Differential TLR7-mediated cytokine expression by R848 in M-CSF- versus GM-CSF-derived macrophages after LCMV infection

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Abstract

Granulocyte-macrophage colony-stimulating factor (GM-CSF) and macrophage colony-stimulating factor (M-CSF) play an important role in macrophage (MΦ) development by influencing their differentiation and polarization. Our goal was to explore the difference between M-CSF- and GM-CSF-derived bone marrow MΦ responsiveness to TLR7-mediated signalling pathways that influence cytokine production early after infection in a model of acute virus infection. To do so, we examined cytokine production and TLR7-mediated signalling at 1 h post-lymphocytic choriomeningitis virus (LCMV) Armstrong (ARM) infection. We found that R848-induced cytokine expression was enhanced in these cells, with GM-CSF cells exhibiting higher proinflammatory cytokine expression and M-CSF cells exhibiting higher anti-inflammatory cytokine expression. However, R848-mediated signalling molecule activation was diminished in LCMV-infected M-CSF and GM-CSF macrophages. Interestingly, we observed that TLR7 expression was maintained during LCMV infection of M-CSF and GM-CSF cells. Moreover, TLR7 expression was significantly higher in M-CSF cells compared to GM-CSF cells. Taken together, our data demonstrate that although LCMV restrains early TLR7-mediated signalling, it primes differentiated MΦ to enhance expression of their respective cytokine profiles and maintains levels of TLR7 expression early after infection.

INTRODUCTION

Macrophages (MΦ) are phagocytic cells that serve important roles in anti-viral immunity. In response to acute virus infection MΦ are excellent producers of cytokines that, if not adequately controlled, can lead to harmful effects. Generally, MΦ are activated by a variety of stimuli, which can lead to their polarization into what was originally defined as classically activated M1 MΦ or alternatively activated MΦ, M2 [1–8]. M1 MΦ typically secrete proinflammatory cytokines, such as IL-12, IL-23 and TNF-α [9], and these cells also exhibit an increase of phagocytic activity, the induction of autophagy and production of nitric oxide [4]. On the other hand, exposure to cytokines such as IL-4, IL-10, or IL-13 shifts MΦ polarization towards an M2 phenotype [10].

MΦ cultured in granulocyte macrophage colony-stimulating factor (GM-CSF) have been found to be closely related to M1 MΦ, based on cytokine expression [6, 7, 11–14]. Monocytes treated with macrophage-colony stimulating factor (M-CSF) differentiate into MΦ, which express anti-inflammatory cytokines such as IL-10 and are characterized by enhanced arginase-1 production over nitric oxide [15]. It should be noted that M1 and M2 MΦ can share markers such as CD11c, CD11b and MHCII [14, 16–18], reflecting the spectrum of MΦ activation [4]. Both M-CSF and GM-CSF play roles in immune responses to virus infection, thus these cytokines may influence the activity of MΦ during virus infection, skewing the cytokine response to either a proinflammatory or an anti-inflammatory profile.

Lymphocytic choriomeningitis virus (LCMV) is a natural mouse pathogen, which infects and replicates in MΦ [14, 19, 20], as well as in dendritic cells (DCs) [21, 22]. LCMV infection in vivo induces M1 activation, as illustrated by the expression of iNOS and YM-1 in splenic MΦ after viral infection [23]. Innate immune responses to LCMV infection are led by a variety of pattern recognition receptors (PRRs). Of these, TLR7 is a key endosomal PRR that recognizes ssRNA. With respect to LCMV infection, TLR7 knockout mice (TLR7−/−) are unable to induce type I IFN in high levels in response to the acute LCMV-WE strain (LCMV-WE) [24], which indicates the importance of TLR7 during viral infection. Although TLR7−/− mice clear LCMV-Armstrong...
We used a model in M-CSF- and GM-CSF-derived MФ in infection [25], indicating a requirement for TLR7-mediated LCMV clone 13 (LCMV CL13; model of chronic infection) used for mock infection conditions. Johnson detection of LCMV NP by flow cytometry as described by (Hamburg, Germany). Virus titration was carried out with 10 % FCS, originally obtained from F. Lehmann-Grube. The LCMV ARM strain was propagated in baby hamster fibroblast cells, purchased from JAX Labs (Bar Harbor, ME, USA) were used. All procedures were carried out in accordance with the guidelines of the Canadian Council of Animal Use and Queen's University animal ethics procedures. Media [RPMI or Dulbecco’s modified Eagle’s medium (DMEM), 10 % foetal calf serum (FCS)] for cell culture were purchased from Invitrogen (Ontario, Canada).

