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Modulation of host innate and adaptive immune defenses by cytomegalovirus: timing is everything

A. Loewendorf and C. A. Benedict

Division of Molecular Immunology, La Jolla Institute for Allergy and Immunology, La Jolla, CA, USA

Abstract

Loewendorf A, Benedict CA (La Jolla Institute for Allergy and Immunology, La Jolla, CA, USA). Modulation of host innate and adaptive immune defenses by cytomegalovirus: timing is everything (Symposium).

Human cytomegalovirus (HCMV) (HHV-5, a β -herpesvirus) causes the vast majority of infection-related congenital birth defects, and can trigger severe disease in immune suppressed individuals. The high prevalence of societal infection, the establishment of lifelong persistence and the growing number of immune-related diseases where HCMV is touted as a potential promoter is slowly heightening public awareness to this virus. The millions of years of co-evolution between CMV and the immune system of its host provides for a unique opportunity to study immune defense strategies, and pathogen counterstrategies. Dissecting the timing of the cellular and molecular processes that regulate innate and adaptive immunity to this persistent virus has revealed a complex defense network that is shaped by CMV immune modulation, resulting in a finely tuned host–pathogen relationship.

Keywords

cytokines; herpes virus; immunity; immunology; infectious disease; virology

Cytomegalovirus

The herpesviruses have co-evolved with their vertebrate hosts for more than one hundred million years to establish lifelong infections [1]. Accordingly, all herpesviruses employ a multitude of strategies to modulate the host immune response, facilitating this persistence in the face of a robust innate and adaptive immune response. Human cytomegalovirus (HCMV/HHV-5, a β -herpesvirus) is highly prevalent in most populations (50–90% seropositive in the United States, and virtually 100% in the developing countries) and is usually acquired early in life as an asymptomatic, subclinical infection in immune competent persons. However, if primary infection occurs in the developing foetus or neonate (before full immune system development) the consequences can be severe, and HCMV is the most common infectious cause of congenital birth defects [2]. HCMV establishes latency/persistence in monocyte precursors and diverse populations of tissue stromal cells [3,4]. This is unlike its cousins, herpes simplex virus and Epstein Barr virus (α and γ herpesviruses), whose latency is exclusively restricted to neurons and B cells, respectively. Rapid reactivation of HCMV from this ‘systemic latency’ occurs upon immunosuppression (transplant recipients, AIDS patients, etc.),

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Correspondence: Chris A. Benedict, Division of Molecular Immunology, La Jolla Institute for Allergy and Immunology, 9420 Athena Circle, La Jolla, CA 92037, USA, (fax: 858 752 6986, benedict@liai.org).

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supporting the notion that constant immune surveillance is required to keep persistent infection in check, and can result in morbidity and/or mortality if not controlled by antiviral drug therapy. Significant evidence associates HCMV to vascular disease, and recent work has revealed potential mechanisms underlying these links [5]. HCMV is also linked to the development of several other chronic inflammatory disorders [6], and specific human malignancies [7,8]. However, in many of these cases whether the presence of HCMV is causative or merely reactivated/detectable due to altered host immune control in these disease settings remains an open question.

CMV replication is species restricted, and therefore no natural animal model exists for examining HCMV pathogenesis, although a few studies have been performed in SCID-hu mice [9,10]. Consequently, CMV has been studied extensively in the mouse model (MCMV), which provides several advantages due to the availability of genetically characterized inbred strains. Much work has also been performed in the rat, guinea pig and rhesus monkey models of CMV infection, all of which utilize genetically unique CMVs. All CMVs show significant homology/organization in their genomes of >200 kB, exhibit conserved tissue tropism and temporal regulation of gene expression and display similar pathogenesis. However, significant primary genomic sequence diversity exists between CMVs, and <50% of HCMV orfs have identifiable homologues in MCMV [11,12]. The greatest sequence divergence is seen in the genomic termini of the CMVs, the area encoding the highest concentration of genes dedicated towards immune modulation. This genomic divergence is not unexpected, as this virus has been evolving in diverse hosts since the appearance of the primordial CMV more than 10^8 years ago [1]. In turn, CMV has almost certainly impacted the diversification of host immune defense genes, with more than 3% of the mouse genome composing the 'resistome' to this virus [13]. Therefore, although the sequences of immune modulatory orfs and their precise modes of action often differ between the CMVs, overall the immune mechanisms that are targeted are largely conserved.

The level of cross-talk between innate and adaptive immune cells that is required for the development of effective immunity is a complex, pathogen-specific matter. In this review, we will focus on: (i) information gleaned from the MCMV model regarding the timing of the innate immune response upon initial infection and subsequent viral spread *in vivo*, (ii) how this translates to the development of adaptive immunity and (iii) what strategies MCMV utilizes to modulate these host defenses. When possible, we will highlight where viral and host strategies are conserved (or diverge) during HCMV infection. Importantly, as this review focuses on the timing of immune responses, MCMV completes its replication cycle in ~30 h in both cultured fibroblasts and most organs, but HCMV replication takes considerably longer (72–96 h).

The innate response to CMV infection

NK cells and type I interferons (IFN $\alpha\beta$) play a major role in innate control of MCMV replication [14], and not surprisingly MCMV encodes several gene products which target these defenses [15,16]. NK cells are also critical for controlling HCMV infection [17]. Within a decade after the identification of IFN $\alpha\beta$ in classical studies more than half a century ago [18], the importance of IFN $\alpha\beta$ signalling in regulating MCMV replication *in vivo* and *in vitro* was studied by several groups [19-23]. In turn, NK cells were suspected to contribute to MCMV host defenses more than 30 years ago [24,25], and were definitively shown to be critical by adoptive transfer or depletion studies in the mid 1980s [26,27]. Since these early studies, the MCMV model has proven to be fertile ground for dissecting the cellular and molecular mechanisms involved in regulating these innate defenses, which themselves are also intertwined due to the ability of IFN $\alpha\beta$ to activate NK cell effector functions. We will start by reviewing, what regulates IFN $\alpha\beta$ production and NK cell function during the first 2 days of MCMV infection *in vivo*, a

biphasic response composed of innate recognition of the initial viral inoculum (peaks ~8 h) followed by a second phase triggered by the initiation of viral spread within the infected host (starts ~36 h).

CMV induction of the initial IFN $\alpha\beta$ response: the ‘kick start’

Early studies identified a rapid burst of IFN $\alpha\beta$ in the serum detectable ~6 h after intraperitoneal (ip) MCMV infection and waning by 24 h (i.e. ‘systemic’ IFN $\alpha\beta$), and the magnitude of this response varied between mouse strains in a MHC independent manner [22]. As MCMV takes ~30–36 h to complete its replication cycle *in vivo* [28], it is intuitive that initial IFN $\alpha\beta$ production occurs in response to the injected virus inoculum. More recently, the source of this systemic IFN $\alpha\beta$ has been shown to be derived from splenic stromal cells, and is dependent upon B cells that express lymphotoxin(LT) $\alpha\beta$ [a ligand of the tumour necrosis factor (TNF) family] and signal to LT β -receptor(R) expressing stroma [28,29] (Fig. 1). This mechanism accounts for ~80–90% of the entire IFN $\alpha\beta$ production at 8 h. Importantly, although B cell-expressed LT $\alpha\beta$ promotes this first wave of IFN $\alpha\beta$ production in the spleen, the liver does not require LT β R signalling to mount the same response at this time [28], and it is currently unknown what cell type/mechanism produces the first IFN $\alpha\beta$ in this organ. The initial IFN $\alpha\beta$ coming from the stroma is consistent with marginal zone stromal cells being the first target of MCMV infection in the spleen after ip infection ([30] and Fukuyama *et al.* unpublished data).

What is the consequence of this initial IFN $\alpha\beta$ production? Because the earliest that significant MCMV production can be measured in infected organs is ~36 h after infection, it is somewhat difficult to directly quantify the antiviral consequences of this first wave of innate cytokine production *in vivo*. However, several indications for its ‘kick-starting’ innate immune control do exist. IFN $\alpha\beta$ can act directly on infected cells to inhibit virus production, and can also activate NK and other immune effector cells. Many elegant studies have analysed, how IFN $\alpha\beta$ and NK cells regulate MCMV infection at times well after the initial 8 h IFN $\alpha\beta$ peak (36 h and later) [31–33], when MCMV has spread within the infected organ and triggered a second wave of IFN $\alpha\beta$ by distinct mechanisms (discussed below). However, early work indicated the first wave of MCMV-induced IFN $\alpha\beta$ in the spleen can promote NK cell cytotoxicity at 12 h [25]. Furthermore, injection of neutralizing anti-IFN $\alpha\beta$ antibody substantially reduced NK cell cytotoxicity when measured at this time [22], confirming the first burst of IFN $\alpha\beta$ from splenic stromal cells does have functional consequences. More recent work identified the activation of invariant NK-T-cells (*i*NKT) during initial MCMV infection, which peaked at 12 h and waned at 24 h in both the spleen and liver, and was accompanied by upregulation of *i*NKT CD25 and CD69 expression but no production of IFN γ [34]. *In vitro* studies, where human dendritic cell (DC) were exposed to HSV or HCMV also suggested that human NK-T-cells can be activated in response to herpesvirus ‘danger signals’ [35].

