A chemokine-like viral protein enhances IFN-α production by plasmacytoid dendritic cells but delays CD8+ T cell activation and impairs viral clearance

Short title: Viral chemokine dampens CD8+ T cell immunity

Matthew E. Wikstrom *†§, Peter Fleming *†§, Iain Comerford ‡, Shaun R. McColl ‡, Christopher E. Andoniou *†, Mariapia A. Degli-Esposti *†#

* Immunology and Virology Program, Centre for Ophthalmology and Visual Science, The University of Western Australia, Crawley, Western Australia, Australia
† Centre for Experimental Immunology, Lions Eye Institute, Nedlands, Western Australia, Australia
‡ Chemokine Biology Group, School of Molecular & Biomedical Science; The University of Adelaide, South Australia, Australia
§ These authors contributed equally

# Please address correspondence to:
Mariapia A Degli-Esposti
Lions Eye Institute, 2 Verdun St, Nedlands, Western Australia 6009, Australia
email: mariapia@lei.org.au
Tel: +61 8 9381 0808 Fax: +61 8 9381 0777

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Abstract

Murine cytomegalovirus encodes numerous proteins that act on a variety of pathways to modulate the innate and adaptive immune responses. Here, we demonstrate that a chemokine-like protein encoded by murine cytomegalovirus activates the early innate immune response and delays adaptive immunity, thereby impairing viral clearance. The protein, m131/129 (also known as MCK-2), is not required to establish infection in the spleen, however, a mutant virus lacking m131/129 is cleared more rapidly from this organ. In the absence of m131/129 expression there is enhanced activation of dendritic cells (DC), and virus-specific CD8+ T cells are recruited earlier into the immune response. Viral mutants lacking m131/129 elicited weaker production of IFN-α at 40 hours post-infection, indicating that this protein exerts its effects during early rounds of viral replication in the spleen. Furthermore, while wild-type and mutant viruses activated plasmacytoid dendritic cells (pDC) equally at this time, as measured by the upregulation of costimulatory molecules, the presence of m131/129 stimulated more pDC to secrete IFN-α accounting for the stronger IFN-α response to the wild-type virus. These data provide evidence for a novel immunomodulatory function of a viral chemokine and expose the multi-functionality of immune-evasion proteins. In addition, these results broaden our understanding of the interplays between innate and adaptive immunity.

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Introduction

The study of viral virulence and immunomodulatory factors has provided us with a better understanding of innate and adaptive immune function. We now recognise that viral products can act at multiple levels to sabotage the anti-viral immune response. For example, Epstein-Barr virus, herpes simplex virus, and cytomegalovirus (CMV) encode proteins that interfere with MHC class I expression, proteasome function and/or peptide transport to prevent the processing and presentation of viral epitopes to activated CD8+ cytotoxic lymphocytes (CTL) (23). Viral products also target the innate immune response by inhibiting natural killer (NK) cell activation (31) or interfering with signalling by pattern recognition receptors such as the toll-like receptors (TLR) (12).

CMV is a member of the herpesvirus family that is well known for its elaborate immune modulation and evasion strategies. Acute infection is typically asymptomatic in immunosufficient individuals, but despite an aggressive and ongoing immune response, the virus is able to persist in the host indefinitely thanks to its sophisticated immune evasion strategies. A large proportion of the CMV genome is devoted to immune modulation and evasion and many of these products have been characterised for both mouse CMV (MCMV) and human CMV (HCMV) (reviewed by (33)), though our understanding is far from complete. CMV has been shown to target a variety of pathways including cytokine and chemokine signalling, antibody Fc binding, apoptosis, complement activation, NK cell activation, and antigen processing and presentation. CMV has refined these strategies over millions of years of co-evolution with its host, and
as a consequence, the virus can provide us with valuable insights on how the immune response is regulated (10).

MCMV is attenuated by the deletion of the m131/129 open reading frame (also known as MCK-2) such that fewer inflammatory foci are observed in the liver and viral titres are lower in the salivary glands (20, 44, 45). Interestingly, the m131/129 protein exhibits some sequence homology with the CC-chemokines (32) and appears to act as an agonist for some chemokine receptors (45). Furthermore, m131/129 can recruit myeloid cells to the site of MCMV infection (36), leading to the suggestion that the main function of this protein is to facilitate dissemination of the virus. Interestingly, our earlier study of m131/129 mutant viruses also showed they are cleared from the spleens of susceptible BALB/c mice with faster kinetics than wild-type MCMV (20), indicating that m131/129 may influence the immune response during early acute infection. In this report, we establish that m131/129 inhibits the activation of CD8+ T cells in the spleen via an effect on the innate immune response to MCMV. This function is associated with enhanced levels of IFN-α production by pDC during early infection, inhibition of IL-12 production and antigen presentation by conventional dendritic cells (cDC), and slower generation of virus-specific CTL. Thus, m131/129 modulates pathogen sensing and innate inflammation so as to delay activation of the adaptive immune response.
Materials and Methods

