A CD4⁺ T Cell-NK Cell Axis of Gammaherpesvirus Control

Clara Lawler,^a Philip G. Stevenson^{a,b}

^aSchool of Chemistry and Molecular Biosciences, University of Queensland, Brisbane, Australia

^bChild Health Research Center, University of Queensland, South Brisbane, Australia

ABSTRACT CD4⁺ T cells are essential to control herpesviruses. Murid herpesvirus 4 (MuHV-4)-driven lung disease in CD4⁺ T-cell-deficient mice provides a well-studied example. Protective CD4⁺ T cells have been hypothesized to kill infected cells directly. However, removing major histocompatibility complex class II (MHCII) from LysM⁺ or CD11c⁺ cells increased MuHV-4 replication not in those cells but in type 1 alveolar epithelial cells, which lack MHCII, LysM, or CD11c. Disruption of MHCII in infected cells had no effect. Therefore, CD4⁺ T cells engaged uninfected presenting cells and protected indirectly. Mice lacking MHCII in LysM⁺ or CD11c⁺ cells maintained systemic antiviral CD4⁺ T cell responses, but recruited fewer CD4⁺ T cells into infected lungs. NK cell infiltration was also reduced, and NK cell depletion normalized infection between MHCII-deficient and control mice. Therefore, NK cell recruitment seemed to be an important component of CD4⁺ T-cell-dependent protection. Disruption of viral CD8⁺ T cell evasion made this defense redundant, suggesting that it is important mainly to control CD8-evasive pathogens.

IMPORTANCE Gammaherpesviruses are widespread and cause cancers. CD4⁺ T cells are a key defense. We found that they defend indirectly, engaging uninfected presenting cells and recruiting innate immune cells to attack infected targets. This segregation of CD4⁺ T cells from immediate contact with infection helps the immune system to cope with viral evasion. Priming this defense by vaccination offers a way to protect against gammaherpesvirus-induced cancers.

KEYWORDS CD4 T cell, MHC class II, antiviral immunity, gammaherpesvirus, immune evasion

Neutralizing antibodies bar most viruses from significant reinfection. Herpesviruses continue to transmit despite eliciting antibody, and they emerge after immune cell suppression, implying that they are subject to T-cell-dependent control. Analysis of gammaherpesviruses has focused on CD8⁺ T cell recognition of B cells transformed *in vitro* by Epstein-Barr virus (EBV). However, EBV harms patients lacking CD4⁺ T cells or NK cells rather than CD8⁺ T cells (1), and its control in immunosuppressed patients correlates better with CD4⁺ than with CD8⁺ T cell reconstitution (2). The related murid herpesvirus 4 (MuHV-4) shows more acute lytic replication in CD8⁺ T-cell-deficient mice, but infection is still contained (3), whereas in CD4⁺ T-cell-deficient mice it is not (4). Similar CD4⁺ T-cell-dependent control of murine cytomegalovirus (MCMV) (5) and human cytomegalovirus (HCMV) infections (1) suggests that this is a common theme—perhaps due to viral attack on major histocompatibility complex class I (MHC I)-restricted antigen presentation limiting infection control by CD8⁺ T cells (6, 7).

How CD4⁺ T cells control herpesvirus infections is unclear. Their provision of help to B cells may contribute, as antibody can limit MuHV-4 infection (4, 8). Yet CD4⁺ T cells control MuHV-4 even without B cells—or CD8⁺ T cells (9, 10)—and CD4⁺ T cell deficiency confers greater susceptibility than B cell deficiency to human herpesviruses (1). Therefore, CD4⁺ T cell effector function also seems to be important. Direct,

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Address correspondence to Philip G. Stevenson, p.stevenson@uq.edu.au.

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cytotoxic attack is often assumed to operate (11), and CD4⁺ T cells have been reported to kill EBV-transformed B cells via EBNA-1 recognition (12). However, in other settings EBNA-1 is not recognized (13). Linking a CD4⁺ T cell epitope to the equivalent MuHV-4 open reading frame (ORF), ORF73, did not reduce host colonization (14), whereas equivalent CD8⁺ T cell epitope expression did so severely (15), so even when CD4⁺ T cells recognize latent infection, their capacity for directly controlling it seems to be poor. CD4⁺ T cell deficiency leads mainly to more MuHV-4 lytic infection (4), and the CD4⁺ T cells associated with EBV control recognize mainly lytic antigens (11). Therefore, CD4⁺ T cell killing of latently infected cells seems unlikely.

CD4⁺ T cells could potentially limit host colonization by killing lytically infected cells (16, 17). However, a fundamental problem with this idea is that MHCII presents mostly cell-exogenous antigens (18). B cells take up antigens via their surface immunoglobulin, so those presenting MHCII-bound viral peptides should be mostly virus specific. As few virus-specific B cells are virus infected (19), killing viral antigen-positive B cells would seem counterproductive. Perhaps for this reason, CD4⁺ T cells tend to activate rather than kill engaged presenting cells and have limited cytotoxic machinery: CD8⁺ T cells and NK cells directly damage target cells via perforin, whereas CD4⁺ T cells kill mainly through fas signaling (20), which viruses can block (21). Direct CD4⁺ T cell attack is also limited to MHCII⁺ targets. Thus, it seems better suited to immune regulation than to controlling evasive viruses. CD4⁺ T-cell-dependent gammaherpesvirus control is clearest for MuHV-4 lung infection. To understand how this works, we tracked infection in mice with cell-type-specific disruptions of MHCII.

RESULTS

LysM^{cre} MHCII^{f/f} mice lose MHCII from lung myeloid cells and type II alveolar epithelial cells. To remove MHCII from infected lungs without disrupting CD4⁺ T cell development, we crossed a murine LysM-cre transgene (LysM^{cre}) (22) with cre-inactivated MHCII (IA^b floxed exon 1 [MHCII^{f/f}]) (23). We checked the distribution of cre expression by separately crossing LysM^{cre} mice with a reporter strain in which cre turns on TdTomato expression from a ROSA26 promoter (TdT) (24). The main cell types in lung alveoli are podoplanin (PDP)⁺ type 1 alveolar epithelial cells (AEC1 cells), which have a flattened morphology for gas exchange, surfactant protein C (SP-C)⁺ type 2 alveolar epithelial cells (AEC2 cells), which produce surfactants, and CD68⁺ alveolar myeloid cells (AM cells). In uninfected LysM^{cre} TdT mice, most AM and AEC2 cells were TdT⁺, while AEC1 cells were TdT[−] (Fig. 1a and b), so AEC2 and AM cells expressed LysM^{cre}, while AEC1 cells did not.

Most AM cells are macrophage-like: CD11c^{lo} and phagocytic, with poor migration to lymph nodes (LNs) (25). A subset smaller is dendritic cell (DC)-like: CD11c^{hi}, weakly phagocytic, and readily migrating to LNs. LysM^{cre} MHCII^{f/f} mice substantially lost MHCII from AEC2 and macrophage-like AM cells, but largely retained it in CD11c^{hi} AM cells (Fig. 1e). This was consistent with LysM^{cre} being expressed in most mature macrophages and granulocytes and in relatively few dendritic cells (DCs) (22). Figure 1f shows representative images. Flow cytometry of LysM^{cre} MHCII^{f/f} spleens showed a <15% decrease in CD4⁺ T cell numbers (Fig. 1g), so CD4⁺ T cell development was largely unaffected.