Initial IFN $\alpha\beta$ induction during CMV infection, a role for NF κ B

LT $\alpha\beta$ -LT β R-regulation of initial IFN $\alpha\beta$ production during CMV infection has also been examined in a model of HCMV infection of cultured fibroblasts. Triggering LT β R signalling within the first 4 h of HCMV infection resulted in substantially higher levels of IFN β production, which in turn inhibited HCMV spread within the culture via a noncytolytic mechanism [36]. The addition of IL-2-activated NK cells at low effector: target ratios 4 h after infection similarly inhibited HCMV spread by inducing IFN β from the fibroblasts, with IFN γ produced by the NK cells also contributing [37]. The ability of LT β R signalling to induce higher levels of IFN β from HCMV-infected cell cultures was strictly dependent upon activation of NF κ B [36], perhaps not surprisingly as NF κ B is a key component of the IFN β transcriptional ‘enhancesome’ [38]. LT β R ‘cosignals’ delivered during the first few hours of HCMV or

MCMV infection do not appear to alter the activation of IRF3 in cultured fibroblasts (Benedict, unpublished data), another key component of the IFN β enhancosome [39]. Notably, enhanced induction of IFN β upon initial HCMV infection is not unique to LT β R signalling, as TNFR1 signalling [36,37] and overexpression of RIP2/RICK/CARDIAK [40] also induced higher IFN β via a NF κ B-dependent mechanism. Prolonged treatment of fibroblasts with IL-1 β prior to HCMV infection also induced higher IFN β levels, although a direct connection between IL-1 β and NF κ B was not demonstrated in this study [41]. A role for NF κ B in LT β R-regulation of initial IFN $\alpha\beta$ production from splenic stromal cells is also supported, as *aly/aly* mice have a severely compromised response at 8 h after MCMV infection (*aly/aly* mice contain a spontaneous mutation in the NF κ B interacting kinase (NIK) [42]) [28]. Co-injecting an agonistic anti-LT β R antibody 4 h prior to MCMV infection of LT $\alpha\beta$ deficient mice restores IFN $\alpha\beta$ production to some degree, suggesting that triggering NIK-dependent NF κ B signalling may also function as a cosignal *in vivo*. However, anti-LT β R does not completely restore IFN $\alpha\beta$ (~50%), and injecting anti-LT β R antibody 4 h prior to MCMV infection is more efficacious than co-injecting it with the virus (Schneider and Benedict, unpublished data). Consequently, it is possible that altered differentiation of marginal zone stromal cells in mice lacking either LT β R or noncanonical NF κ B signalling also contributes to the defective IFN $\alpha\beta$ response observed at 8 h after MCMV infection [43].

HCMV and MCMV differ in their restriction of initial IFN $\alpha\beta$ induction

IFN $\alpha\beta$ mRNA peaks in the spleen and liver at ~8 h after ip infection, and returns to baseline levels at 24 h [28]. MCMV gene expression is just becoming detectable at 4 h [28], indicating IFN $\alpha\beta$ mRNA peaks in splenic stromal cells within ~4–6 h after encountering the MCMV particle. For HCMV, infection of cultured fibroblasts with ultraviolet light inactivated virus (UV) induces dramatically higher IFN β mRNA levels within the first 4–6 h when compared with replication-competent HCMV [36,44,45]. This block is likely due to the inability of UV HCMV to express the immediate-early 2 protein (IE2/IE86), which blocks induction of IFN β and other inflammatory chemokines within hours after HCMV infection [45,46]. Interestingly, IE2 does not block nuclear translocation of the RelA/p50 NF κ B heterodimer induced by HCMV infection, but instead blocks its DNA binding activity by an unknown mechanism [46]. In addition to blocking NF κ B DNA binding induced by HCMV, overexpression of IE2 can also block extrinsic activation of NF κ B by TNF [46]. As discussed earlier, LT β R/TNFR1 and other NF κ B inducing stimuli can enhance IFN β transcription when they signal concurrently with HCMV infection (or within 2–4 h) [36]. This is an important point, as IE2 would potentially moderate the ability of these signals to amplify IFN β production by blocking extrinsic NF κ B activation. In fact, a careful timecourse analysis of TNF-induced NF κ B DNA binding activity in HCMV-infected cells showed a modest decrease by 8 h post infection, but not by 4 h [47]. A much more dramatic block to the ability of TNF and IL-1 β to induce NF κ B activation is seen at later times of HCMV infection (48–72 h) [47,48], but at this time significant downregulation of TNFR1 from the surface of infected cells also occurs [47, 49].

MCMV also restricts NF κ B activation by extrinsic TNF signalling in macrophages at 18 h after infection [50], but downregulation of TNFR1 cell surface levels was postulated to be responsible for this block [50]. If MCMV utilizes mechanism(s) similar to HCMV to restrict the initial transcription of IFN $\alpha\beta$, injection of UV MCMV *in vivo* should result in significantly higher levels of IFN $\alpha\beta$ mRNA in splenic stromal cells. However, no differences in the level of splenic IFN $\alpha\beta$ mRNA at 8 h is seen when injecting UV MCMV [28], suggesting MCMV does not employ a similar rapid mechanism to dampen the initial induction of IFN $\alpha\beta$ transcription. This is consistent with results indicating that UV MCMV induces similar peak levels of IFN β mRNA in cultured fibroblasts 3–4 h after exposure to the virus ([51] and Benedict unpublished data). This does not mean; however, that MCMV does not employ

alternate mechanisms to attenuate IFN $\alpha\beta$ transcription. In the same studies, where UV MCMV and wildtype virus induced an equivalent peak of IFN β mRNA at 3–4 h, a transcription-dependent mechanism(s) restricted the duration of IFN β transcription over the next 6 h [51]. A plausible explanation for this result might be that MCMV attenuates IFN β mRNA expression in fibroblasts by inhibiting the IFN $\alpha\beta$ ‘positive feedback loop’ [52]. However, studies using several MCMV mutants lacking specific orfs known (M27 [53]), or thought (M83 and M84), to restrict IFN $\alpha\beta$ signalling in infected cells suggested this is not the case.

Importantly, there are a variety of mechanisms employed by mouse, human and rhesus CMV to block the downstream effects of IFN $\alpha\beta$ signalling in infected cells. These have been recently reviewed [16,54], and therefore we will not dwell on them extensively. Although, these mechanisms could in theory restrict the maximal induction level of IFN $\alpha\beta$ by restricting the feedback loop, there is no direct evidence for this to date. Notably, the protein derived from the M27 orf is the only known MCMV protein to inhibit IFN $\alpha\beta$ signalling by inducing degradation of STAT2 [53], but M27 does not alter IFN $\alpha\beta$ induction upon infection [51]. M27 protein is detectable within 4 h, and by 8 h IFN α signalling is already dampened in MCMV-infected cell cultures.

CMV restricts cell death upon initial infection

Programmed cell suicide (i.e. apoptosis) has the potential to serve as an effective strategy to restrict viral replication/spread at very early points in the first populations of infected cells. Consequently, virtually all viruses have developed strategies to block apoptosis at multiple levels, including CMV. CMV encodes several gene products restricting the activation of both the intrinsic (i.e. mitochondria/Bcl-2 dependent) and extrinsic (i.e. death-receptor mediated) pathways, and these have been recently reviewed [55-57]. To highlight very recent data, the M45 protein of MCMV targets the newly characterized ‘necroptotic’ cell death pathway [58] by binding to receptor interacting protein 1 (RIP1) and RIP3 [59-61]. M45 contains both a RIP homotypic interaction motif (RHIM) domain as well as a ribonucleotide reductase homology domain, and was originally shown to be absolutely critical for MCMV to replicate both in cultured endothelial cells and to establish a productive infection *in vivo* [62]. A functional M45 RHIM domain is required to block MCMV-induced endothelial cell death [60], indicating that MCMV infection triggers the initiation of necroptosis, but M45 subsequently blocks it. Very recent results suggest that RIP3 is the critical adaptor that mediates MCMV-induced necroptosis, and is inhibited by M45 (Upton and Mocarski, personal communication). Additionally, M45 binding of RIP1 and RIP3 via its RHIM domain can sequester these adaptors and restrict their ability to bind to DAI/ZBP1 [61], a DNA-sensing receptor localized to the cell cytoplasm that induces IFN $\alpha\beta$ production [63]. Consequently, M45 has the potential to restrict DAI-mediated induction of IFN $\alpha\beta$ in MCMV-infected target T-cells, but no evidence of DAI regulating the cell-intrinsic response to MCMV infection currently exists. Very recently, however, DAI has been implicated in the induction of IFN β during HCMV infection of cultured fibroblasts [64].

