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Pathogenic Old World Arenaviruses Inhibit TLR2/Mal-Dependent Proinflammatory Cytokines *In Vitro*

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Lymphocytic choriomeningitis virus (LCMV), the prototype arenavirus, and Lassa virus (LASV), the causative agent of Lassa fever (LF), have extensive strain diversity and significant variations in pathogenicity for humans and experimental animals. The WE strain of LCMV (LCMV-WE), but not the Armstrong (Arm) strain, induces a fatal LF-like disease in rhesus macaques. We also demonstrated that LASV infection of human macrophages and endothelial cells resulted in reduced levels of proinflammatory cytokines. Here we have shown that cells infected with LASV or with LCMV-WE suppressed Toll-like receptor 2 (TLR2)-dependent proinflammatory cytokine responses. The persisting isolate LCMV clone 13 (CL13) also failed to stimulate interleukin-6 (IL-6) in macrophages. In contrast, nonpathogenic Mopeia virus, which is a genetic relative of LASV and LCMV-Arm induced robust responses that were TLR2/Mal dependent, required virus replication, and were enhanced by CD14. Superinfection experiments demonstrated that the WE strain of LCMV inhibited the Arm-mediated IL-8 response during the early stage of infection. In cells transfected with the NF-κB–luciferase reporter, infection with LCMV-Arm resulted in the induction of NF-κB, but cells infected with LCMV-WE and CL13 did not. These results suggest that pathogenic arenaviruses suppress NF-κB-mediated proinflammatory cytokine responses in infected cells.

The Old World (OW) group of the *Arenaviridae* includes the prototypic lymphocytic choriomeningitis virus (LCMV) and Lassa virus (LASV), the causative agent of Lassa fever (LF), which is the most prevalent viral hemorrhagic fever (VHF) in West Africa (20). In addition, the OW group contains LCMV-related Dandenong virus and African arenaviruses, including Mopeia, Mobala, Ippy, and Morogoro viruses (52). Mopeia virus (MOPV) is a nonpathogenic relative of LASV. Both viruses are hosted by the native multimammate rat *Mastomys natalensis* and can produce live reassortants after the coinfection of cells *in vitro* (34). In 2008, an LF-like outbreak in South Africa uncovered a novel highly pathogenic OW arenavirus, Lujo virus (12), bearing some features of New World arenaviruses (Tacaribe serocomplex) circulating in South and North America (52).

LASV and LCMV have extensive strain diversity, with remarkable genetic and biological variations. LCMV infection in humans results in a wide range of outcomes, from asymptomatic infection to aseptic meningitis and even death (3, 10, 11, 21, 30). An LF-like disease in immunocompromised transplant patients infected with LCMV through donor tissues has been documented (21). Previously, we showed (18, 35–37) that rhesus macaques infected with the WE strain of LCMV developed a fatal LF-like disease, providing a valuable model for the study of LF pathogenesis and vaccine development (25, 62). The Armstrong (Arm) strain of LCMV (LCMV-Arm) shares 88% amino acid homology with WE (17) but does not induce disease in monkeys.

Two major risk factors that predict a fatal outcome for LF patients and experimentally infected primates are high-level viremia and elevated plasma aspartate transaminase (AST) levels (44). In contrast to filovirus VHF (23), in LF patients, the profound shock, vascular damage, and hemorrhage in late-stage victims of Old World arenaviruses are not associated with a "cytokine storm." In fact, we (15, 35, 38) and others (5–7, 27, 39, 40) showed that LASV replication *in vitro* and *in vivo* is associated with the suppression of proinflammatory responses, impaired activation of innate immune cells, and delayed cytotoxic T cell responses, contributing to unchecked viremia and fatal outcomes.

Toll-like receptors (TLRs), the retinoic acid-inducible gene I (RIG-I)-like helicases (RLHs), RIG-I, and melanoma differentiation-associated gene 5 (MDA5) are the major pattern recognition receptors (PRR) of animal cells recognizing RNA viruses during the early stage of infection (2, 45, 57). Recent studies also showed that TLR2-MyD88-dependent signaling is involved in the induction of proinflammatory cytokines in tissue cultures and innate and adaptive immune responses during acute LCMV infection (31, 63–65). The main goal of this study is to gain insights into the immunosuppressive phenotype of pathogenic OW arenaviruses, comparing viruses with different pathogenic potentials for humans (LASV versus MOPV) and for nonhuman primates (LCMV-WE versus LCMV-Arm), in the context of the TLR2-MyD88-mediated signaling recently described for LCMV (65).

MATERIALS AND METHODS

Cells and viruses. THP-1, HEK293T, HEK293T-TLR2, and HEK293T-TLR4 cells were provided by A. Medvedev and were cultivated as previously described (50). Immortalized bone marrow-derived macrophages (iBMDM) from wild-type (WT) C57BL6/J mice (NR-9456), TLR2 knockout (KO) mice (NR-9457), CD14 KO mice (NR-9570), and Mal KO mice (NR-9459) were obtained through the NIH Biodefense and Emerging Infections Research Resources Repository (BEI Resources). HEK293T, Vero, and BHK21 cells were maintained in 1× minimal essential medium

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(MEM), supplemented with 10% fetal bovine serum (FBS), in a humidified chamber at 37°C in 5% CO_2. THP-1 and iBMDM cells were maintained in complete RPMI, supplemented with 10% FBS, in a humidified chamber at 37°C in 5% CO_2. Cells were infected with LCMV-Arm strain 5, LCMV-Arm clone 13 (CL13) (a persisting isolate of LCMV-Arm5), LCMV-WE strain 54, MOPV (AN20410), or LASV (Josiah) at a multiplicity of infection (MOI) of 1 PFU/cell for 1 h at 37°C. Virus stocks (5×10^6 PFU/ml) were harvested in cell-free medium and kept at −80°C before use. The stocks did not contain detectable levels of cytokines, as measured by a multiplex immunoassay (Bio-Rad). Simultaneously, control cells were treated with serum-free medium as a mock infection control or with 100 μg/ml of Pam_3CSK4 (P3C) as a positive control (TLR1/2 ligand). In some experiments, lipoteichoic acid (LTA), a TLR2/6 agonist, was used as well (10 μl/ml). At 24, 48, and 72 h postinfection (hpi), unless otherwise stated, medium was removed and stored at −80°C for analysis. Viral titers were determined by a standard plaque assay. Briefly, a monolayer of Vero E6 cells was inoculated with 10^1-fold- to 10^6-fold-diluted virus in a 6-well culture plate for 1 h in a humidified chamber at 37°C in 5% CO_2 with periodic rocking. A solution containing 1× MEM, 4% FBS, and 1% agarose was applied onto each well and incubated for 5 days. Cells were fixed with 12% paraformaldehyde, and the agarose was removed. Cells were stained with crystal violet. LCMV-Arm and LCMV-WE were inactivated by incubation under a UV light source for 30 min on ice. Alternatively, LCMV-Arm and LCMV-WE were inactivated by heating at 56°C for 30 min and then cooling on ice. The residual infectivity of inactivated virus was assessed by a standard plaque assay. No plaques or cytopathic effects were observed.

All experiments with infectious LASV were performed in a biosafety level 4 (BSL-4) facility at the Texas Biomedical Research Institute, San Antonio, TX.

