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Cytokine and Chemokine Networks: Pathways to Antiviral Defense

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Abstract The complex interplays between cytokines and chemokines are emerging as key communication signals in the shaping of innate and adaptive immune responses against foreign pathogens, including viruses. In particular, the virus-induced expression of cytokine and chemokine profiles drives the recruitment and activation of immune effector cells to sites of tissue infection. Under the conditions of infection with murine cytomegalovirus (MCMV), a herpesvirus with pathogenic potential, early immune functions are essential in the control of virus replication and virus-induced pathology. The coordinated MCMV-induced cytokine and chemokine responses promote effective natural killer (NK) cell recruitment and function, and ultimately MCMV clearance. The studies highlighted in this chapter illustrate in vivo pathways mediated by innate cytokines in regulating chemokine responses that are vital for localized antiviral defenses.

Abbreviations

MCMV	Murine cytomegalovirus
NK	Natural killer
MIP-1α	Macrophage inflammatory protein 1α
Mig	Monokine induced by interferon-γ
MCP-1	Monocyte chemoattractant protein-1
rIFN-α	Recombinant interferon-α

1 Introduction

The host response to microbial pathogens necessitates the integrated action of both the innate and adaptive arms of the immune system. Innate immunity is largely dependent on granulocytes, macrophages, dendritic cells, and natural killer (NK) cells, whereas adaptive immune responses require T and B lymphocytes. It is becoming increasingly evident that an effective immune response requires crosstalk among the various immune cells for linkage of innate and adaptive immunity. This intercellular network of communication signals is mediated in part by cytokine and chemokine responses generated against infectious agents, including viruses.

Cytokines constitute a group of low molecular weight soluble proteins that modulate various immune functions upon induction by various stimuli, such as bacterial or viral components [10–12, 32, 92]. Virtually all cells produce these proteins, including leukocytes and infected or activated nonimmune cells such as fibroblasts, endothelial cells, and a variety of tissue parenchymal cells [10, 11]. Cytokines control the magnitude and kinetics of immune responses by inducing or inhibiting the activation, proliferation, and/or differentiation of various target cells, and they also regulate the production of antibodies and other cytokines including chemokines [1, 11, 54].

Chemokines are small heparin-binding secreted cytokines that are functionally appearing to be more diverse than used to be thought. Housekeeping chemokines are generally constitutively expressed under physiological conditions, and they function in development and homeostasis [20, 56, 73, 81, 95, 104]. Inducible inflammatory chemokines function in the directed trafficking and localization of effector leukocytes within tissue compartments during inflammation, infection, and trauma [5, 58, 73, 82, 91, 95, 104]. There is considerable evidence highlighting the importance of chemokine-mediated inflammatory responses to pathogens as being crucial to survival during infections [18, 53–55, 57, 77, 78, 80, 83]. Additionally, it has become evident that infiltrating leukocytes may sometimes play a role in disease pathogenesis [18,

54, 55, 83, 98]. Nevertheless, as chemokines are central to the routing of key immune cells, they have emerged as key players in host defense mechanisms [54–58, 62, 80, 95, 104]. These proteins mediate their biological effects through selective interactions with seven-transmembrane G protein-coupled receptors on the surface of target cells [7, 9, 59, 74, 90]. Inflammatory chemokines are selective for a broad range of receptors. Nonetheless, in vivo studies have demonstrated specific functions for various chemokines [18, 19, 21, 25, 27, 37, 58, 80, 93].

Protective immunity to viruses is dependent on the activation and interplay between cytokines and chemokines to enhance or regulate innate or adaptive (or both) effector functions. Studies have demonstrated that the cytokine milieu induced by pathogens may determine the cellular constituents that get activated, and thus the nature of the immune response, by selectively inducing specific chemokines in infected tissue compartments [18, 37, 55, 57, 77, 78, 80]. The murine cytomegalovirus (MCMV) model of infection in the liver has been used to demonstrate the coordinated effects of activated cytokine and chemokine responses on the recruitment of NK cells to sites of infection to maintain early control of virus replication. This chapter presents a brief overview of the role of NK cells in antiviral defense, the molecular mechanisms regulating the innate cytokine and chemokine networks promoting NK cell inflammation into liver, and the conceivable role of these pathways in promoting downstream adaptive responses for effective antiviral defense.

