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Lisa P. Daley-Bauer, Emory University
Grace Wynn, Emory University
Edward S Mocarski, Emory University

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Cytomegalovirus impairs antiviral CD8\(^+\) T cell immunity by recruiting inflammatory monocytes

Lisa P. Daley-Bauer, Grace M. Wynn, and Edward S. Mocarski

Department of Microbiology and Immunology, Emory Vaccine Center, Emory University School of Medicine, Atlanta, GA 30322

SUMMARY

Inflammatory monocytes are key early responders to infection that contribute to pathogen-host interactions in diverse ways. Here, we report that the murine cytomegalovirus-encoded CC chemokine, MCK2, enhanced CCR2-dependent recruitment of these cells to modulate antiviral immunity, impairing virus-specific CD8\(^+\) T cell expansion and differentiation into effector cytotoxic T lymphocytes, thus reducing the capacity to eliminate viral antigen-bearing cells and slowing viral clearance. Adoptive transfer of inflammatory monocytes into \(Ccr2^{-/-}\) \(Ccl2^{-/-}\) mice impaired virus antigen-specific clearance. Cytomegalovirus therefore enhances a natural CCR2-dependent immune regulatory network to modulate adaptive immunity via nitric oxide production, reminiscent of the monocytic subtype of myeloid-derived suppressor cells primarily implicated in cancer immunomodulation.

Keywords

CMV; MCK2; dissemination; herpesvirus; cytotoxic T lymphocyte; pathogenesis

INTRODUCTION

Monocytes are crucial in host defense against a broad range of microbial and viral pathogens, differentiating into macrophages and dendritic cells (DCs) that shape innate as well as adaptive immunity. In mice, CD11b\(^+\)CD115\(^+\) F4/80\(^+\) blood monocytes, initially characterized by CX\(_3\)CR1, Gr-1 and CCR2 expression, are divided into bone marrow (BM)-resident CX\(_3\)CR1\(^{int}\)CCR2\(^{-}\)Ly6C\(^{hi}\) inflammatory monocytes (IMs) and long-lived CX\(_3\)CR1\(^{hi}\)CCR2\(^{-}\)Ly6C\(^{lo}\) patrolling monocytes (Geissmann et al., 2003; Serbina and Pamer, 2006; Sunderkotter et al., 2004). CCR2 signaling controls IM egress from BM into the bloodstream and tissues (Serbina and Pamer, 2006) whereas constitutive CX3CR1-mediated signals provided by endothelial cells sustain patrolling monocytes (Geissmann et al., 2003; Landsman et al., 2009; Sunderkotter et al., 2004). Paralogous human subsets have been proposed based on the differential expression of the markers CD14 and CD16 (Robbins and Swirski, 2010; Serbina and Pamer, 2006), although functional parallels with mice remain to be fully elucidated. The dramatic activation of murine IMs from BM is a hallmark of the...
host response to localized or systemic insult suggesting that these cells help shape adaptive immunity.

Macrophages, tumor necrosis factor (TNF) and inducible nitric oxide (NO)-producing DCs (TipDCs; (Serbina and Pamer, 2006)) and mononuclear myeloid-derived suppressor cells (MDSCs; (Movahedi et al., 2008)) all develop from mobilized IMs, exhibiting diverse roles including direct control of microbes, immunopathology, immunostimulation and immunosuppression. IM-derived macrophages and TipDCs protect by directly killing microbes (Shi and Pamer, 2011). The role of IMs in viral infections seems context-specific, protecting from West Nile virus (Lim et al., 2011), mediating disease pathology by influenza virus (Lin et al., 2008), and suppressing T cells responses to Thielers murine encephalomyelitis virus (Bowen and Olson, 2009). IM-like MDSCs have long been associated with suppression of CD8+ T cell function in models of tumor immunity (Movahedi et al., 2008). In cytomegalovirus (CMV) infection, IM mobilization is a hallmark of the inflammation that stimulates a robust anti-viral natural killer (NK) cell response to limit disease consequences (Crane et al., 2009; Hokeness et al., 2005); however, any contribution to shaping adaptive immunity remains unexplored.

CMVs are highly species-specific betaherpesviruses encoding modulators that target cell-intrinsic, innate and adaptive immunity (Crane et al., 2009; Mocarski et al., 2007). The biological similarities between human and murine CMV (HCMV and MCMV, respectively) as well as their common targeted pathways have facilitated understanding of key host and virus-encoded factors modulating the host-pathogen balance (Babic et al., 2011; Hengel et al., 1998; Hengel et al., 1999; Mocarski et al., 2007). Viral modulators dampen host clearance mechanisms and sometimes exploit the response. Monocytic cells play a central role ferrying virus from initial sites of infection via the blood stream (Stoddart et al., 1994). The proinflammatory factor encoded by m131/129, MCMV CC chemokine (MCK)2, mobilizes monocytes for dissemination to salivary glands (SGs) (Fleming et al., 1999; Saederup et al., 2001; Saederup et al., 1999). MCK2 enhances leukocyte recruitment and viral dissemination in C57BL/6 mice, where innate NK cell control predominates (Crane et al., 2009; Hokeness et al., 2005), as well as in BALB/c mice (Noda et al., 2006), where adaptive immunity is crucial for host control of infection (Biron et al., 1999; Koszinowski et al., 1990). MCK2 also impedes NK and T cell-mediated clearance of infection (Fleming et al., 1999), without altering peak viral replication levels (Saederup et al., 2001). Thus, it is important to understand the extent of MCK2-enhanced IM recruitment and the role of these cells in shaping immunity.

In the C57BL/6 model of CMV infection where the immunostimulatory virus-encoded gene product, m157, activates NK cells via Ly49H (Arase et al., 2002), IFNαβ drive CCR2-dependent IM mobilization that promotes NK cell-mediated viral clearance (Crane et al., 2009; Hokeness et al., 2005). The resulting robust NK cell response reduces viral replication levels and damps adaptive immunity (Andrews et al., 2010; Su et al., 2001). The Ly49H-m157-NK axis is absent in BALB/c mice, as well as most wild mice, where viral clearance is predominantly CD8+ T cell-mediated. A Gr-1intCD11b+, BM-resident, IM-like monocytic population (Noda et al., 2006) is mobilized during infection of BALB/c mice. Given the extent to which immune control, pathogenesis and dissemination have been tied to the behavior of monocytic lineage cells (Hokeness et al., 2005; Klemola and Kaariainen, 1965; Noda et al., 2006; Presti et al., 2001; Saederup et al., 1999; Sissons et al., 1991; Stoddart et al., 1994), we set out to determine how IMs shape host defense in BALB/c mice. We report that CCR2-dependent, BM-derived IMs inhibited CD8+ T cell activation and cytotoxic activity via an iNOS-mediated NO production pathway, contributing to slower viral clearance. In this way, viral MCK2 synergized with host CCR2-signaling to enhance the natural IM mobilization and sustain viral persistence.
RESULTS