Mice and Viruses

Inbred BALB/c mice were obtained from the Animal Resources Centre (Perth, WA, Australia) and congenic BALB.B6-CT6 mice (H-2^d, I-A^d, NK1.1^+, Ly49H^-) (47) were bred in the Animal Services Facility of the University of Western Australia. All mice were housed under specific-pathogen-free conditions at the Animal Services Facility of the University of Western Australia. Experiments were performed with the approval of the Animal Ethics and Experimentation Committee of the University of Western Australia and according to the guidelines of the National Health and Medical Research Council of Australia. The viruses used in this study were as follows: MCMV-K181-Perth (K181), and the mutant virus lacking the m131/129 gene, Δm131-129-stop (20). The virus stocks used for in vivo studies were propagated in the salivary glands of 3-week-old BALB/c mice.

Treatment of mice

Mice were infected intraperitoneally (i.p.) with salivary gland-propagated virus (SGV) diluted in phosphate-buffered saline-0.05% fetal bovine serum. 5 x 10^3 plaque-forming units (pfu) of SGV was used for all experiments except for the experiment shown in Figure 2A where 1 x 10^4 pfu was used. To deplete CD4 and CD8 T cells, mice were inoculated i.p. with 100 μg of the GK1.5 (anti-CD4), YTS 169 (anti-CD8), or PKH 136 (anti-NK1.1) monoclonal antibodies diluted in phosphate-buffered saline-0.05% fetal
bovine serum at days -2, 0 and +2 relative to MCMV infection. Depletion of relevant cell subsets was confirmed by flow cytometry.

**Determination of viral titres**

Spleen, liver, lungs and salivary glands were collected on the indicated days post-infection and processed to determine viral titres by standard plaque assay using M210B4 cells, as previously described (2).

**Histochemistry**

Spleens were collected at the indicated days post-infection, formalin-fixed and processed for tissue histology using standard methods. For detection of the immediate-early 1 (IE1) protein, spleen tissue was collected and processed for immunofluorescence according to the method of Hsu et al. (24). The monoclonal antibody 6/58/1 was used to detect IE1 (27).

**Flow cytometry**

Single-cell suspensions prepared from the spleen were preincubated on ice for 30 min with PBS-2% fetal calf serum containing 10% normal goat serum and then stained with specific antibodies. Propidium iodide was incorporated in the final wash at 1 μg/ml and then the labeled cells were analyzed on a FACSCanto (Becton Dickinson, San Jose, CA).

Antibodies used: anti-CD8α (53-6.7; Biolegend, San Diego, CA, USA), anti-TCR-β (H57-597; Biolegend), PE-conjugated H-2Ld-168-YPFMPTNL-176 MCMV IE1 tetramer (ImmunoID Tetramers, Melbourne, VIC, Australia), anti-CD11b (M1/70;
Biolegend), anti-CD11c (N418; Biolegend), anti-F4/80 (BM8; eBioscience, San Diego, CA, USA), anti-IA/IE (M5/114.15.2; Biolegend), anti-Ly6C (AL-21, BD Biosciences), anti-CD80 (1610A1, BD Biosciences), and anti-CD86 (GL1, BioLegend). Anti-IL-12 (C17.8, BioLegend) was used for intracellular staining after cells were fixed and permeabilised with cytofix/cytoperm solution (BD Biosciences, San Diego, CA, USA).

Type I IFN was detected *ex vivo* using a mixture of two unconjugated rat monoclonal antibodies against IFN-α (F18, Hycult Biotech, The Netherlands; RMMA-1, PBL Interferon Source, Piscataway, NJ, USA) and one unconjugated rat monoclonal antibody against IFN-β (RMMB-1, PBL Interferon Source) followed by detection with a biotinylated anti-rat (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) and streptavidin conjugates (BD Biosciences) after cells were fixed and permeabilised with cytofix/cytoperm solution. After blocking with 10% normal rat serum, pDC were identified using 120G8 (prepared in-house) in conjunction with antibodies against IA/IE, CD11b and CD11c. Appropriately stained controls were used to check compensation for all fluorochromes used. Analysis of the FACS data was performed with the FlowJo software (Ashland, OR, USA).