2
NK Cells and Antiviral Defenses

NK cells are innate effector cells that respond quickly to a variety of pathogens before the onset of adaptive immunity [11, 96, 103]. These cells originate from bone marrow precursors and predominate in peripheral blood and spleen. However, they can be induced to traffic into other compartments including the liver during infection [77, 99]. Classical NK cells are $CD3^-$ and do not express rearranged antigen-specific receptors [11, 42, 71, 97, 103]. Instead, it is now widely accepted that NK cells express a complex repertoire of activating and major histocompatibility complex (MHC) class I-specific inhibitory receptors on their surfaces that interact with ligands on target cells [23, 101–103]. Engagement of activation receptors with ligands on infected cells permits NK cell-mediated killing and cytokine production, while the inhibitory receptors restrain NK cell activation [4, 23, 43, 101, 102]. The central role of NK cells as mediators of antiviral defenses has long been appreciated [11, 96].

Furthermore, viruses that evade immune detection by restricting the function of MHC class I T lymphocytes are more susceptible to NK cell-mediated protective responses [101–103]. These cells are quick to respond and can be activated to produce high levels of the cytokine interferon (IFN)-γ, as well as additional antiviral and immunoregulatory cytokines [11, 12]. NK cells have been demonstrated to mediate protection against a number of viral infections (reviewed in references [11, 12]), most notably is the extensive data documenting their importance in innate defenses against MCMV infection [6, 11, 15, 63, 64, 88].

2.1 NK Cell Responses in MCMV Infection

Resistance to MCMV infection is critically dependent on the activation of NK cells for early control of viral replication in target organs such as spleen and liver [11, 12, 14, 63, 77, 94], although T lymphocyte responses do get activated and contribute to viral clearance late in infection [35, 39, 69, 70]. The profound antiviral effects of NK cells against acute MCMV infection have been exemplified with a variety of experimental systems, including in vivo depletion of NK cells [14, 15, 63, 86], infection of beige mutant mice [66, 88], and most recently infection of mice deficient in NK cell, but not NKT or T cell, functions [50]. Together, these studies have demonstrated increased viral titers, liver pathology, and enhanced mortality upon elimination of functional NK cell responses. Conversely, resistance to MCMV infection has been demonstrated when NK cells were adoptively transferred into susceptible mice [16].

The mechanisms of NK cell-mediated defenses against MCMV infection in vivo have not been completely elucidated, although there is evidence that disparate NK cell responses are used in the spleen and liver for protection against MCMV infection. In the spleen, resistance seems to be mainly through perforin-dependent mechanisms, suggesting that NK cell-mediated cytotoxicity [94] and NK cell surface expression of an activating receptor [4, 23, 43, 101, 102], found on resistant strains of mice [4, 101], is required. In contrast, there is evidence that NK cell effector function in the liver is mediated through production of IFN-γ [52, 64, 77, 78, 94].

2.2 NK Cell Inflammatory Responses in Liver During MCMV Infection

The liver is a common target organ of MCMV infection [29, 61, 65, 87], and the rapid control of virus-induced disease is essential for survival [64, 65,

78, 80, 87]. As shown in Fig. 1a, the liver responds to MCMV infection by inducing a profound accumulation of inflammatory cells into the parenchyma that peaks between 48 and 72 h after infection of C57BL/6, 129SvEV, and T- and B cell-deficient C57BL/6-SCID mice [65, 77, 79]. Studies have identified NK cells as the major cellular constituents of the inflammatory foci [3, 22, 77]. As NK cells surround and sequester sites of MCMV antigen expression (Fig. 1b), it is highly conceivable that the inflammatory response limits the spread of infection to neighboring hepatocytes, thus minimizing virus-induced pathology. The precise effector mechanism used by inflammatory NK cells remains to be elucidated, although localization of IFN-γ within inflammatory sites ([77] and Sect.3) is critical to antiviral defense.