Recruitment of CCR2-dependent IMs during MCMV infection

MCMV induces a dramatic viral chemokine-dependent inflammatory response at the inoculation site (Noda et al., 2006; Saederup et al., 2001). We set out to define the myeloid cells recruited to the footpad (FP) site of infection previously characterized as Gr-1<sup>+</sup>CD11b<sup>+</sup>CD45<sup>+</sup> leukocytes. The developing understanding of IMs prompted the use of Ly6C as a marker, which facilitated the identification of a Ly6C<sup>hi</sup>CD11b<sup>+</sup> population recruited to infected FPs (Fig. 1A). Surprisingly, we found that these Ly6C<sup>hi</sup> IMs had low levels of Gr-1 compared to Ly6C<sup>int</sup> polymorphonuclear leukocytes (PMNs), which showed the expected Gr1<sup>hi</sup>Ly6G<sup>+</sup>Ly6C<sup>int</sup>CD11b<sup>+</sup> phenotype (Fig. 1B and S1). We therefore relied on Ly6C and CD11b as defining markers for IMs (Crane et al., 2009; Serbina and Pamer, 2006; Sunderkotter et al., 2004), an approach found useful by others (Dolcetti et al., 2010; Movahedi et al., 2008; Nagendra and Schlueter, 2004). IM recruitment continued as infection proceeded but declined in mock-inoculated mice (Fig. S1D and E). When purified, IMs (gate R1) displayed the characteristic morphology of large (~15 μm), granular, mononuclear cells with high cytoplasm to nucleus ratios (Geissmann et al., 2003) (Fig. S1F), consistent with previous studies of this population in MCMV-infected C57BL/6 mice (Crane et al., 2009). IMs and PMNs (R2) together represented the majority (~63%) of CD45<sup>+</sup> FP infiltrates at 3 days post-infection (dpi). R2 cells exhibited the features of PMNs (Fig. S1F). The remaining populations represented NK cells, DCs, B cells, T cells, and patrolling monocytes (data not shown). The recruited IMs expressed CD115 (macrophage colony stimulating factor receptor; CSF-1R or c-Fms), F4/80, low expression of Ly6G with the expected high expression of the adhesion molecules CD31 and CD62L as well as the stem cell antigen (Sca-1) (Fig. 1C) but lacked lineage-specific markers for DCs (CD11c), NK cells (CD49b), B cells (CD19) or T cells (CD3<sup>ε</sup>, CD4 and CD8<sup>α</sup>) (data not shown). Thus, the phenotype Gr1<sup>lo</sup>Ly6C<sup>hi</sup>CD11b<sup>+</sup>CD115<sup>+</sup>F4/80<sup>+</sup>Ly6G<sup>lo</sup>CD11c<sup>−</sup>CD49b<sup>−</sup> identified an IM population prominently recruited during MCMV infection of BALB/c mice.

As expected (Serbina and Pamer, 2006), the emigration of IMs from BM was tightly regulated by CCR2-mediated signaling. Whereas wild-type (WT) BALB/c mice exhibited a sustained IM response at the inoculation site (Fig. 1D, S1E and F), increasing fivefold between 1 and 3 dpi and continuing to accumulate through 5 dpi (Fig. 1E), IMs were poorly recruited in Ccr2<sup>−/−</sup>Ccl2<sup>−/−</sup> BALB/c mice (Fig. 1D and E). CCR2 deficiency severely compromised IM recruitment, but did not alter trafficking of the other leukocyte populations, including PMNs, Ly6C<sup>−</sup>CD11b<sup>−</sup>, Ly6C<sup>−</sup>CD11b<sup>+</sup> and Ly6C<sup>+</sup>CD11b<sup>−</sup> cells or Ly6C<sup>−/lo</sup>CD11b<sup>+</sup> patrolling monocytes (Fig. 1D). Accordingly, IM recruitment was substantially reduced in the absence of CCR2 and CCL2 (Fig. 1D, bottom panels), even at later times (5 dpi), suggesting that additional signals contribute to recruitment. Reduced IM recruitment correlated with reduced FP swelling in Ccr2<sup>−/−</sup>Ccl2<sup>−/−</sup> mice at 3 dpi (Fig. 1F). Overall, IMs were the predominant mononuclear population naturally recruited to the initial infection sites.

The distribution of IMs in blood and lymphoid tissues was evaluated. In contrast to their patterns in WT mice, IMs accumulated in the BM of infected Ccr2<sup>−/−</sup>Ccl2<sup>−/−</sup> mice and did not appear in blood, spleen or other sites (Fig. 1G–K and S1G). Together, these results indicated that BM was the source and site of turnover of IMs, and that the innate response to infection was marked by CCR2-dependent recruitment of these cells out of the BM via the bloodstream to sites of infection. Importantly, the late myeloid progenitors identified previously without the benefit of a Ly6C-specific antibody (Noda et al., 2006) are clearly Ly6C<sup>hi</sup> IMs.
Viral chemokine controls recruitment of IMs to infected footpads

MCK2 is a proinflammatory CC chemokine that promotes monocyte-associated viremia and leukocyte recruitment to initial sites of infection (Fleming et al., 1999; Noda et al., 2006; Saederup et al., 2001). To evaluate the impact of the viral chemokine on the recruitment of IMs, we compared responses at the FP inoculation site in WT mice infected with Mck2-expressing, rescued virus (RQ461) or Mck2 null mutant (RM461) (Saederup et al., 2001; Stoddart et al., 1994). Infection with the chemokine-deficient virus resulted in a 50% decrease in IM frequency at 3 dpi (Fig. 2A, top panels), as well as fewer IMs in peripheral blood and spleen (Fig. S2). At the same time, greater numbers of IMs were detected in draining popliteal lymph node (pLN). Mutant virus also recruited fewer PMNs but similar numbers of other leukocyte populations. Next, we inoculated Ccr2−/−Ccl2−/− mice with Mck2 mutant or rescued viruses in order to evaluate whether the viral and host chemokines collaborate in the recruitment of IMs. CCR2-CCL2 deficiency reduced IM numbers by 100% (3 dpi) and 87% (5 dpi) (Fig. 2B). The combination of host CCR2-CCL2 and viral MCK2 deficiency abolished IM recruitment at all times (Fig. 2A and B). Notably, poor IM recruitment was not due to poor replication, as both viruses showed similar peak titers in WT and mutant mice (Fig. S2B–F) and (Fleming et al., 1999; Noda et al., 2006; Saederup et al., 2001). Although fewer IMs were recruited in the absence of either host CCR2 signaling or the viral chemokine (Fig. 2B), the time course of recruitment during infection with chemokine-expressing virus in Ccr2−/−Ccl2−/− mice was slower than the chemokine-deficient virus in WT mice. Although, expression of the viral CC chemokine precipitated IM recruitment to the inoculated site, MCK2 does not stimulate cells via CCR2 (data not shown). Thus, MCK2 cooperates with host CCR2-mediated signaling to assure IMs are mobilized from BM to sites of infection.

