A little cooperation helps murine cytomegalovirus (MCMV) go a long way: MCMV co-infection rescues a chemokine salivary gland defect

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Cytomegaloviruses (CMVs) produce chemokines (vCXCLs) that have both sequence and functional homology to host chemokines. Assessment of vCXCL-1’s role in CMV infection is limited to in vitro and in silico analysis due to CMVs species specificity. In this study, we used the murine CMV (MCMV) mouse model to evaluate the function of vCXCL-1 in vivo. Recombinant MCMVs expressing chimpanzee CMV vCXCL-1 (vCXCL-1CCMV) or host chemokine, mCXCL1, underwent primary dissemination to the popliteal lymph node, spleen and lung similar to the parental MCMV. However, neither of the recombinants expressing chemokines was recovered from the salivary gland (SG) at any time post-infection although viral DNA was detected. This implies that the virus does not grow in the SG or the overexpressed chemokine induces an immune response that leads to suppressed growth. Pointing to immune suppression of virus replication, recombinant viruses were isolated from the SG following infection of immune-ablated mice [i.e. SCID (severe combined immunodeficiency), NSG (non-obese diabetic SCID gamma) or cyclophosphamide treated]. Depletion of neutrophils or NK cells does not rescue the recovery of chemokine-expressing recombinants in the SG. Surprisingly we found that co-infection of parental virus and chemokine-expressing virus leads to the recovery of the recombinants in the SG. We suggest that parental virus reduces the levels of chemokine expression leading to a decrease in inflammatory monocytes and subsequent SG growth. Therefore, aberrant expression of the chemokines induces cells of the innate and adaptive immune system that curtail the growth and dissemination of the recombinants in the SG.

INTRODUCTION

Human cytomegalovirus (HCMV) is a ubiquitous β-herpesvirus that is an important pathogen in immune-compromised individuals and newborns (Arvin et al., 2004; Mocarski et al., 2013; Nigro & Adler, 2011). It infects between 50–90 % of the population resulting in largely asymptomatic infections (Mocarski et al., 2013). However, primary or reactivated HCMV is a frequent cause of retinitis in AIDS patients (Cheung & Teich, 1999), and increases the incidence of organ rejection and graft-versus-host disease in transplant recipients (Ljungman, 1996; McCarthy et al., 1992). Central nervous system damage due to congenital HCMV infection affects 5000–8000 newborns in the USA each year (Adler, 2005). As a result, HCMV is the leading cause of infectious hearing loss and non-hereditary mental retardation (Mocarski et al.,
Cytomegaloviruses (CMVs) encode numerous proteins that modulate the host immune system. HCMV infection not only alters the expression of host chemokines (Chan et al., 2008b), but also encodes viral homologues of host chemokines and their receptors (Dogra & Sparer, 2014; McSharry et al., 2012; Miller-Kittrell & Sparer, 2009; van Cleef et al., 2006). The virulent strain of HCMV, Toledo (Quinnan et al., 1984), produces a functional CXC chemokine, vCXCL-1_Tob which binds the chemokine receptors CXCR1 and CXCR2 inducing chemotaxis and calcium flux in freshly isolated human peripheral blood neutrophils (PBNs) (Lüttichau, 2010; Penfold et al., 1999). In our previous studies, we have shown that the viral chemokine from chimpanzee CMV (vCXCL-1_CCMV), which is 22 % identical and 52 % similar to the vCXCL-1_Tob protein, triggers calcium release and chemotaxis of PBNs (Miller-Kittrell et al., 2007). Both viral chemokines were shown to upregulate the expression of adhesion molecules on PBNs and downregulate neutrophil apoptosis, albeit with different potencies (Miller-Kittrell et al., 2007). These findings provide circumstantial evidence for a role of vCXCL-1 in activating and recruiting neutrophils to facilitate CMV dissemination. Clinical evidence, including the recovery of HCMV from neutrophils of immunocompromised patients (Saltzman et al., 1988; van der Bij et al., 1988a, b) and the presence of neutrophilic infiltrates in CMV-associated retinitis (Pepose et al., 1983), supports the role of neutrophils, as well as monocytes (Chan et al., 2008a, 2012a, b), in HCMV dissemination. While the species specificity of CMVs limits direct evaluation of vCXCL-1 in HCMV dissemination, the function of vCXCL-1_CCMV was used as a surrogate for the HCMV vCXCL-1 homologue. We chose vCXCL-1_CCMV for our experiments because it is more conserved in CCMV isolates unlike vCXCL-1 from HCMV, which is one of the most variable in HCMV (Davison et al., 2003; Dolan et al., 2004; Heo et al., 2008). In addition, we wanted to extend previous in vitro vCXCL-1_CCMV findings into an in vivo system.

Murine CMV (MCMV) is a well-established animal model of CMV infection and has similar cellular tropism and disease manifestations to HCMV (Hudson, 1979). MCMV expresses a C-C chemokine homologue, MCK2, which increases the inflammatory response in mice and enhances dissemination of MCMV to the salivary gland (SG) (Fleming et al., 1999; Saederup et al., 2001). Therefore, MCMV was chosen to characterize the role of vCXCL-1_CCMV in vivo. To test this in the mouse system, we generated recombinant MCMVs expressing vCXCL-1_CCMV and host chemokine, mCXCL1 (i.e. KC). Our results show that expression of both the host and viral chemokines is detrimental to the recombinants in the SG. We show that there is an increase in inflammatory monocytes that contributes to a blockade of viraemia and growth in the SG. Surprisingly, co-infection with a non-chemokine-expressing strain of MCMV restored growth of the chemokine-expressing recombinants in the SG by tipping the immune response to a more favourable environment for normal growth of the recombinants.

