Tick-Borne Flaviviruses Antagonize Both IRF-1 and Type I IFN Signaling To Inhibit Dendritic Cell Function

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*J Immunol* published online 14 February 2014
http://www.jimmunol.org/content/early/2014/02/14/jimmunol.1302110
Tick-Borne Flaviviruses Antagonize Both IRF-1 and Type I IFN Signaling To Inhibit Dendritic Cell Function

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Tick-borne encephalitis virus (TBEV), a member of the Flaviviridae family, is a leading cause of viral encephalitis in Europe and Asia. Dendritic cells (DCs), as early cellular targets of infection, provide an opportunity for flaviviruses to inhibit innate and adaptive immune responses. Flaviviruses modulate DC function, but the mechanisms underpinning this are not defined. We examined the maturation phenotype and function of murine bone marrow–derived DCs infected with Langat virus (LGTV), a naturally attenuated member of the TBEV serogroup. LGTV infection failed to induce DC maturation or a cytokine response. Treatment with LPS or LPS/IFN-γ, strong inducers of inflammatory cytokines, resulted in enhanced TNF-α and IL-6 production, but suppressed IL-12 production in infected DCs compared with uninfected “bystander” cells or mock-infected controls. LGTV-mediated antagonism of type I IFN (IFN-I) signaling contributed to inhibition of IL-12p40 mRNA expression at late time points after stimulation. However, early suppression was still observed in DCs lacking the IFN-I receptor (Ifnar−/−), suggesting that additional mechanisms of antagonism exist. The early IFN-independent inhibition of IL-12p40 was nearly abolished in DCs deficient in IFN regulatory factor-1 (IRF-1), a key transcription factor required for IL-12 production. LGTV infection did not affect Ifr-1 mRNA expression, but rather diminished IRF-1 protein levels and nuclear localization. The effect on IRF-1 was also observed in DCs infected with the highly virulent Sofjin strain of TBEV. Thus, antagonism of IRF-1 is a novel mechanism that synergizes with the noted ability of flaviviruses to suppress IFN-α/β receptor–dependent signaling, resulting in the orchestrated evasion of host innate immunity. The Journal of Immunology, 2014, 192: 000–000.

Tick-borne encephalitis (TBE), a single-stranded, positive-sense RNA virus belonging to the family Flaviviridae. Approximately one third of TBEV infections cause an acute febrile illness that progresses to severe neurologic disease including meningitis and meningoencephalitis with mortality rates ranging from 0.5 to 40% depending on the TBEV subtype (1, 2). Endemic throughout Europe and Asia, TBEV is considered an emerging pathogen because of its recent expansion into new geographical areas and increased incidence of human infections (3). Approximately 10,000 clinical cases of TBE are reported annually. Other emerging tick-borne flaviviruses include Kyasanur Forest disease virus and Omsk hemorrhagic fever virus that cause hemorrhagic fever, also with relatively high mortality rates (4). There are currently no specific therapies for the treatment of flaviviral infections, underscoring the importance of identifying therapeutic targets for development of novel antivirals.

Dendritic cells (DCs) represent a fundamental bridge between innate and adaptive immunity. In peripheral tissues such as the skin, immature DCs recognize RNA virus infection through expression of pathogen recognition receptors (PRRs) in the cytosol, such as the RNA helicases retinoic acid inducible gene I and melanoma differentiation associated gene-5, and in the endosome, through TLR3, TLR7, and TLR8 (5). After virus recognition, DCs migrate to local lymphoid tissues and undergo a process of maturation that involves cytokine production and Ag presentation to activate naïve T cells and shape adaptive immunity. DCs also represent early targets of TBEV infection following the bite of an infected tick (6), providing the virus with opportunities to manipulate DC functions as a means of evading host immunity. Indeed, many flaviviruses, including dengue virus (7–9), West Nile virus (WNV) (10), and Japanese encephalitis virus (11–13), infect DCs, resulting in impaired DC maturation and T cell priming/proliferation. This manipulation of DC function is thought to be important in virus pathogenesis, although the molecular mechanisms are unknown.