**Methods**

**Mice and media**

Mice were used as a source for bone marrow-derived MФ (BMDMs). Specifically, 6–8-week-old C57BL/6 (H-2b) purchased from JAX Labs (Bar Harbor, ME, USA) were used. All procedures were carried out in accordance with the guidelines of the Canadian Council of Animal Use and Queen's University animal ethics procedures. Media [RPMI or Dulbecco’s modified Eagle’s medium (DMEM), 10% foetal calf serum (FCS)] for cell culture were purchased from Invitrogen (Ontario, Canada).

**Virus preparation**

The LCMV-ARM strain was propagated in baby hamster kidney (BHK) fibroblast cells, in DMEM supplemented with 10% FCS, originally obtained from F. Lehmann-Grube (Hamburg, Germany). Virus titration was carried out by detection of LCMV-NP by flow cytometry as described by Johnson et al. [30]. Media from uninfected BHK cells were used for mock infection conditions.

**Macrophage preparations**

Bone marrow was flushed from femurs and tibia with phosphate-buffered saline (PBS). Cells were then resuspended in lysis buffer (1.66% ammonium chloride) for 5 min to lyse red blood cells. Cells were cultured in six-well tissue culture plates with RPMI supplemented with 10% FCS (Fisher Scientific, USA), 50 µg ml⁻¹ gentamycin and 20% supernatant from GM-CSF-secreting X63Ag8 cells or 20% supernatant from M-CSF-secreting L929 fibroblasts. After 3 days, non-adherent cells were removed from both GM-CSF- and M-CSF-conditioned media. BMDMs were generated and the adherent cells were harvested on day 7 post-culture. The phenotypic characteristics of M-CSF or GM-CSF MФ were evaluated by flow cytometry.

**Infection and stimulation of MФ**

BMDMs (M-CSF MФ or GM-CSF MФ) were infected with LCMV-ARM [multiplicity of infection (m.o.i.)=3] or mock control media for times ranging from 1 to 6 h. MФ were also infected with LCMV-ARM for 1 h, followed by stimulation with R848 (resiquimod, 1 µg ml⁻¹ or 5 µg ml⁻¹), and cultured in RPMI with 5% FCS at 37°C for various time points. Cell pellets were collected for flow cytometry or Western blotting and cell-free supernatant was collected for enzyme-linked immunosorbent assay (ELISA).

**Flow cytometry analyses**

Flow cytometry analyses of M-CSF MФ and GM-CSF MФ were performed after 7 days in culture. For surface marker staining, cells were stained for 20 min at 4°C with fluorochrome-labelled anti-mouse Abs (Biolegend, USA) specific for CD11b (clone M1/70-PE) and F4/80 (clone BM8-PE/Cy5). Detection of TLR7 was done by intracellular staining; cells were fixed with 1% paraformaldehyde (PFA) for 20 min, washed twice in PBS and then permeabilized with 0.1% saponin for 25 min before incubation with PE-conjugated anti-mouse TLR7 (clone A94B10; BD Pharmingen, USA) for 25°C at room temperature. Flow cytometry analysis was performed using the CytoFLEX flow cytometer (Beckman Coulter, USA) and analysed using FlowJo software. The geometric mean fluorescent intensity values are indicated for each histogram.