RESULTS

Construction of the chemokine-expressing MCMVs

Strict species specificity of CMVs requires the generation of a recombinant MCMV to assess the function of viral chemokines in the mouse model. Therefore, we generated a recombinant MCMV expressing vCXCL-1_CCMV (RMvCXCL1_CCMV) to study the role of this chimpanzee CMV viral chemokine in viral dissemination in vivo. MCMV expressing mCXCL1 was also generated as a control to evaluate the specific effects of the viral chemokine on dissemination. mCXCL1 is the murine equivalent of human CXCL-1 (Gro-α) with a high affinity for CXCR2. MCMV RM4511 was chosen as the parental strain of these recombinants because RM4511 lacks a functional MCMV viral chemokine, MCK2. This will allow us to analyse the contribution of vCXCL-1_CCMV and mCXCL1 in the dissemination of MCMV in the absence of the endogenously encoded chemokine, MCK2 (Fig. S1a, available in the online Supplementary Material).

Recombinant plasmid, L120.1, was modified to contain either the vCXCL-1_CCMV or mCXCL1 coding sequence under the control of HCMV immediate early (HCMV IE) promoter (Fig. S1b). This promoter has been used to drive expression of other genes inserted into the MCMV genome and was chosen to ensure high levels of chemokine expression. We chose to insert the chemokine cassette into the ie2 region as it is dispensable for growth of MCMV in vivo (Manning & Mocarski, 1988). The chemokine cassette displaces the puromycin-GFP segment present in RM4511, such that loss of GFP expression allowed visual selection of recombinant viruses.

Following transfection of NIH3T3 cells with Drd-linearized L120.1+vCXCL-1_CCMV or L120.1+mCXCL1 and subsequent infection with RM4511, recombinant viruses were passaged twice in medium containing mycophenolic acid and xanthine to select for recombinant viruses expressing gpt. The loss of GFP expression identified recombinant viruses and each virus was plaque purified three times. Recombination and correct insertion of the chemokine cassette was confirmed using PCR (Fig. S1c).

Recombinant MCMVs have similar in vitro growth kinetics and chemokine expression

To determine whether insertion of the vCXCL-1_CCMV or mCXCL1 cassette affected replication or spread of the recombinant viruses in cell culture, we set up both single-step (m.o.i.=5) and multi-step (m.o.i.=0.05) growth curves. RMvCXCL1_CCMV and RMmCXCL1 replicated as well as RM4511 in both assays (Fig. S1d), indicating no deleterious
effects of the insertion on growth of the viruses in cell culture. Immunoblotting Ni-NTA (nickel-nitriotriacetic acid)-concentrated supernatants from each time point of the single-step growth curve was used to detect the temporal expression of the chemokines in the supernatants of virally infected cells. Both RMvCXCL-1<sub>CCMV</sub> and RMmCXCL1 proteins were detected in the supernatants beginning at the second day post-infection (p.i.) and continued for the duration of the experiment (Fig. S1e).

**Dissemination of chemokine-expressing viruses in vivo**

The contribution of vCXCL1 to the replication and dissemination of recombinant viruses in vivo was evaluated after infecting mice with RM4511, RMmCXCL1 and RMvCXCL1<sub>CCMV</sub> in the foot pad (FP) and measuring virus in the organs with a plaque assay. Recombinants reached similar titres as parental virus in FP and in organs of primary dissemination (i.e. lymph node, lung and spleen) (Fig. 1a). However, no recombinant virus was detected in SG at day 7 and 14 p.i., when the parental virus usually reaches peak titres (Fig. 1a). To exclude the possibility that the dissemination of the recombinant virus to the SG was only delayed, viral load in the SG was also measured at day 21 p.i. No recombinant virus was detected even at this later time point (data not shown). Similar experiments were performed using intraperitoneal (i.p.) infection to determine if the route of infection had any effect on viral dissemination. Mice infected i.p. also had no chemokine-expressing recombinants in the SG (data not shown). The lack of dissemination of these recombinants to the SG was accompanied by an absence of detectable viraemia during secondary dissemination at day 4 p.i. (Fig. 1b). Surprisingly, we found equivalent amounts of viral DNA in the SG at day 7 in spite of the differences in viraemia, but less viral DNA for the recombinants only at day 14 p.i. (Fig. 1c). These data point to a viraemia defect that still allows for enough recombinant virus to disseminate to the SG early after infection, but the recombinant viruses cannot continue to grow in the SG.

**NK, T- and B-cell-mediated blockade of SG growth of the chemokine-expressing recombinants**

Both vCXCL-1<sub>CCMV</sub> and mCXCL1 bind and activate neutrophils via CXCR2 (Kobayashi, 2006; Miller-Kittrell et al., 2007). The recruitment and activation of CXCR2<sup>+</sup> cells could lead to an inflammatory environment that prevents normal SG growth. Although there was a slight increase in the number of neutrophils recruited to the site of inoculation at day 3 p.i., in mice infected with the chemokine-expressing recombinants compared with RM4511, it was not statistically significant (Fig. 2a). In addition, we observed more NK cells early (day 5) in both the spleen and the lung for the mCXCL1-expressing virus, but only the lung at day 7 continued with significant increases in both recombinants (Fig. 2b). Early in infection (day 5 p.i.), there was a two- to threefold increase in inflammatory and patrolling monocytes in the lungs of chemokine-expressing recombinant viruses compared with RM4511-infected mice (Fig. 2b). There was no difference in the CD4 and CD8 numbers in the sites of primary dissemination or in the SG (Fig. 2b, c).