IMs influence MCMV clearance, but are not vehicles of dissemination

We infected WT or Ccr2−/−Ccl2−/− mice and followed viral titers in organs known to be involved in viral pathogenesis to assess whether IM recruitment altered viral clearance. Virus was detected in spleen, liver and lungs independent of CCR2 signaling (Fig. 3A–C), as previously reported (Noda et al., 2006), peaking at the same time (3 dpi) in the spleen and liver of WT and mutant mice (Fig. 3A and B). Here, virus titers peaked earlier in the lungs of mutant (5 dpi) than in WT mice (7 dpi) (Fig. 3C). Overall, virus was cleared earlier from lungs and more rapidly from spleens of Ccr2−/−Ccl2−/− mice (7 dpi), suggesting a compromise in immunity when IMs were recruited (Fig. 3A and B). Concurrent with rapid viral clearance in Ccr2−/−Ccl2−/− mice, acute host-associated disease pathogenesis was more pronounced, whether assessed by weight loss (Fig. 3D) or coat appearance (data not shown). Additionally, Mck2-deficient virus was cleared more rapidly from spleen and lungs of WT mice (Fig. S2B), revealing the impact of fewer IMs in the absence of viral chemokine. These results point to a role of IM-derived cells in modulating immune clearance.

MCMV disseminates within a monocytic cell via the blood stream to the SG (Stoddart et al., 1994), an important site of prolonged replication and source of transmitted virus. The efficiency of viral dissemination to SG is a recognized predictor of the success of this pathogen in the host (Lagenaur et al., 1994; Manning et al., 1992), as recently highlighted with bacmid-derived virus (Jordan et al., 2011). Although viral persistence in lungs and spleen was reduced in the absence of IM recruitment in Ccr2−/−Ccl2−/− mice, this did not compromise dissemination to SGs (Fig. 3E), as previously established in Ccr2+/−, Ccl2+/− and Ccr2+/−Ccl2−/− mice (Noda et al., 2006). The poor dissemination of Mck2-deficient virus in WT (Fig. S2C) and Ccr2−/−Ccl2−/− mice (Noda et al., 2006), suggests that a non-IM blood cell population acts as the vehicle for dissemination. Further, the ability of MCMV to establish latency was not altered by differences in IM recruitment in WT or mutant mice, as reactivation was observed after 7 days in explanted spleen, lung or SGs, with 100% of the
explants becoming virus-positive by 35 days of culture (Fig. 3F). Although viral clearance from lungs and spleen is influenced by IM recruitment, viral persistence levels and latency set points are not compromised. Thus, IM lineage cells do not play a direct role in dissemination or latency.

**IM mobilization impairs MCMV-specific CD8+ T cell response**

To investigate IM-associated reduced clearance of MCMV, we evaluated the antiviral CD8+ T cell response. In BALB/c mice, many cytotoxic T lymphocytes (CTLs) recognize a major MHC class I Ld epitope within the viral IE1 (pp89) protein to clear virus-infected cells (Koszinowski et al., 1990). When the splenic immune response in infected Ccr2−/− Ccl2−/− or WT mice was evaluated at 7 dpi, expansion of leukocyte numbers was significantly greater in mutant mice (Fig. 4A). Total numbers of CD8+ T cell did not increase during infection in WT mice, but increased markedly in infected mutant mice (Fig. 4B). Additionally, Ccr2−/− Ccl2−/− mice had twice as many MCMV-specific IE1 tetramer-positive CD8+ T cells as WT mice even though overall frequencies of Ag-specific T cells were similar at this time (Fig. 4C). Infection of WT mice with either Mck2 rescued or mutant virus did not lead to differences in total CD8+ T cells, but mutant virus induced significantly higher frequencies of IE1-specific cells (Fig. S2D and E). Thus, IMs recruitment influences the antigen-driven proliferative response of MCMV-specific CD8+ T cells.

To evaluate the differentiation state of MCMV-specific CD8+ T cells, multiparameter flow cytometric analysis was performed on IE1-specific CD8+ T cells. Both WT and mutant mice exhibited Ag-specific T cells with a KLRG1hiCD44+CD127−CD62L− short-lived effector CTL phenotype; however, there was a disparity in KLRG1 expression (Fig. 4D). Whereas the Ccr2−/− Ccl2−/− Ag-specific CD8+ T cells were uniformly KLRG1hi, a phenotype that is expected at 7 days following an acute viral infection (Joshi et al., 2007; Sarkar et al., 2008), cells from WT mice contained equal proportions of KLRG1− and KLRG1hi subsets (Fig. 4D, left panel) consistent with a delayed activation state (Sarkar et al., 2008). Similar to Ccr2−/− Ccl2−/− mice, IE1-specific CTLs induced during Mck2 mutant virus infection of WT mice were uniformly KLRG1hi (Fig. S2E). The presence of IE1 tetramer-positive CD8+ T cells lacking both KLRG1 and CD127 (IL7Rα) at 7 dpi in Mck2 rescue virus-infected WT mice was consistent with fewer cell divisions following activation (Joshi et al., 2007). The aggregate data provided the initial key evidence that IM recruitment alters proliferation as well as quality of antiviral CD8+ T cells.