In vivo cell trafficking studies using fluorescently labeled bone marrow cells from either C57BL/6 or C57BL/6-SCID donor mice have demonstrated the rapid deployment and collective mobilization of NK cells between portal areas and central hepatic veins in MCMV-infected recipient mice [77]. Transfer studies using bone marrow donor cells from mice depleted of NK cell subsets using specific antibodies or from mice genetically deficient in NK cells and T lymphocytes demonstrated the accumulation of cells only in the liver sinusoids [77]. Thus, in vivo, NK cells are induced to migrate to sites in a pattern similar to the inflammatory foci observed in histological liver sections (see Fig. 1a).

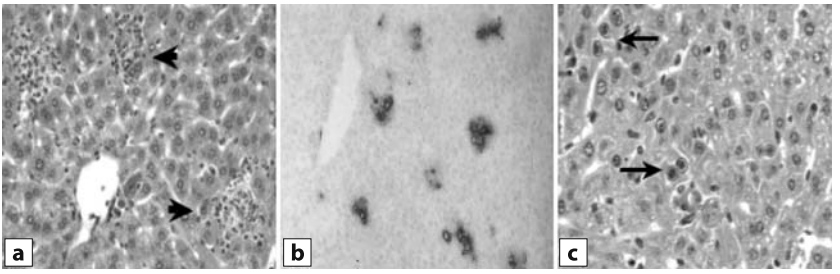

Fig. 1a–c Characterization of liver inflammatory foci during MCMV infection. **a** and **b** Paraffin-embedded or frozen livers sections were prepared from C57BL/6 mice infected with MCMV for 48 h and (**a**) stained with hematoxylin and eosin or (**b**) stained with immune serum antibodies to MCMV. Bound antibodies were detected with NBT/BCIP, followed by methyl green counterstain to highlight localization of inflammatory nuclei. *Arrowhead* denotes focus of inflammatory cells. **c** Paraffin-embedded liver sections were prepared from C57BL/6-MIP-1α deficient mice infected with MCMV for 48 h and stained with hematoxylin and eosin. *Arrow* denotes cytomegalic inclusion bodies

3
CCL3/MIP-1α: Primary Mediator of NK Cell Inflammation

To understand the key mechanisms governing the in vivo recruitment of NK cells to liver during MCMV infection, it is important to appreciate the chemokine expression profile. Of particular interest is the inflammatory chemokine CCL3 or macrophage inflammatory protein-1α (MIP-1α). Mice rendered genetically deficient in MIP-1α (MIP-1α knockouts) have been shown to exhibit reduced lung inflammation and delayed clearance of influenza virus when compared to control animals [19]. Moreover, MIP-1α promoted pulmonary inflammation and antiviral defense in a model of paramyxovirus infection [24]. During MCMV infection, MIP-1α messenger (m)RNA and protein can be detected in liver at times consistent with peak NK cell inflammation (48 h after infection) [77, 78]. Results obtained from in vivo cell trafficking studies demonstrated that the induction of NK cell inflammation in liver during MCMV infection was dependent on MIP-1α, as NK cell trafficking is dramatically impaired in MIP-1α knockout mice [77]. It is notable that monocyte and macrophage trafficking to liver is not affected in MIP-1α knockout mice, suggesting that although other chemokines are induced in these mice, MIP-1α responses are required for NK cell recruitment. Furthermore, MIP-1α knockout mice lacked detectable inflammatory foci in liver sections by histological evaluation, and displayed a profound increase in the number of cytomegalic inclusion bodies, or MCMV-infected cells (Fig. 1c) [77]. Flow cytometric analysis demonstrated that in the absence of MIP-1α function the absolute numbers of NK cells become significantly elevated in the blood but are dramatically reduced in the liver when compared to control mice [78]. These results imply that although NK cells get activated in response to infection, the communication signals directing their migration to tissue sites of virus replication are impaired in the absence of MIP-1α. Collectively, these studies define a prominent and unique role for MIP-1α in the initial influx of NK cells into the liver during MCMV infection.