The second phase of the innate response during acute CMV infection: responding to virus spread

A large body of elegant work exists characterizing the molecular and cellular regulation of innate defenses to MCMV infection at ~36 h after initial infection, a time-point after MCMV has replicated and spread once within infected organs *in vivo* [28]. It has been known for some time that NK cell activation is biphasic over the first 36 h of MCMV infection [25], and additional work suggested the same for the IFN $\alpha\beta$ response [32]. The genetic polymorphism existing between inbred mouse strains has allowed for the characterization of specific molecular and cellular requirements that regulate innate defense to MCMV infection, and these

have been reviewed recently elsewhere [14,65]. Importantly, the function of the Ly49H NK cell activating receptor in the commonly used C57BL/6 (B6) mice, and the lack of it in Balb/c mice, contributes at multiple levels to the development of innate defenses. Ly49H binds to its MCMV expressed 'ligand', m157, resulting in robust activation of the ~50% of NK cells that express this activating receptor in select mouse strains [66]. Consequently, the Ly49H and m157 'status' must always be considered when interpreting experimental results. We have not discussed the functional consequences of the Ly49H-m157 axis to this point in the review, as we consider it unlikely to contribute significantly during the first 12 h of *in vivo* infection. However, as we will now discuss how IFN $\alpha\beta$ and NK cells regulate the first round of MCMV spread *in vivo*, it clearly must be considered.

DC production of IFN $\alpha\beta$ in response to MCMV spread within the spleen

Starting at ~36 h after ip infection of B6 mice with MCMV, DC are major producers of IFN $\alpha\beta$ via an LT β R-independent mechanism in the spleen. When splenocytes (excluding stromal cells) were harvested at this time and cultured *ex vivo*, dendritic cell populations produced >95% of the IFN $\alpha\beta$ [67]. Subsequent *ex vivo* analysis revealed that plasmacytoid DC (pDC) produced significantly more IFN α and other innate cytokines (IL-12, TNF, MIP-1 α) on a per cell basis when compared to conventional DC (cDC) subsets in the spleen at 36 h [68]. The first *in vivo* pDC depletion studies (utilizing an anti-Ly6G/C antibody) indicated that systemic IFN $\alpha\beta$ levels at 36 h after MCMV infection are derived from pDC (>95% reduction) [67], and this result was confirmed using a more specific depleting antibody (anti-PDCA1, >85% reduction) [69]. Detailed flow cytometry studies confirmed that splenic pDC are the major producers of IFN $\alpha\beta$, TNF and IL-12 on a per cell basis at 36 h in B6 mice [70]. Importantly, liver-resident pDC do not produce significant levels of IFN $\alpha\beta$ at 36 h after MCMV infection [70], even though IFN $\alpha\beta$ is present at high levels in the liver at this time. Consequently, the data indicates that systemic IFN $\alpha\beta$ levels are largely derived from the spleen at 36 h, and is consistent with results indicating that the spleen is also the major source of systemic IFN $\alpha\beta$ at 8 h after MCMV infection [28].

The Toll-like receptors (TLR)-dependent pathways that regulate IFN $\alpha\beta$ production in response to MCMV spread

The explosion of research in recent years examining the TLR has certainly impacted the CMV field [71], and much is known regarding how TLRs promote innate immunity during MCMV infection. Two initial studies identified TLR9 as regulating the innate response to MCMV infection *in vivo* [69,72]. The first utilized TLR9^{CpG1} ENU mutant B6 mice [72] and the other examined Balb/c mice genetically deleted for TLR9 expression (i.e. $-/-$ mice) [69]. In this work, >95% and ~75–80% reduction in systemic IFN $\alpha\beta$ levels was observed 36 h after MCMV infection, respectively. Another study confirmed the decrease in systemic IFN $\alpha\beta$ levels in TLR9 $-/-$ B6 mice at 36 h (~70% reduction) [73], and two other groups observed that systemic IFN $\alpha\beta$ levels trended lower in TLR9 $-/-$ mice at 36 h, although the results did not achieve statistical significance [74,75]. It is possible these relatively minor discrepancies seen in TLR9 $-/-$ mice may be due to different doses or preps/strains of MCMV utilized in these experiments. Recently, TLR7 has been shown to function redundantly with TLR9 to promote systemic IFN $\alpha\beta$ production in B6 mice infected with MCMV, with TLR7 $-/-$ TLR9 $-/-$ double-deficient mice having >95% reduction in systemic IFN $\alpha\beta$ levels at 36 h. This is intriguing, as both these TLRs interact directly with the ER-resident membrane protein UNC93B [76], a protein critical for the 36-h systemic IFN $\alpha\beta$ response to MCMV (>95% reduction in an ENU-generated mutant) [77]. MCMV infection of MyD88 $-/-$ B6 mice, the adaptor required for both TLR7 and TLR9 signalling, resulted in >90% reduction in systemic IFN $\alpha\beta$ levels at 36 h in four independent studies [69,72,74,75].

TLR3s role in promoting systemic IFN $\alpha\beta$ production at 36 h after MCMV infection is still somewhat un-clear, as initial studies in TLR3^{-/-} B6 mice have shown a modest reduction (~60%) [72], but subsequent studies show no defect [74]. This discrepancy was not resolved, when examining the contribution of the essential adaptor for TLR3 signalling, TRIF, as TRIF^{LPS2} B6 ENU mutant mice show >95% reductions in serum IFN $\alpha\beta$ levels at 36 h [78], whilst B6 TRIF^{-/-} mice are reported to be normal [74]. It is possible that the generation of the TRIF^{LPS2} mice on a 'pure' B6 background might explain this difference, as the 129 strain of mice (which compose a certain percentage of the B6 TRIF^{-/-} mice genome, even after extensive backcrossing) encode an 'alternative' pathway to respond to TLR3/TRIF signals [79]. Additionally, TLR3 also interacts with UNC93B to signal appropriately [76,77]. Consequently, all three of these TLRs (3, 7 and 9) may function redundantly to some degree to promote MCMV-induced serum levels of IFN $\alpha\beta$ at 36 h. TLR2^{-/-} mice have also been reported to produce ~50% less IFN $\alpha\beta$ in the spleen at 36 h after MCMV infection [80]. Although systemic IFN $\alpha\beta$ levels were not examined in this study, they would likely be similarly decreased given the defect in the spleen [75]. Although a direct molecular signalling pathway from TLR2^{-/-} leading to IFN $\alpha\beta$ transcription is not known [71], a study published as this review went to press indicates that TLR2 may regulate IFN $\alpha\beta$ production by 'inflammatory monocytes' upon MCMV infection [81].

Timing and tissue specific issues during the first round of MCMV spread

The TLR-dependent mechanisms discussed above that regulate splenic and systemic production of IFN $\alpha\beta$ are all operational at 36 h after MCMV infection, and the compiled data are consistent with a model, where splenic pDC are the major producers at this time via a TLR9/MyD88 dependent mechanism. However, again illustrating the extreme complexity and flexibility of the IFN $\alpha\beta$ system, systemic IFN $\alpha\beta$ levels are normal only at 8 or 12 h later in mice deficient in TLR9, MyD88 or depleted of pDC [69,73,74]. The source of this 44–48 h IFN $\alpha\beta$ has not been strictly defined, but cDC or stromal cells are likely contributing sources. All the studies depleting pDC or using TLR9 signalling-deficient mice show some residual level of systemic IFN $\alpha\beta$ at 36 h, it is simply that pDC contribute the majority of the response at this specific time-point. When infected in culture with MCMV, CD11b+ 'cDC' generated from bone marrow (BM) with GM-CSF (GM-CSF BM-DC) produce copious amounts of IFN $\alpha\beta$ in a TLR-independent fashion [73]. The division of labour between TLR-dependent recognition of viruses by pDC, and TLR-independent recognition by cDC or stromal cells, is a common theme seen for many RNA viruses, where cytoplasmic receptors like RIG-I/MDA-5 trigger IFN $\alpha\beta$ production in non pDC [82]. To date, the TLR-independent pathway(s) that regulate innate sensing of DNA viruses (such as CMV) have not been defined as well as for RNA viruses. Nevertheless, some strides have been made towards identifying the signalling pathways that contribute to TLR-independent regulation of IFN $\alpha\beta$ during CMV infection [28,61,64].