To further characterize the response, virus-specific CD8+ T cells from WT and mutant mice were stimulated ex vivo with the immunodominant nonapeptide, IE1YPHMPTNL (Reddehase et al., 1989), and assessed by intracellular cytokine staining. Numbers of IFNγ-producing CD8+ T cells from WT mice peaked at 5 dpi and decreased by 10 dpi, whereas, cells from Ccr2−/− Ccl2−/− mice increased dramatically by 7 dpi and continued to accumulate through 10 dpi (Fig. 4E and S3). In contrast to mutant mice, WT mice generated high numbers of TNF+ IE1-specific CD8+ T cells (Fig. 4E, F and S6), including both single-positive and IFNγ+TNF+ bifunctional cells (Fig. 4G, top, left panel) peaking at 5 dpi (Fig. 4F). In contrast, CD8+ T cells from Ccr2−/− Ccl2−/− mice yielded a pattern associated with functional antiviral T cells (Seder et al., 2008) with a predominance of IFNγ single-positive along with IFNγ+TNF+ bifunctional cells (Fig. 4G, bottom, left panel). Thus, TNF single-positive T cells appeared to retain a naïve phenotype (Brehm et al., 2005) despite coming from virus-infected animals. Single positive T cells from WT mice contained lower amounts of cytoplasmic TNF than bifunctional T cells (Fig. 4G, top and bottom, left panels). Thus, IM recruitment delays T cell differentiation towards an effector phenotype.

In addition to differences in intracellular cytokines, there was a significant defect in the cytolytic potential of T cells from WT mice (Fig. 4I). Only few (~15 to 30 %) TNF+ CD8+ T
cells expressed the degranulation marker, CD107a, compared to the ~70 to 90 % of the bifunctional cells from Ccr2<sup>−/−</sup>-Ccl2<sup>−/−</sup> mice (Fig. 4I and G, middle panels). A substantial proportion (~60 – 90 %) of bifunctional CD8<sup>+</sup> T cells in either WT or mutant mice expressed this cytolytic marker; although expression in Ccr2<sup>−/−</sup>-Ccl2<sup>−/−</sup> mice was generally higher (Fig. 4I). Consistent with the trend of reduced total CD8<sup>+</sup> T cells, there were fewer perforin-producing cells in WT than mutant mice, even though frequencies were similar (Fig. 4J). Finally, CD69 and PD-1 were not detected on cytokine-producing CD8<sup>+</sup> T cells from WT or mutant mice (Fig. 4G, right panels and data not shown). Thus, IM recruitment compromises the generation of fully functional, virus-specific T effector cells.

The overall differences between WT and mutant mice were even more striking when virus-specific CD8<sup>+</sup> T cells from pLNs that drain inoculated FPs were examined (Fig. 4H and S3). Most (~70 %) CD8<sup>+</sup> T cells in WT mice were TNF single-positive cells whereas the majority (~74 %) were bifunctional in Ccr2<sup>−/−</sup>-Ccl2<sup>−/−</sup> mice (Fig. 4H). Combined, the spleen and pLN data suggest that viral persistence observed in the lungs and spleen of WT BALB/c mice is due to a global suppression of virus-specific CTL proliferation and differentiation.

**IMs impair CD8<sup>+</sup> T cell effector function**

The conspicuous differences observed in WT and Ccr2<sup>−/−</sup>-Ccl2<sup>−/−</sup> mice prompted direct evaluation of effector activity of virus-specific CD8<sup>+</sup> T cells. To assess function, in vivo CTL assays were conducted to compare clearance of adoptively transferred CFSE-labeled, IE1 peptide-loaded target cells in infected WT and mutant mice. When performed at 7 dpi, CTL-mediated lysis was significantly more complete in Ccr2<sup>−/−</sup>-Ccl2<sup>−/−</sup> (87% specific killing) than in WT (60% specific killing) mice within 3 h post transfer (Fig. 5A and B). Even following an extended period, WT mice remained less efficient at clearing Ag-loaded cells (Fig. 5B). Thus, the recruitment of IMs compromised cytolytic functions of antiviral CTLs.

To directly assess the impact of IM mobilization on CTL-mediated killing in vitro, increasing doses of Ccr2<sup>+</sup> IMs from 7 dpi spleens were incubated with IE1-stimulated, CFSE-labeled splenocytes from 7 dpi Ccr2<sup>−/−</sup>-Ccl2<sup>−/−</sup> mice. At 3 d post-incubation, Ag-stimulated proliferation, as measured by CFSE dilution, revealed a direct correlation between IM numbers and the CD8<sup>+</sup> T cell proliferative response (Fig. 6A). As the proportion of IMs in the cultures was increased from 1.5% through 24%, proliferation was suppressed from 10% to over 40% (Fig. 6B), coinciding with a 30% – 100% increase in frequency of T cell death (7-AAD<sup>+</sup>; Fig. 6C). To evaluate the physiological relevance of the suppression observed in vitro, purified Ccr2<sup>+</sup> IMs were adoptively transferred into Ccr2<sup>−/−</sup>-Ccl2<sup>−/−</sup> recipients within 1 h of FP inoculation, and CD8<sup>+</sup> T cell activation (expansion) and functional differentiation (ability to clear virus from spleens) were assessed at 7 dpi. Transfer of only 6.7 × 10<sup>4</sup> IMs into infected Ccr2<sup>−/−</sup>-Ccl2<sup>−/−</sup> mice was sufficient to limit T cell expansion (Fig. 6D) and increase viral titers (Fig. 6E) to those of WT mice. Transfer of either 2 × 10<sup>5</sup> or 6 × 10<sup>5</sup> IMs prevented T cell expansion and delayed viral clearance, recapitulating the phenotype in WT mice while also supporting a modulatory role for IMs on T cells. Overall, IMs were sufficient to restrict Ag-specific CD8<sup>+</sup> T cell expansion and delay virus clearance from tissues.