**Western blotting**

M-CSF MФ and GM-CSF MФ were infected with mock or LCMV for 1 h, and then stimulated with R848 for 15 min. Cells were then harvested and pellets were incubated in lysis buffer (1 M HEPES, 0.5 M NaF, 0.5 M EGTA, 2.5 M NaCl, 1 M MgCl₂, 10% glycerol, 1% Triton X-100) with PhosSTOP phosphatase inhibitor (Roche, Switzerland). The Bradford assay (BioRad Laboratories, USA) was used to determine protein concentration. Lysates were subjected to electrophoresis on 10% polyacrylamide SDS-PAGE and transferred to polyvinylidene difluoride membrane (BioRad Laboratories). Membranes were probed with the following primary antibodies: rabbit anti-phospho-NF-κB (Santa Cruz Biotechnology, USA), anti-phospho-p42/44, anti-phospho-p38 (Cell Signaling Technologies, USA) and...
secondary antibody: goat anti-rabbit-HRP (Santa Cruz Biotechnology). Membranes were stripped and reprobed with rabbit anti-NF-κBp65, anti-pan p38, or anti-p42/44 (Santa Cruz Biotechnology). All membranes were visualized with Clarity Western ECL Substrate (Bio-Rad, USA) and imaged and quantified using an Alpha Innotech FluorChem HD2 system using AlphaView software version 3.1.0.0. To obtain fold change data, the densitometry of phospho-specific bands was first normalized to respective pan bands and the mock medium control for each of M-CSF and GM-CSF cells was used to calculate the fold change.

ELISA
ELISA was performed on cell supernatants as per the manufacturer’s instructions using mouse Invitrogen ELISA kits specific for IL-12p70, IL-12/23p40, TNF-α, IL-6 and IL-10 (Thermo Fischer Scientific, USA). Absorbance was read at 450 nm using a BioTek EL800 Plate Reader (Thermo Fisher Scientific) using Gen5 software version 1.0.4.5. Data were analysed using GraphPad Prism 8 and are shown as the average±sd from a minimum of three different experiments.

RESULTS
M-CSF- and GM-CSF-derived MΦ express F4/80 and CD11b surface markers and are readily infected with LCMV
Throughout this study, we used bone marrow-derived cells differentiated in the presence of either M-CSF or GM-CSF, resulting in differentially polarized MΦ. Initially, we wanted to confirm that the cells express the well-characterized MΦ markers F4/80 and CD11b. As expected, M-CSF-derived cells show higher levels of F4/80 expression compared to GM-CSF MΦ (Fig. 1) [12, 14, 16, 18].

To confirm that M-CSF and GM-CSF cells are permissive to LCMV infection, we cultured the MΦ with LCMV-ARM at an m.o.i. of 3, and tested for de novo synthesis of LCMV-NP. We incubated the virus with M-CSF MΦ and GM-CSF MΦ for 60 min to allow for absorption and infection to occur. We then cultured the cells for 24 h at 37 °C to ensure sufficient time for LCMV-NP to accumulate to detectable levels [31, 32]. Following staining for LCMV-NP, flow cytometry was used to measure the percentage of LCMV-NP-positive cells (Fig. 2). The data show that LCMV infected 93.3% of M-CSF MΦ (Fig. 2a; MED MFI: 6950, LCMV MFI: 9600), compared to 66.1% of the GM-CSF MΦ (Fig. 2b; MED MFI: 7640, LCMV MFI: 4550). Thus, the majority of both types of MΦ were infected with LCMV.
M-CSF- and GM-CSF-derived MΦ exhibit disparate cytokine responses early post-LCMV infection

We previously examined the influence of LCMV infection on GM-CSF and M-CSF MΦ after 6 and 24 h of infection [14]. Herein, we assessed the rapidity of the cytokine response to infection by examining earlier time points of 1 and 3 h and included the 6 h time point as a control (Fig. 3). We confirmed that GM-CSF cells displayed a more inflammatory cytokine profile compared to M-CSF cells. At time points as early as 1 h, GM-CSF MΦ exhibited enhanced TNF-α and IL-12/23p40 and by 3 h, enhanced IL-6 expression (Fig. 3a–c). In contrast, M-CSF MΦ cells under the same conditions exhibited higher levels of the pro-inflammatory cytokine, IL-10, by the 3 h time point (Fig. 3d). As expected, GM-CSF cells exhibited greater TNF-α, IL-6 and IL-12/23p40 levels compared to M-CSF cells (Fig. 3a–c). At the 6 h time point, LCMV infection induced a low, but significant, amount of IL-10 from GM-CSF cells (Fig. 3d). Taken together, these results indicate that as early as 1 h post-infection, changes in cytokine secretion levels are detectable; therefore, we chose this time point for analysis of TLR7 responsiveness during very early LCMV infection in subsequent experiments.