3.1
MIP-1α and Antiviral Defenses: The Cytokine and Chemokine Networks

As discussed above, NK cells are an essential source of IFN-γ production that is required for early antiviral defense in liver. During MCMV infection, this cytokine is maximally elevated in spleen and serum within 40 h but subsides by 48 h after viral challenge [75, 78]. It has been established that an essential function of the MIP-1α-dependent NK cell inflammatory response is to sustain IFN-γ production in liver beyond the systemic kinetics of the cytokine, or further than 48 h [78]. MIP-1α knockout mice, with their

inability to mount an effective NK cell inflammatory response, do not sustain sufficient levels of IFN-γ in the liver. The result is a profound elevation in spleen and liver viral titers followed by death on day 5 of MCMV infection. Interestingly, mice genetically deficient in interleukin (IL)-18 do not succumb to MCMV infection, although they are severely compromised in systemic, but not hepatic, IFN-γ responses [68]. Thus, MIP-1α functions promote viral resistance by mediating the recruitment of NK cells and the delivery of IFN-γ in a localized site of infection.

3.2 The Type 1 Interferons (IFN-α/β) Association

Early infection with MCMV induces production of multiple innate cytokines including the type 1 interferons, IFN-α/β [1, 11, 12]. These cytokines are potent activators of antiviral pathways [12, 36, 46, 84, 50] as well as mediators of multiple immunomodulatory functions, including induction of MHC class I expression, activation of NK cell cytotoxicity, and modulation of cytokines and cytokine receptors, including regulation of other type 1 interferon genes [12, 84]. IFN-α/β expression has also been shown to affect leukocyte trafficking [33, 38, 76, 79]. During MCMV infection, bone marrow-derived macrophages and NK cells have been shown to migrate to secondary sites in response to IFN-α/β production [76]. Recent studies have demonstrated production of IFN-α/β in MCMV-infected livers [79]. Histological analysis of liver sections in mice unable to respond to functions induced by IFN-α/β as a result of mutation in the receptor for the cytokines (IFN-α/βR knockouts) illustrated the lack of inflammatory foci but the presence of extensive virus-induced pathology in liver [79]. Moreover, IFN-α/βR knockout mice had reduced levels of MIP-1α protein in liver. Accordingly, IFN-α/βR knockout mice exhibit profound decreases in the accumulation of NK cells in liver. Furthermore, these mice exhibit mortality by day 5 of MCMV infection [79]. It is notable that NK cell accumulation is not significantly affected when uninfected MIP-1α knockout mice are treated with recombinant IFN-α (rIFN-α), indicating that IFN-α/β mediate immunoregulatory events upstream of MIP-1α.

In vivo cell trafficking studies demonstrated that leukocyte migration to the liver is dependent upon the effects of IFN-α/β, as only donor-derived fluorescent-labeled cells from immunocompetent—but not IFN-α/βR knockouts—accumulated extensively in liver [79]. Additionally, IFN-α/β mediated the recruitment of macrophage populations that were identified as a major source of MIP-1α production [79]. Altogether, these studies identify IFN-α/β as key mediators in the production of MIP-1α, and they define a cellular delivery mechanism driven by innate cytokines and chemokines for regulation of NK cell inflammation.