Cytokine assays. Cytokine secretion was analyzed by using BD OptEIA human enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's instructions (BD Biosciences). Briefly, enzyme immunoassay (EIA) flat-bottom plates were coated with primary capture antibody specific for interleukin-8 (IL-8) or interferon-inducible protein 10 (IP-10) in carbonate-bicarbonate buffer overnight. Plates were blocked in 10% FBS for 1 h at 4°C. Samples were diluted with 1× phosphate-buffered saline (PBS)–10% FBS to reach a linear range of ELISA standards. Samples were incubated in plates for 2 h, followed by extensive washing. Plates were then incubated with secondary antibody specific for IL-8 or IP-10 and a streptavidin conjugate. Results were developed by incubation with a peroxidase substrate for 30 min, and reactions were stopped by the addition of a 1% HCl solution. Plates were read within 15 min at 450 nm, with the background at 570 nm subtracted. Alternatively, a multiplex cytokine analysis was done by using a customized BioPlex assay, a fluorescent, magnetic bead-based, multiplex immunoassay (Bio-Rad). Culture supernatants were analyzed at 24 hpi for IL-8, IL-6, IP-10, tumor necrosis factor alpha (TNF-α), and IL-1β on a Luminex system and quantified by using BioPlex Manager software. Levels of alpha interferon (IFN-α) in culture medium samples were measured by using a mouse eBioscience Platinum ELISA kit (catalog number BMS6027) and a VeriKine human IFN-α ELISA kit (catalog number 41100).

Quantitative RT-PCR. RNA was isolated from cells by using TRIzol-chloroform. cDNA was generated by using a reverse transcription (RT) system (Promega) and random primers. RNA was then quantified by using the following primers and probes specific for IL-8 and β-actin: probe 5′-AAGACATACTCCAAACCTTTCCACCCC-3′ and primers 5′-TCCTGATTTCTGCAGCTCTG-3′ (primer 1) and 5′-GTCCACTCTCA ATCACTCTCAG-3′ (primer 2) for IL-8 and probe 5′-ATCTGGGTCAT CTTCTCGCGGTTG-3′ and primers 5′-ACCTTCTACAATGAGCTGC G-3′ (primer 1) and 5′-CCTGGATAGCAACGTACATGG-3′ (primer 2) for β-actin. Quantitative PCRs (qPCRs) were run by using Maxima SYBR green-fluorescein qPCR master mix (Fermentas). IL-8 values were normalized to β-actin values.

Functional blocking antibody assay. HEK293T-TLR2 cells were seeded into a 12-well culture plate and grown overnight to 2.5×10^6 cells/well. Infected or control cells were pretreated with functional blocking anti-TLR2 antibody TL2.1 (Santa Cruz Biotechnology) or medium for 1 h. Antibody was removed, and cells were washed with warmed 1× PBS. THP-1 cells were also used in these experiments. The cells were pretreated with functional blocking antibodies for TLR2, TLR1, TLR6, or a combination of TLR2 and TLR1 or TLR2 and TLR6 for 1 h prior to infection with LCMV-Arm or LCMV-WE.

CD14 transfection. A plasmid carrying the human CD14 gene in a pcDNA3 mammalian expression vector was the gracious gift of A. Medvedev. HEK293 cells were seeded at 1×10^6 cells in a T25 culture flask and grown overnight. Three micrograms of plasmid was transfected by using FuGENE HD transfection reagent (Roche) and observed for 48 h for gene expression by flow cytometry. The efficacy of transfection was confirmed by staining with human anti-CD14 antibodies conjugated to allophycocyanin (APC). Samples were gated on live cells, and fluorescence was measured and compared to that of the corresponding isotype control.

Coinfection assay. A total of 2.5×10^5 THP-1 cells were seeded into each well of a 12-well culture plate. Cells infected with LCMV-Arm were superinfected at different time points with the WE strain for 1 h. After WE infection, the virus inoculum was removed, and cells were washed and incubated for an additional 24 h. As controls, single infections as well as mock infection and incubation with P3C or with LTA were conducted. Single infections (Arm or WE) were done according to standard protocols (infection was done for 1 h, and inoculates were removed and replaced with fresh medium at time zero [T_0]). The treatment of cells with P3C and with LTA or mock treatments were done in the same way, with exposure for 1 h and then replacement with fresh medium. At 13 h after T_0 infection, the culture medium from all conditions was replaced with fresh medium to normalize all conditions before the final collection (36 h after infection/treatment) of culture medium samples for determinations of IL-8 production. Final culture supernatant samples were collected at 36 h and analyzed for IL-8 production by an ELISA. For the inhibition of WE growth by UV treatment, cells infected with LCMV-Arm or treated with 100 ng/ml of P3C were exposed to UV-inactivated LCMV-WE 1 h prior to infection, concurrently, or at various times postinfection. After the final exposure, cells were washed and incubated for an additional 24 h. For LTA treatment, THP-1 cells infected with LCMV-WE or LCMV-Arm were treated with LTA (10 μl/ml) concurrently or at 1, 3, 6, or 12 hpi. Culture medium was collected at 36 hpi and analyzed by an IL-8 ELISA.

NF-κB luciferase reporter. A total of 2×10^6 HEK293T-TLR2 cells were transfected with 1 μg of the pNFκB-MetLuc2-Reporter vector (catalog no. 631743; Clontech), using Amaxa Nucleofector Kit V (Lonza). Cells were rested for 24 h and then infected, as described above, with LCMV-Arm, LCMV-WE, or LCMV-CL13 at an MOI of 1. Control cells were treated with P3C (100 ng/ml) or with LTA (10 μg/ml). Luciferase secretion in culture medium samples was monitored at 24 and 48 hpi by using the Ready-To-Glow luciferase assay (Clontech) and a Veritas luminometer.

RESULTS

LCMV-Arm, but not LCMV-WE, induces the chemokines IL-8 and IP-10 in THP-1 cells. Previously, we showed (38) that the replication of LASV, but not MOPV, in human monocyte-derived macrophages and endothelial cells failed to stimulate TNF-α and IL-8 genes and even downregulated lipopolysaccharide (LPS)-inducible cytokine responses. We also showed that LCMV-WE infection of rhesus macaques resulted in a fatal LF-like hepatitis with IL-8 downregulation in infected tissues (35). To determine if the OW arenaviruses of differing pathogenic potentials induced different proinflammatory responses, THP-1 cells, a human monocytic cell line, were infected with the WE and Arm strains of