**In Vivo CTL assay**

Viral specific CTL-mediated cytotoxicity was assessed by measuring the elimination of targets pulsed with the ie1 viral peptide YPHFMPTNL. The *in vivo* CTL assay quantifies CTL activity by measuring the loss of specific peptide-pulsed targets in comparison to targets that have not been pulsed with peptide. Here, target cell lysis was measured using a modification of the previously described *in vivo* CTL assay (7). IE1 peptide-pulsed
(0.02 μg/ml) splenocyte targets were labelled with a low concentration (final [0.025μM]) of 5,6-carboxyfluorescein diacetate, succinimidyl ester (CFSE, Invitrogen, Carlsbad, CA, USA), whereas control targets, not pulsed with peptide, were labelled with a high concentration (final [0.25μM]) of CFSE. Labelled cells were washed to remove free CFSE and differential labelling confirmed by flow cytometry. CFSE-low IE1-pulsed targets and CFSE-high unpulsed control targets were resuspended in PBS, mixed in equal proportions, and a total of 4 x 10⁷ cells per mouse transferred intravenously into syngeneic mice that had been infected with MCMV for various periods of time. Mice were sacrificed 4 hours later and single cell suspensions from spleen analysed by flow cytometry. Transferred cells serve as targets for peptide-specific CTL. The frequency of unpulsed targets serves as an internal control for cell trafficking, recovery, and non-specific cell loss. The loss of specific peptide-pulsed targets is a measure of CTL activity against targets pulsed with the ie1 viral peptide. Specific lysis was determined using the following formula: Percentage specific lysis of CFSE-labelled target cells in each mouse is calculated as follows: [1- (runinfected control mouse / r infected test mouse)] x 100 where r = (frequency of unpulsed targets / frequency of peptide-pulsed targets). The assay is a very sensitive and specific method to measure MHC class I-restricted, CD8⁺ T cell-dependent cytotoxicity in vivo.

**Quantitation of cytokines and chemokines by ELISA**

Serum and spleen cytokine levels were measured after infection with 5 x 10³ PFU of MCMV. IFN-γ and IL-12 were measured by standard sandwich ELISA with BD Biosciences antibodies. Detection was achieved with poly-horseradish peroxidase (poly-
HRP) conjugated to streptavidin (CBL, Amsterdam, Netherlands) and K-Blue (Elisa Systems, Brisbane, Australia). IFN-α was quantitated with the Verikine Mouse Interferon Alpha ELISA Kit (PBL Interferon Source, Piscataway, NJ, USA). CCL2 and CCL21 were also measured by ELISA, as previously described (28), using commercial antibodies (R&D Systems, Minneapolis, MN, USA). Absorbance was measured at 450 nm with an automated ELISA reader (SpectraMAX 250; Molecular Devices, Sunnyvale, CA, USA).

Statistical Analysis

For statistical analysis, the Mann-Whitney or student’s unpaired t-test was performed using the statistical software package InStat® (GraphPad Software, San Diego, CA, USA). All data are shown as mean ± standard error or mean ± standard deviation, as indicated.
Results

**m131/129 contributes to the virulence of MCMV infection in the spleen**

Upon infection with MCMV, high viral titres are detected in the spleens of BALB/c mice from day 2 post-infection and viral replication peaks around day 4, then declines from day 6 onwards (Figure 1A, and (52)). When BALB/c mice were infected with recombinant MCMV lacking m131/129 (hereafter referred to as Δm131), similar viral titres were seen in the spleen over the first five days, however, by day 6 post-infection, viral titres were significantly lower (p=0.028) (Figure 1A). We subsequently examined spleen tissue sections for expression of the immediate-early 1 (IE1) protein and found that at day 2 post-infection, expression of IE1 in the spleens of mice infected with Δm131 was comparable to that observed in mice infected with wild-type virus (Figure 1B-C). However by day 5 post-infection, when IE1 was readily detectable in mice infected with the wild-type virus, fewer infected cells were found in mice inoculated with Δm131 (Figure 1B-C).

The improved clearance of Δm131 was associated with reduced tissue pathology. On day 4 post-infection, a time when there was little difference in viral titres (Figure 1A), mice infected with Δm131 exhibited far less disorganisation of the spleen and very little disruption of the lymphoid follicles compared to mice infected with wild-type virus (Figure 2A). This observation prompted us to assay local production of two chemokines, CCL2 and CCL21, which are known to play a role in tissue inflammation and organisation of the lymphoid tissues. CCL2 was produced in the spleen in response to...
infection and levels were similar for both viruses on days 2 and 3, however significantly higher levels were detected on days 4 and 5 (p=0.0018 and p=0.0049, respectively) in mice infected with the wild-type virus compared to mice infected with Δm131 (Figure 2B). In contrast, production of CCL21 declined upon infection and the decline was faster after infection with wild-type virus compared to Δm131 (Figure 2C). Consistent with the ELISA results, by day 5 post-infection RT-PCR revealed that the wild-type virus had induced an ~30-fold reduction in CCL21 mRNA expression compared to only an ~3-fold reduction for Δm131 (data not shown). Therefore, changes in spleen architecture induced by MCMV infection correlate with a steady decline in CCL21 production and a late increase in CCL2 production, both of which are moderated in the absence of m131/129 expression.