LCMV infection primes MΦ to secrete cytokines in response to subsequent TLR7 stimulation

To evaluate how GM-CSF MΦ and M-CSF MΦ respond to TLR7 ligation very early after virus infection, differentiated cells were either infected with LCMV or mock for 1 h and then stimulated with R848 for an additional 1, 3 and 6 h. Cell-free supernatants were collected and analysed by ELISA (Fig. 4). Overall, GM-CSF cells expressed higher levels of the proinflammatory cytokines TNF-α, IL-6, IL-12/23p40 and IL-23 in response to R848 in the presence or absence of LCMV compared to M-CSF cells (Fig. 4a–d). Interestingly, IL-12p70 was not detected in any condition (Fig. 4e). However, differential responses to R848 treatment in mock- and LCMV-infected cells with respect to proinflammatory cytokine expression were observed. M-CSF MΦ produced low levels of TNF-α in response to virus infection alone and also in response to LCMV infection followed by R848 stimulation (Fig. 4a). However, R848 stimulation of LCMV-infected GM-CSF MΦ induced TNF-α to significantly higher levels compared to R848-treated GM-CSF MΦ mock controls as well as untreated (MED) and R848-uninfected GM-CSF MΦ controls (Fig. 4a). GM-CSF MΦ produced significantly higher levels of TNF-α and IL-6 in both mock- and LCMV-infected cells in response after 3 and 6 h of R848 stimulation.

IL-6 exhibited differential responses to R848 stimulation in M-CSF and GM-CSF cells (Fig. 4b). In M-CSF cells, LCMV infection followed by R848 stimulation resulted in the induction of IL-6 expression, but expression of this cytokine was not detected in response to LCMV alone. In contrast, in GM-CSF cells, LCMV infection alone induced IL-6 expression, and in response to R848 alone, these cells produced significantly higher levels of IL-6. LCMV-infected cells at 1 h post-R848 stimulation exhibited significantly higher levels of IL-6; however, these cells exhibited significantly reduced IL-6 production by 6 h post-R848 stimulation compared to mock-infected cells (Fig. 4b). Similar to TNF-α, GM-CSF MΦ produced significantly higher levels of IL-6 in both mock- and
LCMV-infected cells in response to R848 stimulation (3 and 6 h).

IL-12/23p40 was produced by M-CSF MΦ in response to LCMV infection (Fig. 4c), while in GM-CSF MΦ, LCMV infection resulted in significantly elevated levels compared to mock control. The combination of virus infection and R848 treatment primed GM-CSF MΦ to significantly elevate IL-12/23p40 levels compared to media-treated cells (Fig. 4c). In comparison to M-CSF cells, GM-CSF cells expressed significantly higher levels of IL-12/23p40 in cells infected with LCMV in the presence and absence of R848 stimulation. In contrast to IL-12/23p40, IL-23 was only induced by R848-treated GM-CSF MΦ cells, where it was expressed at significantly higher levels compared to media- and mock-infected controls (Fig. 4d).

Expression of the anti-inflammatory cytokine IL-10 was significantly higher in LCMV-infected M-CSF cells compared to mock infection and further stimulation with R848 significantly enhanced the production of IL-10 in LCMV-infected M-CSF cells (Fig. 4f). Surprisingly, R848 and the combination of virus infection and R848 treatment primed GM-CSF MΦ to express similar amounts of IL-10 and these levels reached comparable levels to those for LCMV-infected M-CSF cells stimulated with R848 (Fig. 4f). Taken together, these results indicate that GM-CSF cells are more responsive to R848 stimulation with respect to both proinflammatory and anti-inflammatory cytokine expression, while M-CSF cells are more responsive to R848 stimulation with respect to anti-inflammatory cytokine expression.

R848-mediated signalling is blocked in LCMV-primed cells

Since we observed differential regulation of cytokine expression in response to LCMV infection and R848 stimulation in the two cell types, we examined whether LCMV infection could influence the TLR7 responsiveness to R848.