**Nitric oxide production facilitates IM impairment of anti-viral CD8<sup>+</sup> T cells**

The IM-dependent impairment of anti-viral T cell function appeared reminiscent of the mononuclear MDSCs implicated in regulation of tumor immunity (Movahedi et al., 2008). To test whether these effects were similarly dependent on IFN<sub>γ</sub> regulated gene products, iNOS, ROS or arginase-1 (Movahedi et al., 2008), we assessed the impact of inhibitory drugs, L-NMMA (NOS/iNOS), superoxide dismutase (ROS) and nor-NOHA (arginase-1) on
the ability of IMs to suppress the CD8+ T cell proliferative response. The suppressive activity of IMs correlated with iNOS-dependent production of NO2− as L-NMMA was the only drug that modulated the response (Fig. 7A and B). Inhibition of ROS or arginase-1 activity had no impact on IM-mediated suppression. Any combination of drugs that included L-NMMA exhibited inhibition whereas nor-NOHA and superoxide dismutase, even together, did not. To evaluate the impact of NOS activity in vivo, WT mice were treated with pan NOS inhibitors (L-NMMA or L-NAME) or L-NIL (iNOS-specific) 3 days prior to and during the course of MCMV infection and weighed daily to monitor for any additional adverse effects. Mice were analyzed at 7 dpi for tissue virus loads along with splenic CD8+ T cell responses (Fig. 7C and D). In contrast to L-NMMA-treated (Tay and Welsh, 1997) or iNOS-deficient MCMV-infected C57BL6 mice (Zhang et al., 2007) where virus titers increased in the absence of NOS activity, treatment of BALB/c with this NOS inhibitor reduced virus titers in lungs, spleen and pLN but not SG when compared to untreated, infected mice (Fig. 7C). L-NAME and L-NIL treatment yielded similar results with the exception that lower virus titers were observed in SGs. None of the drugs caused additional weight loss or reduced virus titers at the inoculation site (data not shown). CD8+ T cell proliferation appeared delayed in spleens of mice treated with the NOS inhibitory drugs yet viral replication was effectively controlled (Fig. 7D). The reduced expansion contrasted in vitro observations where an increase in T cell proliferation occurred when NOS was inhibited. This discrepancy may be attributed to potential off-target drug effects; although, the absence of the restrictive effect of NO on T cell function likely results in a more efficacious control of viral burden in these mice negating the requirement for large CTL numbers. These data illuminate the significance of CD8+ T cell quality over quantity, suggesting that NO production impacts the effectiveness of the anti-viral response. Overall, the data are consistent with a key role for the iNOS-NO pathway in IM-dependent impairment of the anti-viral T cell response.

To determine the relationship of MCMV-induced IMs to MDSCs, we evaluated Ly6C^hi monocytes in BM, blood, spleen, pLN and inoculated FPs for markers that have been used to define the MDSC population. Consistent with previous studies (Movahedi et al., 2008), IMs upregulated the expression of iNOS and IL-4Rα, a hallmark receptor on mononuclear MDSCs (Gallina et al., 2006), while trafficking from BM to infected tissues (Fig. 7E). Additionally, TNF and IL-10 production developed as IMs reached tissues, reflecting an immature, dual M1-M2 phenotype of mononuclear MDSCs (Umemura et al., 2008) (Fig. 7E).

To address whether IM-induced expansion of regulatory CD4+ T (Treg) cells (Huang et al., 2006) contributed to the suppression of anti-viral CD8+ T cells, we evaluated CD25+CD4+ lymphocytes in WT and Cer2+/−Ccr2+ animals. Mutant mice controlled infection better than WT mice despite having markedly greater numbers of splenic CD25+CD4+ T cells (data not shown), indicating little or no contribution of regulatory CD4+ T cells to IM-dependent virus-specific CD8+ T cell impairment. Thus, the suppressive effect of IMs on MCMV-specific CD8+ T cells is mediated primarily through the iNOS-NO axis.

DISCUSSION

Myelomonocytic cells have long been implicated in CMV pathogenesis. Here, we have shown the importance of CCR2-mediated emigration of IM out of the BM to sites of viral infection where they regulate the quality of the T cell response in a mouse strain where adaptive immunity dominates. The viral chemokine, MCK2, collaborates with CCR2 to affect this regulation. Despite being aggressively recruited by the combination of host CCR2 signaling and the elaboration of MCK2, IMs do not contribute to MCK2-enhanced viral dissemination, which is likely to be carried out by another susceptible monocyctic lineage.
such as patrolling monocytes. When recruited to sites of infection, IM lineage cells modulate adaptive immunity in a manner reminiscent of monocytic MDSCs, undermining the effectiveness of the antigen-viral CD8+ T cell response by restricting cytokine polyfunctionality and cytolytic activity, and delaying virus clearance. The overall increase in quality of virus-specific CTLs during infection of Ccr2−/−Ccl2−/− mice as well with viral chemokine-deficient virus serve to reinforce this role.

IM-mediated modulation occurs through the iNOS metabolic pathway, which is exploited by MCMV to subvert anti-viral CD8+ T cell immunity. For many years, efforts to link viral modulators to compromised virus-specific T cell responses have focused on MHC class I downregulation (Doom and Hill, 2008). Instead of altering the characteristics of the T cell response, MHC class I downmodulation appears to be most relevant in dictating the susceptibility of virus-infected cells to clearance. In contrast, the impact of increased IM recruitment is on the quality of the anti-viral CD8+ T cell immunity in lymphoid organs as well as in other virus-infected tissues that contribute to viral pathogenesis.

In C57BL/6 mice, CCR2-dependent IMs promote NK cell activity due to the Ly49H-m157 NK axis, leading to an early reduction in viral levels (Crane et al., 2009; Hokeness et al., 2005). While this is a distinct impact compared to BALB/c mice, greater viral persistence results from IM recruitment in either strain. Increased IM numbers in BALB/c mice reduce CTL-mediated clearance. In addition to the early reduction afforded by an IM-driven NK response, NK-mediated cytolysis of APCs in C57BL/6 mice reduces CTL levels as assessed by in vivo killing (Andrews et al., 2010). MCK2 drives the dramatic mobilization of IMs to infection sites in either mouse strain. This behavior has additional implications because the bacterial artificial chromosome-derived MCMV Smith strain, pSM3fr, initially proposed to exhibit WT properties (Wagner et al., 1999) and used widely to study host immunity (Ruzsics and Koszinowski, 2008), encodes a nonfunctional MCK2 protein (Jordan et al., 2011). As a result of the mutation, reduced IM recruitment would be expected to alter both antiviral NK cell as well as T cell control in ways that will need careful examination. Besides this issue, future efforts will need to evaluate more precisely the extent to which IMs directly regulate CTLs in C57BL/6 mice. The fact that fully competent MCMV infection promotes pluripotent IMs regardless of mouse strain indicates that immune regulation through the iNOS-NO axis likely combines with the indirect impact of NK cell-mediated killing of infected APCs to aid persistent viral replication.

Whether the strategies identified in MCMV are at play during HCMV infection is unknown, although both a virus-encoded chemokine and a role for CTLs in host defense are well documented (Mocarski et al., 2007). Potentially, CXCR2-specific viral chemokine vCXCL1 encoded by HCMV UL146 (Penfold et al., 1999) may impact human IMs to regulate CD8+ T cell-specific immunity. Given the existing parallels in MHC class I downmodulation strategies to escape CTL surveillance common to MCMV and HCMV (Mocarski, 2004), additional immunomodulatory similarities may occur. On the one hand, viral modulation of CTL activity has remained elusive despite significant studies on the regulation of MHC class I expression. On the other hand, MCK2-recruited IMs facilitate a robust anti-viral NK response that negatively regulates T cell responses to promote MCMV persistence. The studies reported here reinforce the importance of CD8+ T cells as a target of viral immune modulation right from the start of infection. As such, the diverse therapeutic strategies being applied in clinical settings where HCMV remains a medical problem will need to consider the impact of promoting IM responses that could potentially antagonize efforts to boost adaptive immunity.