**Activation of anti-viral CTL is improved in the absence of m131/129 expression**

BALB/c mice limit MCMV infection by generating an effective anti-viral CTL response. The immunodominant epitope recognised by CTL in these mice is derived from the IE1 protein (40). Using MHC class I (H-2Ld) tetramers containing this epitope, we enumerated and characterised the antigen-specific CD8+ T cell response to MCMV, as described in our previous studies (4, 5). IE1-specific CD8+ T cells were detectable in the spleen 4 days after MCMV infection, with the numbers being higher (~3-fold) in mice infected with Δm131 compared with mice infected with wild-type virus (Figure 3A). By day 6, however, there were ~2.5-fold more IE1-specific CD8+ T cells in mice infected with wild-type virus, though the total number had increased in both groups of mice (Figure 3A). IE1-specific CD8+ T cells displayed an activated/effector phenotype in

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response to both viruses, where the majority of cells expressed CD69 and PD1, upregulated CD44, and lost CD62L expression in mice infected with either virus on day 4 (Supplementary Figure 1). Interestingly, IE1-specific CD8+ T cells that were CD127- KLRG1+ were readily detectable on day 4 in mice infected with Δm131 but not wild-type virus, though by day 6 both groups exhibited similar numbers (Supplementary Figure 1).

Next, we measured CTL activity against IE1 peptide-pulsed targets using an in vivo killing assay. CTL activity was higher in mice infected with Δm131 on day 4 post-infection (73.82 ± 1.044 versus 49.80 ± 1.792, mean ± SEM) (p<0.0001) (Figure 3B). Taken together, these data demonstrate that CD8+ T cell activation was faster and more efficient in mice infected with Δm131 compared to those infected with wild-type virus.

**CD8+ T cells are responsible for early clearance of MCMV lacking m131/129**

In our previous study, we found that the improved control of Δm131 in the spleen was prevented by pre-treatment with anti-asialo GM₁ or a combination of antibodies depleting both CD4+ and CD8+ T cells (20). In order to better define the cell populations required for improved control of the Δm131 virus in the spleen, CD8+ or CD4+ T cells were individually depleted prior to infection with Δm131 or wild-type virus and viral titres were determined at day 6 post-infection. As expected, Δm131 titres were over 10-fold lower than wild-type virus in undepleted mice (Figure 3C). CD8-depletion completely abrogated the difference between Δm131 and wild-type titres (Figure 3D). CD4-depletion did not alter Δm131 viral titres (Figure 3E). The potential role of NK cells was examined using BALB/c.CT6 congenic mice where NK cells can be specifically depleted using an
antibody against the NK1.1 determinant. In these mice, Δm131 titres were also over 10-fold lower than wild-type virus in the spleen on day 6, and NK cell-depletion did not alter this phenotype (Figure 3F). Thus, early control of Δm131 in the spleen can be attributed solely to CD8+ T cells.

_Cytokine production is influenced by m131/129_

Cytokines that are produced during MCMV infection can have a profound effect on the course of the disease (16, 22, 41, 52). Since the course of infection was altered in the spleen by the absence of m131/129 expression, we compared the local expression of IFN-γ, IL-12, and IFN-α following infection with wild-type and Δm131 viruses.

BALB/c mice infected with wild-type virus exhibited a sharp peak of IFN-γ production on day 2, a trough on day 3 followed by a second peak on day 5 (Figure 4A). The IFN-γ response to Δm131 was identical to wild-type virus until day 5 where production plateaued rather than rising to a second peak (Figure 4A). There was a single peak of IL-12 production on day 2 post-infection, that was ~2-fold higher for Δm131 when compared to wild-type virus and sustained at later times post-infection (Figure 4B). A peak of IFN-α production was observed at 40 hours post-infection and largely lost by 50 hours (Figure 4C). Both viruses elicited a similar kinetic, however IFN-α peak levels were ~2-fold lower in response to infection with Δm131 (Figure 4C). Therefore, wild-type MCMV expressing m131/129 induced high levels of IFN-α and moderate levels of IL-12 production during early infection, followed by rising levels of IFN-γ; in contrast, a
stronger IL-12 response and weaker production of IFN-α and IFN-γ were elicited in the absence of m131/129 expression.