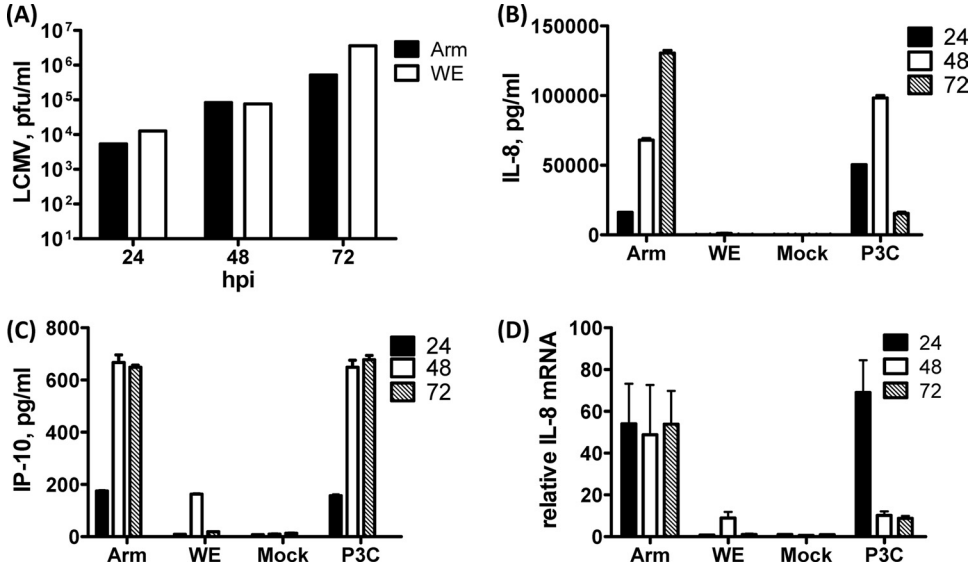

FIG 1 Replication of LCMV in THP-1 cells and induction of proinflammatory chemokines. In all experiments, plaque-purified virus stocks harvested in cell-free medium with undetectable levels of proinflammatory cytokines were used. Control cells were treated with serum-free medium as a mock infection or with 100 μg/ml of P3C as a positive control for the induction of IL-8 and IP-10. At 24, 48, and 72 hpi, culture supernatants were collected and analyzed by a plaque assay using a monolayer of Vero E6 cells. (A) THP-1 cells were infected with LCMV-Arm5 or LCMV-WE54 (MOI = 1) for 1 h. (B and C) Production of IL-8 (B) and IP-10 (C) assayed by an OptEIA human ELISA (BD Biosciences). (D) Real-time IL-8 PCR. RNA from infected cells was reverse transcribed into cDNA, and primers and probes for IL-8 and β-actin were used to quantify IL-8 mRNA by using Maxima SYBR green-fluorescein qPCR master mix (Fermentas).

LCMV (MOI = 1), and the levels of production of IL-8 and IP-10 in infected cells were measured.

As shown in Fig. 1, both strains of LCMV had similar replication kinetics. However, the replication of LCMV-Arm, but not LCMV-WE, was associated with a strong induction of IL-8 and IP-10 at the protein level, as measured by an ELISA (Fig. 1B and C), and at the mRNA level, as quantitated by RT-PCR (Fig. 1D; not shown for IP-10 mRNA).

We also measured IFN-α levels in samples collected from THP-1 cells infected with LCMV-Arm or LCMV-WE or treated with P3C. As expected, P3C did not induce IFN-α, and there was no detection of basal levels of IFN-α in mock-infected cells. Neither WE nor ARM induced IFN-α to detectable levels.

IL-8 production correlates with LCMV-Arm replication in THP-1 cells. Since the level of cytokine production appears to increase with increasing virus replication, we wanted to confirm this observation with experiments using a wide range of MOIs, from 0.001 to 1 PFU/cell. At the lowest MOI, both strains produced titers below the detection limit, 100 PFU/ml, during 72 hpi. Infection with an MOI of >0.001 resulted in the effective replication of both viruses with similar kinetics, reaching the highest titers at 48 to 72 hpi depending on the MOI (Fig. 2A and B). This illustrates that THP-1 cells are equally permissive to both LCMV strains, negating the possibility that the observed differences in cytokine induction might be due to differences in cell permissibility and viral replication. The replication kinetics of LCMV-Arm mimicked IL-8 secretion patterns in the culture medium, and they were clearly MOI dependent (Fig. 2C). Interestingly enough, at the lowest MOI (0.001), the level of IL-8 in the culture medium at 24 hpi was at the detection limit, but during further incubation, the level of IL-8 production rapidly increased and peaked at 60 hpi. Importantly, No IL-8 was detected in WE-infected cell cultures at any tested MOI (Fig. 2D).

TLR2-mediated signaling is required for induction of proinflammatory cytokine responses in cells infected with the OW nonpathogenic arenaviruses. A growing body of evidence indicates that virus-TLR2 interactions are important for the induction of immune responses (8, 47, 64, 65). Particularly, it has been discovered that TLR2-mediated responses are important for innate immunity to LCMV and that MyD88 is essential for the control of LCMV infection and for the maturation/activation of virus-specific $CD8^+$ T cells (65). We set out to determine the importance of virus recognition by TLR2 for the induction of proinflammatory responses induced by OW arenaviruses with different pathogenic potentials.

Wild-type HEK293T cells (HEK-WT cells) and cells stably expressing TLR2 (HEK-TLR2 cells) were infected with virus (MOI = 1), and the levels of production of IL-8 and IP-10 were measured by an ELISA at 24 hpi. As shown Fig. 3A, LCMV infection with the Arm or WE strain in HEK-WT cells did not induce IL-8. Both strains of LCMV failed to induce IL-8 in HEK-TLR4 cells as well, while bacterial LPS induced a strong TLR4-dependent response in these cells (not shown). In contrast, the infection of HEK-TLR2 cells with LCMV-Arm induced a robust IL-8 response comparable with those induced by the TLR2 ligand P3C. Similarly, in HEK-TLR2 cells, MOPV infection induced strong IL-8 production, which was inhibited after cell pretreatment with monoclonal antibodies capable of blocking TLR2 function (Fig. 3B, inset).

We confirmed these results with THP-1 cells. In addition, LCMV-Arm-infected cells pretreated with antibodies against TLR6 (but not against TLR1) also reduced IL-8 production. Notably, the combined anti-TLR2 and anti-TLR6 treatment had a strong cumulative effect, with a 15-fold reduction (not shown). Anti-TLR antibody treatment did not affect the infectivity of either LCMV-Arm or LCMV-WE, indicating that these receptors do not play a role in virus uptake.

The IL-8 results were confirmed by a customized BioPlex assay

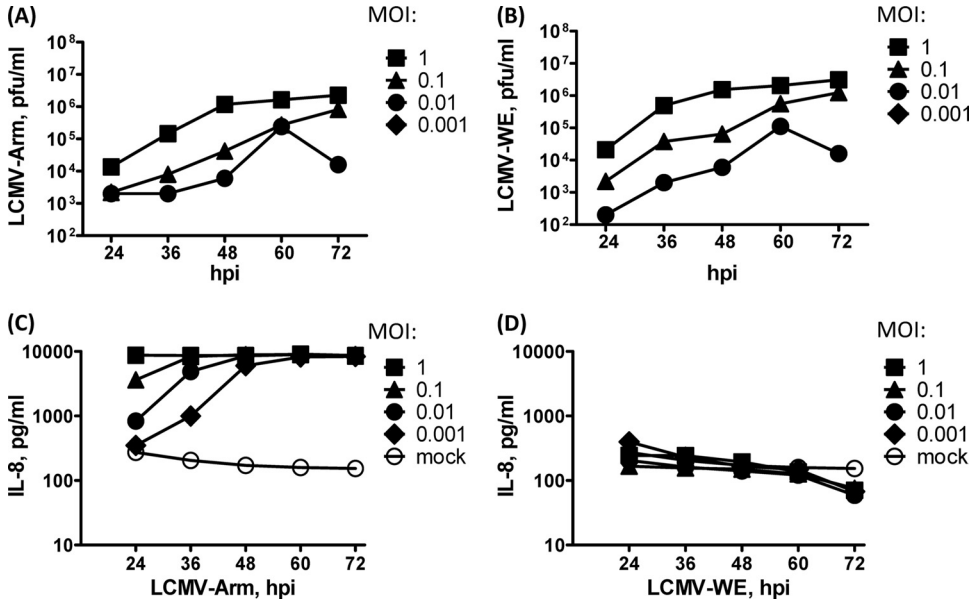

FIG 2 Effects of MOI on IL-8 production. THP-1 cells were infected with LCMV-Arm (A and C) or LCMV-WE (B and D) at different MOIs, and culture supernatants were analyzed for IL-8 secretion (C and D) and infectious virus production (A and B). At an MOI of 0.001, the virus production level was less than 2 $\log_{10}$ PFU/ml (undetectable).

for IL-8, IP-10, IL-6, TNF-α, and IL-1β (Bio-Rad). As shown in Fig. 3C to E, with the exception of TNF-α and IL-1β, cytokines were strongly upregulated in Arm-infected cells. Levels of the cytokines TNF-α and IL-1β were lower than the detection limits for all samples tested. In agreement with the results described above (Fig. 2), the production of IL-8 in HEK-TLR2 cells infected with LCMV-Arm was dependent on the MOI (not shown). LCMV-WE did not induce IL-8 in these cells at any tested MOI.