IFNγ, in a STAT1-dependent fashion, activates iNOS (Samardzic et al., 2001), resulting in metabolism of L-arginine to produce citrulline and NO (Bogdan, 2001). NO-mediated
MDSC suppression of T cell activation and expansion occurs through the disruption of downstream signaling initiated through IL-2R binding IL-2 whereby phosphorylation of JAK1, JAK3, STAT5, ERK and AKT is precluded (Mazzoni et al., 2002). The NO-dependent immnosuppressive behavior of the MCK2-targeted, CCR2-dependent IMs in this study is reminiscent of the phenotypically similar mononuclear MDSCs studied primarily in tumor immunity. CX3CR1intCCR2+Ly6Chi cells are a functionally heterogeneous population containing precursors that follow discrete differentiation pathways depending on the inflammatory cue. It is evident by the increasing numbers of reports that microbial infections elicit suppressor CX3CR1intCCR2+Ly6Chi cells (De Santo et al., 2008; Delano et al., 2007; Mencacci et al., 2002). The implication of a NO-mediated pathway as a mechanism of IM immunomodulation elicited during acute MCMV infection being consistent with monocytic MDSC suggests an overlap in the inflammatory cues that promote differentiation of these cells during acute and chronic inflammatory states, including tumorigenesis. IFNγ is considered crucial for limiting viral replication (Biron et al., 1999; Koszinowski et al., 1990). T cells and NK cells likely contribute the IFNγ that activates iNOS in IMs during MCMV-infection. Accordingly, limiting IFNγ production would prove beneficial to the establishment of infection. The core activity contributing to the development of IM suppressive mechanisms can be addressed more comprehensively in settings where IFNγ is regulated. Further parallels to tumor MDSCs might emerge from investigations in settings where such key cytokines are absent or restricted.

In conclusion, we have shown that IMs dampen immune control by CD8+ T cells in a virus infection setting where CTLs dominate clearance, identifying a specific benefit IM-mediated impairment of CD8+ T cell function that results from the collaboration of viral and host control. The resulting diminished CTL immunity facilitates greater viral persistence. This study elucidates how diverse immunological settings prompt the evolution of distinct viral modulatory strategies that achieve persistence, highlighting major aspects of host defense that are critical to the clearance of infection. Previously, the immunosuppressive effect of IMs on CD8+ T cells emerged from mouse tumor models engineered to carry exogenous antigens to facilitate analysis. Our approach benefited from the existence of a natural antigen-specific T cell response focused on a particular viral epitope. The environment that favors MCMV persistence that results from IM-mediated impaired T cell function will help inform immune tolerance as conceptualized in persistent virus infection as well as tumor progression.

EXPERIMENTAL PROCEDURES

Viruses, mice, experimental infection and plaque assay

MCMV K181+-derived recombinant Mck2 mutant (RM461) and rescued (RQ461) viruses (Stoddart et al., 1994) were propagated on NIH3T3 murine fibroblasts (ATCC CRL-1658) grown antibiotic-supplemented DMEM medium (Manning et al., 1992). Virus stocks were prepared from clarified infected medium (Saederup et al., 1999) and stored at −80 °C until used to infect 8 to 16 week-old BALB/cJ (Jackson Laboratories) or Ccr2−/−Ccl2−/− BALB/c (Noda et al., 2006) mice used in this study. One hind FP was inoculated with 1 × 106 plaque-forming units (PFU) of virus diluted in medium or virus-free medium (mock-infected). Mice were maintained at the Emory University Division of Animal Resources and experimental procedures were conducted in accordance with National Institutes of Health and Emory University Institutional Animal Care and Use Committee guidelines. Plaque assays were conducted on tissues homogenized in medium (Manning et al., 1992) using 3T3 Swiss Albino murine fibroblasts (ATCC CCL-92). Virus titers were calculated at 4 d when cells were fixed with methanol and stained with Giemsa to visualize plaques.
Antibodies

Antibodies to CD16/32 (FcγRII/III; Clone 2.4G2), Ly6C (Clone AL21), Ly6G (Clone 1A8), CD279 (Clone J43), TNF-α (Clone MP6-XT22), CD4 (Clone RM4–5), CD11b (Clone M1/70) and CD3e (Clone 500A2) were purchased from BD Pharmingen; CD45 (Clone 30F11), CD69 (H1.2F3) and F4/80 (Clone BM8) were purchased from Invitrogen, and IFNγ (Clone XMG1.2), CD62L (Clone MEL14); CD115 (Clone ASF98) and CD107a (Clone 1D4B) were purchased from Biolegend. The following Abs were purchased from eBioscience: CD4 (Clone GK1.5), CD11c (Clone N418), Ly-6A/E (Sca-1; Clone D7), CD19 (Clone 1D3), CD31 (PECAM-1; Clone 390), CD8α (Clone 53–6.7), CD49b (Clone DX5), CD8a (53–6.7), Siglec H (Clone eBio440c), CD3e (Clone 17A2), and Ly6C/6G (Gr-1; Clone RB6.8C5).

Tissue collection, leukocyte preparation and flow cytometry

Tissues were collected from euthanized mice for single-cell suspension preparation and analysis by flow cytometry. FP and lung tissue were minced into ~3 mm sections then digested with collagenase D (1.5 mg/ml; Sigma) in PBS. Cells were collect from lung digest, spleen and pLN by mashing through a metal sieve, femurs by flushing BM and from blood collected in EDTA-loaded syringes by Histopaque-1119 (Sigma) density medium separation. Erythrocytes were lysed where applicable, cell preparations filtered (40 μm mesh) then viable cells counts were performed on a hemacytometer using trypan blue dye exclusion. Cell surface FcγRII/III was blocked prior to incubating with Abs for nine-color flow cytometric analyses. Data were acquired by flow cytometry (BD LSRII cytometer and FACSDiva Software; BD Biosciences), analyzed using FlowJo (TreeStar), and graphed with Prism (GraphPad). For morphological assessments, cells were sorted (purity >97%; FACSVantage, BD Biosciences), centrifuged onto microscope slides and stained with hematoxylin and eosin (Hema3 stain kit; Fisher Scientific).

For ICCS, cells isolated from tissues were incubated with 10−9 M MCMV IE1 peptide (YPFMPTNL, purity >80%; JPT Peptide Technologies) in RPMI medium containing brefeldin A along with the CD107a Ab for 5 h at 37 °C then prepared for cytokine detection using the BD GolgiPlug kit (BD Biosciences).