3.3
The CXCL9/Mig Association

One downstream consequence of the MIP-1α-dependent NK cell inflammatory response is induction of the IFN-γ-inducible chemokine CXCL9/monokine-induced by interferon (Mig), a potent T cell chemoattractant [2, 47, 49, 100]. Mig production is severely reduced in MIP-1α knockout mice when compared to control mice [78]. Furthermore, treatment of immunocompetent mice with immune serum against Mig resulted in highly significant increases in viral titers in both spleen and liver [78]. Consistent with results obtained using mice deficient in IFN-α/β and MIP-1α functions, Mig was required for survival, as neutralization of the chemokine led to death by day 5 of infection [78]. Studies have identified a prominent T cell response in liver by day 5 after MCMV challenge [28, 39]. It is therefore plausible that early expression of MIP-1α by MCMV-induced IFN-α/β recruits NK cells into the liver, and through localization of IFN-γ and subsequent Mig induction, these cytokine and chemokine networks provide an important link of innate and adaptive immune responses for overall antiviral defenses in tissue compartments. Ongoing studies in our laboratory are evaluating this likely scenario under the conditions of MCMV infection in the liver.

4
CCL2/MCP-1: The First Link

As discussed above, IFN-α/β clearly plays a role in promoting the recruitment of MIP-1α-producing macrophages into liver, but the primary molecular mechanisms guiding this process have only recently been examined during MCMV infection. Studies have identified CCL2, or monocyte chemoattractant protein-1 (MCP-1), as a key intermediate of IFN-α/β activity for regulation of inflammatory responses in liver [30]. MCP-1 production can be induced at this site as early as 24 h after MCMV infection. Furthermore, MCP-1 production precedes that of MIP-1α in a temporal fashion (Fig. 2) [30]. IFN-α/βR knockout mice show dramatic decreases in the levels of MCP-1 protein when compared to immunocompetent mice. In addition, the treatment of uninfected immunocompetent mice with rIFN-α results in a significant release of MCP-1 protein [30]. In vitro stimulation of naïve liver leukocytes obtained from immunocompetent mice with rIFN-α was shown to generate a dose-dependent induction of MCP-1. Together, these studies clearly establish that the induction of MCP-1 protein in liver is dependent on IFN-α/β-mediated functions.

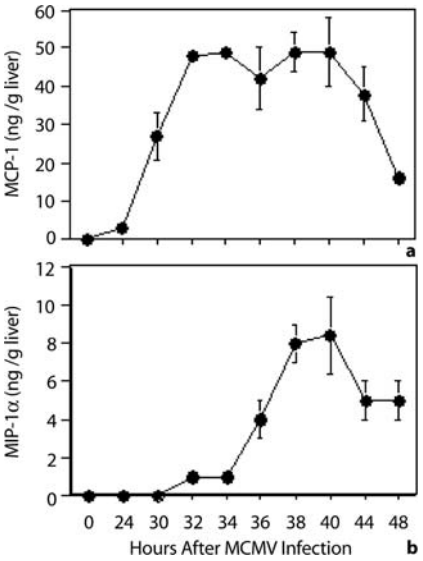

Fig. 2a, b Kinetics of MCP-1 and MIP-1α during MCMV infection in liver. Infected intraperitoneally with 5×10^4 plaque-forming units (pfu) MCMV were 129 mice. Livers were harvested from uninfected (0 h) or infected mice at the indicated time points. MCP-1 (**a**) and MIP-1α (**b**) protein levels in liver homogenates were determined by sandwich enzyme-linked immunosorbent assay (ELISA) [30]. The levels of detection were 0.08–0.2 and 0.02–0.05 ng/g liver for MCP-1 and MIP-1α, respectively. Data are the means±SE (n=3 mice tested individually for each time point). (Figure used with permission from [30]. Copyright 2005. The American Association of Immunologists)

4.1
Resident Macrophages: MCP-1 Producers

In multiple models of hepatic injury, resident macrophages have been shown to contribute to MCP-1 production [8, 44, 72]. The experiments highlighted in Sect. 4) strongly suggest that naïve liver leukocytes can be stimulated to release MCP-1 in the presence of IFN-α/β. Additional studies using enriched F4/80-positive and F4/80-negative cell populations—F4/80 being a mouse macrophage-restricted marker [45]—from uninfected immunocompetent mice demonstrated a clear induction of MCP-1 protein following stimulation with r-IFNα [30]. A similar induction of MCP-1 protein was observed with enriched F4/80-positive, but not F4/80-negative, cell populations from immunocompetent mice infected with MCMV for 24 h [30]. Thus, resident macrophages are early responders to the effects of IFN-α/β and are major producers of MCP-1 protein in liver.