These data show that the chemokine-expressing recombinants induce differential recruitment of inflammatory monocytes and NK cells suggesting an immune-mediated blockade of the dissemination/growth of the recombinants in the SG. To investigate the extent of this blockade, mice were administered cyclophosphamide (cyclo) to deplete immune cells prior to infection (Mayo et al., 1977; Rager-Zisman et al., 1990; Selgrade et al., 1981) and dissemination of the recombinants was measured in the different organs. We observed no difference in the primary dissemination between the chemokine-expressing recombinants and the parental virus in untreated and cyclo-treated mice, in spite of a significant decrease in NK cells in the spleen and lung (Fig. S2). However, the chemokine-expressing recombinants were recovered from the SG of cyclo-treated mice albeit at a much lower titre compared with the parental (Fig. 2d), with detectable viraemia measured at day 4 p.i. (Fig. 2i, left panel).

Data from the above experiment provides further evidence for an immune-mediated blockade of recombinant virus dissemination/growth in the SG. Activated neutrophils or NK cells could trigger an inflammatory response capable of clearing recombinant viruses from the SG or suppressing their ability to grow. Both can express CXCR2 and can respond to both of the CXC chemokines overexpressed in these viruses (Stacey et al., 2014) and NK cells form an important arm of the innate immune response to MCMV infection (Bukowski et al., 1984; Sacher et al., 2012; Tessmer et al., 2011). To parse out the contribution of these immune cell subsets in this blockade, we depleted neutrophils or NK cells. Virus was measured in the SG at day 14 p.i. Despite a slight enhancement of neutrophils to the site of infection and increase in NK cells (Fig. 2a, b), depletion of either cell type did not restore MCMV recombinants in the SG (Fig. 2e, f). These results show that neither neutrophils nor NK cells are responsible for decreased viraemia of the recombinants or growth defect in the SG observed in vivo.

Another approach to map the immune response responsible for the phenotype of the chemokine-expressing recombinants in vivo is to use mice that have genetic defects that alter their immune capabilities. First, we utilized the NSG [NOD (non-obese diabetic) SCID (severe combined immunodeficiency) gamma] mouse model, which lacks NK cells and T and B lymphocytes (Shultz et al., 2005). NSG mice were infected with the recombinant or parental viruses, and the viral load in the SG at day 14 p.i. was measured. We recovered recombinant virus from the SG of NSG mice at day 14 p.i., albeit much less than the parental virus (~3 log) (Fig. 2g). Note that these recombinant viral titres are similar to those in the cyclo-treated mice (Fig. 2d) and that BALB/c mice show the same recombinant virus SG defect as our

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**Co-infection rescues MCMV dissemination defect**
transgenic or knock in mice (Fig. S3a). In addition, we observed viraemia for the chemokine-expressing recombinants as well as parental virus in NSG mice (Fig. 2i, right panel). Data from this experiment suggests that NK and/or T- and B-cells play a partial role in preventing the dissemination of the recombinant virus to the SG, but in light of
Fig. 2 (a, b). Absence of cellular subsets permits dissemination/growth of chemokine-expressing recombinants to the SG. hCXCR2KI mice were infected in the FP with 1 x 10⁶ p.f.u. RM4511, RMmCXCL1 or RMvCXCL1. Flow cytometry was used to measure the number of neutrophils (CD11b⁺, Ly6G⁺) infiltrating the FP at day 3 p.i. (a); NK cells (CD3⁺, CD49b⁺); patrolling (CD11b⁺, Gr1⁺, CD11c⁺) and inflammatory monocytes (CD11b⁺, Gr1⁺, CD11c⁺) recruited (b) into the spleen and the lung at day 5 and 7 p.i.
Fig. 2 (Cont.) (c–i). (c) CD4+ (CD3+ CD4+) and CD8+ (CD3+ CD8+) cells from 14 day p.i. mice. (d) Mice were treated with cyclo for systemic immune ablation prior to infection with the parental and recombinant viruses alone. Viral titre in SG were measured at days 3, 7, 14 and 18 p.i. (e) Viral titres in SG of mice (a) depleted of neutrophils using anti-Ly6G antibody, (f) NK cells using anti-asialo GM1 antibody (on days 1 and 0), or control treated, (g) NSG mice, which lack T-, B- and NK cells, and (h) SCID mice at day 14 post i.p. infection with parental or recombinant virus alone. (i) Viraemia as measured via leucocyte enumeration and is representative of two or more experiments. Bars represent the mean virus titre (±SD). One-way ANOVA followed by Tukey’s multiple comparison test was used to compare the data. *** = P < 0.001, ** = P < 0.01, * = P < 0.05. NS, Not significant.
Co-infection rescues MCMV dissemination defect

Results of the NK depletion experiment (Fig. 2f), T- or B-cells are more likely.

To address the role of the T- and B-cells in the lack of dissemination of chemokine-expressing recombinants, we used SCID mice, which lack both T- and B-cells. In particular, T-cells play an important role in controlling MCMV infection. While CD8+ T-cells effectively clear MCMV from organs in the periphery (Reddehase et al., 1987), viral clearance from the SG is dependent on CD4+ T-cells (Campbell et al., 2008; Lucin et al., 1992). To determine if the adaptive immune response could be clearing RMvCXCL1 and RMmCXCL1 from the SG, SCID mice were infected with recombinant or parental virus and viral load was measured in the SG at day 14 p.i. Here again, the chemokine-expressing recombinants were recovered from the SG of SCID mice as seen in the NSG mice, although not to the same levels as parental virus (Fig. 2h). This data suggests that the absence of RMvCXCL1 and RMmCXCL1 from the SG may be a result of an amplified adaptive immune response to the recombinants as well as a dissemination defect. Together, data from these experiments suggest that the chemokine-expressing recombinants’ aberrant production of the chemokines induces an innate (i.e. IM mediated) and adaptive (i.e. T-/B-cell mediated) immune response that is responsible for the lack of viraemia and/or clearance from the SG.