The number of antigen-presenting cells is not influenced by m131/129

In order to learn more about the effects of Δm131 on the spleen, we enumerated a range of cell types 2 to 4 days after infection. The number of conventional dendritic cells (CD11c<sup>+</sup>MHCII<sup>hi</sup>) is not affected by infection with wild-type MCMV until day 3 when a 50% reduction in DC numbers was noted (Figure 5A). pDC (CD11c<sup>lo</sup>SiglecH<sup>−</sup>CD11b<sup>+</sup>) were lost from the spleen within 2 days of infection with wild-type virus (Figure 5B), as were red pulp macrophages (CD11c<sup>hi</sup>CD11b<sup>lo</sup>F4/80<sup>hi</sup>) and monocytes (CD11b<sup>hi</sup>CD115<sup>−</sup>Ly6G<sup>lo</sup>) (Figures 5C and 5D, respectively). Infection with Δm131 depleted antigen-presenting cell populations with the same kinetics and to the same extent as infection with the wild-type virus. We also found that both viruses had similar effects on NK cell and granulocyte numbers (data not shown).

m131/129 influences activation of both pDC and cDC

Since infection with a virus lacking m131/129 led to significant differences in early cytokine production, particularly IFN-α and IL-12, along with enhanced recruitment of anti-viral CD8<sup>+</sup> T cells, we investigated activation and cytokine production in pDC and cDC. Both viruses stimulated upregulation of CD86 on the surface of pDC and cDC to a similar extent at 40 hours post-infection (Figure 6A). Similarly, we observed no differences in the upregulation of CD40 (data not shown). We then examined the production of type I interferons (IFN-I) using a combination of three monoclonal
antibodies against IFN-α and IFN-β and intracellular cytokine detection. At 40 hours post-infection, IFN-α/β was only detected in pDC (data not shown), and the number of pDC producing IFN-α/β was significantly higher (p<0.0003) in spleens of mice infected with wild-type MCMV compared to Δm131 (Figure 6B), as was the frequency of pDC producing IFN-α/β (WT: 16.57 ± 1.396 N=6; Δm131: 8.695 ± 0.6365 N=4). We also found that the frequency (WT: 19.23 ± 2.290 N=6; Δm131: 10.47 ± 1.439 N=4) and number of pDC producing IL-12 was similarly elevated in response to wild-type MCMV (Figure 6C). In contrast, there was no difference in the number of cDC producing IL-12 (Figure 6D) though the frequency of IL-12-producing cDC was mildly elevated at this timepoint in mice infected with Δm131 (WT: 5.352 ± 0.3170 N=6; Δm131: 7.538 ± 0.8932 N=4, p=0.0271). These results demonstrate that expression of m131/129 directly enhances both IFN-I and IL-12 production by pDC during acute infection. In addition, enhanced IFN-I production coincided with significantly lower titers of the wild-type virus in the spleen 40 hours post-infection (Figure 6E). Taken together, these data indicated m131/129 directly enhances the anti-viral response of pDC in the spleen, leading to better control of early replication of MCMV.

We then went on to examine cDC activation after the peak of IFN-α production (50 hours post-infection) and found higher levels of CD80 and CD86 were expressed by CD8α+ cDC, but not CD11b+ cDC, in mice infected with Δm131 compared to those infected with wild-type virus (Figure 6F). We also detected a larger number of IL-12+ cells amongst both CD8α+ and CD11b+ cDC in mice infected with Δm131 (Figure 6G). Therefore cDC, particularly CD8α+ cDC, appeared to be activated more efficiently by Δm131 than wild-type virus.
type virus during acute infection, potentially accounting for the improved anti-viral CD8+ T cell response observed in the absence of m131/129.
Discussion

The chemokine-like protein m131/129 was originally identified for its effects on inflammatory cell recruitment and dissemination of MCMV (20, 44, 45). In this report, we describe a novel effect of this viral protein on CD8+ T cell activation via interplay with the innate response to the virus. A recombinant MCMV encoding a mutation within the m131/129 open reading frame productively infects the spleen and replicates as efficiently as wild-type virus, but infection is controlled more rapidly. We demonstrate that the early control of Δm131 infection of the spleen can be attributed to the faster generation of virus-specific CD8+ T cell responses. Improved T cell responses were associated with increased activation of cDC in the context of a weaker IFN-α response by pDC. This is the first time the m131/129 viral protein has been shown to enhance IFN-I production by pDC and influence DC and CD8+ T cell activation, thereby accounting for its effect on the efficacy of the adaptive immune response to MCMV.