5
MCP-1 and Macrophage Recruitment

It has been clearly established that MCP-1 effectively promotes the mobilization of inflammatory macrophages to sites of tissue damage [17, 26, 31, 51, 72] by preferential binding to the chemokine receptor CCR2 [13, 34, 40, 41, 67]. Recent studies have shown a dynamic impairment in liver macrophage and NK cell accumulation in mice deficient in MCP-1 (MCP-1 knockout) or CCR2 (CCR2 knockouts)—when compared to control mice—during infection with MCMV. Furthermore, MCP-1 and CCR2 knockout mice have decreased levels of both MIP-1α and IFN-γ proteins [30]. These results establish a central role for MCP-1 in promoting the recruitment of macrophages and NK cells. Moreover, they agree with previous observations that trafficking macrophages contribute to the initial release of MIP-1α, and subsequently the delivery of NK cell-derived IFN-γ in the liver [79, 80]. As CCR2 knockout mice displayed comparable responses, the results define MCP-1 as a key factor in initiating critical innate inflammatory events.

5.1
MCP-1 and Antiviral Defenses

It is clear from previous studies that MCP-1 is an important innate chemokine because it is uniquely necessary for monocyte and macrophage migration and the establishment of host defense against various pathogens [13, 18, 51, 31, 34, 40, 41, 85]. During MCMV infection, MCP-1 knockouts exhibit a marked elevation in spleen and liver viral titers by day 4 that remains prominent into day 5 of infection [30]. Comparable results were evident in CCR2 knockout mice. Increased viral burden was associated with increases in the circulating levels of the liver enzyme alanine aminotransferase, indicating liver damage. Accordingly, histological evaluation of liver sections prepared from uninfected control, MCP-1 and CCR2 knockout mice, did not show variability in appearance within the groups of mice (Fig. 3a–c). In contrast, on day 5 post-MCMV infection, MCP-1 or CCR2 knockout mice revealed large areas of necrosis as well as numerous cytomegalic inclusion bodies (Fig. 3e and f). Mortality in these mice coincided with virus-induced liver disease, as they succumbed to infection by day 5. This marked pathology was not evident in control mice. Instead, control mice displayed evidence of intermittent clusters of inflammatory foci, viral clearance, and were remarkably similar to the liver sections from the uninfected mice (Fig. 3a and d). These studies define a role for MCP-1, through interactions with CCR2, in promoting antiviral defense and protection from virus-induced liver disease.