Co-infection rescues dissemination of chemokine-expressing recombinants

If this chemokine-mediated response leads to an increased immune response that masks normal growth, a simple co-infection experiment could reveal its function in trans. We hypothesized that during co-infection (i.e. parental+recombinant), the dissemination/growth defect would dominate, leading to an inhibition or reduction in the dissemination/growth of parental virus in the SG. To test this hypothesis, mice were infected in the same FP with a mixed inoculum at a 1:1 ratio of the parental and chemokine-expressing recombinant virus. SGs from these mice were harvested 14 days p.i. and the viral load determined. Surprisingly, chemokine-expressing recombinant viruses were recovered from the SG of mice infected with the mixed inoculum albeit at reduced titres (Fig. 3a). Because there was no difference between vCXCL1 and mCXCL1 in these co-infection experiments and the chemokine receptors for mCXCL1 are well characterized, we carried out the remaining co-infection experiments only with mCXCL1-expressing virus. Dissemination of the chemokine-expressing recombinants to the SG was also rescued in mice that were co-infected i.p. demonstrating that the route of infection was not critical (Fig. 3b). This rescue is also independent of MCMVs expression of its endogenous C-C chemokine (MCK2), as both the MCK2 expressing RM4503 and mutated RM4511 (MCK2-) rescue the dissemination of the recombinants to the SG (Fig. 3c). Higher replication of the virus in the FP, spleen or lung post-co-infection (Fig. S3d) does not explain the increase in virus in the SG during co-infections. In addition, the timing of the infections was critical. For example, no recombinants were recovered in the SG when the infection of the two viruses was separated by 2 or 7 days (Fig. S2e). Taken together, these data indicate that the adverse effects of aberrant chemokine expression are nullified during co-infection and the SG phenotype rescue is temporally restricted requiring the viruses to interact early during infection.

Requirement for interaction at the site of infection during co-infection rescue

To explore how co-infection rescues the dissemination of recombinant viruses to the SG, mice were infected with the two viruses in separate FPs on the same day. This would eliminate the possibility of the viruses interacting at the site of infection, but still allow them interact at the sites of primary dissemination (i.e. spleen and lung). Both the viruses grew to similar levels in the FP and primary dissemination sites (Fig. S3d). However, chemokine-expressing recombinant viruses were recovered from the SG of these mice at 14 days p.i. (Fig. 3b).

The requirement of the close proximity of the two viruses to mediate the rescue phenotype suggests that the viruses may co-infect the same cell at the site of infection. To test this possibility, we infected mice with a low p.f.u. mixed inoculum (1:1 ratio of 100 p.f.u. each of RM4511 and RMmCXCL1). Infection with a low p.f.u. inoculum reduces the probability of co-infection by greater than 1000-fold and subsequently reduces the dissemination rescue (Fig. 3c). In order to directly address whether the viruses co-infect cells at the site of infection, we performed infectious centre assay with plastic adherent leucocytes isolated from the FP at day 3 p.i. from mice infected with high titre mixed inoculum of 1:1 mix of parental and recombinant virus, schematically described in Fig. 3d. During the amplification step, we were able to detect GFP-positive and -negative plaques from ~49% of purified GFP-positive plaques (Fig. 3e). All purified GFP-negative plaques yield only GFP-negative plaques during the amplification step. These results indicate that the two viruses need to co-infect the same cell at the site of infection.

Live parental virus is required to rescue the isolation of chemokine-expressing recombinant virus from the SG

A possible contribution to the rescue of dissemination of the recombinants to the SG during co-infection is that the presence of the parental virus particles stimulates an immune response that overcomes the blockade of dissemination. To explore this possibility, we infected mice in the same FP with a mixed inoculum containing a 1:1 mixture of viable RMmCXCL1 or RM4511 and either UV-inactivated RM4511 or RMmCXCL1, respectively. Infection of mice with an inoculum containing UV-inactivated

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RMmCXCL1+Rm4511 did not alter the dissemination of RM4511 to the SG (Fig. 4). However, UV inactivation of RM4511 completely abolished the rescue of the dissemination of RMmCXCL1 to the SG. These data demonstrate that live-replicating parental virus is necessary to mediate the rescue of the chemokine-expressing recombinants to the SG.

The need for recombinant and live parental virus to co-infect the same cells suggests that the chemokine-expressing viruses might have undergone DNA recombination with the parental virus. This could lead to the loss of expression of the chemokine gene and allow for subsequent dissemination/growth. PCR amplification and subsequent DNA sequencing of the chemokine gene from recombinants isolated from the SG following co-infection shows that they all carry an unmutated chemokine gene (Fig. S4a). Additionally, RFLP analysis of these isolates showed no overt recombination (Fig. S4b). According to this data, there is no alteration of the chemokine-expressing recombinants, but a genuine rescue of the SG phenotype.

Co-infection alters the immune response to chemokine-expressing recombinant viruses

To see how the immune response profile changes following co-infection, immune cells were characterized from co-infected mice from the different organs. The major reduction between RMmCXCL1 and the co-infected mice is the inflammatory monocytes found in the lung (Fig. 5). These cell types may interfere with the normal secondary dissemination/growth of the chemokine-expressing recombinants to the SG. During co-infection, the reduction in their recruitment to sites of infection would allow for increased dissemination to the SG of the chemokine-expressing recombinants.
titre from the experiment. The dashed line indicates the DL of the

The asterisk (*) indicates viral titres in the SG from mice infected

day 14 p.i. Each symbol indicates the titre from individual mice.

RM4511: live RMmCXCL1. Viral titre was measured in the SG at

mix) of UV inactivated (UV ia) RMmCXCL1: live RM4511 or UV ia

RM4511 or RMmCXCL1 virus alone, or a mixed inoculum (1 : 1

Co-infection reduces mCXCL1 production in vitro

In the absence of any recombination or mutation of the

chemokine gene, a reduction in chemokine production during

co-infection could lead to the SG recovery of the recombinants. To test this possibility, we carried out an in vitro co-infection assay. Cells in culture were infected with recombinant or parental virus alone or with a mixed inoculum. Supernatants were harvested every 24 h and silver stained to measure chemokine protein levels. We observed a reduction in the relative expression of the chemokine during co-infection in vitro compared with recombinants alone (Fig. 6a). In spite of these differences in chemokine expression, there was no difference in the viral titres (Fig. 6b). These data suggest that during co-infection, less chemokine is produced, without an effect on viral load. This reduction could be sufficient for dissemination of the recombinants to the SG.