LysM^{cre} MHCII^{f/f} mice poorly control MuHV-4 lytic infection. We infected LysM^{cre} MHCII^{f/f} mice and MHCII^{f/f} controls intranasally (i.n.) with MuHV-4, delivering virus to the nose and to the lungs (Fig. 2). In the lungs, MuHV-4 infects type I alveolar epithelial (AEC1) cells and alveolar myeloid (AM) cells (26). In the nose, it infects the olfactory epithelium (27). At day 3, infected LysM^{cre} MHCII^{f/f} and MHCII^{f/f} mice had equivalent virus titers in the lungs (Fig. 2a) and equivalent titers also in noses (Fig. 2b). MHCII^{f/f} mice then cleared infection faster. Lung infections of MHCII^{f/f} mice were equivalent to those of wild-type C57BL/6 (MHCII^{+/+}) mice (Fig. 2c).

The need for MHCII expression in LysM⁺ cells to clear MuHV-4 rapidly from the lungs possibly reflected a limit on antiviral CD8⁺ T cell function. To test this, we infected LysM^{cre} MHCII^{f/f} and MHCII^{f/f} mice with MuHV-4 lacking its K3, which degrades MHCII

FIG 1 Floxed MHCII disruption by *LysM^{cre}*. (a) Lung sections of *LysM^{cre}* TdT mice were stained for MHCII with MAb M5/114. Nuclei were stained with DAPI. More than 80% of MHCII⁺ cells were TdT⁺, implying *LysM^{cre}* expression. Arrows show examples. (b) Summary of TdT⁺ cell counts in lungs of naive *LysM^{cre}* TdT mice. TdT expression was triggered by *LysM^{cre}* in surfactant protein C (SP-C)⁺ AEC2, CD68⁺ AM, and MHCII⁺ cells, but not in podoplanin (PDP)⁺ AEC1 cells. Bars show mean $\pm$ standard deviation (SD) of 5 fields of view per section for 3 sections per lung from each of 3 mice. (c) Lung sections of control MHCII^{fl/fl} (*cre*⁻) and *LysM^{cre}* MHCII^{fl/fl} (*cre*⁺) mice were stained for MHCII. Counts of MHCII⁺ cells are shown for 10 to 11 sections from 3 mice per group. Circles show counts for individual sections, bars show means. *Cre*⁻ mice had significantly more MHCII⁺ cells than *cre*⁺ mice. (d) Lung sections as in panel c were stained for MHCII and CD68. Not all CD68⁺ cells lost MHCII. Nonetheless *cre*⁻ mice had significantly more CD68⁺ MHCII⁺ cells than *cre*⁺ mice ($P < 10^{-4}$). Bars show mean $\pm$ SD counts for 12 fields of view from 3 mice per group. (e) Lung sections as in panel c were stained for MHCII and CD11c. CD11c⁺ MHCII⁺ cell numbers were not significantly different between *cre*⁻ and *cre*⁺ mice. Bars show mean $\pm$ SD counts for 12 fields of view from 3 mice per group. (f) Representative images from panels c to e, with arrows showing dual-positive cells. PDP⁺ AEC1 cells lacked MHCII. SP-C⁺ AEC2 and CD68⁺ AM cells showed MHCII loss in *LysM^{cre}* MHCII^{fl/fl} mice, while CD11c^{hi} AM cells did not. (g) Flow cytometry of splenic T cells showed only a small contraction of the CD4⁺ subset in *cre*⁺ mice, with a corresponding small expansion of the CD8⁺ subset. Circles show individual mice, and crosses show means.

heavy chains and the associated peptide transporter (28). Unlike with wild-type MuHV-4, *LysM^{cre}* MHCII^{fl/fl} and MHCII^{fl/fl} mice now showed no significant differences in lung infection at day 7 or day 9 (Fig. 2d). Therefore, viral CD8⁺ T cell evasion seemed to be why lung infection control depended on MHCII⁺ *LysM*⁺ cells.

The levels of seeding of wild-type MuHV-4 from the lungs to mediastinal LN (MLNs) (Fig. 2e) and spleens (Fig. 2f) were similar between *LysM^{cre}* MHCII^{fl/fl} and MHCII^{fl/fl} mice. More lung infection might be expected to give more MLN infection. However, experiments reducing lung infection through a low inoculation dose (29) or vaccination (30) have shown that

FIG 2 Poor MuHV-4 clearance from LysM^{cre} MHCII^{fl/fl} mouse lungs. (a) LysM^{cre} MHCII^{fl/fl} and MHCII^{fl/fl} mice were infected i.n. with MuHV-4 (10⁴ PFU). The titer of lung virus was determined by plaque assay. Symbols show individual mice, and bars show means. Day 3 titers were equivalent between cre⁺ and cre⁻ mice. Titters in cre⁻ mice were then significantly lower. (b) LysM^{cre} MHCII^{fl/fl} and MHCII^{fl/fl} mice were infected i.n. with MuHV-4 (10⁴ PFU) in a low volume without anesthesia to restrict inoculation to the upper respiratory tract. The titer of infectious virus in noses was determined by plaque assay. Symbols show individuals, and bars show means. Day 3 titers were equivalent between cre⁺ and cre⁻ mice. At day 7, cre⁻ titers were significantly lower. (c) MHCII^{fl/fl} and parental C57BL/6J (MHCII^{+/+}) mice were infected i.n. with MuHV-4 (10⁴ PFU). Plaque assays of lungs 7 days later showed equivalent titers in both group, so the unrecombined loxP sites of MHCII^{fl/fl} mice did not affect virus replication. (d) LysM^{cre} MHCII^{fl/fl} and MHCII^{fl/fl} mice were infected i.n. with K3⁻ MuHV-4 (10⁴ PFU). Plaque assays of lungs 7 and 9 days later showed no significant difference in titers between mouse groups. (e) LysM^{cre} MHCII^{fl/fl} and MHCII^{fl/fl} mice were infected as in panel a. The titer of reactivatable virus in MLNs was determined by infectious center (i.c.) assay, with no significant difference between groups. (f) The titer of reactivatable virus in spleens of the mice in panel e was determined by infectious center (i.c.) assay, again without significant difference between groups. (g) Mice infected as in panel a were analyzed after 7 days by staining lung sections for MuHV-4 lytic antigens (MHV). Symbols show counts of positive cells for individual fields of view across 3 sections per mouse for 3 mice per group. Bars show means. MHV⁺ cell counts were significantly higher in LysM^{cre} MHCII^{fl/fl} mice than in MHCII^{fl/fl} controls. (h) Lung sections of mice infected as in panel g were costained for viral antigens and cell-type-specific markers. Nuclei were stained with DAPI. Arrows show representative MHV⁺ marker⁺ cells. Scale bars show 50 μm. (i) Staining of lung sections as in panel h was quantitated across 3 sections per mouse for 3 mice per group. Symbols

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even large reductions in primary lytic infection only transiently reduce MuHV-4 spread to lymphoid tissue. Thus, experimental lung infection may normally exceed the level required for efficient spread.