The roles of IFN $\alpha\beta$ and other innate cytokines in regulating innate MCMV defenses in the liver have been largely delineated by the groups of Biron and Salazar-Mather [83]. Although splenic IFN $\alpha\beta$ levels were modestly reduced in TLR9^{-/-} mice at 40 h following MCMV infection in their studies (~40%), IFN $\alpha\beta$ production was completely independent of TLR9 in the liver at 40 h [84]. However, IFN $\alpha\beta$ levels were ~75% decreased in both these organs at this time in MyD88^{-/-} mice. The authors concluded that the responsible cell in the liver producing IFN $\alpha\beta$ was a pDC due to its high level of Ly6C expression [84], however, inflammatory monocytes express similarly high levels of Ly6C [81]. Consequently, at the time of this review, it is known that a TLR-dependent, MyD88-independent mechanism regulates IFN $\alpha\beta$ production in the liver at 40 h, and could be potentially derived from redundant TLR7 signalling in a pDC [75], TLR2-dependent signalling in a liver-recruited inflammatory monocyte [81] or another currently unknown source. It is interesting to note that the 40-h time-point is centred exactly,

when the source of IFN $\alpha\beta$ production in the spleen is 'switching' from being TLR9/MyD88/pDC-derived (36 h) to MyD88/pDC-independent (44 h) [74].

NK cell activation in response to MCMV spread: Day two

IFN $\alpha\beta$ promotes activation of NK cell cytotoxicity, and is a primary mechanism by which MCMV replication/spread is controlled at ~36–48 h. Additionally, IL-12 promotes NK cells to produce IFN γ starting at around the 36-h time-point [31]. These two NK effector functions are molecularly separable events, with the IFN $\alpha\beta$ /cytotoxicity axis being dependent upon STAT1 and IL-12/IFN γ requiring STAT4 [85]. NK cells utilize both these pathways to restrict acute MCMV replication in all peripheral organs [86,87], although some organ-specific preferences may exist [88]. IL-18 can also regulate splenic NK cell production of IFN γ at ~1.5 days, but was not found to contribute to activation of liver NK cells [89]. Notably however, these studies did not account for the fact that *i*NKT-cells comprise the majority of NK1.1+ cells in the liver at 36 h after MCMV infection [90]. Infection of GM-CSF BM-DC with MCMV results in secretion of IL-12 and IL-18, resulting in the production of IFN γ by co-cultured NK cells in an IL-18-dependent fashion [73]. Infection of Flt3-ligand generated BM-DC with MCMV (i.e. a mix of pDC and cDC subsets) activates *i*NKT-cells to produce IFN γ in a similar co-culture setup, and IL-12 (not IL-18) is the promoting cytokine in this scenario [34]. IL-12 is required for *i*NKT cell production of IFN γ in the spleen and liver at 36 h after MCMV infection, and still contributes at 40 h in the liver [34,90]. Interestingly, IFN $\alpha\beta$ also promotes IFN γ production by *i*NKT-cells at 36 and 40 h, highlighting a potential difference in how *i*NKT and NK are regulated by type I interferons. Importantly, mice deficient for IFN $\alpha\beta$ signalling (or depleted for pDC) show both increased levels and altered cellular sources of IL-12 at 36 h after MCMV infection [67,69,91].

In general, the DC subsets that produce the bulk of the IFN $\alpha\beta$ at 36–48 h after MCMV infection *in vivo* also secrete the IL-12 that is responsible for NK and *i*NKT cell activation, with a few notable exceptions. Although pDC produce the vast majority of IFN $\alpha\beta$ in a TLR9/MyD88 dependent manner at 36 h in the spleen (>90%), they only produce ~60–70% of the IL-12 at this time, with the remaining IL-12 being produced largely by the cDC subsets [69,70,72]. Consequently, the numbers of splenic NK and *i*NKT-cells producing IFN γ at 36 h is decreased ~4–5-fold in both TLR9^{-/-} and MyD88^{-/-} mice [69,72,74], and at 40 h were reduced by ~50% in the livers of MyD88^{-/-} mice, but were normal in livers of TLR9^{-/-} mice [84]. Splenic NK cells isolated at 36 h after MCMV infection of MyD88^{-/-} mice exhibited a modest defect in cytolytic activity [69], but no defect was seen in a second study [74], with the potential difference being the dose of infection (no differences were seen when injecting 10-fold more MCMV). In general, this suggests that the residual production of IFN $\alpha\beta$ in the absence of pDC/MyD88 function is still sufficient to activate some NK effector functions during the second day of infection. This is substantiated by the fact that both TLR9^{-/-} and MyD88^{-/-} B6 mice control MCMV replication quite well at 48 h after infection in the spleen [74]. However, NK control breaks down by 3–5 days after infection of these deficient mice, with 100–1000-fold more virus being present in their spleens [69,72,74]. Interestingly, TLR9^{-/-} Balb/c mice show no increases in splenic MCMV replication at day 3 or 6 after infection, completely different from what is observed in TLR9^{-/-} B6 mice, suggesting it is the Ly49H-expressing NK cells that are the ultimate effectors activated by the pDC/TLR-dependent pathway [69]. Finally, MyD88^{-/-} B6 mice showed increased levels of MCMV replication in the liver at day 5 after infection, whilst TLR9^{-/-} B6 mice showed no analogous defect [74], perhaps explainable by the inability of TLR2-expressing inflammatory monocytes to function appropriately in the absence of MyD88 [81]. Importantly, as the timing of cellular recruitment to the MCMV-infected liver is dramatically altered in MyD88^{-/-} mice, it is difficult to directly link control of MCMV replication at day 5 to NK cell activation events occurring around day 2 of infection.

This is why we have tried to focus largely on NK cell activation that occurs simultaneously with innate cytokine production in this review.

CMV-modulation of the NK cell response

Even though, NK cells are activated by IFN $\alpha\beta$ and IL-12/IL-18, MCMV utilizes a variety of strategies to restrict NK cell effector functions, and these have been extensively reviewed recently [15,92]. Four MCMV gene products are known to inhibit expression of ligands for activating NK cell receptor NKG2D in infected cells (m138, m145, m152 and m155) (Fig. 2): m155 decreases the expression of H60, m145 inhibits mouse UL16-binding protein-like transcript (MULT)-1, m152 inhibits RAE-1 and H60 expression and m138 inhibits mult-1, H60 and RAE varepsilon. Deletion of each of these genes results in reduced viral replication *in vivo* in an NKG2D and NK cell-dependent fashion, indicating that the viral gene products directly protect infected cells from NK-mediated effect or functions [92]. To the best of our knowledge, none of the various NKG2D ligand modulating genes of MCMV have been analysed *in vivo* with respect to the precise timing of when these genes dampen NK effector function over the course of the first few days of infection. Nevertheless, elegant work has demonstrated an ultimate effect of all these genes in restricting NK-mediated control by day 3–4 [93-96]. The m144 protein (homologous to MHCI) of MCMV also restricts NK cell control [97], but its binding partner is unknown. Aside from the NK-activating function of m157-Ly49H interactions, m157 also binds the Ly49i inhibitory NK cell receptor [98], strong support for the hypothesis that CMV actively drives the evolution of NK cell effector mechanisms in its host [99]. In turn, m04, in complex with H-2D^K, also enhances NK cell-recognition of infected cells by binding the activating NK cell receptor Ly49P [100]. Analogously, HCMV encodes six separate gene products and a micro RNA capable of restricting the expression of NK cell activating ligands (UL16, UL18, UL40, UL83, UL141, UL142 and miR-UL112), and the mechanisms by which these gene products function have also been recently reviewed [101]. Hence it is clear that the ‘sum total’ of the host attack and CMV retort at the level of NK cell activation is a very complex process.