DISCUSSION

In our previous study we characterized the CCMV chemokine homologue, vCXCL-1$_{CCMV}$, and demonstrated that it is a functional chemokine, activating and recruiting human neutrophils similar to the HCMV chemokine vCXCL-1$_{HCMV}$ (Miller-Kittrell et al., 2007). Due to the species specificity of CMV, the in vivo function of vCXCL-1$_{CCMV}$ is unknown. We have gained significant knowledge about HCMV dissemination using the MCMV mouse model (Dogra & Sparer, 2014; Mocarski et al., 2013). MCMV has similar tropism to HCMV and both viruses demonstrate a cell-associated viraemia in myelomonocytic lineage cells such as neutrophils, monocytes and their precursors (Bale & O’Neil, 1989; Daley-Bauer et al., 2014; Dogra & Sparer, 2014; Hudson, 1979; Mocarski et al., 2013; Saltzman et al., 1988; Stoddart et al., 1994). Although the mechanism and relative contribution of each of these cell types to MCMV dissemination in vivo has been studied in some detail, the role of host and viral chemokines on this dissemination remains unclear (Daley-Bauer et al., 2014; Dogra & Sparer, 2014; Mocarski et al., 2013; Noda et al., 2006). In this study, we used MCMV RM4511, which does not express functional MCK2, to generate recombinant MCMVs expressing viral and host CXC chemokines in order to evaluate the impact of vCXCL-1$_{CCMV}$ on viral dissemination.

The primary dissemination pattern of RMvCXCL-1$_{CCMV}$ and RMmCXCL1 was similar to RM4511 (Fig. 1a). However, the chemokine-expressing recombinant viruses were not recovered from the SG (i.e. secondary dissemination) (Fig. 1a). There was no difference in viral growth of the recombinants compared to the parental virus at the site of inoculation in the FP or the primary dissemination organs [i.e. popliteal lymph node, spleen and lung (Fig. 1a)]. However, viral DNA was detected in the SG from our recombinants (Fig. 1c). It is possible that our recombinants are unable to replicate in the SG and carry a mutation in the sgl1 gene, which has been shown for other MCMV recombinants with a SG growth defect (Lagenaur et al., 1994; Manning et al., 1992). However, this is unlikely for several reasons. First, the independently generated recombinants show a similar SG phenotype (data not shown). Moreover, when the recombinants reach the SG, as is the case of SCID mice, NSG mice, cyclo-treated mice or co-infected mice, they are able to replicate in the SG (Fig. 2d, g, h). Therefore, the recombinants are capable of SG replication when immune cells are depleted. However, we did observe defective viraemia for the recombinants pointing to at least a partial dissemination defect (Fig. 1b).

It is possible that overexpression of the chemokine leads to an overactive immune response against the recombinants, which results in their increased clearance from the SG. Viral clearance from the SG is CD4$^+$ T-cell mediated (Campbell et al., 2008; Lucin et al., 1992). Chemokine-expressing recombinants were found in the SG of SCID mice, lacking B and T lymphocytes, supporting this possibility (Fig. 2h). Chemokine-expressing recombinant viruses were also found in the SG in NSG mice and after systemic immune ablation with cyclo treatment, implicating both the innate/adaptive immune system for the SG dissemination defect/clearance (Fig. 2d, g). Interestingly, recovery of recombinants in the SG in both cases paralleled an increase in viraemia still implicating a dissemination defect (Fig. 2e). Thus, although the recombinants may be susceptible to adaptive immune-mediated clearance or growth suppression in the SG, they also show an innate immune-mediated defect in

Fig. 4. Rescue of the dissemination phenotype requires live virus.

hCXCR2KI mice were infected with $5 \times 10^5$ p.f.u. of either RM4511 or RMmCXCL1 virus alone, or a mixed inoculum (1 : 1 mix) of UV inactivated (UV ia) RMmCXCL1: live RM4511 or UV ia RM4511: live RMmCXCL1. Viral titre was measured in the SG at day 14 p.i. Each symbol indicates the titre from individual mice. The asterisk (*) indicates viral titres in the SG from mice infected with a mixed inoculum. The horizontal line represents the median titre from the experiment. The dashed line indicates the DL of the assay.
SG dissemination. This points to a multifunctional defect: cells in the blood carrying MCMV and immune clearance in the SG.