MuHV-4 drives infected B cell proliferation in lymphoid germinal centers. Germinal center function requires CD4⁺ T cells, so CD4⁺ T-cell-deficient mice poorly amplify latent MuHV-4 infection (31). LysM^{cre} MHCII^{f/f} mice showed no such defect (Fig. 2f). Thus, the capacity of LysM^{cre} MHCII^{f/f} CD4⁺ T cells to provide B cell help was unimpaired. Nor did LysM^{cre} MHCII^{f/f} mice show more latent infection than MHCII^{f/f} controls at days 9, 14, and 21 postinfection (Fig. 2f). Thus, CD4⁺ T cell engagement of LysM⁺ cells did not contribute to clearing established latent infection. As with complete MHCII loss (4), MHCII loss from LysM⁺ cells chiefly increased lytic infection.

LysM^{cre}-driven MHCII loss increases MuHV-4 replication in LysM^{cre}-negative cells. Immunostaining of lung sections at day 7 after infection showed significantly more viral antigen-positive cells in LysM^{cre} MHCII^{f/f} mice than in MHCII^{f/f} mice (Fig. 2g), consistent with the LysM^{cre} MHCII^{f/f} mice having higher plaque titers (Fig. 2a). Surprisingly, the additional infected cells were not AEC2 or CD68⁺ AM cells, but podoplanin-positive (PDP⁺) AEC1 cells (Fig. 2h and i). AEC1 cells of naive mice lacked both LysM^{cre} (Fig. 1b) and MHCII (Fig. 1f). MuHV-4-infected LysM^{cre} MHCII^{f/f} mice (Fig. 2j) had more MHCII⁺ PDP⁺ lung cells than their naive counterparts (Fig. 1f), presumably because of inflammation, but PDP⁺ AEC1 cells remained MHCII[−]. Therefore, MHCII loss increased infection but not in the cells losing MHCII, implying that CD4⁺ T cells controlled MuHV-4 indirectly.

MuHV-4 control is unimpaired by MHCII loss from infected cells. We tested further the idea of indirect CD4⁺ T cell control by infecting MHCII^{f/f} mice with cre⁺ MuHV-4 (MHV-cre) (32), thereby disrupting MHCII just in infected cells (Fig. 3). Cre was expressed from an HCMV IE1 promoter, from a neutral site between ORFs 57 and 58. HCMV IE1 promoters operate independently of MuHV-4 lytic infection (33) and so can mark latently infected cells. For example, they mark many more infected myeloid cells *in vitro* than does viral lytic antigen expression, because this infection is largely latent (33). Thus, HCMV IE1-driven cre expression potentially allowed loxP recombination in infected myeloid cells to disrupt MHCII before lytic cycle initiation. To test MHV-cre-driven recombination, we transduced fibroblasts with a retroviral vector that encodes floxed mCherry upstream of green fluorescent protein (GFP) (Fig. 3a). MHV-cre switched them from mCherry⁺ to GFP⁺. Thus, it could effect a cre-mediated genetic switch in viable cells.

Multiple antibodies failed to detect viral cre expression on fixed sections, so to identify it we gave i.n. MHV-cre to floxed TdT reporter mice. After 9 days, TdT⁺ cells were evident in the lungs (Fig. 3b). TdT⁺ cells showed little lytic antigen expression, consistent with HCMV IE1 expressing cre independently of viral lytic genes, but they were in the same lung areas as lytic antigen⁺ cells. Therefore, to identify MHV-cre-driven MHCII loss, we quantified MHCII expression in lytic antigen⁺ lung areas (Fig. 3c and d). At 5 days after i.n. MHV-cre, infected areas of MHCII^{f/f} lungs showed a strong reduction in MHCII expression compared to MHCII^{+/+} controls. Thus, MHV-cre was remarkably effective at rendering MHCII^{f/f}-infected cells MHCII[−]. Lung virus titers at day 7 postinfection nonetheless showed no significant difference between MHCII^{f/f} mice and MHCII^{+/+} controls (Fig. 3e). Therefore, disruption of MHCII expression in infected cells did not compromise MuHV-4 control, supporting the idea that protective CD4⁺ T cells did not engage infected targets.

Immune responses in MHCII-depleted LysM^{cre} mice. We tested next whether LysM^{cre} MHCII^{f/f} mice had normal systemic immune responses. At 9 days after i.n. administration of wild-type MuHV-4, LysM^{cre} MHCII^{f/f} and MHCII^{f/f} mice had equivalent numbers of virus-specific, gamma interferon (IFN-γ)-producing cells in LNs and spleens (Fig. 4a). Virus-specific serum IgM and IgG titers (Fig. 4b) were lower in LysM^{cre} MHCII^{f/f} mice, but not significantly so. Different IgG isotypes showed similar trends (Fig. 4c). B

FIG 2 Legend (Continued)

show counts for individual fields of view. Bars show means. LysM^{cre} MHCII^{f/f} mice showed significantly more infection of PDP⁺ AEC1 cells but not of CD68⁺ AM cells. (j) Sections of MHCII^{f/f} and LysM^{cre} MHCII^{f/f} mice infected as in panel a were stained after 9 days for PDP and MHCII. Nuclei were stained with DAPI. No PDP⁺ cells were MHCII⁺.

FIG 3 Infection of MHCII^{fl/fl} mice by cre⁺ MuHV-4. (a) NIH 3T3 cells transduced with a retrovirus expressing floxed mCherry upstream of GFP were infected with either wild-type MuHV-4 or a recombinant expressing cre from an intergenic HCMV IE1 promoter (MHV-cre) (0.1 PFU/cell). Cell switching from mCherry⁺ to GFP⁺ was evident after exposure to MHV-cre but not the wild type. (b) Cre⁻ TdTomato mice were infected i.n. with MHV-cre. Nine days later, lung sections were stained for MuHV-4 antigens (MHV). TdTomato fluorescence, visualized directly, was absent from uninfected control mice. In infected mice, MHV⁺ and TdTomato⁺ cells showed little or no overlap, but they were in the same lung regions. Gray arrows show representative single-positive cells. (c) MHCII^{fl/fl} and control MHCII^{+/+} mice were infected i.n. with MHV-cre. Five days later, lung sections were stained for MuHV-4 antigens (MHV), cell-type-specific markers (PDP for AEC1 cells and CD68 for AM cells) and MHCII. Lytic antigen staining was equivalent between MHCII^{fl/fl} and MHCII^{+/+} mice. Arrows show examples of MHV⁺ AEC1 and AM cells. Scale bars show 50 μm. MHCII^{fl/fl} mice showed a marked loss of MHCII⁺ cells in the vicinity of MHV⁺ AEC1 cells. (d) Quantitation of MHCII staining in areas of lytic infection showed a significant decrease in MHCII^{fl/fl} mice. Symbols show individual fields of view, and bars show means for 1 pair of mice. Equivalent results were obtained in 3 such pairs, with MHCII^{fl/fl} mice showing a consistent, significant reduction in MHCII staining of infected lung areas ($P < 10^{-6}$). (e) MHCII^{fl/fl} and control MHCII^{+/+} mice were infected i.n. with MHV-cre. Seven days later, lungs were plaque assayed for infectious virus. Symbols show individual mice, and bars show means. There was no significant difference in titers between mouse groups.