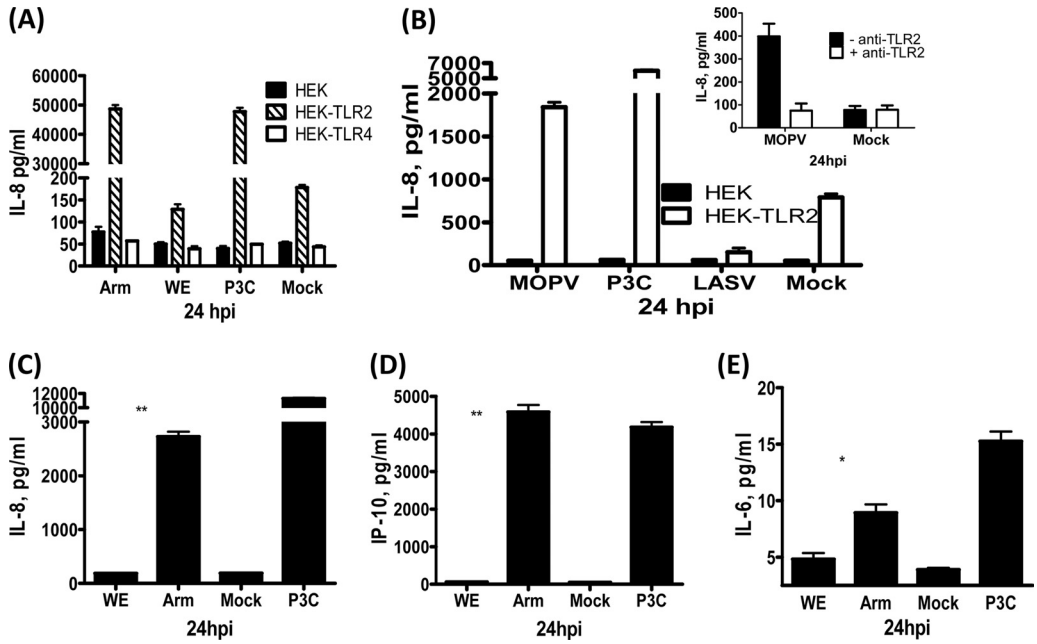

FIG 3 TLR2-mediated signaling is required for induction of proinflammatory cytokine responses in cells infected with OW nonpathogenic arenaviruses. (A and B) Wild-type HEK293T (HEK) cells or HEK293T cells stably expressing TLR2 (HEK-TLR2) or TLR4 (HEK-TLR4) were infected (MOI = 1) with LCMV-Arm and LCMV-WE (A) or with MOPV and LASV (B). Control cells were treated with medium (mock infection) and with P3C as a positive TLR2 control. At 24 hpi, culture supernatants were removed and analyzed by an IL-8 ELISA and a plaque assay (not shown). (B, inset) Inhibition of IL-8 production by anti-TLR2 monoclonal antibodies. HEK-TLR2 cells were pretreated with a blocking anti-TLR2 antibody or medium (mock) for 1 h, and cells were subsequently infected with MOPV (MOI = 1). (C to E), BioPlex analysis. A customized BioPlex assay (Bio-Rad) was used to evaluate multiple cytokines in response to LCMV infection. Culture supernatants from LCMV-Arm- and LCMV-WE-infected cells were analyzed for the production of IL-8 (C), IP-10 (D), and IL-6 (E). Levels of TNF-α and IL-1β in the infected cells were lower than the detection limits for all samples tested. *, $P < 0.05$; **, $P < 0.001$.

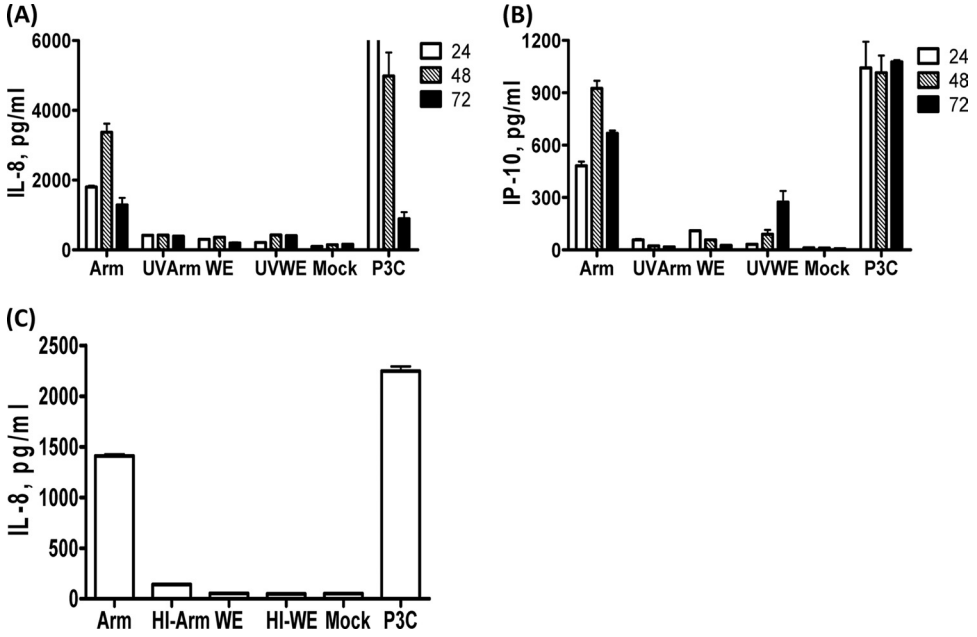

FIG 4 Live replicating virus is required to induce TLR2-dependent chemokine induction. LCMV-Arm and LCMV-WE were inactivated by incubation under UV light for 30 min on ice or by heating at 56°C for 30 min. The residual infectivity of inactivated virus was assessed by a plaque assay. HEK-TLR2 cells were infected (MOI = 1) or treated with inactivated virus for 1 h. At 24, 48, and 72 hpi, culture supernatants were collected and analyzed for IL-8 (A and C) and IP-10 (B) production by an ELISA and for infectivity (by a plaque assay). All tested samples did not show infectious activities. UVArm and UVWE indicate viruses inactivated by UV, and HI-Arm and HI-WE indicate viruses inactivated by heating.

Induction of proinflammatory cytokines requires live, replicating virus. Given that replication appears to be closely intertwined with the induction of proinflammatory responses in cells infected with LCMV-Arm or MOPV, we wanted to determine if nonreplicating virus could induce a response. LCMV was inactivated by UV exposure or by temperature (56°C for 30 min). After the confirmation of inactivation by a plaque assay, HEK-TLR2 cells were exposed to inactivated viruses. UV-inactivated or heat-inactivated viruses did not induce IL-8 or IP-10 production (Fig. 4), confirming the link between viral replication and cytokine induction.

CD14 expression enhances TLR2-dependent induction of IL-8 and IP-10 in HEK-TLR2 cells infected with LCMV-Arm. CD14, a coreceptor for TLR2/TLR4, interacts with TLR to present the microbial agonist, leading to increased TLR-initiated responses (1). To determine how the coexpression of CD14 in HEK293T cells contributes to virus-induced proinflammatory responses, WT and HEK-TLR2 cells were transfected with a plasmid encoding human CD14.