The IFN $\alpha\beta$ response upon HCMV infection of DC

The ability of HCMV to infect DC subsets has been examined by many groups, including pDC, myeloid-DC (mDC, both from human blood and GM-CSF/IL-4 derived) and Langerhans-like DC. Much of this work has been focused on how HCMV alters the ability of these cells to prime/activate T-cells, as this is a major function of DC, and this aspect has been recently reviewed [102]. In general, this work has shown that mDC and Langerhans-like DC can be infected in culture with ‘clinical isolates’ of HCMV at high MOI, resulting in the decreased expression of MHC and costimulatory molecules which then restricts the allogeneic activation of co-cultured T-cells [103-108]. Importantly, this inhibition requires productive HCMV replication within the antigen presenting cell (APC), allowing for expression/function of viral immune modulatory genes. In more recent studies, where pDC isolated from peripheral blood have been infected with HCMV, efficient productive infection has not been observed [108-111]. However, these pDC still produce large amounts of IFN $\alpha\beta$ when exposed to HCMV particles, likely through a TLR7 or TLR9-dependent pathway [110], and very little IL-12 is produced concurrently. The production of innate cytokines by HCMV-exposed pDC, in combination with the inability of the virus to replicate in these cells, is consistent with what has been observed *in vivo* for MCMV and pDC [68]. Interestingly, pDC exposed to HCMV display a compromised ability to activate B, T or NK cells [110,111], suggesting HCMV may alter pDC functions even when it cannot replicate efficiently in these cells. Finally, adding further complexity to the story, if human pDC are isolated from tonsil tissue as opposed to peripheral blood, HCMV infects these cells efficiently, restricts their ability to produce IFN $\alpha\beta$ and inhibits expression of MHC and costimulatory molecules [112]. Taken together, it

seems the field is only beginning to grasp what role specific subsets of human pDC might play in the context of HCMV infection. Notably, as chronic viral infections in mice can result in the 'exhaustion' of pDC function [113], it is intriguing to speculate that reactivation of HCMV during immune suppression may cause a similar problem, and this possibility has been recently discussed[114].

Transitioning from innate to adaptive immunity during CMV infection

Up to this point, we have focused almost entirely upon the timing of innate defense mechanisms that control CMV infection during the first few days. However, the development of adaptive immunity is needed to ultimately control primary CMV infection. Furthermore, sustained adaptive immunity is crucial in maintaining long-term control of CMV, highlighted by the fact that CMV reactivates and causes serious clinical problems in patients who are immune suppressed. We will now discuss how the robust innate defenses operable during primary MCMV infection can help to promote the development of adaptive immunity. In very general terms, as MCMV-induced innate cytokines (e.g. $IFN\alpha\beta$ and TNF) can promote upregulation of MHC and cosignalling ligands on bystander APC, this represents a link between innate and adaptive immunity that is conserved amongst most pathogens [115]. As most of the key cellular immune players are not productively infected by CMV (e.g. NK, *i*NKT, T and B cells), this limits CMV's ability to modulate these responses to two general strategies: (i) altering their function by infecting/modulating the cells they interact with (e.g. infection of myeloid lineage or stromal cells) or (ii) promoting the secretion of host or viral cytokines/chemokines that can act upon these cells.

The relationship between virus 'load', $IFN\alpha\beta$ production and CMV-specific adaptive immunity

The functional consequences of $IFN\alpha\beta$ signalling can vary greatly depending upon the relative amount produced, with low levels often being immune stimulatory and higher levels capable of promoting immune suppression. The role that varying levels of $IFN\alpha\beta$ production play in promoting the development of MCMV-specific T-cell responses has been examined in mice containing or lacking Ly49H-expressing NK cells [116]. When infecting Ly49H-mice with modest doses of MCMV (5×10^3 pfu), splenic pDC produced high levels of $IFN\alpha\beta$ at 36 h in response to the first round of MCMV spread, not unexpectedly based on previously published work. However, when Ly49H+ mice were infected with the same low MCMV dose, splenic pDC did not produce $IFN\alpha\beta$. Importantly, if 5-fold more MCMV was injected, splenic pDC from both Ly49H+ and Ly49H- mice produced high amounts of $IFN\alpha\beta$. This highlights the fact that critical 'thresholds' exist in genetically distinct mouse strains, and these must be considered when comparing MCMV-specific innate immune responses (reviewed in [65]). Subsequently, the authors observed that the maintenance of cDC numbers in the spleen correlated with the production of $IFN\alpha\beta$ by the pDC, with Ly49H+ mice maintaining 2–3-fold higher numbers of cDC at day 2–3 after infection. Consequently, when the numbers of MCMV-specific CD8 T-cells were examined in the spleen at day 4 after infection of Ly49H+ mice, ~5–10-fold higher numbers were seen in comparison with Ly49H- mice (Fig. 2). However, 1 day later, MCMV-specific CD8 T-cell numbers in Ly49H- mice achieved similar levels to that seen in Ly49H+ mice, suggesting that the 'advantage' gained by maintaining cDC numbers and accelerating the development of pathogen-specific adaptive immunity is rather short lived, at least in this system. Nevertheless, this work revealed that when CMV infection approaches specific threshold levels of innate immune control, which undoubtedly exist in the polymorphic human population, significant differences in the overall shape of innate and adaptive immunity can result. Interestingly, at very high doses of MCMV infection in Ly49H+ mice, $IFN\alpha\beta$ promotes the survival of splenic cDC at day 2–3 [29], highlighting the complexity of these different thresholds.

What are other possible consequences of high levels of early IFN $\alpha\beta$ in MCMV infection? In the LCMV model, NK cells exposed to high IFN $\alpha\beta$ levels are unable to produce IFN- γ , an effect not described for MCMV infection [117,118]. LCMV infection can also induce a transient lymphopenia caused by IFN $\alpha\beta$ -mediated attrition of memory and 'memory phenotype' (CD44 high) CD8 T-cells [119-121]. In turn, IFN α can desensitize naïve CD8 T-cells to IL-2, IL-7 and IL-15-induced proliferation [118], altering the development of LCMV-specific T-cell responses. However, it is currently not known whether similar effects of IFN $\alpha\beta$ might be operable during CMV infection.

Perforin-deficient mice reveal roles for NK cells in regulating MCMV-specific adaptive immunity

In addition to potentially promoting MCMV-specific adaptive immunity by regulating IFN $\alpha\beta$ -dependent cDC survival, a second function for NK cells in regulating adaptive immunity has been revealed by infecting perforin^{-/-} mice with MCMV (Perf^{-/-}) [87,122,123]. Perf^{-/-} mice cannot control acute MCMV replication and succumb to infection in ~1 week, displaying excessive levels of TNF and IFN γ in the serum and severe immunopathology in the liver. In Ly49H⁺ mice, CD11b⁺ myeloid cells (DC and macrophages) produce the majority of the detrimental TNF, with some contribution by CD4 T-cells. The increased levels of systemic IFN γ production were found to be due to the expansion of specific NK subsets and MCMV-specific CD8 T-cells [123]. Perf^{-/-} mice show a preferential proliferation and expansion of Ly49H⁺ NK cells, with more than a 5-fold increase compared to wild-type mice by day 4 after infection [123]. This is almost certainly because Ly49H-expressing NK cells are stimulated by m157 to a higher degree in Perf^{-/-} mice due to increased MCMV replication levels. Notably, these hyperactivated, Ly49H⁺ NK cells produce IL-10, which in turn limits the expansion/activation of MCMV-specific CD8 T-cells at day 7 of infection. Supporting this model, Ly49H⁻Perf^{-/-} mice have even higher levels of MCMV-specific CD8 T-cells and lower levels of systemic IL-10, resulting in the death of these mice due to CD8 T-cell-mediated immunopathology. Importantly, this data indicates that direct stimulation of NK cells through an activating receptor (Ly49H in this case) can modulate their proliferation and cytokine production, distinct from roles for these receptors in promoting cytotoxic functions.

NK-DC cross-talk in the promotion of T-cell responses

A positive role for NK cells in promoting adaptive immunity was first suggested in 1987 [124]. NK cells have a unique capability to further activate DC when suboptimal levels of 'danger signals' are present, a function that is especially important for the elicitation of immune responses against tumours [125-128]. NK-DC cross-talk requires NK-derived cytokines (TNF α /IFN- γ) and cell-cell contact, although the specific cell surface molecules are currently uncharacterized [125,129]. In various experimental systems, including CMV, the operable DC-derived cytokines promoting IFN γ production by NK cells are IL-12 and IL-18 [68,130,131], whilst IFN $\alpha\beta$, IL-2 and IL-15 regulate NK cytotoxicity and proliferation [68,130-135]. During MCMV infection, Ly49H⁺ NK cells maintain the numbers of CD8 α ⁺ cDC in the spleen of B6 mice at day 4-6 of infection [136]. When Ly49H⁺ NK cells are depleted, CD8 α ⁺ cDC numbers decrease dramatically at day 4, but MCMV replication also increases concurrently by ~100-fold. Consequently, it is somewhat difficult to separate the relative roles that cross-talk and restriction of MCMV replication mediated by Ly49H⁺ NK cells have in protecting this splenic cDC population. However, there does appear to be some selectivity for CD8 α ⁺ cDC in regulating the Ly49H⁺ NK population, as expansion of Ly49H⁺ but not Ly49G⁺ NK cells requires their presence, in combination with IL-12 and IL-18 [136]. Consistent with this selectivity, Ly49H⁺ NK cells proliferate and acquire effector functions during MCMV infection in the absence of IL-15 [137], suggesting that the m157-Ly49H interaction can functionally substitute for host pathways that normally regulate NK cell differentiation and

function. Finally, previously discussed results indicated that NK-DC cross-talk via NKG2D-NKG2DL contributes to NK acquisition of cytotoxicity [73].