Latent infections and explant reactivation

Groups of five Ccr2−/−Ccl2−/− or WT mice were inoculated as described above and at ~6 months post-infection SG, lung and spleen were collected, divided into three sections and cultured as intact sections with DMEM. Supernatant was collected from culture wells and replaced with fresh DMEM at weekly intervals for 5 weeks. Viral titers were obtained from the collected supernatant by plaque assays.

In vivo cytolytic T lymphocyte (CTL) assay

Target cells were prepared from naïve WT or Ccr2−/−Ccl2−/− mouse splenocytes incubated 1 h at 37 °C with 1 mM IE1 peptide, labeled 10 min at 37 °C with 5 μM CFSE (CFSEhi, CellTrace; Molecular Probe) then combined (1:1 ratio) with peptide-free splenocytes labeled with 0.5 μM CFSE (CFSElo). Twenty million target cells (~1 × 107 each CFSElo and CFSEhi) were injected via tail veins into syngeneic recipients at 7 dpi. Recipients were euthanized at 3 or 15 h post transfer and CFSE+ cell numbers in spleens were analyzed. Percent specific killing was calculated as: (1−[r naïve/r infected]) × 100, where r = (% CFSElo cells)/(% CFSEhi cells).
Adoptive transfer
Monocytes were isolated from 3 dpi WT mouse spleens using the EasySep® Mouse Monocyte Enrichment Kit and magnet system (Stemcell Technologies). Purity was assessed at ≥90% Ly6C<sup>hi</sup>Gr-1<sup>lo</sup>CD11b<sup>+</sup> IMs. Purified cells were adoptively transferred by tail vein injections into groups of n = 3 – 5 recipient Ccr2<sup>−/−</sup>Ccl2<sup>−/−</sup> mice that were subsequently inoculated via the FP route with MCMV. Controls were infected Ccr2<sup>−/−</sup>Ccl2<sup>−/−</sup> mice that received PBS prior to virus inoculation and infected WT mice. Spleens were harvested from euthanized mice at 7 dpi and ~50% (based on weight) was assessed for virus burden and the remaining portion was used in flow cytometry to phenotype virus-specific CD8<sup>+</sup> T cells.

In vitro CD8<sup>+</sup> T cell suppression assay
Mice were infected as described above. Ccr2<sup>−/−</sup>Ccl2<sup>−/−</sup> mouse splenocytes (responders) were isolated at 7 dpi and labeled with 5 μM CFSE, combined with IE1 peptide (stimulus; 10<sup>−9</sup> M) in RPMI medium supplemented with 5% FBS and 50 μM 2-mercaptoethanol before plating in 96-well microtiter plates (Costar) at 5 × 10<sup>5</sup> cells per well. Purified IMs (suppressors) were immediately added in twofold dilutions except for control wells. Monocytes were purified from 7 dpi WT mouse spleens using the EasySep® kit. At 72 h, culture supernatants were assayed for NO<sub>2</sub> using the Greiss reagent assay (Promega) and CD8<sup>+</sup>CD3<sup>+</sup> T cell proliferation was evaluated by CFSE dilution. Dead cells were identified as 7-AAD<sup>+</sup>. In some instances, N<sup>ω</sup>-Methyl-L-arginine acetate salt (L-NMMA, 0.5 mM; Sigma-Aldrich), N<sup>ω</sup>-Hydroxy-nor-L-arginine, diacetate salt (nor-NOHA, 0.5 mM; Calbiochem), and superoxide dismutase (SOD, 200 U/mL; Sigma-Aldrich) inhibitors were added to the culture wells. Suppression was calculated as: 100 × [1 – (proliferation with IMs)/(proliferation without IMs)]

In vivo inhibition of NOS-dependent IM function
WT mice were treated with 4 μM of L-NMMA, N<sup>G</sup>-Nitro-L-arginine-methyl ester. HCl (L-NAME; Enzo Life Sciences) or N<sup>6</sup>-(1-iminoethyl)-L-lysine, dihydrochloride (L-NIL; Cayman Chemicals) administered ad libitum in drinking water starting 3 days prior to infection. Control group received no drugs. Drinking water with or without drugs was refreshed every other day coincident with the weighing of mice. At 7 dpi, tissues were collected and assess for viral loads. Half the spleen was used to evaluate CD8<sup>+</sup> T cell responses by flow cytometry.

Statistical analysis
Experimental groups contained a minimum of three mice. Statistical differences between groups of mice were calculated using Student’s t test or one-way ANOVA with Bonferroni’s or Dunnett’s Multiple Comparison post tests with p ≤0.05 considered significant using the GraphPad Prism 5 software.

Supplementary Material
Refer to Web version on PubMed Central for supplementary material.