As shown in Fig. 5, the infection of HEK-WT-CD14 cells did not result in the induction of IL-8 by either LCMV-WE or LCMV-Arm, confirming the lack of a signal transducing function for CD14 itself, as reported previously (55). In contrast, the expression of CD14 in HEK-TLR2 cells resulted in a 5-fold increase in the levels of production of IL-8 and IP-10 in response to LCMV-Arm infection compared to cells expressing TLR2 alone. LCMV-WE infection of HEK-TLR2-CD14 cells did not produce detectable levels of IL-8.

TLR2 and Mal are required for the induction of proinflammatory responses in LCMV-Arm-infected cells but not in LCMV-WE- or CL13-infected cells. To further confirm the TLR2 requirement for the induction of proinflammatory responses by the nonpathogenic OW arenaviruses, we used immortalized bone

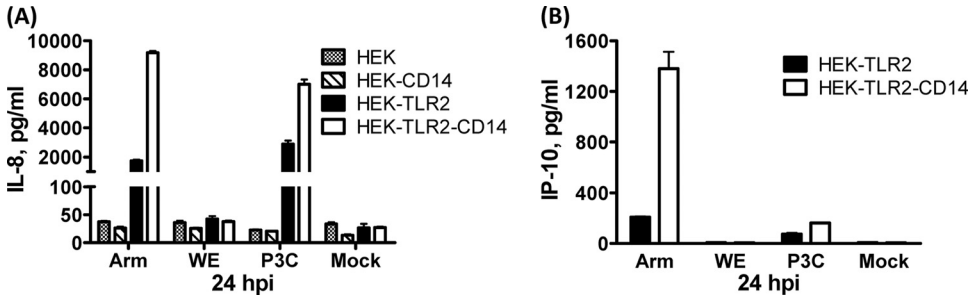

FIG 5 Chemokine production is enhanced by CD14 transfection in HEK-TLR2 cells. A plasmid carrying the human CD14 gene in the pcDNA3 plasmid was transfected into HEK293T and HEK-TLR2 cells by using FuGENE HD transfection reagent (Roche). The efficacy of CD14 expression (>80%) was confirmed by flow cytometry using an anti-CD14 antibody conjugated to APC (PharMingen). WT, HEK-TLR2, and CD14-transfected cells were infected with LCMV-Arm or with LCMV-WE (MOI = 1). Culture supernatants were collected at 24 hpi and assayed for IL-8 (A) and IP-10 (B) levels and virus titers (not shown).

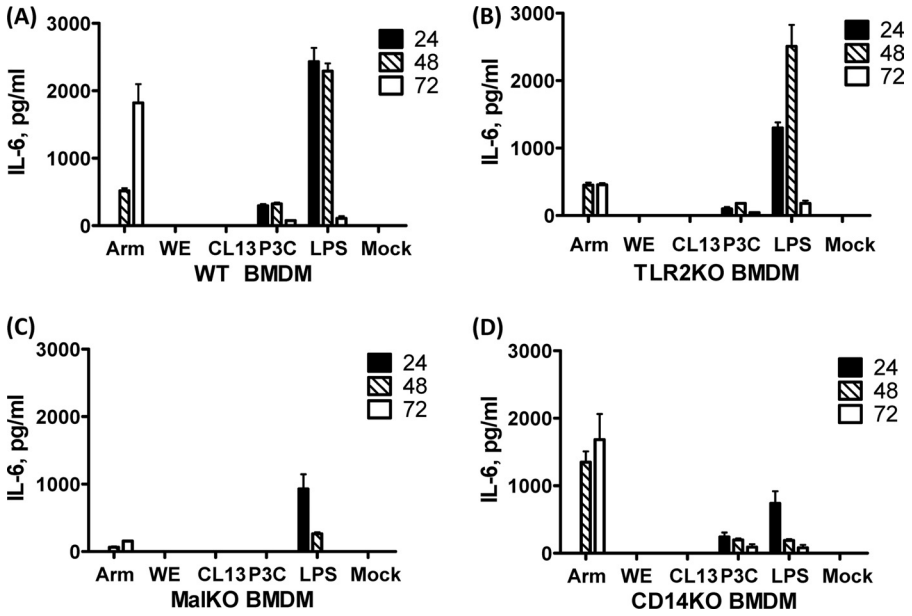

FIG 6 TLR2 and Mal are required for induction of IL-6 in LCMV-Arm-infected cells. Immortalized bone marrow-derived macrophages (iBMDM) from C57BL6/J mice (wild type) and from TLR2 (NR-9457), CD14 (NR-9570), and Mal (NR-9459) knockout mice were purchased from BEI Resources. iBMDM from WT or KO mice were infected with LCMV-Arm or LCMV-WE (MOI = 1) or were treated with medium (mock infection), P3C, or *Escherichia coli* K-12 LPS (induction control) for 1 h. Culture supernatants from WT (A), TLR2 KO (B), Mal KO (C), and CD14 KO (D) iBMDM cultures were collected at 24, 48, and 72 hpi and analyzed for IL-6 by an ELISA (BioLegend).

marrow-derived macrophages (iBMDM) from C57BL6/J mice (WT) and from TLR2, CD14, and Mal KO mice. The iBMDM derived from WT and KO mice were infected with LCMV strains (MOI = 1), and culture supernatant samples were collected at 24, 48, and 72 hpi and analyzed for the production of murine IL-6 (BioLegend). As expected, in WT macrophages, Arm, but not WE, induced a strong IL-6 response (Fig. 6A). At 24 h posttreatment, P3C induced very little cytokine production. In subsequent studies looking at earlier time points, however, the induction of cytokines by P3C in iBMDM was rapid and short lived, peaking at 8 h posttreatment. As shown in Fig. 6B, in TLR2 KO-derived macrophages, the Arm- and P3C-induced IL-6 responses were significantly reduced at 72 h as well as at the 8-h time point for P3C-treated cells. Notably, all LCMV strains replicated with the same kinetics in iBMDM (not shown).

Previous studies of KO mice showed that TLR2 and MyD88 are essential for the induction of proinflammatory cytokine/chemokine responses to LCMV (65). Mal (MyD88 adaptor-like) is a bridging adaptor molecule that binds to the intracellular domains of dimerized TLRs. Mal recruits MyD88, which subsequently recruits IL-1 receptor (IL-1R)-associated kinases (IRAKs), leading to IRAK activation, triggering signalosome assembly and the activation of downstream adapters and kinases, and resulting in NF-κB activation. As shown in Fig. 6C, in Mal KO-derived macrophages, the IL-6 response induced by LCMV-Arm and the TLR2-specific ligand P3C was undetectable in all tested samples (with the exception of LPS-stimulated mock-infected cells at the 24-h time point).

In CD14 KO-derived macrophages (Fig. 6D), LCMV-Arm infection induced the IL-6 response at levels seen for WT iBMDM cells, confirming that the CD14 coreceptor is not critically required for TLR2-dependent proinflammatory responses but may enhance these responses depending on the cell type (see also Fig. 5). It is interesting that the LCMV-Arm induction of IL-6 in TLR2 KO iBMDM was nearly equivalent to that in WT iBMDM but that this induction of cytokines was unable to increase at 72 hpi. We hypothesize that there may be some alternative mechanism of cytokine induction with the viral ligand that may be more dependent on Mal than TLR2. Along the same idea, Zhou et al. (65) reported previously that MyD88 was more critical than TLR2 for generating LCMV-specific $CD8^+$ T cells.