cell enzyme-linked immunosorbent spot (ELISpot) assays (Fig. 4d) showed some reduction in IgG-producing cells in the spleens of LysM^{cre} MHCII^{fl/fl} mice, but no reduction in LNs and no significant reduction in IgM-producing cells. Thus, LysM^{cre} MHCII^{fl/fl} and MHCII^{fl/fl} mice made similar systemic CD4⁺ T cell and B cell responses. Nonetheless, infected LysM^{cre} MHCII^{fl/fl} lung sections of mice showed significantly less CD4⁺ T cell recruitment than in MHCII^{fl/fl} controls (Fig. 4e and f). More CD8⁺ T cells were recruited, presumably due to the higher viral loads of LysM^{cre} MHCII^{fl/fl} mice at days 7 and 9 (Fig. 2). Flow cytometry of lymphocytes recovered from lung homogenates (Fig. 4g) confirmed the poor recruitment of CD4⁺ T cells relative to CD8⁺. Thus, normal recruitment depended on local MHCII expression by LysM⁺ cells.

CD11c^{cre} MHCII^{fl/fl} mice also show increased lung infection. As both AM and AEC2 cells express LysM and MHCII, LysM^{cre} MHCII^{fl/fl} mice did not distinguish which cell type was important to engage infiltrating CD4⁺ T cells. In CD11c^{cre} TdTomato mice, AM cells but not AEC2 cells are TdTomato⁺ (34), so we used CD11c^{cre} MHCII^{fl/fl} mice to distinguish the antiviral contributions of AM and AEC2 cells (Fig. 5). Lungs of naive CD11c^{cre} MHCII^{fl/fl} mice had similar total MHCII⁺ cell numbers to MHCII^{fl/fl} controls (Fig. 5a and b), consistent with most MHCII⁺ lung cells being CD11c⁻ SP-C⁺ AEC2 (Fig. 5a). MHCII was lost from essentially all CD11c⁺ lung cells (Fig. 5a and c). While few AM cells were CD11c^{hi} by immunostaining, >90% of the AM cells identified by CD68 staining in lungs of CD11c^{cre} TdTomato mice are TdTomato⁺ (34), so most AM cells seem to express sufficient CD11c^{cre} during development to recombine genomic loxP sites. CD11c^{cre} MHCII^{fl/fl} mice accordingly showed >90% reduction in CD68⁺ MHCII⁺ lung cell numbers, compared

FIG 4 Immune responses in *LysM*^{cre} *MHCII*^{fl/fl} mice. (a) *LysM*^{cre} *MHCII*^{fl/fl} (*cre*⁺) and *MHCII*^{fl/fl} (*cre*⁻) mice were infected i.n. with MuHV-4 (10⁴ PFU). Nine days later, spleen and MLN cells were assayed for IFN- γ production by ELISpot, using MuHV-4-exposed targets. Although other cell types can make IFN- γ , this assay detects only CD4⁺ T cells (55). Bars show the mean $\pm$ SD from 4 mice per group, with no significant difference between *cre*⁺ and *cre*⁻ mice. Uninfected targets elicited <10 IFN- γ ⁺ spots/10⁶ cells. (b) Sera from mice infected as in panel a were assayed for MuHV-4-specific IgM and IgG by ELISA. "naive" indicates sera from uninfected mice. Graphs show the mean

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to cre^{-} controls (Fig. 5a and d). Like $LysM^{cre} MHCII^{f/f}$ mice, they showed a modest decrease in splenic $CD4^{+}$ T cell numbers and a modest, compensatory increase in $CD8^{+}$ T cell numbers (Fig. 5e).

Infected $CD11c^{cre} MHCII^{f/f}$ mice showed the same poor MuHV-4 clearance from lungs and noses as $LysM^{cre} MHCII^{f/f}$ mice (Fig. 5f). Thus, the $MHCII^{+}$ lung cells promoting MuHV-4 clearance appeared to be AM cells rather than AEC2 cells. The equivalent defects of $CD11c^{cre} MHCII^{f/f}$ and $LysM^{cre} MHCII^{f/f}$ mice argued that the key AM subset was $LysM^{+} CD11c^{lo} MHCII^{+}$ —that is macrophage-like rather than DC-like AM cells, as few $CD11c^{hi}$ lung cells expressed $LysM^{cre}$ (Fig. 1e). Similar macrophage-like cells presumably functioned in noses. While inflammation drives DC migration to LN, macrophages remain in inflammatory sites and so may be better placed to interact with infiltrating $CD4^{+}$ T cells. As with $LysM^{cre} MHCII^{f/f}$ mice, lung sections of $CD11c^{cre} MHCII^{f/f}$ mice showed significantly more infected cells than in $MHCII^{f/f}$ controls (Fig. 5g), and again they were PDP^{+} AEC1 cells rather than $CD68^{+}$ AM cells (Fig. 5h and i), supporting the idea that protective $CD4^{+}$ T cells act indirectly.

Immune responses of $CD11c^{cre} MHCII^{f/f}$ mice. At day 9 after i.n. infection, spleens of $CD11c^{cre} MHCII^{f/f}$ mice showed equivalent antiviral IFN- γ responses to $MHCII^{f/f}$ controls (Fig. 6a). This result was confirmed at day 13 (Fig. 6b), with parallel $CD8^{+}$ T cell IFN- γ assays also showing no defect. Efficient $CD4^{+}$ T cell priming in $CD11c^{cre} MHCII^{f/f}$ mice was surprising, as this should require $MHCII^{+}$ DCs. A likely explanation is that while the $CD11c^{cre}$ transgene efficiently targeted $CD11c^{+}$ lung cells (Fig. 5c), it does not target all LN DCs: 20 to 40% of $CD11c^{+}$ cells in lymphoid tissue of $CD11c^{cre}$ TdT mice are not TdT $^{+}$ (34). Thus, in $CD11c^{cre} MHCII^{f/f}$ mice, resident $MHCII^{+}$ LN DCs could still prime $CD4^{+}$ T cells. $CD11c^{cre} MHCII^{f/f}$ mice, like $LysM^{cre} MHCII^{f/f}$ mice, nonetheless poorly recruited $CD4^{+}$ T cells into infected lungs (Fig. 6c and d). Again there was again a small increase in recruitment of $CD8^{+}$ T cells, due presumably to their higher viral loads (Fig. 5).

$CD4^{+}$ T cell-NK cell interdependence in defense against MuHV-4. Despite the higher viral loads of $CD11c^{cre} MHCII^{f/f}$ mice, staining lung sections showed significantly fewer $NKp46^{+}$ NK cells than in $MHCII^{f/f}$ controls (Fig. 7a). As infection was patchy and increased in the cre^{+} mice, we allowed for differences in inflammation by normalizing $NKp46^{+}$ cell counts according to the $CD8^{+}$ T cell counts (Fig. 6) of adjacent sections (Fig. 7b). This confirmed a relative reduction in NK cell recruitment to infected $CD11c^{cre} MHCII^{f/f}$ lungs. Flow cytometry of lung cells for $CD49b$, the NK cell marker recognized by monoclonal antibody (MAB) DX5, also showed a significant reduction in NK cell recruitment relative to $CD8^{+}$ T cells for $CD11c^{cre} MHCII^{f/f}$ mice, while spleen cells showed no significant difference (Fig. 7c).