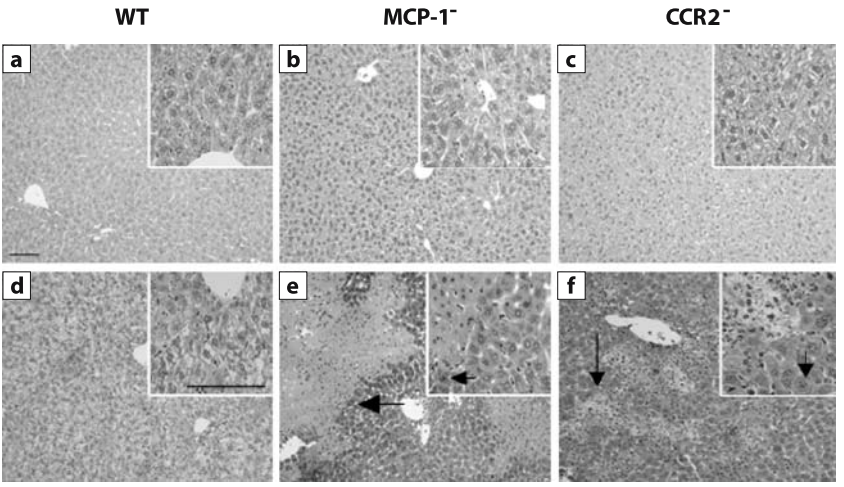

Fig. 3a–f Characterization of MCMV-induced liver damage in MCP-1- and CCR2-deficient mice. C57BL/6 (WT) (**a** and **d**) or mice genetically deficient in MCP-1 (MCP-1^{-}) (**b** and **e**) or CCR2 (CCR2^{-}) (**c** and **f**) were either uninfected (**a–c**) or infected with MCMV (5×10^4 PFU) for 5 days (**d–f**). Livers were harvested, paraffin was embedded, and they were sectioned for hematoxylin and eosin staining. *Bar* represents 100 μm. *Arrows* indicate necrotic lesions. *Arrows within insets* indicate cytomegalic inclusion bodies. Figure was used with permission from reference [30]. Copyright 2005. The American Association of Immunologists

6 Conclusions

The studies highlighted in this chapter define a network of cytokine and chemokine pathways that promote the activation, coordination, and shaping of the most effective immune response directed against a virus infection establishing itself in tissues. Specifically, the response elicits the migration of macrophages and NK cells by the selective induction of inflammatory chemokines (Fig. 4). The importance of chemokines to antiviral defense is underscored by the exploitation of the chemokine system by various human and mouse viruses, including herpesviruses, poxviruses, adenoviruses, and cytomegaloviruses [48, 53, 56, 60, 89]. Future in vivo studies dissecting the complex interactions between cytokines, chemokines, immune cell populations, and viruses will add to our understanding of immune regulation and perhaps the development of new therapeutic strategies for defense mechanisms against viral infections.

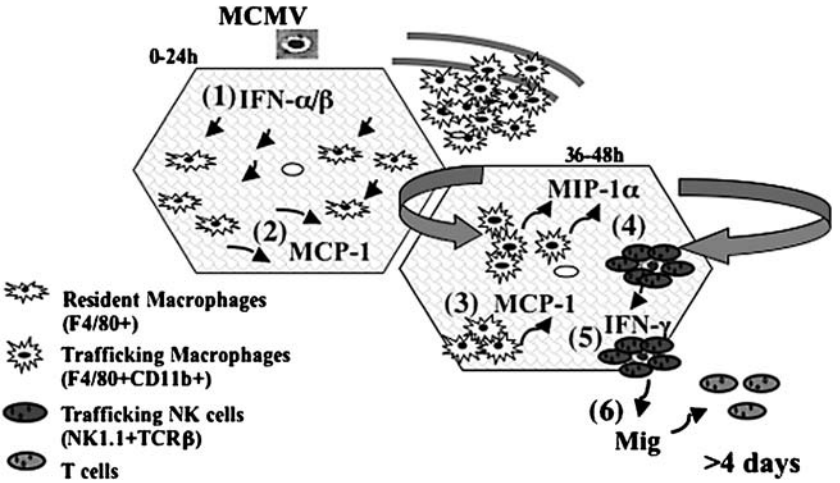

Fig. 4 Model of cytokine and chemokine interactions critical to antiviral defense during MCMV infection in liver. (*1*) The expression of the cytokine IFN-α/β is locally induced in response to MCMV challenge and (*2*) promotes the early release of MCP-1 from resident (F4/80+) macrophage populations. (*3*) MCP-1 initiates the recruitment of MIP-1α-producing macrophages from the periphery to the liver. (*4*) Subsequently, MIP-1α promotes the initial mobilization of NK cells from the periphery to the liver where they surround and sequester MCMV-infected cells to form characteristic clusters of inflammatory foci. (*5*) These events localize production of IFN-γ and (*6*) promote the induction of the IFN-γ-inducible chemokine Mig, a known potent chemoattractant of T lymphocytes. Together, these innate cytokine and chemokine interactions provide vital antiviral defenses in liver, and conceivably play a role in linkage of innate and adaptive immune responses

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