An accelerated CD8+ T cell response against MCMV has been reported in Cmv1r congenic BALB/c mice that express the Ly49H NK cell receptor derived from C57BL/6 mice (41). Ly49H facilitates recognition and killing of MCMV-infected cells by NK cells and dramatically improves control of the virus in the spleen and lung (13, 19, 47, 48). Under these conditions, where NK cells mediate a reduction in early viral load, Cmv1r congenic BALB/c mice were found to produce significantly lower levels of both IFN-α and IL-12 than wild-type BALB/c mice after MCMV infection. Cmv1r congenic BALB/c mice also exhibited faster activation of CD8+ T cells that was associated with the...
conservation of DC numbers in the spleen (41). The authors of this study concluded that
NK cells promoted the CD8⁺ T cell response to MCMV by limiting the early viral load, which in turn limited the production of IFN-α, thereby ensuring DC populations were preserved (41). Our data for Δm131 infection of wild-type BALB/c (ie lacking Ly49H) demonstrate a reduction in IFN-α production despite a high viral load; a finding that has been confirmed using an independent mutant virus lacking m131 expression generated by the Mocarski laboratory (L Daley-Bauer and E Mocarski, personal communication). Furthermore, we found that a qualitative, rather than a quantitative, difference in the DC compartment of the spleen was associated with an accelerated CD8⁺ T cell response against MCMV.

Mocarski and colleagues recently reported that inflammatory monocytes recruited by m131/129 suppress the CD8⁺ T cell response to MCMV 7 days after infection (15). We now show that m131/129 also influences early CD8⁺ T cell activation (from day 4 post-infection), and this occurs in the absence of monocyte recruitment to the spleen. It should be noted that our results are not directly comparable with those of Daley-Bauer et al (15) since we employed a different route of infection (intraperitoneal versus subcutaneous). Nevertheless, when taken together, these results indicate that MCMV is capable of modulating both the initiation and the duration of the anti-viral CD8⁺ T cell response.

IFN-I are crucial for host survival, with the lethal dose for MCMV infection being around 100-fold lower in mice lacking IFN-αβ receptors (39). Three phases of IFN-I production have been identified following MCMV infection; the first phase is characterised by an
IFN-I peak at 6-8 hours, the second phase peaks at around 36 hours post-infection, and a third smaller, peak is detected at 48 hours (17, 18, 30, 38, 49). Stromal cells are largely responsible for IFN-I production in the spleen during the first phase (49), pDC are the major source of IFN-I at 36 hours (16, 53, 54), and the third phase is attributed to cDC (3, 18, 30). In addition, IFN-α production at this time point relies on toll-like receptor signals (18, 30). Our results support this notion since IFN-I was only detected in pDC at 40 hours post-infection. Importantly, expression of m131/129 directly influenced the number of pDC producing IFN-I during the second phase, and therefore the magnitude of the IFN-I response. Further investigation of the interaction of MCMV with pDC and the role of m131/129 is likely to provide further insight into the mechanisms that regulate IFN-I production in response to viral infection.

Recent work by Benedict and colleagues has provided more detail on the induction of the first phase of IFN-I production in the spleens of C5BL/6 mice during MCMV infection and demonstrated that early production of IFN-I does not require toll-like receptor signals, which is in contrast to the second phase. Furthermore, early IFN-I production depends on lymphotoxin-αβ production by B cells, while the late phase is unaffected. Finally, stromal cells are responsible for the bulk of early IFN-I production (6, 8, 49). These findings have led to the suggestion that MCMV may target the lymphotoxin pathway, however it is unclear how the virus exerts its effect on lymphotoxin production by B cells. When we examined serum and spleen samples from BALB/c mice 8 hours post-infection, we could not detect IFN-α using a commercial ELISA (data not shown) suggesting that the early phase is either much weaker or lacking in MCMV-susceptible mouse strains.
However, we did find evidence of IFN-I production, since CD69 was upregulated on NK and T cells in the spleen, a phenotype that can be attributed to the non-specific effects of IFN-α (29), and there was no difference between Δm131 and wild-type virus at 30 hours post-infection (data not shown). Therefore, m131/129 appears to only influence the toll-receptor-dependent phase of IFN-I production.

Once produced, IFN-α acts quickly to promote NK cell cytotoxicity, an activity required for early control of MCMV in mouse strains that express Ly49H (eg C57BL/6) (17, 30, 35, 38). IFN-I also activates NK cells in MCMV-susceptible mouse strains (eg BALB/c) ((30) and our unpublished results). Interestingly, the type I interferons can contribute to early control of MCMV in BALB/c since administration of low oral doses of IFN-αβ prior to infection reduces early viral titres in the spleen and liver (11), probably by directly inhibiting viral replication (46). Thus, depending on the dose and timing of secretion, IFN-I can have a profound effect on the course of the host response to MCMV.