Infected $LysM^{cre} MHCII^{f/f}$ mice similarly showed significantly less recruitment of $NKp46^{+}$ cells relative to $CD8^{+}$ T cells on lung sections (Fig. 7d and e) and significantly fewer $CD49b^{+}$ cells relative to $CD8^{+}$ T cells in lungs but not spleens by flow cytometry (Fig. 7f). These results argued that by engaging $CD11c^{lo} LysM^{+} MHCII^{+}$ AM cells, protective $CD4^{+}$ T cells help to recruit NK cells into infected lungs.

$CD4^{+}$ T cell depletion also reduced NK cell recruitment (Fig. 7g). We depleted C57BL/6 mice of $CD4^{+}$ T cells or not with MAB GK1.5, infected them i.n. with MuHV-4,

FIG 4 Legend (Continued)

$\pm$ SD from 4 mice per group. Absorbance was lower in the $LysM^{cre} MHCII^{f/f}$ group, but not significantly so ($P > 0.05$). A repeat experiment gave equivalent results. (c) Sera as in panel b were assayed for MuHV-4-specific IgG by isotype. Again $LysM^{cre} MHCII^{f/f}$ sera gave lower absorbance than $MHCII^{f/f}$ sera, but the difference was not statistically significant ($P > 0.05$). The groups showed no difference in isotype distributions of antiviral IgG. (d) Mice infected as in panel a were assayed for MuHV-4-specific IgM- and IgG-producing cells in MLNs and spleens by ELISPOT. Each bar shows the mean $\pm$ SD from 3 pools of 2 mice each. IgM-producing cells were equivalent between the $MHCII^{f/f}$ and $LysM^{cre} MHCII^{f/f}$ groups. IgG-producing cells were equivalent in MLNs, although lower in $LysM^{cre} MHCII^{f/f}$ than in $MHCII^{f/f}$ spleens (*, $P < 0.05$). (e) $LysM^{cre} MHCII^{f/f}$ (cre^{+}) and $MHCII^{f/f}$ (cre^{-}) mice were infected i.n. with MuHV-4 (10^4 PFU). Nine days later, lymphocytic infiltrates were analyzed by immunostaining lung sections. Bars show mean $\pm$ SD counts from 10 fields of view from 3 mice per group. Cre^{+} mice showed significantly less $CD4^{+}$ T cell recruitment and significantly more $CD8^{+}$ T cell recruitment than controls. (f) Representative sections from panel e showing lymphocytic infiltrates of naive and day 9 MuHV-4-infected lungs. Nuclei are stained with DAPI. The scale bar is 100 μ m. (g) $LysM^{cre} MHCII^{f/f}$ (cre^{+}) and $MHCII^{f/f}$ (cre^{-}) mice were infected as in panel e. Lymphocytes recovered from spleens and lungs were analyzed for CD4 and CD8 expression by flow cytometry. Bars show mean $\pm$ SD $CD4^{+}$ T cells as a percentage of all T cells ($CD4^{+}$ plus $CD8^{+}$) for organs from 4 mice per group. Spleens showed no significant difference between groups, but the proportion of $CD4^{+}$ T cells recovered from lungs was significantly lower in cre^{+} mice.

FIG 5 MuHV-4 infection of CD11c^{cre} MHCII^{fl/fl} mice. (a) Lung sections of naive CD11c^{cre} MHCII^{fl/fl} mice were stained for MHCII and markers of AEC1 (PDP), AEC2 (SP-C), and AM (CD68 and CD11c) cells. In the images, arrows indicate representative dual-positive cells. Representative staining of MHCII^{fl/fl} mice is in Fig. 1f. Quantitation is in panels b to d. MHCII was retained on SP-C⁺ AEC2 cells but lost from CD11c⁺ cells and most CD68⁺ cells. AEC1 cells lack MHCII. (b) Total MHCII⁺ cells were quantitated for lungs stained as in panel a. Symbols show individual fields of view, across sections from 3 mice per group. Bars show means. CD11c^{cre} MHCII^{fl/fl} and MHCII^{fl/fl} mice showed no significant difference in total MHCII⁺ cells, reflecting that most MHCII⁺ lung cells are CD11c[−] AEC2 cells. (c) Quantitation of staining as in panel a showed significantly fewer MHCII⁺ CD11c⁺ cells in CD11c^{cre} MHCII^{fl/fl} mice. Mean $\pm$ SD counts are shown from 10 fields of view from 3 mice per group. In MHCII^{fl/fl} mice, $>80\%$ of CD11c⁺ cells were MHCII⁺; in CD11c^{cre} MHCII^{fl/fl} mice, essentially none was. (d) Quantitation of staining as in panel a showed significantly fewer MHCII⁺ CD68⁺ cells in CD11c^{cre} MHCII^{fl/fl} mice. Mean $\pm$ SD counts are shown for 10 fields of view from 3 mice per group. (e) Flow cytometry of splenic leukocytes showed only a small contraction of the CD4⁺ T cell subset in CD11c^{cre} MHCII^{fl/fl} relative to MHCII^{fl/fl} mice, and a small expansion of the CD8⁺ subset. Bars show mean $\pm$ SD counts from 4 mice per group. *, $P < 10^{-2}$; **, $P < 0.05$. (f) CD11c^{cre} MHCII^{fl/fl} and MHCII^{fl/fl} mice were given i.n. MuHV-4 (10^4 PFU). Plaque assays showed significantly impaired infectious virus clearance from CD11c^{cre} MHCII^{fl/fl} lungs. Clearance from noses was also impaired. Symbols show individual mice, and bars show means. Dotted lines show assay sensitivity limits. (g) Lung sections of mice infected as in panel f were stained for viral lytic antigens (MHV). Symbols show individual fields of view from each of 5 mice per group. Bars show means. Infected cell numbers were significantly higher in CD11c^{cre} MHCII^{fl/fl} mice. (h) Lung sections as in panel g were stained for MHV antigens plus PDP and CD68 to identify infected cell types. Symbols show individual fields of view from each of 5 mice per group. Bars show means. The increased infection of CD11c^{cre} MHCII^{fl/fl} mice was entirely in PDP⁺ AEC1 cells. (i) In representative images from panel h, arrows indicate representative viral antigen⁺ marker⁺ cells. Scale bars are 50 μm.