While exploring the effects of chemokine expression in trans in the co-infection model (i.e. parental+chemokine-expressing recombinants), we made the serendipitous discovery that the recombinants were able to disseminate to the SG even in immune-competent mice (Fig. 3a). The rescue of dissemination required the two viruses to infect simultaneously and at the same site, as separating the infection spatially or temporally does not rescue the dissemination to the SG (Figs 3b and S3). This localization is required for the two viruses to infect the same cell, most likely a monocyte/macrophage population at the site of infection (Fig. 3c, d, e). We show that these viruses do not undergo recombination, mutation or deletion of the chemokine gene while replicating within parental virus infected cells (Fig. S4). Our data points to the parental virus reducing the production of the chemokine from the recombinant during co-infection without affecting viral growth (Figs 6 and S6). Therefore, the reduced chemokine level seen during mixed infection is not due to less virus production, but to potentially intracellular resource competition among other possibilities (George et al., 2015; Gyorgy & Vecchio, 2014; Kuo et al., 2013; Li et al., 2014; Rondelez, 2012; Weisse et al., 2015). Unfortunately we were unable to show this reduction in the chemokine levels in vivo due to the limits of chemokine protein detection. How could parental virus interfere with the HCMV IE promoter? Intracellular resource competition or gene expression suppression like that seen with HCMV IE proteins on the major IE promoter (Stenberg et al., 1990) within the infected cells could be responsible for the reduced chemokine levels in vivo and subsequent rescue of SG virus. Currently, these are just several possible explanations that are being explored.
In order to have a decrease in chemokine production, there needs to be co-infection at the site of infection (Fig. 3a, b). Surprisingly, MCMV co-infection in vivo is more common than one would expect (Cicin-Sain et al., 2005). Using both an attenuated virus and a clever Cre–lox system, Cicin-Sain et al. (2005) were not only able to show functional trans-complementation but also co-dissemination to distant organs. They estimate that 1 in 22 of virally infected cells are co-infected in vivo. Many of our findings recapitulate their findings: in order to get complementation, viruses must be infected at the same FP and at the same time. Although their attenuated viruses did have reduced primary growth/dissemination, ours did not. Our findings point to a co-infection requirement implying that this is an internal ‘competition’ suggests a possible resource competition scenario instead of ‘chemokine masking’ that acts in trans. Although we were unable to show a true titration of virus and chemokine production to support resource competition (Fig. S6), this assay is limited by its sensitivity and the fact that we are using viruses instead of plasmids, which would allow for tighter controls on entry and copy number of our chemokine genes. Additional tightly controlled experiments will be needed to ascertain how or whether this competition occurs.

Our data shows that over-produced chemokines may recruit or activate cells of innate or adaptive immune system that are detrimental for the dissemination/growth of the recombinants in the SG. A more physiological level of expression of the viral chemokine could allow for evaluation of the viral chemokine in vivo, such as expression under the control of the MCK2 promoter, which expresses with late kinetics. These data also bring up the interesting observation that the immune system-mediated blockade of dissemination of the chemokine-expressing recombinants to the SG is not very stringent and a little reduction in chemokine levels seems to be sufficient to tip the scales in favour of normal dissemination/growth to the SG. Surprisingly, neutrophil or NK cell depletion did not restore dissemination to the SG (Fig. 2e, f). Therefore, another innate cell type that expresses CXCR2 could be responsible for this phenotype. For example, dendritic cells or a subset
of monocytes can be induced to express CXCR2 and CXCR1 (Bernardini et al., 2013; Inngjerdingen et al., 2001; Takata et al., 2004; Wang et al., 2012), and may be involved in this process. Although an exhaustive analysis of vCXCL1<sub>CCMV</sub> receptor usage is lacking, mCXCL1 receptor usage is well characterized (Lee et al., 1995). As both have the same phenotype in our experiments, this SG dissemination defect does not seem to be exclusively a vCXCL1<sub>CCMV</sub> phenomenon. NK cells play a major role in the antiviral response against MCMV and mCXCL1 increases their presence during primary dissemination (Fig. 2b) (Bukowski et al., 1984; Sacher et al., 2012; Tessmer et al., 2011). BALB/c mice, which do not induce NK cell activation via the m157-Ly49H axis, are susceptible to MCMV infection and show much higher viral titres in peripheral organs (Arase et al., 2002; Mercer & Spector, 1986). In spite of this, depletion of NK cells did not alter isolation of recombinant viruses in the SG.

Recently, Farrell et al. (2015) showed that FP infected MCMVs spread via the lymphatics and that this infection/spread is multi-layered. They show that the subcapsular sinus macrophages act to dampen MCMV growth and without them more fibroblasts are infected resulting in higher titres and increased spread. In our experiments, we hypothesize that an increase in inflammatory cells blocks the normal dissemination of MCMV to the SG. In mice infected with the chemokine-expressing recombinants, we observed more inflammatory monocytes in the lungs compared with mice infected with the parental virus (Fig. 2b). Co-infection reduced the number of inflammatory monocytes (IM) in the lung, while not affecting the number of patrolling monocytes (Fig. 5). This data supports our hypothesis that the monocytes at the site of infection or around the foci of infection are the wrong type when the chemokine is expressed during MCMV infection (i.e. more inflammatory rather than patrolling). Although this might not affect viral growth at the site of infection, it would adversely affect the dissemination of the virus during viraeemia. Preliminary experiments adaptively transferring leukocytes isolated at day 3 p.i. from the FP of mice infected with parental, recombinant or a mixed inoculum show that we can recover virus from the SG of mice receiving cells from mice infected with parental or a mixed inoculum. However, no virus was recovered from the SG of mice that received cells from chemokine-expressing recombinants (Fig. S5).

Therefore we propose a model where the virus is carried out of the FP to the organs of primary dissemination initially by patrolling monocytes. During infection with the chemokine-expressing recombinants alone, the overexpression of the chemokine in the organs of primary dissemination (i.e. spleen, liver, lung) causes an enhanced inflammatory state. This inflammatory state supports the differentiation of inflammatory monocytes to M1 macrophages at the site, which do not disseminate the virus to the SG. The increased number of IM may also interfere with the ability of patrolling monocytes to gain access to virally infected cells. During co-infection, there is a reduction in chemokine levels. The reduced chemokine levels leads to less IM cells at the site of infection and a reduction in the inflammatory environment granting patrolling monocyte access to the foci of infection, allowing for increased dissemination. This model is summarized in Fig. S7.