The persisting LCMV strain CL13 was included in the above-described experiments with iBMDM. This strain, isolated from the spleens of Arm-infected mice, differs from the parental Arm strain by 5 nucleotides, one of which resulted in an amino acid substitution in the viral polymerase and one substitution in the GP1 glycoprotein (51, 56). Importantly, in these experiments, CL13 behaved like LCMV-WE and failed to induce IL-6 in WT cells and in CD14 KO-derived macrophages. A possible contribution of CL13 mutations to the inability of the virus to induce IL-6 expression in macrophages is discussed below.

Levels of murine IFN-α in all tested samples (WT and TLR2 KO iBMDM infected with ARM, WE, or CL13; mock infected; or treated with P3C), measured by an ELISA, were below the level of detection (30 pg/ml).

LCMV-Arm-stimulated IL-8 induction is blocked by LCMV-WE during the early stage of infection. The above-described experiments demonstrated the inability of LCMV-WE to induce proinflammatory responses in human THP-1 cells, HEK-TLR2 cells, and murine macrophages. However, it was unclear if this was an active inhibition or a lack of stimulation. In the next experiment, we asked whether LCMV-WE is able to block already initiated proinflammatory responses. To address this question, THP-1 cells were infected with LCMV-WE before, simultaneously

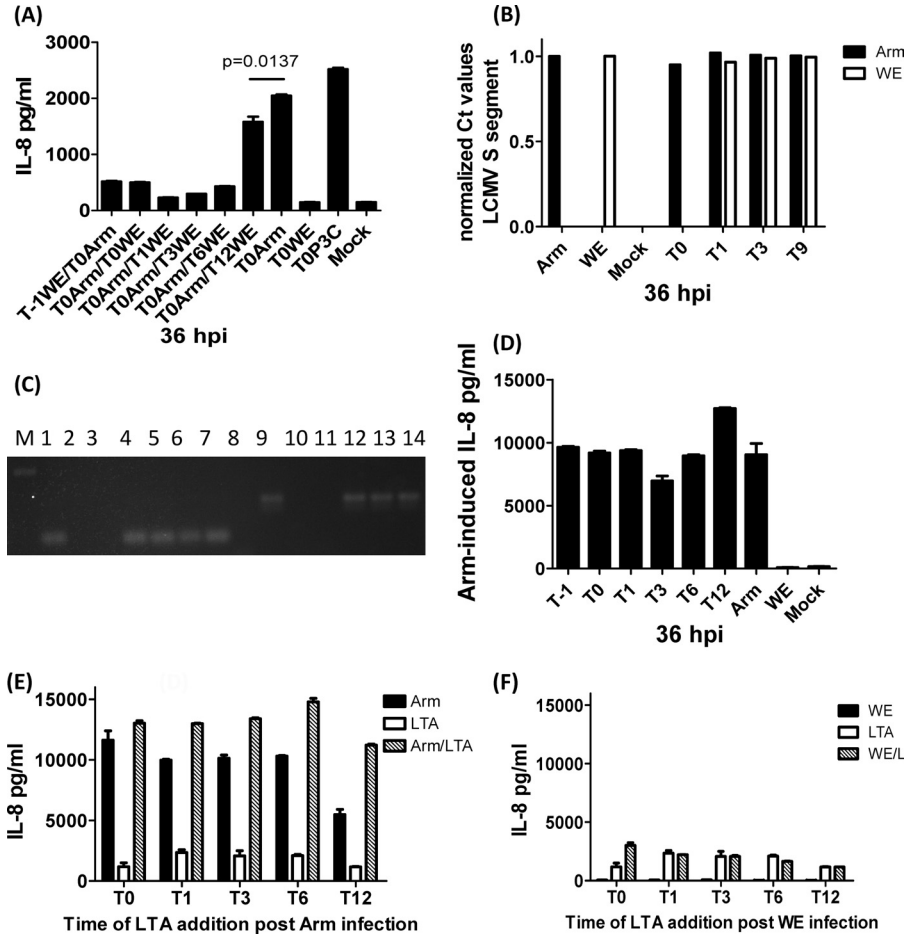

FIG 7 LCMV-WE inhibits the Arm-inducible IL-8 response. (A) THP-1 cells were infected with LCMV-WE 1 h prior to, simultaneously with, or 1 to 12 h after LCMV-Arm infection. All time points are based on LCMV-Arm infection at time zero (T_0). T−1WE/T0Arm indicates WE infection 1 h prior to Arm infection. T0Arm/T0WE indicates simultaneous coinfection with both viruses. T0Arm/T1WE and subsequent time points (T_3, T_6, T_9, and T_{12}) indicate WE infection at 3, 6, 9, and 12 h after Arm infection. Single-infection controls, mock infection, and P3C controls were conducted at T_0. At 13 h post-T_0 infection, the culture medium under all conditions was replaced with fresh medium. Culture supernatant samples were collected at 36 h and analyzed for IL-8 production by ELISA as described above. (B) RNA from cells coinfected with both LCMV strains WE and Arm was extracted with TRIzol, and cDNA was generated by using random primers and a reverse transcription system (Promega) according to the manufacturer's instructions. Quantitative PCR was done to determine the ability of viruses to replicate simultaneously in coinfected cells by using primers and probes specific for divergent sequences targeting the S RNA segment (see Materials and Methods). All threshold cycle (C_T) values were normalized to values for the β-actin control and then normalized to values for a single-infection control. (C) Agarose gel analysis. PCR products were run on a 4% agarose gel in 1× Tris-acetate-EDTA (TAE) buffer to verify results. Lanes: M, marker lane; 1 to 7, Arm-specific amplification; 1, LCMV-Arm infection; 2, LCMV-WE infection; 3, mock infection; 4 to 7, T_0, T_1, T_3, and T_9 infections, respectively; 8 to 14, LCMV-WE-specific amplification; 8, LCMV-Arm infection; 9, LCMV-WE infection; 10 to 14, mock, T_0, T_1, T_3, and T_9 infections, respectively. (D) LCMV-Arm-infected cells were treated with UV-inactivated LCMV-WE at different time points (−1, 0, 1, 3, 6, and 12 h) after infection, and IL-8 levels in the culture medium were measured. (E and F) treatment of THP-1 cells with LTA, a TLR2/6 agonist (10 μg/ml). Mock-infected cells or cells infected with LCMV-Arm (E) or with LCMV-WE (F) were treated with LTA at different time points (1 to 12 h), as described in Materials and Methods, and IL-8 levels were measured by an ELISA.

with, or at different time points after LCMV-Arm infection, and the production of IL-8 was measured by an ELISA.

As shown in Fig. 7A, the pretreatment of cells with LCMV-WE, the coincubation of LCMV-WE with LCMV-Arm, or the treatment of cells up to 6 h after LCMV-Arm infection effectively blocked the induction of IL-8. However, LCMV-WE treatment at 12 h after LCMV-Arm inoculation did not prevent IL-8 induction. The results indicate that LCMV-WE infection actively inhibits the LCMV-Arm-mediated IL-8 response during the early stage of LCMV infection. These results are in line with our previously reported observations showing that LASV infection inhibited LPS-mediated proinflammatory responses in human monocyte-derived macrophages (38). Given the possibility of infection exclusion between similar viruses, we used quantitative PCR to verify the active replication of both LCMV-Arm and LCMV-WE in superinfection experiments. PCR primers and probes were designed for a region of the S RNA segment with sequence variability between Arm and WE. As shown in Fig. 7B and C, these primers/probes were able to distinguish between two infections and to demonstrate that both viruses are replicating equivalently.