FIG 6 Immune responses in CD11c^{cre} MHCII^{fl/fl} mice. (a) CD11c^{cre} MHCII^{fl/fl} (cre⁺) and MHCII^{fl/fl} (cre⁻) mice were given i.n. MuHV-4 (10^4 PFU). Nine days later, ELISpot assays showed no significant difference in the number of spleen cells producing IFN- γ in response to MuHV-4-infected targets. Uninfected targets elicited <10 IFN- γ ⁺ spots/ 10^6 cells. Symbols show results for individual mice, and bars show means. (b) Mice were infected as in panel a. Thirteen days later, IFN- γ production by MLN and spleen cells, in response to syngeneic spleen cells exposed to MuHV-4 (CD4⁺) or pulsed with H2D^b (CD8⁺ [AGPHNDMEI]) or H2K^b (CD8⁺ [TSINFVKI])-binding viral peptides, was assayed by ELISpot. Symbols show results for pooled pairs of mice, and bars show means. Uninfected targets elicited <10 IFN- γ ⁺ spots/ 10^6 cells. The responses were not significantly different between CD11c^{cre} MHCII^{fl/fl} and MHCII^{fl/fl} mice. (c) Mice were infected as in panel a. Nine days later, lymphocytic infiltrates were analyzed by immunostaining lung sections for CD4 or CD8. Nuclei were stained with DAPI. Representative images are shown. (d) Quantitation of staining as in panel c. CD11c^{cre} MHCII^{fl/fl} lungs recruited significantly fewer CD4⁺ T cells than MHCII^{fl/fl} controls and significantly more CD8⁺ T cells. Bars show mean $\pm$ SD counts from 10 fields of view from 3 mice per group.

and 9 days later analyzed spleen and lung cells by flow cytometry. Lungs but not spleens contained significantly fewer CD49b⁺ cells relative to CD8⁺ T cells after CD4⁺ T cell depletion.

We tested the functional significance of the reduction in NK cell recruitment by depleting NK cells from infected cre⁺ and cre⁻ mice with MAb PK136. For both CD11c^{cre} MHCII^{fl/fl} mice (Fig. 7h) and LysM^{cre} MHCII^{fl/fl} mice (Fig. 7i), NK cell depletion normalized lung titers with respect to MHCII^{fl/fl} controls. For LysM^{cre} MHCII^{fl/fl} mice, we also tested CD4⁺ and CD8⁺ T cell depletion (Fig. 7j and k). CD4⁺ T cell depletion abolished the difference in lung virus titers between cre⁺ and cre⁻ mice (Fig. 7j). CD8⁺ T cell depletion increased lung titers, but did not abolish the difference between cre⁺ and cre⁻ groups. Therefore, CD4⁺ T cells did not protect via CD8⁺ T cells. Rather NK cell recruitment provided a mechanistic explanation for CD4⁺ T-cell-dependent MuHV-4 control in MHCII^{fl/fl} cells.

FIG 7 Poor NK cell recruitment in MHCII-disrupted mice. (a) CD11c^{cre} MHCII^{fl/fl} and MHCII^{fl/fl} mice were infected i.n. with MuHV-4 (10⁴ PFU). Nine days later, lung sections were stained for the NK cell marker NKp46. Nuclei were stained with DAPI. Representative images are shown. (b) To allow for differences in inflammation between lung sections, staining as in panel a was quantitated by comparing NKp46⁺ and CD8⁺ T cell numbers on adjacent sections. CD11c^{cre} MHCII^{fl/fl} (cre⁺) lungs showed a significant decrease in the proportion of NKp46⁺ cells compared to MHCII^{fl/fl} controls (cre⁻). Symbols show counts for pairs of sections from 3 mice per group. Bars show means. (c) Mice were infected as in panel a. Nine days later, spleen and lung cells were analyzed by flow cytometry for expression of the CD49b (DX5) NK cell marker. Again to allow for differences in virus load, DX5⁺ cell counts were normalized by CD8⁺ T cell counts for each mouse. Symbols show individual mice, bars show means. Spleens showed no significant difference between cre⁺ and cre⁻ mice in NK cell representation, while lungs showed a significant decrease in cre⁺ mice. (d) LysM^{cre} MHCII^{fl/fl} and MHCII^{fl/fl} mice were infected i.n. with MuHV-4 (10⁴ PFU). Nine days later, lung sections were stained for the NK cell marker NKp46. Nuclei were stained with DAPI. Representative images are shown. Quantitation is in panel e. (e) As in panel b, to allow for differences in total virus loads and the amounts of infection in randomly sampled lung areas, we compared NKp46⁺ and CD8⁺ T cell numbers on adjacent sections. LysM^{cre} MHCII^{fl/fl} (cre⁺) lungs showed a significant decrease in the proportion of NKp46⁺ cells compared to MHCII^{fl/fl} controls (cre⁻). Symbols show counts for pairs of sections from 3 mice per group. Bars show means. (f) As in panel c, spleen and lung cells were analyzed by flow cytometry, normalizing DX5⁺ cell counts by CD8⁺ T cell counts for each mouse. Symbols show individual mice, and bars show means. Spleens showed no significant difference between cre⁺ and cre⁻ mice in NK cell representation, while lungs showed significantly fewer NK cells in cre⁺ mice. (g) C57BL/6J mice were depleted or not of CD4⁺ T cells with MAb GK1.5 and then infected i.n. with MuHV-4. Nine days later, spleen and lung cells were analyzed for DX5 and CD8 expression as in panel f. Spleens showed no significant difference in NK cell representation, while lungs showed significantly fewer NK cells in CD4⁺ T-cell-depleted mice. (h) CD11c^{cre} MHCII^{fl/fl} (cre⁺) and MHCII^{fl/fl} (cre⁻) mice were left undepleted (control) or were depleted of NK1.1⁺ cells with MAb PK136 and then infected i.n. with MuHV-4 (10⁴ PFU). Infectious virus in lungs was plaque assayed after 9 days. Symbols show individual mice, and crosses show means. Undepleted mice showed a significant cre-dependent reduction in infection; NK-depleted mice showed no significant difference between cre⁺ and cre⁻ infections. (i) LysM^{cre} MHCII^{fl/fl} (cre⁺) and MHCII^{fl/fl} mice were depleted of NK1.1⁺ cells with MAb PK136 and then infected i.n. as in panel h. Infectious virus in lungs was plaque assayed after 9 days. Symbols show individual mice, and bars show means. There was no significant difference between the groups. (j) LysM^{cre} MHCII^{fl/fl} and MHCII^{fl/fl} (cre⁻) mice were depleted of CD4⁺ cells with MAb GK1.5 (αCD4) or not (control) and then infected i.n. as in panel h. Nine days later, the titer of infectious virus in lungs was determined by plaque assay. Bars show mean ± SD from 5 mice per group. (k) Mice were depleted of CD8⁺ T cells with MAb 2.43 and then infected as in panel h. Infectious virus in lungs was plaque-assayed after 9 days. Symbols show individual mice, and bars show means. CD8⁺ T cell depletion increased lung virus titers in both groups, but titers in LysM^{cre} MHCII^{fl/fl} mice remained significantly higher than those in MHCII^{fl/fl} mice.