We observed that activation of cDC and the anti-viral CD8⁺ T cell response was improved in the context of a weaker IFN-I response, though it is not clear how these events are related. Δm131 replicates better than wild-type virus in the spleen 40 hours days after infection, which is likely to be a consequence of the weaker IFN-I response. Higher titres of Δm131 could provide greater stimulation for local cDC, accounting for the increase in costimulatory molecule expression and IL-12 production, leading to earlier activation of CD8⁺ T cells. In this scenario, m131/129 appears to protect the virus because it induces more IFN-I during the early stages of infection.
Previous experiments demonstrated that MCMV induces a breakdown in the architecture of the spleen during acute infection involving the downregulation of CCL21 expression (9). This phenomenon occurred in both MCMV-susceptible (BALB/c) and -resistant (C57BL/6) mouse strains, and the extent of tissue disruption increased with viral load (9). Subsequent experiments by Mueller et al. demonstrated that the organisation of lymphoid organs breaks down in a variety of infections, including those caused by vaccinia, influenza, and *Listeria monocytogenes* and determined that these changes are caused by IFN-γ-dependent inhibition of CCL21 production (34). In agreement with these data, we found that an increase in IFN-γ production on day 5 post-infection was associated with a marked reduction in CCL21 and obvious disorganisation of the spleen in mice infected with wild-type MCMV. Similarly, the initial decline in CCL21 production observed on day 2 post-infection also coincided with a peak of IFN-γ production in the spleen. At this point though (days 2-4 post-infection), there was little difference in IFN-γ production in mice infected with either virus, yet downregulation of CCL21 was at least 30% greater for wild-type virus than Δm131. Thus, an additional mechanism may contribute to the inhibition of CCL21 production and the breakdown in splenic architecture observed after infection with wild-type virus (i.e. in the presence of m131/129 expression).

Our understanding of the function of m131/129 is far from complete. Previous studies have established that a homologue of m131/129 is present in rat CMV (26), but absent from guinea pig CMV (GPCMV) (37) and HCMV. Despite the absence of sequence homology, the genes located in this region of the CMV genome may encode functional homologues and, in this regard, there are some striking similarities between MCMV,
GPCMV and HCMV. For example, the GPCMV genes that correspond to m131/129 are critical for viral dissemination and high titres in the salivary glands (37). In addition, one of the GPCMV genes in this region shares weak homology with UL130, a chemokine-like protein crucial for HCMV entry into epithelial and endothelial cells and subsequent transmission to leukocytes (21, 42, 43), which is thought to assist dissemination of the virus. Interestingly, this coding region is highly conserved in MCMV, GPCMV and HCMV passaged in vivo, yet it is lost when viruses are propagated in vitro (14, 25, 37, 51). Such similarities raise the possibility that m131/129 may play a role in cell entry and/or influence cell tropism for MCMV, either on its own or in combination with other proteins encoded by neighbouring genes, as has been observed for UL130 (43). To this end, it is worth noting that early titres of Δm131 were higher than wild-type virus in the spleen, and thus m131/129 is not required by MCMV to establish infection in the spleen. However, m131/129 may be important for spread of the virus to other cell types, and this may account for its effect of IFN-α production during the second phase of production.

Our appreciation for the role of chemokines in immune function has grown over the last decade and with it has come a greater understanding of viral infection and immune evasion strategies (1, 50). The data presented in this paper highlights the success of this strategy for viruses. Importantly, our data demonstrate that the effects of IFN-α on CD8$^+$ T cell activation and CTL function can be mediated by a direct effect on the activation status of DC. These results also deepen our understanding of the potential effects of type I interferons on adaptive immunity, and thus will be relevant to the design of therapeutic strategies for both CMV and other viral infections.
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Figure Legends

Figure 1. Recombinant MCMV lacking m131/129 is controlled in the spleen earlier than wild-type virus. BALB/c mice were infected with 5x10^3 pfu wild-type MCMV or Δm131 i.p. (A) Spleens were harvested at the indicated times and homogenised for plaque assay. Viral titres are plotted as the mean±SEM, n=3-9 mice. (B) On days 2 and 5 post-infection, spleens were collected and frozen for immunohistochemistry to detect IE1 expression. Representative sections are shown for 3 mice per group, objective x20. (C) The number of fluorescent IE1^+ cells was quantified from six fields for each group on days 2 and 5 post-infection using ImageJ. Values are expressed per mm^2.

Figure 2. The architecture of the spleen is preserved in the absence of m131/129. BALB/c mice were infected with 5x10^3 pfu wild-type MCMV or Δm131 i.p. (A) Spleens were collected day 4 post-infection and prepared for tissue histology. Representative sections are shown for 3 mice/group, objective x10. (B) Spleens were harvested at the indicated times and homogenised for CCL2 assay by ELISA. CCL2 concentrations are plotted as the mean±SEM, n=3-5 mice. (C) Spleens were harvested at the indicated times and homogenised for CCL21 assay by ELISA. CCL21 concentrations were pooled for two independent experiments, normalised with respect to uninfected controls, and plotted as the mean±SEM (n=5-8 mice for all time points, except day 3 where n=3).