Although our results may seem to contradict our original hypothesis (i.e. the expression of HCMV CXC chemokines aids dissemination), we have to consider the caveat that our chemokine-expressing recombinants overexpress the chemokines. In reality, the expression of the vCXCL-1 gene in HCMV is tightly regulated and expressed with late expression kinetics (Lurain et al., 2006; Penfold et al., 1999), and not constitutively, as in the case of our recombinants. Because monocytes play an important role in HCMV dissemination in vivo (Smith et al., 2004a; Stevenson et al., 2014), it is conceivable that HCMV has evolved to express vCXCL-1 at the stage when the virus is budding from the infected cell. The chemokine-induced monocyte is at the right place at the right time to pick up the budding virus and once the infected monocyte/neutrophil re-circulates, it carries the virus to distal sites spreading infection within the host.

**METHODS**

**Cells and viruses.** Murine NIH3T3 and M210B4 cells (ATCC) were propagated in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% FetalClone III (FCIII; Hyclone), 1% penicillin/streptomycin (P/S; Hyclone), 1× non-essential amino acid (NEAA) solution, 1% sodium pyruvate (100 mM) and 0.5% HEPEs (1 M). MEF 10.1 cells (ATCC) were propagated in DMEM supplemented with 10% FCIII and 1% P/S and L-Gln. The parental MCMV strain used in this study was MCMV RM4511 (Saederup et al., 2001), which has a 1.7 kb puromycin-GFP cassette inserted into the i2e2 region and a double point mutation in the m131 gene resulting in a non-functional MCK2 protein (Fig. S1a). RM4503 is similar to 4511 except with WT mk2. These viruses were obtained from Dr Edward Mocarski, Emory University. For UV inactivation of the virus, 50 µl of the stock virus were exposed to UV light in a UV crosslinker (Stratagene Stratallinker) at a setting of 1200 for 8 min. Complete inactivation was confirmed via a plaque assay.

**Mice.** Initially, mice that overexpress human CXCR2 (hCXCR2) or replace the murine CXCR2 (mCXCR2) gene with the human CXC chemokine (hCXCR2) gene were used. There was concern that vCXCL-1<sub>CCMV</sub> might not stimulate mCXCR2 as seen with vCXCL-1<sub>FoV</sub> (Saper et al., 2004). The hCXCR2 transgenic BALB/c mice express the hCXCR2 gene under the control of the neutrophil-specific, human myeloid related protein-8 promoter (Saper et al., 2004). These are named hCXCR2 transgensics. In the co-infection experiments, mice that knock in the hCXCR2 gene with the mCXCR2 gene were used (i.e. hCXCR2KI) (Mihara et al., 2005). These mice have normal expression levels of hCXCR2 in all the appropriate murine cell types. We have subsequently shown that vCXCL-1<sub>CCMV</sub> can function in normal mice (data not shown), which allowed us to use SCID and NSG mice with the appropriate parental controls (i.e. BALB/c). The 4–8-week-old BALB/c NSG mice were originally purchased from Jackson Laboratory and SCID/NCr were purchased from Taconic Labs. All mice were housed under specific pathogen free conditions at the University of Tennessee or the University of Cincinnati Medical School. The Institutional Animal Care and Use Committees (IACUCs) at the University of Tennessee or the University of Cincinnati approved all procedures on animals.
Plasmid constructs. An EcoRI/PstI-digested fragment containing the coding region for vCXCL-1 /CCMV or host mCXCL1 (KC) was cloned into the EcoRI/PstI-digested plasmid pDNA3.1/Zeo immediately downstream of the HCMV IE promoter. The 1.2 kb HCMV IE-chemokine fragment was PCR amplified adding the flanking restriction sites and a C-terminal 6-His tag using the primers: MluI HCMV IE (5’-GGACCGGCTGATG TACGGGCACGAGATACCGCTGACATTGATTAT-3’) and SalI 6-His (5’-ACGCGTCGACTGTTGTTGTTGTTGTTGACCTCCTCC-3’). After sequence verification, the HCMV IE-chemokine cassette was digested with MluI and SalI and cloned into the plasmid L120.1. L120.1 has 5’ and 3’ sequences from MCMV IE2 for homologous recombination and a gpt expression cassette used for selection of recombinant viruses (C. Meiering, unpublished data).

Generation of recombinant viruses. Recombinant viruses were generated using a transfection/infection strategy with subsequent selection for the loss of GFP along with gpt expression. NIH3T3 cells were transfected with Drd-linearized L120.1-vCXCL-1 /CCMV or L120.1-mCXCL1 using Lipofectamine 2000 (Invitrogen). Three hours post-transfection, the cells were infected with MCMV RM4511 at m.o.i. = 3. Transfected cells were harvested and passed twice under selection [mycophenolic acid (12.5 µg ml⁻¹) and xanthine (100 µg ml⁻¹)]. Recombinant viruses were identified by the loss of GFP fluorescence and subjected to three rounds of plaque purification. For PCR verification, viral DNA was isolated by phenol/chloroform extraction and used for diagnostic PCR. The primers used were: RM4511 Forward (5’-CATTCAGCTCATTGG TGGGAAAGTACATGGCG-3’), RM4511 GFP Reverse (5’-CCCGACGCGGTGAAGAGAGGACTTCTGACG-3’) and HCMV IE Rev (5’-GAACCTCATATATGGGTGATGAAGAG-3’).

In vitro growth assay. NIH3T3 or MEF 10.1 cells were plated in triplicate in a six-well dish and infected with RM4511, RM-vCXCL-1 /CCMV or RM-mCXCL1 for either a multi-step (m.o.i.=0.5) or single-step (m.o.i.=5) growth analysis. Supernatants were collected at the indicated times p.i. and sonicated prior to assessing the titre. Viruses were titred via plaque assay.