To determine if the inhibition of cytokine induction required WE replication, THP-1 cells were infected with LCMV-Arm and exposed to UV-inactivated LCMV-WE 1 h prior to, concurrently with, or at 1, 3, 6, or 12 h after LCMV-Arm infection. Except for a

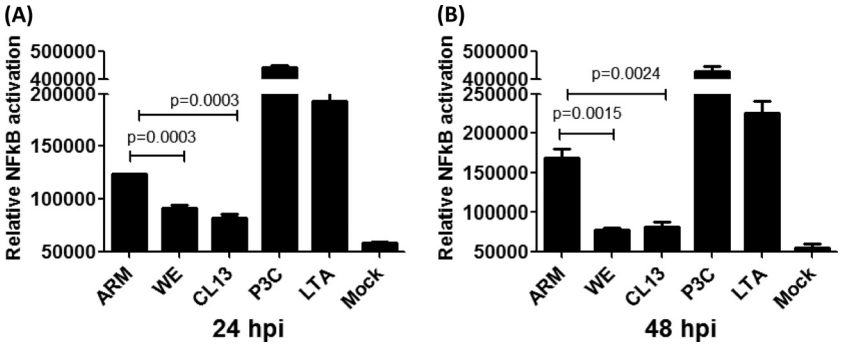

FIG 8 LCMV-Arm but not LCMV-WE or LCMV-CL13 activates NF-κB. HEK293T-TLR2 cells were transfected with the pNFκB-MetLuc2 reporter vector as described in Materials and Methods. Cells rested for 24 h and were then infected at an MOI of 1 with LCMV-Arm, LCMV-WE, or CL13. Controls included cells treated with P3C (100 ng/ml) or LTA (10 μg/ml) and mock-infected cells. At 24 h (A) or 48 h (B) after infection, culture medium samples were collected and analyzed for secreted luciferase in triplicate. Significance was determined by using an unpaired, two-tailed *t* test.

slight inhibition at 3 hpi, it appears that inactivated LCMV-WE is unable to inhibit the LCMV-Arm induction of IL-8 (Fig. 7D).

To determine if LCMV-Arm or LCMV-WE can affect cytokine induction by exogenous TLR2 ligands, LTA was added to LCMV-Arm-infected or LCMV-WE-infected THP-1 cells concurrently or at 1 to 12 hpi. As shown in Fig. 7E, LCMV-Arm induced a robust production of IL-8, while LTA induced more modest IL-8 levels. The combination of LCMV-Arm and LTA resulted in IL-8 levels that were equivalent to the additive value of either LCMV-Arm infection or LTA treatment alone, indicating a lack of interference or competition between LCMV-Arm and LTA for the TLR2-dependent induction of cytokines. As expected, LCMV-WE failed to induce the production of IL-8 (Fig. 7F). Cotreatment with LTA at T_0 resulted in a slight increase in the level of IL-8 production compared to that with LTA treatment alone. However, in contrast to the Arm-infected cells, at all later time points, the LTA treatment of LCMV-WE infected cells did not affect IL-8 production.

NF-κB is differentially activated by LCMV-Arm versus LCMV-WE or CL13. The transcription of IL-8 is classically induced by the activation of NF-κB. Previous studies showed that a virulent strain of Pichinde virus (PICV), a New World (NW) arenavirus, induced the suppressive NF-κB homodimer p50/p50, while the nonvirulent PICV strain induced the activating heterodimer p65(RelA)/p50 (19). To better understand the mechanism by which LCMV-WE inhibits cytokine induction, we used the NF-κB–luciferase reporter transfected into HEK293-TLR2 cells. In mammalian cells transfected with pNFκB-MetLuc2-Reporter (Clontech), the addition of stimulants (e.g., cytokines or TLR ligands) to the culture medium induces transcription factors to bind to the NF-κB enhancer element, thereby initiating the transcription of the secreted luciferase reporter gene. The activation of the NF-κB signal transduction pathway can be monitored simply by sampling the culture medium (Ready-To-Glow; Clontech).

As shown in Fig. 8, the transfection of cells with the NF-κB–luciferase reporter gene and treatment with the TLR2/1 or TLR2/6 ligand, P3C or LTA, respectively, resulted in the activation of NF-κB and the subsequent secretion of luciferase. Similarly, LCMV-Arm infection of transfected cells induced the secretion of luciferase at 24 and 48 hpi. In contrast, the infection of transfected cells with LCMV-WE and CL13 did not stimulate luciferase secretion, and the level of secretion decreased during the incubation (Fig. 8A and B). The differences in NF-κB activation between LCMV-Arm and LCMV-WE as well as between LCMV-Arm and LCMV-CL13 were statistically significant ($P = 0.0003$). Thus, it is likely that during viral replication, LCMV-WE inhibits the pathway leading to NF-κB activation, resulting in the downregulation of cytokine production.

DISCUSSION

We previously showed for the first time that the replication of the OW pathogenic arenaviruses LASV and LCMV-WE, but not nonpathogenic MOPV and LCMV-Arm, was associated with the inhibition of proinflammatory cytokine/chemokine responses *in vitro* and *in vivo* (35, 36, 38). The detection of proinflammatory cytokines in LF patients and in experimentally infected nonhuman primates showed no evidence for a "cytokine storm" observed previously for cases of filovirus-induced hemorrhagic fever (HF) (46). In contrast, in good correlation with our results, in progressed LF patients, fatal infection was correlated with low or undetectable levels of proinflammatory cytokines/chemokines, including IL-8 and IP-10 (39). At present, the immunosuppressive phenotype of progressed LASV infection in humans or in nonhuman primates is a well-accepted concept (7, 15, 46, 49, 62). Our previously reported gene expression analysis of human peripheral blood mononuclear cells (PBMC) from healthy donors exposed to LASV has shown that IFN-related genes and genes involved in apoptosis, NF-κB, and coagulation pathways were among the genes most affected by LASV (61). The production of proinflammatory cytokines results in the activation of antigen-presenting cells and T cell stimulation in the adaptive immune response. Indeed, recent results showed that MOPV infection of human dendritic cells (DCs) induced strong T cell responses and memory cells, while LASV infection did not (49).

TLR2-mediated cytokine responses are also involved in innate immunity against the NW arenaviruses. While the manuscript was in review, Cuevas et al. (16) presented results showing that Junin virus (JUNV), the causative agent of Argentine HF, induced TLR2-dependent proinflammatory cytokines in mouse macrophages. Interestingly enough, in human monocytes/macrophages, nonpathogenic Tacaribe virus (TCRV), a close genetic relative of JUNV, induced the production of proinflammatory cytokines, and this production required TCRV replication. In contrast, JUNV infection did not have an effect on proinflammatory cytokines. The authors of that report, Groseth et al. (26), referred to

our previous results for LASV versus MOPV infection of human macrophages (38) and concluded that "strong cytokine activation during macrophage infection is a feature of nonpathogenic arenavirus infection, regardless of whether the virus in question is an Old World or New World arenavirus." We agree with this statement. An increasing body of evidence suggests that an early induction of cytokines enhances innate immune responses and promotes effective adaptive immune responses to prevent the development of HF during arenavirus infection.