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**Fig. 3.** Infection with LCMV causes differential cytokine production in M-CSF- and GM-CSF-derived MΦ. Murine bone marrow-derived MΦ cultured in M-CSF or GM-CSF for 7 days were exposed to media conditions, mock infection, or LCMV infection for 1, 3, or 6 h before the supernatants were harvested. Supernatants were harvested for cytokine analysis by ELISA: TNF-α (a), IL-6 (b), IL-12/23p40 (c) and IL-10 (d). Data are representative of at least three independent biological replicates and are presented as the mean±sd of three technical replicate wells. Statistical significance for differences in cytokine expression between mock- and LCMV-infected cells was calculated using a two-way ANOVA with Tukey’s multiple comparisons test. *P≤0.05, **P≤0.01, ***P≤0.001, ****P≤0.0001.
Fig. 4. GM-CSF MΦ exhibit enhanced proinflammatory cytokine expression in response to LCMV infection and R848 stimulation. GM-CSF MΦ and M-CSF MΦ were either infected with LCMV (m.o.i.=3) or mock and then stimulated with R848 (1 µg ml\(^{-1}\)) for 1 h, 3 h and 6 h. GM-CSF and M-CSF cell-free supernatants were collected for the measurement of TNF-α (a), IL-6 (b), IL-12/23p40 (c), IL-23 (d), IL-12p70 (e) and IL-10 (f) by ELISA. Data presented are the mean±sd of three independent biological replicates, each with three technical replicate wells. Statistical significance for differences in cytokine expression between R848 treated and untreated cells was calculated using Tukey’s multiple comparisons test. To determine statistical significance between mock- and LCMV-infected cells, Bonferroni’s multiple comparisons were performed. *P≤0.05, **P≤0.01, ***P≤0.001, ****P≤0.0001.
Differentiated cells were infected with LCMV or mock for 1 h, followed by stimulation with R848 for 15 min. Immunoblots were used to evaluate the phosphorylation and expression of p38, p42/44 and NF-κBp65 (Fig. 5). As expected, mock-infected M-CSF and GM-CSF cells exhibited significant upregulation of phosphorylated p38, p42/44 and NF-κBp65 in response to R848 stimulation, indicating that both cell types are capable of signalling in response to R848.

In LCMV-infected M-CSF and GM-CSF cells, R848-mediated phosphorylation of p38 and p42/44 was significantly reduced compared to mock conditions, although not completely abolished. Similarly, R848-mediated phosphorylation of NF-κBp65 was significantly reduced in LCMV-infected GM-CSF cells and abolished in M-CSF cells. Taken together, these results indicate that LCMV infection dampens TLR7-mediated p38, p42/44 and NF-κB signalling in M-CSF and GM-CSF cells.

**LCMV infection enhances TLR7 expression in GM-CSF cells, while R848 reduces TLR7 expression in GM-CSF and M-CSF cells**

Since we observed that R848 treatment and the combination of virus infection and R848 treatment modulated cytokine production and TLR7-mediated signalling, we next examined if these parameters could influence TLR7 expression. Therefore, cells were either infected with LCMV or mock, treated with R848 or left in media for 1, 3, or 6 h followed by intracellular staining for TLR7. Analysis of differentiated cells cultured in media or mock control conditions revealed that M-CSF cells expressed significantly higher levels of TLR7 compared to GM-CSF cells (Fig. 6a, b, top 2 rows, and c). M-CSF cells infected with LCMV maintained relatively high TLR7 levels, whereas the levels in M-CSF cells were lower across all time points (Fig. 6a, b, row 4). In LCMV-infected M-CSF and GM-CSF cells, TLR7 expression was higher.
Fig. 6. Flow cytometry analyses of TLR7 expression in M-CSF MΦ and GM-CSF MΦ. M-CSF (a) and GM-CSF MΦ (b) cells were fixed and permeabilized prior to intracellular staining for TLR7 (shaded grey histogram). The geometric mean fluorescence intensity (MFI) of TLR7 expression is indicated in the top right corner of each histogram. Isotype controls are indicated by white histograms. Data shown are representative of three independent biological replicates. The average MFI±sd (c) was calculated from three biological replicates. Statistical significance for differences in TLR7 expression between R848-treated and untreated cells was calculated using Tukey’s multiple comparisons test. To determine statistical significance between mock- and LCMV-infected cells, Bonferroni’s multiple comparisons were performed. *P≤0.05, **P≤0.01, ***P≤0.001, ****P≤0.0001.
compared to mock cells (Fig. 6a, b, bottom row), but these differences in expression do not reach statistical significance (Fig. 6c). These results indicate that LCMV infection primes the cells to retain TLR7 expression even in the presence of R848.