FIG 8 A model of CD4⁺ T-cell-dependent MuHV-4 control in the lungs. (Step 1) Epithelial cells support MuHV-4 lytic replication. Viral debris from this infection can be taken up by local (macrophage) and migratory (DC) antigen-presenting cells (APC). (Step 2) In lymph nodes, debris-loaded APCs prime virus-specific CD4⁺ T cells. (Step 3) Primed CD4⁺ T cells then migrate to inflammatory sites such as infected lungs. Here, they engage local LysM⁺ APCs and drive their activation via IFN- γ . (Step 4) The activated local APCs recruit and activate NK cells. IL-12 and IL-18, plus IL-2 from CD4⁺ T cells, could all contribute (35). (Step 5) The activated NK cells can then kill virus-infected cells and suppress viral replication via IFN- γ . CD4⁺ T-cell-dependent antibody responses can also promote recognition of infected cells via IgG FcR engagement (43). CD8⁺ T cells also attack infected epithelial cells, but appear to do so independently of CD4⁺ T cells (56).

DISCUSSION

CD4⁺ T cells are essential to control herpesviruses, but how they act has been unclear. Direct cytotoxicity is generally assumed to operate. Here, cell type-selective MHCII disruption increased MuHV-4 lytic infection, but not in the cells losing MHCII. Rather infection increased in cells that were already MHCII[−] and lacked the disrupting transgene, while disrupting MHCII in infected cells had no effect. Therefore, CD4⁺ T cells protected indirectly. CD8⁺ T cells contributed to infection control, but not to its CD4⁺ T-cell-dependent component. Rather MHCII-disrupted mice showed less NK cell recruitment, and NK cell depletion normalized their infection relative to MHCII⁺ controls. Therefore, CD4⁺ T-cell-dependent control seemed to work through NK cells.

The need to control MuHV-4 via MHCII expression in lung cells marked by LysM^{cre} and CD11c^{cre} implicated macrophage-like (CD11c^{lo}) AM cells, as CD11c^{cre} marked both these and DC-like (CD11c^{hi}) AM cells, while LysM^{cre} efficiently marked only the CD11c^{lo} subset. A role for LysM⁺ AEC2 was not completely excluded, but the equivalent phenotypes of CD11c^{cre} MHCII^{f/f} and LysM^{cre} MHCII^{f/f} mice argued against a large role, as AEC2 cells were not marked by CD11c^{cre}. Because DCs migrate to LNs for immune priming, macrophages may be better placed to interact locally with primed CD4⁺ T cells when they extravasate into the lungs.

NK cell recruitment seemed to be an important consequence of the CD4⁺ T cell-AM cell interaction. NK cells can be activated by CD4⁺ T-cell-derived interleukin-2 (IL-2) (35), but they lack MHCII for direct interaction. DCs can engage NK cells directly and activate them via IL-12/IL-18 (36), so AM cells activated by CD4⁺ T cells might also fulfill this role. By engaging both CD4⁺ T cells and NK cells, they could also promote NK activation by CD4⁺ T-cell-derived IL-2 (Fig. 8). CD4⁺ T cell-NK cell cooperation against *Leishmania*, cancers, and pneumocystis (35) suggests that such a defense might be widely employed. It could have particular importance against pathogens that evade CD8⁺ T cells.

IFN- γ activates macrophages and has a central role in MuHV-4 control by CD4⁺ T cells (9, 10). Without IFN- γ signaling, infection causes alternative macrophage activation, chronic fibrosis, and splenic atrophy (37). IFN- γ is important also to control MCMV (38) and human herpesviruses (1), and the shared susceptibilities of CD4⁺ T-cell-deficient, NK-cell-deficient, and IFN- γ -deficient hosts to these viruses possibly reflect different defects in a common defense. CD4⁺ T-cell-dependent control of *Leishmania* in MHCII[−] cells was attributed to IFN- γ reaching them (39). However, CD4⁺

T cells secrete IFN- γ predominantly into immune synapses (40); whether there is sufficient spillage to influence distant infected cells is unclear. As NK cells can independently locate and recognize infected cells, their involvement seems a more straightforward explanation for CD4⁺ T cell recognition of MHCII⁺ cells controlling infection in MHCII⁻ cells. NK cells could also deliver IFN- γ directly to infected cells (Fig. 8).

Herpesviruses evade immune responses mainly by disrupting the presenting functions of infected cells. Therefore, CD4⁺ T cell engagement of uninfected cells provides relatively evasion-resistant immune activation, and CD4⁺ T cells control MCMV in the salivary glands even though infected cells degrade MHCII (41). (This degradation probably avoids MCMV-disseminating DCs being delayed in LNs by CD4⁺ T cell engagement [25], rather than avoiding cytotoxicity.) While herpesviruses evade NK cells (42), the multiplicity and polymorphism of NK cell receptors make full evasion difficult. CD4⁺ T-cell-driven NK cell activation could also counter viral evasion by lowering response thresholds. Antibody protects against MuHV-4 by IgG Fc receptor engagement (43, 44), and NK cells mediate IgG Fc receptor-directed cytotoxicity, so CD4⁺ T-cell-driven antibody responses likely synergize with NK cell activation to reduce response thresholds still further.

Although NK cells have been reported to exert little control over MuHV-4 (45, 46), possibly unrealistic expectations were raised by the very strong attack of Ly49H⁺ NK cells on m157⁺ MCMV (47). MCMVs recovered from Ly49H⁺ mice lack Ly49H binding (48), so this large NK cell effect seems to result from inoculating MCMV into the “wrong” mouse strain. Without Ly49H/m157, NK cells control MCMV less dramatically (49), and comparable MuHV-4 infections show comparable NK-cell-dependent control (50). Most NK cell analysis has focused on preadaptive responses. Recruitment by CD4⁺ T cells gave them an impact with adaptive immune kinetics. Associations between human NK receptor polymorphisms and chronic disease (51) suggest the possibility of further, long-term contributions to infection control.

MATERIALS AND METHODS

Mice. The LysM^{cre} (22) and CD11c^{cre} (52) transgenes were back-crossed onto C57BL/6J mice with floxed MHCII exon 1 (MHCII^{fl/fl}) (23) and then bred as cre^{+/+} MHCII^{fl/fl} $\times$ cre^{-/-} MHCII^{fl/fl} mice to generate MHCII-disrupted cre^{+/+} mice and cre^{-/-} littermate controls. LysM^{cre} and CD11c^{cre} mice were also crossed with floxed ROSA26-TdTomato (TdT) reporter mice (24). C57BL/6 mice were from the Animal Resources Centre (Perth, Australia). Mice were infected when 6 to 12 weeks old, either i.n. in 30 μ l (10⁴ PFU) under isoflurane anesthesia for lung infection, or i.n. in 5 μ l without anesthesia for nose infection. We depleted lymphocyte subsets by intraperitoneal injection of MAbs GK1.5 (anti-CD4), 2.43 (anti-CD8 α), or PK136 (anti-NK1.1), at 200 μ g/mouse/48 h, from 48 h before infection. Antibodies were from Bio X Cell. T cell subset depletion, measured by flow cytometry of spleen cells with antibodies to a distinct CD4 epitope (Mab RM4-4) or CD8 β (Mab H35-17.2) was >95% complete. NK cell depletion, monitored by flow cytometric staining of spleen cells for CD49b (Mab DX5), was >80% complete. Statistical comparisons were by Student's two-tailed unpaired *t* test unless otherwise stated. Experiments were approved by the University of Queensland Animal Ethics Committee (projects 301/13, 391/15, 479/15, 196/18, 207/18), in accordance with the *Australian Code for the Care and Use of Animals for Scientific Purposes*, from the Australian National Health and Medical Research Council, Canberra, Australia.