Arenaviruses are rodent-borne viruses (with the notable exception of the bat-borne TCRV). Immunological differences between natural hosts and human hosts probably account for the remarkably different infectious outcomes. In murine models knocked out for CD4, CD8, or B cell production, LASV did not induce any signs of disease. However, the inoculation of Stat-1 KO mice induced a lethal outcome in 67% of mice, indicating that the innate type I IFN response plays a major role in resistance to LASV infection in rodents and perhaps contributes to the establishment and/or maintenance of persistent infection in natural hosts (60). Recent *in vitro* studies implicated the arenavirus NP protein in the blocking of IFN regulatory factor 3 (IRF3), a major downstream regulator of RIG-I-inducible type I IFN responses (41, 42). Particularly, it was shown previously that LASV NP D389A/G392A and D389T/G392A mutations were critically involved in the suppression of IFN in murine macrophages and DCs (14).

However, this anti-type I IFN activity was found for both pathogenic and nonpathogenic arenaviruses, with the exception of TCRV NP, implying that NP-mediated anti-IFN activity is a feature of rodent-carried arenaviruses (41). This activity seems to contribute to the delicate balance between the magnitude of the innate response and the level of viral replication rather than to pathogenicity in humans. Another interpretation is that anti-IFN responses and the suppression of IL-8 production are both examples of necessary but insufficient components of pathogenesis. For example, an additional component *in vivo* could be the replicative capacities of the viruses, although this was uniform in our previous cell culture studies (38). Interestingly, effective $CD8^+$ cytotoxic T lymphocyte (CTL) responses against early LCMV NP-derived epitopes played a key role in acute virus control in a murine model (53). Finally, a dual role for T cells in both the clearance of infection and the enhancement of pathogenesis (22) cannot be excluded. Vaccine design toward the activation of a T cell response should take this into consideration.

In humans, LASV is a poor type I IFN inducer, LASV itself is relatively resistant to IFN, and the IFN sensitivity of LASV isolates does not correlate with disease progression (4). Severe LASV infection is characterized by unchecked viremia, functional liver damage, and immunosuppression (43, 46). Recovery and protection are dependent on T cell responses and are not associated with the production of specific IgG. In human DC-T cell cocultures, LASV induced only weak memory phenotype markers, while MOPV strongly stimulated $CD8^+$ and $CD4^+$ T cells, activation markers, proliferative responses, and CTL activities (49). We speculate that the TLR2-mediated stimulation of cytokines in MOPV-infected cells (Fig. 3B) (see also reference 38) contributed to the development of strong adaptive immune responses. Similarly, in LCMV-Arm-infected mice, TLR2/Mal/MyD88 signaling played an essential role in antiviral $CD8^+$ T cell responses. In the absence of MyD88, naive $CD4^+$ T cells failed to differentiate to LCMV-specific CD4 T cells (64). Whether the inhibited induction of cytokines is a direct or indirect outcome of virulent virus infection, the lack of innate stimulation likely positions the host to have a delayed adaptive immune response that would clear the infection.

In most cases, proinflammatory responses elicited through TLR2 occur via the stimulation of the receptor at the cell surface (8). However, UV-inactivated or heat-inactivated LCMV-Arm was unable to elicit a TLR2-dependent response (Fig. 4), demonstrating a need for virus internalization and replication in order to stimulate a TLR2-dependent cytokine response. The coinfection data presented in Fig. 7 indicate that LCMV-WE is able to inhibit cytokine induction by LCMV-Arm up to 6 h after Arm infection, after virus entry and fusion and during replication but prior to the release of new virus particles. At this point in the virus life cycle, nucleoproteins, glycoproteins, zinc proteins, genomic RNAs, and mRNAs are being synthesized. Any one of these components may be involved in the suppression of the production of cytokines. In fact, given that LCMV-WE replication was necessary for the inhibition of Arm-induced cytokines (Fig. 7A and D) and that LCMV-WE and CL13 block the activation of NF-κB (Fig. 8), it is possible that viral proteins and/or RNA produced during the replication cycle interrupts the pathway leading to NF-κB activation.

The results of the treatment of Arm-infected cells with LTA corroborate the results for the activation of NF-κB and explain cumulative effects on IL-8 production. Surprisingly, IL-8 inhibition was not observed for WE-infected cells treated with LTA. This indicates that in contrast to Arm-induced responses (Fig. 7A), LCMV-WE does not inhibit exogenously induced TLR2-dependent cytokine responses. The different effects of LCMV-WE on Arm-induced versus LTA- and P3C-induced responses will be an intriguing target for future research.

The major cellular receptor for LASV and LCMV is α-dystroglycan (α-DG), a cell surface receptor for extracellular matrix (ECM) proteins (13). In the spleen, over 99% of α-DG was associated with DCs, and less than 1% was associated with $CD4^+$ and $CD8^+$ T cells. LASV, LCMV-WE54, and Arm-derived CL13 bind to α-DG with high affinities, preferentially infect DCs, effectively compete with and displace ECM molecules of DCs, and alter their ability to initiate an effective immune response (33, 48). In this respect, these strains of LCMV behave like LASV in acute infection (48). A single-point mutation in the CL13 polymerase, K1079Q, was necessary and partially sufficient for viral persistence and, perhaps, for immunosuppression. The GP1 mutation F260L, which is responsible for increased α-DG affinity and DC targeting, played an accessory role in enhancing the duration of persistence and generalized immunosuppression (9). Our findings showing that LASV, LCMV-WE, and CL13 infections suppressed TLR2/Mal-dependent cytokine responses provide additional evidence for immunosuppressive phenotypes of the OW pathogenic arenaviruses. Mice infected with LCMV CL13 have persistent high-level viremia and a dysfunctional immune response resembling that of fatal LF (48). Numerous parallels of CL13 infection with chronic viral infections caused by HIV, hepatitis C virus (HCV), and hepatitis B virus (HBV) have also been described (59). The downregulation of NF-κB in murine macrophages infected with CL13 (Fig. 8) provides additional evidence of how this infection broadly affects immune responses.

The TLR2/Mal-dependent induction of cytokines in cells infected with LCMV-Arm and MOPV required a live replicating virus and was dependent on the MOI (Fig. 1 to 3). In contrast to

many enveloped viruses, LASV and LCMV use a novel pathway of endocytosis and enter host cells via a cholesterol-dependent and clathrin-, caveolin-, and actin-independent pathway via the multivesicular body and dependent on the endosomal sorting complex required for transport (29, 32). Interestingly, MOPV proved to be 10 times more sensitive to the effect of lysosomotropic agents than LASV (24). Recent studies also indicated a possible role for the glycosyltransferase LARGE, the enzyme complex crucial for ECM protein posttranslational modifications and for the virus-binding capacity of α-DG (33), at the intracellular replication level of the virus rather than at the virus-host cell receptor-binding step (58). Similarly, we speculate that the induction of TLR2-dependent chemokine responses also occurs intracellularly, since replication correlated with chemokine production, and inactivated virus was unable to elicit a response.

Several findings suggested that that α-DG is not the sole receptor for LCMV and LASV (28, 33). Indeed, in addition to α-DG, two C-type lectin family members, DC-SIGN and LSECtin, and two members of the receptor tyrosine kinase family of cell surface receptors, Axl and Tyro3, were recently identified as LASV receptors (54). Viral tropism *in vivo* seems to be associated with the expression levels of α-DG, DC-SIGN, LSECtin, Axl, and Tyro3 in different tissues. Interestingly enough, Axl/Tyro3-mediated LASV infection required intracellular signaling via the tyrosine kinase activity of Axl and Tyro3, whereas DC-SIGN/LSECtin-mediated infection and binding were dependent on a specific carbohydrate and on ions. The endocytosis pathways and the role of TLR2, α-DG, ECM ligands, and newly described LASV receptors in cells infected with pathogenic and nonpathogenic OW arenaviruses will be interesting subjects for further studies.

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