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HIGHLIGHTS

- Viral chemokine enhances inflammatory monocyte recruitment from bone marrow.
- Recruitment depends upon host CCR2 signaling.
- Recruitment impairs the antigen-specific CD8+ T cell immunity.
- Recruitment facilitates viral persistence.
FIGURE 1.
MCMV recruits CCR2-dependent IMs during infection. A – C, Flow cytometric analysis of myeloid cells in inoculated FPs at 3 dpi. A, Resolution of mononuclear Gr-1<sup>lo</sup>Ly6C<sup>hi</sup>CD11b<sup>+</sup> (R1) cells from Gr-1<sup>hi</sup>Ly6C<sup>+</sup>CD11b<sup>hi</sup> (R2) PMN. B, Efficiency of the Gr1 marker in resolving CD11b<sup>+</sup> subsets. C, Expression of monocyte subset defining markers on R1 gated cells. Solid line histograms, IMs; dashed lines, positive control; closed lines, negative control. D – F, Recruitment of Gr-1<sup>lo</sup>Ly6C<sup>hi</sup>CD11b<sup>+</sup> IMs into inoculated FPs of WT and Ccr2<sup>−/−</sup>Ccl2<sup>−/−</sup> (DKO) mice. D, Scatterplots comparing cellular infiltrates. E, Total IMs. F, FP swelling. G – K, Distribution of IMs in tissues at 5 dpi. G, Scatterplots of leukocytes. IM frequencies in BM (H), blood (I), spleen (J), pLN (K). Mice were inoculated
via the FP route with $10^6$ PFU MCMV. Scatterplots represent groups of three mice. Elliptical gates outline IMs. Histograms show one representative mouse per group (gray; negative control, dash line; positive control, solid line; IMs). Data points are means ± SE. *, p < 0.05; **, p < 0.01; ***, p < 0.001. Panels show one representative of at least two independent experiments.
FIGURE 2.
Virus-encoded MCK2 cooperates with host CCR2 to recruit IMs. A, Scatterplots of FP cellular components at 3 dpi. Elliptical gate, IMs. B, Total IMs. Mice were inoculated with Mck2-expressing (rescued) or Mck2-deficient (mutant) MCMV. At specified time points leukocytes were isolated and evaluated by flow cytometry. Plots show one animal representative of groups of three mice. Symbols on graph represent mean ± SE. Panels show one of at least two independent experiments.
FIGURE 3.
IM recruitment impacts MCMV clearance but not establishment of infection and latency. A – C, Viral titers at 1, 3, 5, 7 and 14 dpi in tissues of WT (triangle, black lines) and DKO (circles, gray lines) mice. Symbols represent individual mice and lines connect mean values at each time point. *, p < 0.05; ***, p < 0.001; n.s., not significant (p > 0.05). D, Weight change. Symbols represent mean ± SE in groups of three mice. E, Viral titers in SGs at 14 dpi showing log_{10} mean titers ± SE. F, Latency and reactivation of MCMV in explanted tissues. Tissues were collected at 6 months post-infection, sectioned into three portions and cultured for 35 days. Supernatant was sampled at weekly and viral titers were obtained by plaque assay. Bars represent the percentage ± SE of animals whose tissue yielded virus. Groups of 4–5 mice were evaluated and experiment was replicated twice. Dashed lines represent the limit of detection of the assay.
Figure 4.
Recruitment of IMs modulates the differentiation of virus-specific CTLs. Total numbers of lymphocytes (A) and CD8+ T cells (gated as CD3+CD4−) (B) in spleen. Open bars; mock infected, filled bars; MCMV-infected. C and D, IE1-tetramer(tet)+ CD8+ T cells. C, Total numbers (left panel) and frequency (right panel) as mean values ± SE. D, Expression of differentiation markers. Splenocytes were collected from naïve or infected mice at 7 dpi. IE1 tet+ CD8+ T cells were analyzed for the expression of effector and memory differentiation markers. Five mice were evaluated per group. Data are representative of seven experiments. Open histograms, naïve; gray filled, solid line, WT mice; dashed line; DKO mice. E – J, Quality of the splenic CD8+ T cell response following peptide stimulation. Expansion and contraction phases of (E) IFNγ- and (F) TNF-producing cells. Showing mean ± SE. Differentiation state of cells in (G) spleen and (H) pLN. I, Frequency of CD107a+ TNF+ and IFNγ+TNF+ CD8+ T cells. J, Levels of perforin (Prf+) expressing cells. Cells were evaluated at 7 dpi by ICCS following IE1 peptide stimulation in vitro. *, p < 0.05; **, p < 0.01; ***, p < 0.001.
Figure 5.
IMs mobilized in WT mice during MCMV infection inhibit CTL killing. A and B. In vivo CTL assay. A. Frequency of CFSE\textsuperscript{hi} cells post adoptive transfer into mock or infected mice at 3 h post transfer. Histograms of CFSE-labeled cells prior to transfer (top) or from one representative mock (middle) or MCMV-infected (bottom) recipient mouse from groups of five mice. B. Percent specific killing shown as mean ± SE. *, p < 0.05; **, p < 0.01. Splenocytes from naïve DKO or WT mice were CFSE-labeled (CFSE\textsuperscript{hi}), pulsed with IE1 peptide, combined with CFSE\textsuperscript{lo} peptide-free cells then adoptively transferred into syngeneic mock or infected mice at 7 dpi. Spleens from recipient mice were evaluated at 3 or 15 h post-transfer. Experiments were replicated twice yielding similar results.
Figure 6.
MCMV induced IMs impair CD8+ T cell response to antigen. A – C In vitro suppression of Ag-specific CD8+ T cell proliferation by IMs. A. Proliferation in response to IE1 peptide stimulation in the presence of increasing numbers of IMs. B. Percent suppression of proliferation. C. Frequency of dead (7-AAD+) CD8+ T cells per well. Splenocytes were collected from infected mice at 7 dpi. CFSE-labeled DKO splenocytes (5 × 10^5 cells per well) were stimulated with IE1 peptide along with increasing frequencies of IMs purified from WT spleens. At day 3 of incubation, CD8+ T cells were harvested and analyzed for CFSE dilution and 7-AAD detection. Assay was done in triplicate and repeated three times with similar results. D and E. In vivo suppression of CD8+ T cells by IMs. D. Total CD8+ T cells recovered at 7 dpi from spleens of DKO mice adoptively transferred with IMs from
WT mice. E, Viral burden in spleens. IMs were purified from spleens of infected WT mice at 3 dpi and adoptively transferred via tail vein injections into DKO mice within 1 h of FP inoculations with MCMV. At 7 dpi, tissues were harvested and assessed by plaque assay showing \( \log_{10} \) mean ± SE. *, \( p < 0.05 \); **, \( p < 0.01 \); ***, \( p < 0.001 \). Plots are of one animal representative of each experimental group, \( n = 5 \) mice. Similar results were obtained from five independent experiments.
Figure 7.
iNOS mediates MCMV-induced IM suppression of virus-specific CD8+ T cell proliferation. 
A and B, Suppression of CD8+ T cell proliferation by IMs in the presence of metabolic 
inhibitors. A, Percent suppression with inhibitors L-NMMA (NOS), nor-NOHA (arginase-1) 
and SOD (ROS), showing mean ± SE for n = 3 mice. CFSE-labeled DKO splenocytes were 
stimulated in the presence of purified Ccr2+/+ IMs (24% of DKO splenocyte numbers) as 
described above. CD8+ T cells were harvested on day 3 of incubation and analyzed by flow 
cytometry. B, NO$_2^-$ detection using the Greiss reagent assay. Assays were done on groups 
of three mice and repeated three times with similar results. C and D, Effect of NOS-
inhibition on MCMV infection in vivo. C, Virus titers and D, Total (left) and IE1-tet+ (right) 
CD8+ T cell numbers. Mice were given sterile drinking H$_2$O with or without L-NMMA, L-
NAME or L-NIL 3 days prior to inoculation and throughout the course of infection then 
evaluated at 7 dpi. Data are of treatment groups, n = 5. E, Functional phenotype of MCMV-
induced IMs. IMs in blood and tissues from 3 dpi WT mice were analyzed for expression of
MDSC markers and cytokines. Data shown for one mouse representative of group size, n = 3 (solid line histogram, IMs; dashed lines, PMNs; closed histograms, naïve lymphocytes). Similar results were obtained in two independent experiments.