Taken together, the use of Ly49H⁺ and Ly49H⁻ mouse strains has revealed that NK cells have the capacity to regulate the development of MCMV-specific CD8 T-cell responses through both direct and indirect mechanisms. The challenge(s) moving forward are to determine the mechanisms by which NK cells can shape CMV-specific adaptive immunity in the absence of a dominant, Ly49H-like NK cell response. This was revealed to some degree in the studies using Ly49H^{-/-}Perf^{-/-} mice [123], but of course using mice deficient in a major component of innate defense also has some drawbacks. Other work outside the CMV field has elucidated additional 'adaptive' functions for NK cells. NK cell 'helper' functions mediated by skewing CD4 T-cell differentiation towards a Th1 phenotype, NK memory-like responses and finally Th17-like NK cells (NK-22's) are a few specific examples of this [137-146]. These observations suggest that the diverse populations of NK cells which clearly play a role in regulating CMV infection may play additional, currently unappreciated roles in shaping the CMV-specific adaptive response.

The role of IFN $\alpha\beta$ and chemokines in the sequential recruitment of NK and CD8 T-cells to the liver during MCMV infection

An elegant series of papers from the groups of Biron and Salazar-Mather over the last decade have elucidated the role that innate cytokines and chemokines play in regulating the sequential recruitment of innate cell populations to the liver during MCMV infection (Fig. 3). MyD88-dependent, IFN $\alpha\beta$ production by a Ly6G/C expressing cell in the liver (discussed above [84]), in combination with IFN $\alpha\beta$ signalling in the BM, promotes induction of CCR2 ligands (MCP-1/CCL2 and MCP-5/CCL12) by F4/80⁺ myeloid cells. These CCR2 ligands are required for egress of inflammatory monocytes from the BM into the blood and their subsequent entry into the liver, where they then produce MIP-1 α /CCL3 and promote the recruitment of NK cells [147,148]. These liver-recruited NK cells then acquire effector functions in response to locally produced IFN $\alpha\beta$ and IL-12, and function to restrict MCMV spread. In turn, NK-produced IFN γ induces production of CXCR3-ligands in the liver, promoting the recruitment of naïve CD8 T-cells which are primed by MCMV antigens, expand and acquire effector functions [149]. Importantly, both human and mouse CMV encode their own viral chemokines and chemokine receptors which have been shown to regulate various aspects of acute and persistent replication and dissemination, and this has been reviewed recently in detail elsewhere [150].

CMV-modulation of T-cell priming

As already discussed, direct infection of APC by CMV results in profound phenotypic and functional alterations in these cells, including the restriction of MHC expression [151] and inhibition of co-stimulatory molecules, and has been the topic of several excellent recent reviews [102,152]. These alterations have dramatic consequences on the priming of naïve CD8 and CD4 T-cells [153], and MCMV was originally said to promote a 'paralysed' phenotype in infected DC [154]. However, unless experiments are designed to analyse relatively pure populations of CMV-infected DC, in the absence of 'contaminating' uninfected APC, interpreting the outcome of CMV immune modulation on T-cell responses is complicated. Notably, MCMV productively infects only a small fraction of the cDC subsets *in vivo* in B6 mice, even at times of peak acute replication [68], thereby subjecting only a fraction of APC to direct immune modulation by the virus. However, the remaining uninfected APC mature in response to bystander cytokine production (e.g. IFN $\alpha\beta$), which promotes efficient cross-presentation of viral antigens [155] and subsequent priming of MCMV-specific CD8 T-cell responses [156]. This model would explain why direct infection of mice with an MCMV mutant unable to modulate expression of MHCI expression produced no differences in the

immunodominance hierarchy or magnitude of the MCMV-specific CD8T-cell response [157].

To attempt and address this issue, one study utilized a B6 mouse strain containing a mutation in the H-2K^b MHC molecule (Kbm1) to dissect the relative effects that priming naïve T-cells by infected or uninfected DC can have on shaping the MCMV-specific CD8 T-cell response [156]. Interestingly, these studies revealed that naïve T-cells encountering a DC directly infected with MCMV are highly subject to negative cosignalling mediated by PD-L1/PD-1 interactions, and that this interaction could account for a large percentage of the T-cell 'stunting' or 'paralysis' seen by several groups. Again consistent with a model where the majority of MCMV-specific CD8 T-cells are cross-primed by uninfected APC *in vivo*, administration of a blocking anti-PD-L1 antibody barely altered the profile of this response in wild-type mice infected with MCMV. This was in stark contrast to the dramatic restoration of T-cell proliferation and effector function (both *in vitro* and *in vivo*) that was observed when PD-L1 was blocked in an experimental setup where naïve T-cells could only be primed by DC that were productively infected with MCMV [156].

The CMV-specific memory T-cell pool

The nature of the CMV-specific memory T-cell pool has garnered significant attention, as HCMV infection is coincident with every known case of a severe immunosenescence phenotype in old individuals referred to as the immune risk profile (IRP). IRP develops in persons >80 years old, where the circulating CD4: CD8 T-cell ratio inverts (i.e. CD4: CD8 <1) and is predictive of decreased immune function and poor patient survival [158]. The gradual increase (or maintenance) of the numbers of CMV-specific memory T-cells over the many decades of persistent infection is largely unique to this virus (termed 'memory inflation' [159]), and has been postulated to play a role in promoting the IRP. HCMV-specific T-cells can reach strikingly high numbers in select older individuals (>45% has been documented), but normally comprise ~10–20% of the pool [160,161]. This large, diverse CMV-specific T-cell memory pool is also seen during rhesus CMV infection [162]. The inflationary, MCMV-specific memory responses appear to be analogous to the oligoclonal expansion of HCMV-specific CD8 T-cells seen in people (reviewed in [163]). MCMV-specific inflationary CD8 T-cells display an effector-memory phenotype, suggesting they may have recently reencountered antigen [164–166]. This would be consistent with observations that MCMV production from the salivary gland (SG) is detectable at late times after primary infection (>100 days), but occurs in a sporadic, semi-random manner and varies based on the strain of virus and mice (A. Loewendorf, personal observation). Interestingly, the recent characterization of MCMV epitope-specific CD4 T-cell responses revealed that they also can vary greatly with respect to their kinetics of expansion and contraction [167]. Although some progress has been made towards understanding the mechanisms underlying memory inflation [168,169], there is still much to be learned.

Continued CMV shaping of adaptive immunity during persistence/latency

Several elegant studies from Reddehase et al. have indicated that low-level, sporadic MCMV reactivation from latency in the lung of Balb/c mice contributes to the inflation of memory CD8 T-cells specific for an epitope derived from the IE1-protein [170]. However, as these same CD8 T-cells restrict further reactivation of MCMV gene expression, this does not explain how CD8 T-cells specific for nonIE antigens also inflate in Balb/c and B6 mice. Presumably, there is a tissue site of MCMV reactivation, or chronic persistence, which functions as an antigen source and promotes the maintenance/expansion of CMV-specific T-cells. The SG is an immune-privileged, mucosal organ that is a potential candidate for this site. Interestingly, HCMV can shed for years from the SG of infected children [171], and rhesus CMV may shed

for life from the SG [172]. In mice, immune control of MCMV in the SG is quite unique, and includes an immune suppressive role for IL-10 and a strict requirement for CD4 T-cells [173-175]. Strikingly, the HCMV IL-10 orthologue is the only gene known to be expressed during latent infection of myeloid precursor cells [176]. Interestingly, NK cells can promote DC function and T-cell priming under suboptimal conditions of trying to mount effective anti-tumour immunity [125-128]. As NK cells may contribute to control MCMV replication at early time-points in the SG [87], this may represent another link between innate and adaptive immune responses operable during persistent CMV replication in the SG.