Figure 3. Early control of recombinant MCMV lacking m131/129 is mediated by an improved CD8^+ T cell response in the spleen. BALB/c mice were infected with 5x10^3 pfu wild-type MCMV or Δm131 i.p. (A) Spleens were collected at the indicated times
post-infection and prepared for flow cytometry so that the frequency of CD8+ T cells that could bind IE1-tetramer could be determined. The absolute number of IE1-specific CD8+ T cells was calculated using the absolute cell count and plotted as the mean±SEM, n=6 mice per group, 1 representative of 3 independent experiments is shown. (B) On the indicated days, mice were administered CFSE-labelled target cells loaded with IE1 peptide and the proportion of specific killing was determined for the spleen 4 hours later using flow cytometry. Values are plotted as the mean±SEM, n=6 mice per group. (C-E) BALB/c mice were either (C) left undepleted, (D) treated with three doses of anti-CD8, or (E) three doses of anti-CD4, then infected with 5x10^3 pfu wild-type MCMV or Δm131 i.p. Viral titres were determined in the spleen 6 days post-infection and plotted for each mouse per group. 1 representative of 2 independent experiments is shown. (F) BALB/c.CT6 congenic mice were either left undepleted or treated with three doses of anti-NK1.1 to deplete NK cells, then infected with 5x10^3 pfu wild-type MCMV or Δm131 i.p. Viral titres were determined in the spleen 6 days post-infection and plotted for each mouse per group.

**Figure 4. m131/129 modulates cytokine production in the spleen.** BALB/c mice were infected with 5x10^3 pfu wild-type MCMV or Δm131 i.p. Spleens were harvested at the indicated times and homogenised for cytokine assay by ELISA. (A) IFN-γ concentrations are plotted as the mean±SEM, n=3-6 mice. (B) IL-12 concentrations are plotted as the mean±SEM, n=3-6 mice. (C) IFN-α concentrations are plotted as the mean±SEM, n=5-7 mice.
Figure 5. m131/129 does not influence the number of antigen-presenting cells in the spleen. BALB/c mice were infected with $5 \times 10^3$ pfu wild-type MCMV or $\Delta$m131 i.p. Spleens were collected at the indicated timepoints and prepared for flow cytometry. (A) The number of cDC was calculated using the absolute cell count and the frequency of cells that were CD11c<sup>hi</sup>MHC class II<sup>hi</sup>. (B) The number of pDC was calculated using the absolute cell count and the frequency of cells that were CD11b<sup>-</sup>CD11c<sup>int</sup>SiglecH<sup>+</sup>. (C) The number of red pulp macrophages was calculated using the absolute cell count and the frequency of cells that were CD11b<sup>-</sup>CD11c<sup>hi</sup>F4/80<sup>hi</sup>. (D) The number of monocytes was calculated using the absolute cell count and the frequency of cells that were CD11b<sup>hi</sup>CD11c<sup>lo</sup>Ly6G<sup>-</sup>Ly6C<sup>-</sup>.

Figure 6. m131/129 influences the activation of pDC and cDC. BALB/c mice were infected with $5 \times 10^3$ pfu wild-type MCMV or $\Delta$m131 i.p. (A-D) Spleens were collected 40 hours post-infection and (A) the expression of CD86 (as measured by mean fluorescence) was determined for pDC, CD8α<sup>+</sup> cDC and CD11b<sup>+</sup> cDC in the spleen using flow cytometry. At the same time, the frequency of pDC producing IFN-α and IL-12 was determined using intracellular cytokine staining to calculate the number of (B) IFN-α<sup>+</sup> pDC (C) IL-12<sup>+</sup> pDC using the absolute cell count. (D) The number of cDC producing IL-12 was also calculated. (E) Spleens were harvested at 40 hours post-infection and homogenised for plaque assay. Viral titres are plotted as the mean±SEM, n=7 mice. (F-G) Spleens were collected 50 hours post-infection and (F) the expression of CD80 and CD86 (as measured by mean fluorescence, MFI) was determined for CD11b<sup>+</sup> and CD8α<sup>+</sup> cDC in the spleen. (G) At the same time, the frequency of CD11b<sup>-</sup> and CD8α<sup>-</sup> cDC producing...
IL-12 was determined using intracellular cytokine staining. The number of CD11b+ and CD8α+ cDC that were IL-12+ was calculated from the absolute cell count. All values are plotted as the mean±SEM, n=3-5 mice per group, 1 representative of at least 2 independent experiments is shown.
A

Number cDC/spleen

Days post-infection

B

Number pDC/spleen

Days post-infection

C

Number red pulp
macrophages/spleen

Days post-infection

D

Number monocytes/spleen

Days post-infection

WT MCMV

Δm131

p=0.0023

p=0.0084