**DISCUSSION**

In this report, we demonstrate that LCMV infection at an early time point of 1 h can influence the MΦ response to further TLR7 activation. We observed that although LCMV infection partially reduces TLR7-mediated signalling in M-CSF and GM-CSF, TLR7 expression levels are maintained. Furthermore, we found that MΦ primed by LCMV infection expressed higher levels of cytokines in response to R848 stimulation. Specifically, GM-CSF cells expressed higher levels of the proinflammatory cytokines TNF-α, IL-6, IL-12/23p40 and IL-23, and the anti-inflammatory cytokine IL-10, while M-CSF cells expressed higher levels of IL-10 and only low-to-undetectable levels of TNF-α, IL-6, IL-12/23p40 and IL-23.

Under homeostatic conditions, M-CSF is expressed in circulation and produced by diverse cells, such as endothelial cells, fibroblasts and MΦ, while GM-CSF is induced under inflammatory conditions, such as arthritis and atherosclerosis [3–7, 12]. M-CSF and GM-CSF expression is also relevant to the control of ssRNA virus infection, where they are being considered as potential therapeutics for viruses such as HIV and influenza A [3, 33]. For instance, high levels of GM-CSF in the lungs are protective against influenza A infection, and can moderate M1 MΦ-mediated inflammation [33].

Typically, GM-CSF and M-CSF have the ability to differentiate MΦ into opposing phenotypes, whereby M-CSF MΦ tend to have an M2 MΦ phenotype, and GM-CSF MΦ tend to have an M1 MΦ phenotype [12, 14, 16, 17, 20, 23, 34, 35]. Previously, LCMV infection was shown to increase expression of M1 MΦ markers such as iNOS and YM-1 expression, indicating that LCMV infection promotes M1 differentiation [23]. However, M2 MΦ were represented in a high percentage of total MΦ population during chronic LCMV infection [36]. Our data measuring LCMV-NP expression demonstrate that M-CSF MΦ exhibited greater levels of LCMV infection and replication compared to GM-CSF MΦ; however, despite this, both MΦ types expressed higher levels of R848-induced cytokines in the presence of LCMV infection. This indicates that the virus infection primes the cells for TLR7 responsiveness, supporting the notion that LCMV may promote cytokine induction from pre-existing M1- and M2-polarized cells. In other words, M-CSF MΦ and GM-CSF MΦ are primed by LCMV infection for enhanced TLR7-mediated induction of anti-inflammatory and proinflammatory cytokines.

Recently, we compared cytokine production by MΦ at later time points (6–24 h) in response to LCMV-CL13 and LCMV-ARM, which cause chronic and acute infection in vivo, respectively. This study demonstrated that LCMV-CL13 induced lower levels of IL-6 and IL-10 in comparison to LCMV-ARM in GM-CSF MΦ and M-CSF MΦ, respectively, but induced higher levels of TNF-α in GM-CSF MΦ [14]. Overall, GM-CSF MΦ responded to LCMV infection by producing a stronger proinflammatory cytokine response compared to M-CSF MΦ. In contrast, the anti-inflammatory cytokine IL-10 was significantly induced at high levels by M-CSF MΦ [14]. Our current study expands on these results by demonstrating that early after LCMV infection, enhanced sensitivity to TLR7-mediated induction of proinflammatory and anti-inflammatory cytokines from GM-CSF MΦ and anti-inflammatory cytokine expression from M-CSF MΦ occurs.