Conclusions

We have attempted to paint a chronological picture of how anti-CMV immune defenses fit together to combat and control CMV infection. In turn, we have discussed a number of the known CMV immune modulating strategies, and have attempted to present them in the context of when we feel they are most likely to have the greatest effect(s) on shaping host defenses. As CMV infects >50% of the world's population, and a high priority for the scientific community is the development of an anti-CMV vaccine for use in combating congenital infection [177], we hope that this review will allow for easier conceptualization of what is needed to accomplish this goal.

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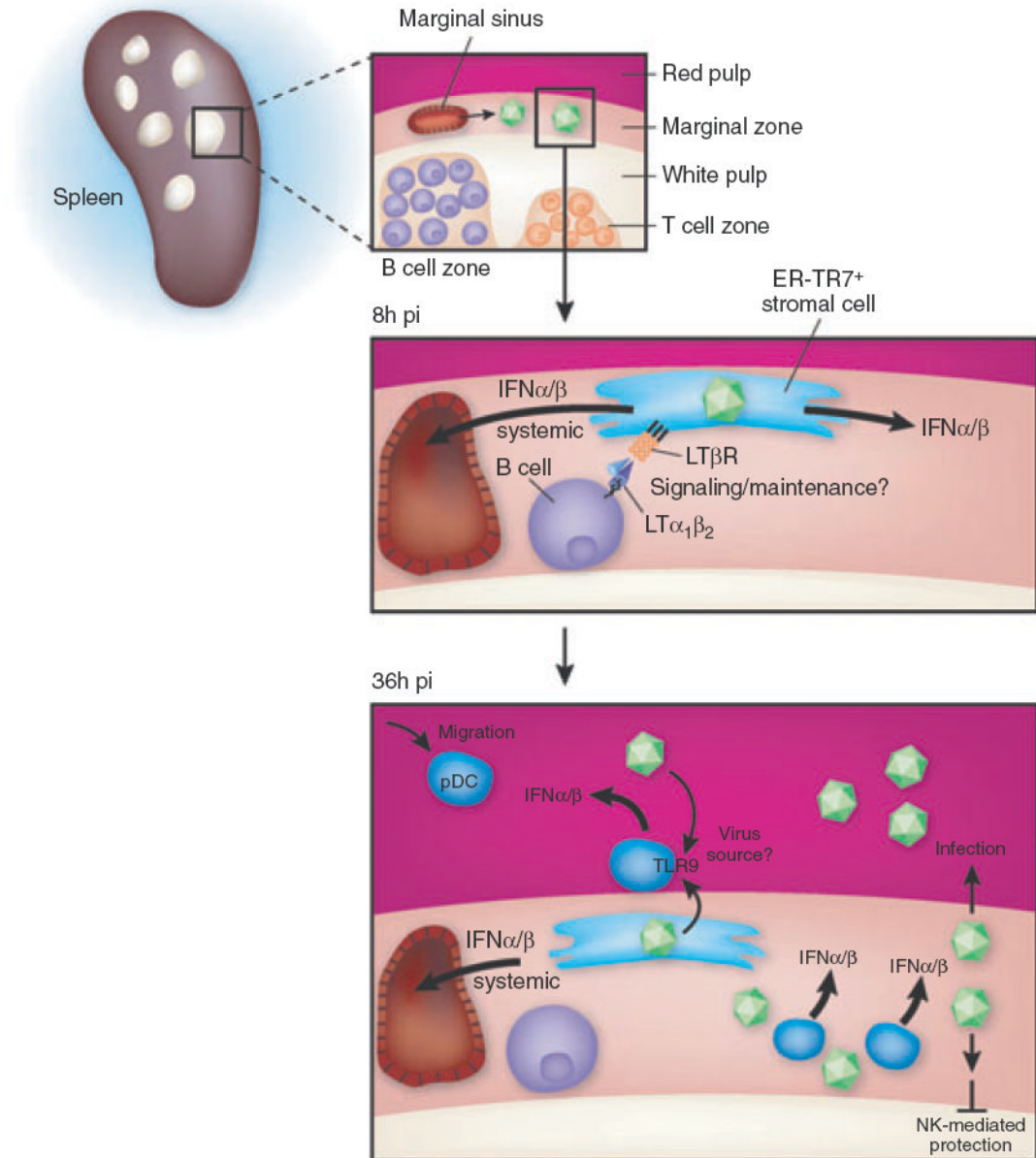


Fig. 1. Regulation of the splenic IFN $\alpha\beta$ response: both during initial MCMV infection, and in response to the first round of viral spread. At 8 h postinfection (pi), MCMV virus enters the spleen largely via the marginalzone (MZ) sinus where it predominantly infects MZ stromal cells expressing ER-TR7 and LT β R. The stromal cells require LT $\alpha_1\beta_2$ -expressing B cells to maintain their differentiation state, as well as to promote activation of noncanonical NF κ B signalling, and these stromal cells secrete the first detectable wave of IFN $\alpha\beta$ mRNA peaking at ~8h after infection. The stroma is also the source of the majority of systemic IFN $\alpha\beta$ peaking at ~8–12 h. At 36 h pi, pDC preferentially localize to the MZ from the red pulp and produce the vast majority of the splenic and systemic IFN $\alpha\beta$ detectable when MCMV is initiating its first round of spread in vivo. pDC utilize a TLR9/MyD88 dependent mechanism to produce IFN $\alpha\beta$, whilst production by the stroma is TLR-independent. In mice containing Ly49H-

expressing NK cells, the white pulp is protected from MCMV infection at day 2–3 of infection, and this is dependent upon $IFN\alpha\beta$ production as well.

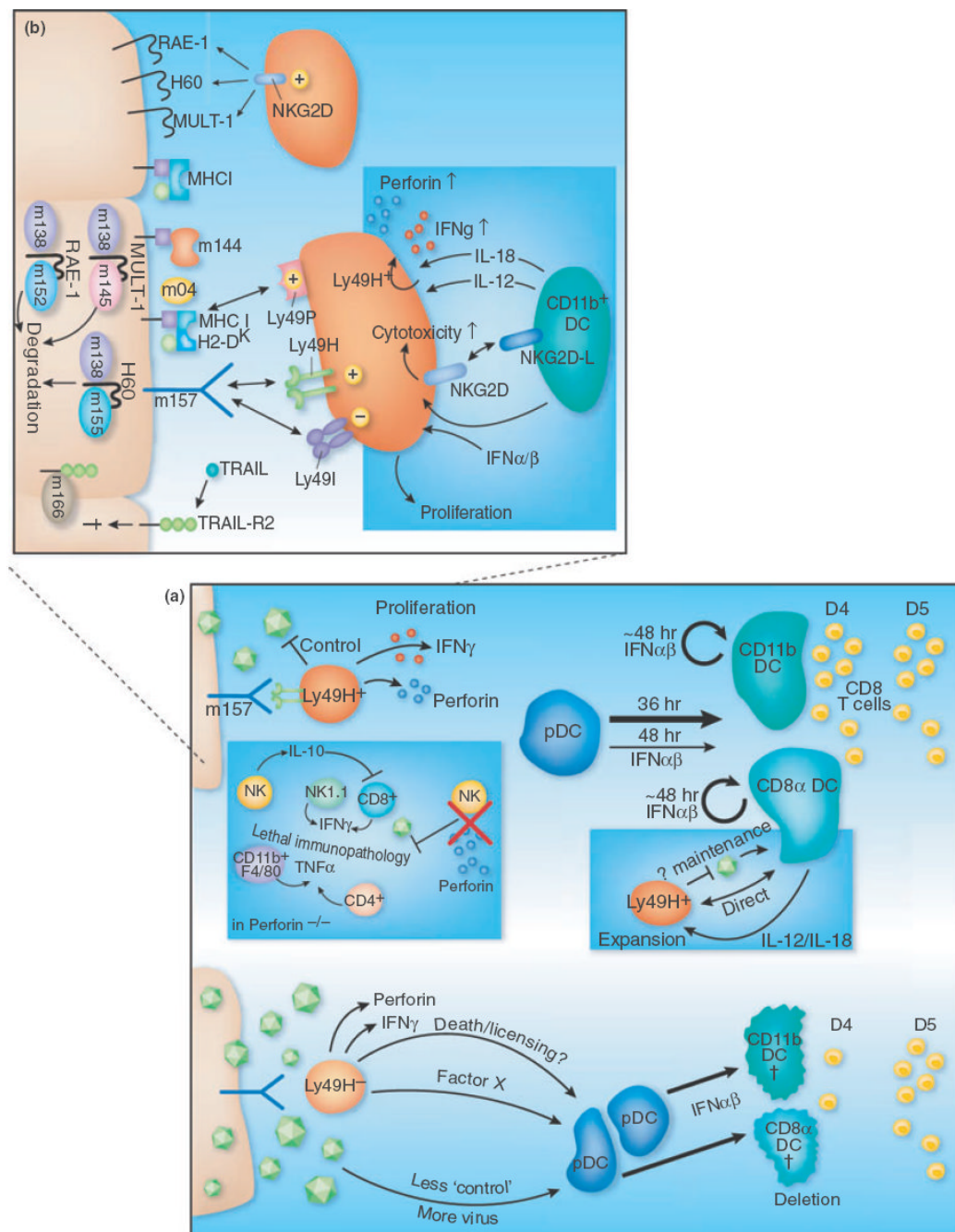


Fig. 2.

