 6. Inefficient NK-mediated control of MCMV results in delayed and CD27-independent accumulation of KLRG1+ SLECs

Representative contour plots show the frequency accumulation of antigen experienced CD8+ T cells (top) and the differentiation of antigen experienced CD8+ T cells into SLECs and MPECs (bottom) for CD27 WT-D^k, CD27 WT, and CD27 KO mice at 6 and 8 d. (B–F) Histograms display the mean (± SD) number of total CD3+ T cells (B), total CD8+ T cells (C), antigen experienced CD8+ T cells (D), SLECs (E), and MPECs (F). (G) CD27 WT-D^k mice were treated with isotype or depleting mAb 4D11 (α-Ly49G2) prior to MCMV infection. Bar graphs show the mean (± SD) frequencies of total CD8+ T cells, CD44+ CD11c+ CD8+ T cells, SLECs, and MPECs. Data are representative of 2 independent experiments (n = 3–4 mice/group). Statistical comparisons were calculated using two-way ANOVA (*P < .05, **P < .01, ***P < .001, ****P < .0001).

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