We demonstrated that IL-23 was only produced by GM-CSF MΦ in response to LCMV infection followed by R848 stimulation, while IL-12p70 was not produced under these conditions. It was unexpected that LCMV infection followed by R848 stimulation did not induce IL-12p70 by MΦ in vitro because GM-CSF MΦ have the capability to express this cytokine in response to LPS stimulation. LPS-mediated TLR4 signalling activates both the MyD88-dependent and MyD88-independent pathways, while R848-mediated TLR7 signalling activates the MyD88-dependent signalling cascade. Therefore, R848 might induce IL-23 expression in response to LCMV infection via a MyD88-dependant pathway but not IL-12p70. Basal IL-12p40 mRNA levels are increased in GM-CSF MΦ during differentiation [12] and our data indicate that in GM-CSF MΦ, IL-12p40 was induced by LCMV infection alone and R848 alone, and was further enhanced in response to R848 in LCMV-infected cells. Furthermore, given that IL-23 is composed of the p19 and p40 subunits and IL-12p70 is composed of p35 and p40 subunits, it is likely that p19 and p35 expression are differentially regulated in response to LPS versus R848.

Our finding that R848 stimulation alone, as well as LCMV infection followed by R848 stimulation of M-CSF MΦ, induced significant levels of IL-10 is supported by a previous study showing that LPS-induced IL-10 was detected in M-CSF MΦ [12]. However, this study also showed that LPS-induced IL-10 expression from M-CSF MΦ was greater than LPS-induced IL-10 expression from GM-CSF MΦ [12]. Interestingly, we show that R848 stimulation enhanced IL-10 production by GM-CSF MΦ after LCMV infection as well as mock treatment to comparable levels to those observed in LCMV-infected, R848-treated M-CSF MΦ. This highlights the differential response between LPS and R848, and indicates that GM-CSF MΦ could be shifted towards an anti-inflammatory or M2 phenotype via TLR7 signalling. Taken together, engagement of TLR7 post-viral infection may play a role in producing anti-inflammatory cytokines such as IL-10, which could lead to a return to homeostasis by controlling the production of proinflammatory cytokines.

MΦ express a variety of TLRs that recognize and respond to LCMV-derived PAMPs. Among them, TLR7 is specific to single-stranded RNA, an indicator of viral infection [37]. TLR7−/− mice lack sufficient type I IFN production in response to acute LCMV-WE infection [24], indicating that TLR7 can
play a crucial role during viral infection. Furthermore, TLR7−/− mice clear acute LCMV-ARM infection, but not the chronic LCMV-CL13 [25], indicating that TLR7 may play different roles in acute versus chronic viral infection. Upon activation, downstream TLR7 signalling leads to the activation of MAPK molecules and NF-κB, in addition to IRF molecules and type I IFN responses [38–41]. TLR7-mediated MAPK and NF-κB signalling typically lead to the production of proinflammatory and anti-inflammatory cytokines by MΦ [42–44]. Activation of p38 is responsible for the induction of the proinflammatory cytokines IL-6, TNF-α and IL-12 in activated MΦ, while ERK controls that of IL-10 [44–46].

Interestingly, our data indicate that TLR7-mediated signalling through the p38 and ERK MAPK pathways as well as the activation of NF-κB are partially inhibited in LCMV-primed cells, although, despite this inhibition, cytokine production in response to TLR7 was maintained at robust levels. Maintenance of TLR7 expression in cells primed with LCMV may account for the availability of R848–TLR7 interactions, allowing for increased cytokine expression in M-CSF and GM-CSF cells. Our data suggesting that R848 treatment of mock-infected cells may decrease TLR7 activity agree with other data showing that treatment of peripheral blood monocytes with R848 decreased TLR7 expression [47].

In summary, we demonstrate that R848 triggered GM-CSF MΦ to induce high levels of proinflammatory cytokines in the presence or absence of LCMV compared to classical M-CSF MΦ. Interestingly, we found that the anti-inflammatory cytokine IL-10 was induced at high levels in response to R848 in GM-CSF MΦ, reaching similar levels to those in M-CSF MΦ. We also showed that LCMV infection partially decreased activation of TLR7-mediated signalling, while maintaining TLR7 expression. These findings provide new insight into how RNA viruses modulate inflammatory responses, with TLR7 ligation rendering a pronounced cytokine response, suggesting that very early exposure to virus infection functions to prime MΦ-led responses.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

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