Cells and viruses. BHK-21 fibroblasts (American Type Culture Collection CCL-10) were grown in Dulbecco's modified Eagle's medium with 2 mM glutamine, 100 IU/ml penicillin, 100 μ g/ml streptomycin, and 10% fetal calf serum (complete medium). Wild-type MuHV-4 (originally from S. Efstathiou, University of Cambridge, United Kingdom), MuHV-4, in which K3 is disrupted by a small deletion and frameshift (K3⁻) (53), and MuHV-4 expressing cre from an HCMV IE1 promoter (32), were grown and the titer determined in BHK-21 cells. Infected cell supernatants were cleared of debris by low-speed centrifugation (200 $\times$ *g*, 5 min). Cell-free virions were then concentrated by ultracentrifugation (30,000 $\times$ *g*, 1.5 h) and stored at -80°C.

Infectivity assays. Infectious virus was quantified by plaque assay (43). Ten-fold dilutions of virus stocks or organ homogenates were incubated with BHK-21 cells (2 h, 37°C), overlaid with complete medium-0.3% carboxymethylcellulose, cultured for 4 days, fixed (1% formaldehyde), and stained (0.1% toluidine blue) for plaque counting. Total virus (latent plus infectious) was quantified by infectious center (i.c.) assay (43). Freshly isolated LN or spleen cells were layered onto BHK-21 cell monolayers and then cultured and processed as for plaque assays.

Immunostaining. Organs were fixed in 1% formaldehyde-10 mM sodium periodate-75 mM L-lysine (18 h, 4°C), equilibrated in 30% sucrose (24 h, 4°C), and then frozen in OCT. Six-micrometer sections were air-dried, washed three times in phosphate-buffered saline (PBS), blocked with 0.3% Triton X-100-5% donkey or goat serum (1 h, 23°C), and then incubated (18 h, 4°C) with combinations of antibodies to CD68 (rat Mab FA-11 [AbCam]), CD4 (rat Mab GK1.5), CD8 (rat Mab 2.43), CD11c (hamster Mab N418

[Abcam]), MHCII (rat MAb M5/114 [Serotec]), NKp46 (rat MAb 29A1.4 [Biolegend]), SP-C (goat polyclonal antibody [pAb] [Santa Cruz Biotechnology]), podoplanin (goat pAb [R&D Systems]), and MuHV-4 (rabbit pAb). The MuHV-4-immune pAb was raised by two subcutaneous inoculations of rabbits with MuHV-4 virions (10^9 PFU). It recognizes a range of virion proteins by Western blotting, including the products of ORF4 (gp70), M7 (gp150), and ORF65 (p20). Sections were washed three times, incubated (1 h, 23°C) with combinations of Alexa 568-conjugated donkey anti-rat IgG pAb, Alexa 488- or Alexa 647-donkey anti rabbit IgG pAb, Alexa 647-goat anti-hamster IgG pAb (AbCam), and Alexa 488-donkey anti-goat IgG pAb (Life Technologies), then washed three times, mounted in Prolong Gold with DAPI (4',6-diamidino-2-phenylindole [Life Technologies]), and imaged with a Zeiss LSM510 confocal microscope. Cell counts were performed on randomly selected fields of view of preset size, across multiple sections for each assay.

ELISA. MuHV-4 was disrupted with 0.05% Triton X-100, cleared of insoluble debris by centrifugation ($500 \times g$, 10 min), diluted in 50 mM sodium carbonate (pH 8.5), and coated (18 h, 4°C) onto Maxisorp enzyme-linked immunosorbent assay (ELISA) plates (Nalge Nunc). Plates were washed three times in PBS–0.1% Tween 20 (PBS-T), blocked with 2% bovine serum albumin (BSA)–PBS-T, incubated with serum dilutions (1 h, 23°C), washed four times in PBS-T, incubated (1 h, 23°C) with alkaline phosphatase-conjugated, Fc-specific goat pAb to mouse IgG, IgG1, IgG2b, IgG2c, or IgM (Southern Biotechnology), washed five times, and developed with nitrophenylphosphate substrate. Absorbance was read at 405 nm (SpectraMax).

T cell assays. Polyvinylidene fluoride-coated 96-well plates (Millipore) were hydrated with 50% ethanol, then washed in PBS, coated (18 h, 4°C) with rat anti-mouse IFN- γ (1 μ g/ml, BD Biosciences), washed three times with PBS, and blocked for 1 h at 37°C with complete medium. Naive target spleen cells were depleted of erythrocytes by Ficoll centrifugation and then incubated (1 h, 37°C) in complete medium, either untreated or treated with MuHV-4 (3 PFU/cell) or with a CD8 $^{+}$ target viral peptide (1 μ M AGPHNDMEI or 1 μ M TSINFVKI) (54). The cells were then irradiated (30 Gy), washed in complete medium, and added to duplicate 3-fold dilutions of responder cells. After incubation (48 h, 37°C) in complete medium, captured IFN- γ was detected with biotinylated rat anti-mouse IFN- γ and streptavidin-alkaline phosphatase (Dako). The plates were washed five times with PBS–0.1% Tween 20, and developed with 5-bromo-4-chloro-3-indolyl phosphate–nitro blue tetrazolium (BCIP/NBT) substrate (Sigma). Spots were counted with a dissecting microscope. CD4 $^{+}$ T cell depletion of mice prior to assay reduced spot formation in response to whole virus by 90%, in accordance with evidence from *in vitro* depletions (55) that this assay is CD4 $^{+}$ T cell specific.

B cell assays. Polyvinylidene difluoride (PVDF)-coated 96-well plates (Millipore) were hydrated with 50% ethanol, then washed in PBS, coated (18 h, 4°C) with MuHV-4 virion lysate, washed four times with PBS, and blocked for 1 h at 37°C with complete medium. Three-fold cell dilutions were added to duplicate wells and incubated for 4 h in complete medium at 37°C. Captured antibody was detected with alkaline phosphatase-conjugated, Fc-specific goat pAb to mouse IgG or IgM (Southern Biotechnology). The plates were washed five times with PBS–0.1% Tween 20, and developed with BCIP/NBT substrate (Sigma). Spots were counted with a dissecting microscope.

Flow cytometry. Lymphocytes were recovered from lungs by fine mincing and then digestion with collagenase-DNase I (Sigma Chemical Co.) and centrifugation on 40% to 60% Percoll (Pharmacia) ($500 \times g$, 20 min). Spleen cells were from organ homogenates. The cells were washed in PBS, blocked with PBS–1% BSA–1 μ g/ml anti-CD16/32 (2.4G2), incubated (1 h, 4°C) with antibodies to CD4 (fluorescein-conjugated RM4-4), CD8 β (phycoerythrin-conjugated H35-17.2), CD49b (phycoerythrin-conjugated Cy7-DX5) (BD Biosciences), washed two times in PBS, and then analyzed with an Accuri flow cytometer (BD Biosciences).

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