MCMV quantitation by qPCR. SYBR Green real-time quantitative PCR (qPCR) was performed to measure the viral load in SG using primers designed to detect MCMV IE1 (Kamimura & Lanier, 2014; IE1 Forward 5’-AGCCACAAACATTGCCAGCCGAC-3’ and IE1 Reverse 5’-GCCCACACCCGAGACGAAACACT-3’). Primers against murine β-actin were used for loading normalization: β-actin Forward 5’-GCTGTATTTCCCTCATGTCG-3’ and β-actin Reverse 5’-CAGCGTGCCGTGAGGTTCA-3’. For setting up the qPCR reaction, 100 ng of DNA extracted from infected SG tissue at indicated time points was mixed with primers (0.5 µM), and 12.5 µL of Power SYBR Green PCR Master Mix (Applied Biosystems, Life Technologies), and adjusted to 25 µL with nuclease-free water. The PCR was performed using a 7900HT Fast Real-Time PCR system (Applied Biosystems) using the following PCR conditions: initial incubation at 95 °C for 10 min, and subsequent 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Relative quantification of viral DNA (IE1) was carried out using SDS software package v2.3 (Applied Biosystems), following normalization with the Cg values of β-actin for the same sample.

In vitro protein expression. Aliquots from the single-step growth assay or co-infection assays were removed and used to verify chemokine expression. Ni-NTA agarose beads were used to isolated vCXCL-1 /CCMV and mCXCL1 proteins from 100 µg of total protein. The eluted protein samples were subjected to Western blot analysis using the primary anti-6-His antibody (Qiagen) diluted 1 : 200 and secondary anti-mouse HRP antibody diluted 1 : 2000. Silver staining for the proteins in the supernatant was carried out using Pierce Silver Staining kit (Thermo Fisher Scientific) as per the manufacturer’s instructions. Relative concentration of the protein was calculated using ImageJ on the captured images (NIH).

In vivo growth of parental and recombinant viruses. The 10⁶ p.f.u. of parental or recombinant viruses were inoculated in the FP or intraperitoneally of hCXCR2 transgenic, hCXCR2KI, BALB/c, NSG or SCID mice. At different times p.i., mice were euthanized and their FP, spleens, liver, lungs, popliteal lymph node and SGs were removed. Organs were individually weighed, homogenized and clarified. Supernatants were titred.

Leucocyte infectious centre assay. Assay was performed as described by Bittencourt et al. (2014). Briefly, peripheral blood was harvested and red blood cells lysed. Leucocytes were plated onto NIH3T3 monolayers. After 6 h, cells were overlaid with carboxy methyl cellulose (CMC) media. Plaques were counted after 7 days.

Plaque formation assay. Plaque formation assay on MEF 10.1 cells was used to determine viral titres in the organs. Briefly, MEF 10.1 cells were plated in a six-well dish. Organs were harvested and homogenized. The homogenate was serially diluted and added to the MEF 10.1 cells and incubated for 1 h. After incubation, the diluted virus was removed and cells were overlaid with CMC media and incubated for 5 days. At the end of the incubation period, CMC was removed and plates were stained with Coomassie blue and plaques counted.

Co-infection experiments. Mice were infected (either in the same FP or separate FPs) or intraperitoneally with 5 × 10⁶ p.f.u. each of chemokine-expressing recombinants (RMmCXCL1/RMvCXCL1) and RM4511 or RM4503 for high titre inoculum experiments or with 100 p.f.u. of each virus for the low titre inoculum experiments. Mice infected separately with chemokine-expressing recombinants or RM4511/RM4503 served as controls for these experiments. SGs were harvested at 14 days p.i. from these mice and virus was titred. Initially the GPF-positive plaques (i.e. parental virus) were visualized under an inverted fluorescence microscope and counted. Following fixation and Coomassie blue staining, all plaques were counted and the number of GPF-positive plaques was subtracted from the total to give the number of GPF-negative plaques (i.e. chemokine-expressing recombinants).

Depletion of cellular subsets. In vivo depletion of cellular subsets was performed using antibodies 1 day prior to MCMV infection and then every 3 days until harvest. Neutrophils were depleted using 1A8 (anti-Ly6G) or RB6C (anti-Ly6C) antibody (1 mg inoculation⁻¹) (BioXcell). For NK cell depletions, mice were injected i.p. with 100 µL of anti-asialo GM1 or PBS (Wako Chemicals) diluted 1 : 10 in PBS on the days indicated in Fig. 2 legend. Flow cytometry was used to confirm depletion of GR-1hi, CD11bhi neutrophils and CD3⁻, CD49b⁻ NK cells.

Flow cytometry. Feet (cut at ankles), lung and SG tissues were minced into small pieces (~3 mm) and incubated on a rotatory shaker at 37 °C for 1 h in a 0.5 % w/v solution of type I collagenase (Worthington). Following collagenase digestion, single-cell suspensions were obtained by passing the feet, lungs, SG and spleens through 40 µm cell strainers (Fisher Scientific). Red blood cells were lysed with ACK (ammonium-chloride-potassium) lysis buffer. Cells were stained for FACS analysis following the following fluorescence-conjugated antibodies to analyse the cellular subsets: anti-CD3 (17A2), anti-CD4 (GK1.5), anti-CD8 (SK1), anti-CD45.2 (104), anti-CD11c (N418), anti-Ly6G (1A8), anti-Ly6C (HK1.4) (all from BioLegend); anti-CD49b (DX5) from eBiosciences and anti-CD11b (M1/70) from BD Pharmingen. Data was acquired on FlowJo Mac software, version 10.1.

Statistical analysis. Statistical significance was calculated using one-tailed Student’s t-test or one-way ANOVA followed by Tukey’s or
Bonferroni’s multiple comparison test in Prism5 (GraphPad) following the recommendations of Vaux (2014) and Vaux et al. (2012). A P<0.05 was considered statistically significant.

ACKNOWLEDGEMENTS

We would like to thank Dr Nathan Schmidt (Department of Microbiology and Immunology, University of Louisville) for helpful discussions. The work was supported through grants from NIH (TES: R01 AI071042-04 and WM: R56 AI095442) and American Heart Association (0431518N).

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