The role of NK–DC interactions and IFN $\alpha\beta$ in regulating both innate and adaptive immune defenses to CMV infection. (a) In mice with Ly49H⁺ NK cells (top left), binding of this activating receptor by MCMV m157 promotes NK proliferation and acquisition of effector functions, which in turn limit viral replication. Whilst pDC are a major source of IFN $\alpha\beta$ at 36 h when infecting with ‘normal’ doses of MCMV, their relative production at 44–48 h is marginal compared to cDC (depicted by arrow thickness). However, if low dose MCMV infection is performed, Ly49H⁺ NK cells restrict the 36 h IFN $\alpha\beta$ production by pDC, resulting in enhanced survival of cDC which promotes increased numbers of MCMV-specific CD8⁺T-cells at day 4 in the spleen. Cross-talk between Ly49H⁺ NK cells and CD8 α DC maintains the numbers of

both these cells, and involves DC-derived IL-12 and IL-18 (blue inset) [136]. Infection of Perforin-deficient mice (blue inset) results in lethal immunopathology mediated by CD11b⁺ F4/80⁺ and CD4⁺ cells secreting TNF α . IL-10, likely secreted by Ly49H⁺ NK cells, dampens immunopathogenic MCMV-specific CD8 T-cell responses that develop in Perforin^{-/-} mice [123]. NK cells are also activated in Ly49H⁻ mice at 36 h (bottom), but in spite of producing IFN γ and TNF α they control viral spread considerably worse than Ly49H⁺ NK cells. Consequently, substantially higher levels of IFN $\alpha\beta$ is produced by pDC at 36h in Ly49H⁻ mice (low MCMV dose), promoting enhanced death of cDC and a 1 day delay in the priming/ expansion of MCMV-specific CD8 T-cells [116]. (b) MCMV m157 interacts with the activating receptor Ly49H on NK cells, resulting in their proliferation and acquisition of effector functions. The cell surface expression of the NKG2D ligands RAE-1, H60 and MULT-1 are restricted by the MCMV proteins m138, m152 and m145 which induce their degradation in infected cells. The mechanism by which the MHC1-homologous protein m144 dampens NK control is unknown. The MCMV m04 protein complexes with H-2D^k and binds the Ly49P NK-activating receptor. MCMV m166 binds TRAIL-R2 and restricts its cell surface expression. Cross-talk between NK cells and CD11b⁺ DC occurs via NKG2D-NKG2D-L interactions, and in concert with IFN $\alpha\beta$ induces enhanced NK cell cytotoxicity. DC-derived IL-12 and IL-18 promote IFN γ production by the NK cells (blue inset).

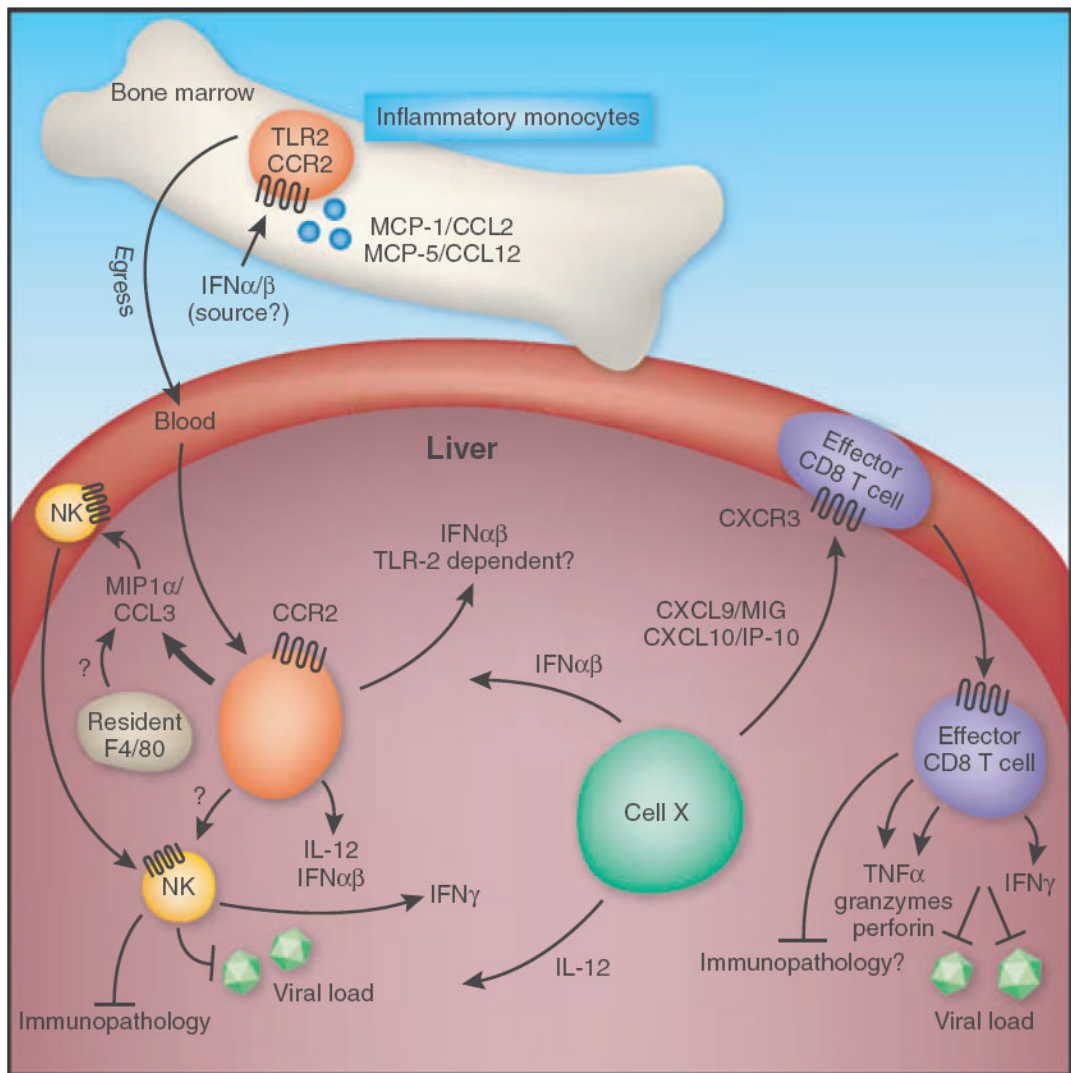


Fig. 3.

Innate MCMV defenses in the liver promote the development of adaptive immunity. $IFN-\alpha/\beta$ signalling is required for the induction of CCR2 ligands in the bone marrow (BM) (MCP-1/CCL2 and MCP-5/CCL12). Upon CCR2 signalling, inflammatory macrophages egress from the bone marrow into the blood and infiltrate the liver. MIP1 α /CCL3 produced by these inflammatory monocytes/macrophages, potentially in combination with production by liver-resident F4/80+ macrophages, recruits NK cells from the blood into the liver. NK cells are then activated in response to $IFN-\alpha/\beta$ and IL-12, potentially produced via a TLR2-dependent mechanism by inflammatory monocytes or by a currently unknown cell 'X' via a TLR9-independent, MyD88-dependent mechanism. Activated NK cells can then function to control MCMV spread in the liver. In turn, NK-produced $IFN-\gamma$ induces expression of CXCR3-ligands (CXCL9 and CXCL10) by cell 'X', promoting the recruitment of MCMV-specific CD8T-cells from the blood, which function to further restrict MCMV replication and may also promote immune pathology in some settings. Effector CD8T-cells induced by these cytokines and chemokines secrete $IFN-\gamma$ and TNF. Furthermore, they are loaded with cytolytic granules containing granzyme and perforin.