Abbreviations used in this article: DC, dendritic cell; Gbp-2, guanylate-binding protein 2; hpi, h postinfection; IFN-I, type I IFN; IFNAR, IFN-α/β receptor; IRF, IFN regulatory factor; ISG, IFN-stimulated gene; LGTV, Langat virus; MHC II, MHC class II; MOI, multiplicity of infection; PRR, pathogen recognition receptor; TBE, tick-borne encephalitis; TBEV, tick-borne encephalitis virus; WNV, West Nile virus; WT, wild type.

Received for publication August 8, 2013. Accepted for publication January 12, 2014.

This work was supported by the Intramural Research Program of the National Institutes of Health, National Institute of Allergy and Infectious Diseases. Address correspondence and reprint requests to Dr. Sonja Best, Laboratory of Virology, Rocky Mountain Laboratories, National Institute of Allergy and Infectious Diseases, National Institutes of Health, 903 South 4th Street, Hamilton, MT 59840. E-mail address: sbest@niaid.nih.gov

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Ligation of PRRs stimulates activation of multiple transcription factors including IFN regulatory factors (IRFs) that regulate expression of target genes. Examples include IRF-3 and IRF-7 that regulate genes encoding IFN-I, and IRF-1, IRF-8, and IRF-4 that regulate genes encoding proinflammatory cytokines such as IL-12 (24). Recently, IRF-1 was identified as a potent inhibitor of a broad spectrum of viruses including flaviviruses (25, 26). Furthermore, IRF-1 is essential for host protection against lethal WNV infection, shaping both innate and adaptive responses to infection (27). Although IRF-1 is itself an ISG, it regulates a transcriptional profile of antiviral genes unique to that induced by IFN-I (26). Thus, IRF-1 and IFN-I responses appear to cooperatively promote an effective and essential antiviral program.

In this study, we analyzed the interaction between tick-borne flaviviruses and DCs to understand the effect of flaviviruses on DC function. Using Langat virus (LGTG), a naturally attenuated member of TBEV serogroup, we found that virus infection impairs DC maturation by suppressing costimulatory molecule expression and selectively inhibiting IL-12 production. This immature DC phenotype was associated with an impaired functional capacity to induce T cell proliferation. Further investigation of the inhibition of IL-12 expression revealed a coordinated impairment of IRF-1 and IFN-I signaling. LGTG and TBEV infection resulted in reduced levels of IRF-1 protein and nuclear localization without affecting Ifr-1 mRNA expression. These data identify IRF-1 as a novel target of flavivirus-mediated antagonism of innate immunity.

Materials and Methods

Mice

C57Bl/6 (wild type [WT]) mice and C57Bl/6-Tg (TcraTcrb)425Cbn/J (called OTII) mice that express a TCR specific for peptides 323–339 of OVA in the context of I-Ab were purchased from The Jackson Laboratory and housed in the animal facility at Rocky Mountain Laboratories. IFNAR1-deficient mice on a C57BL/6 background (Ifnar1−/−) were kindly provided by Dr. G. Cheng of the University of California at Los Angeles and bred at Rocky Mountain Laboratories. All animal procedures were performed in strict accordance with the regulations and guidelines of the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health.

Viruses

Vero cells were cultured in DMEM containing 10% FCS (Life Technologies, Grand Island, NY), LGTV (strain TP21) and TBEV (Sofjin strain) were propagated by infecting Vero cells at a multiplicity of infection (MOI) of 0.005. Virus stocks were prepared by centrifugation (100,000 g, 4°C) of infected cell culture supernatants collected 72 h postinfection (hpi) and stored at −80°C. Virus titers were determined by a focus-forming (FFU) assay in Vero cells (19). Work with TBEV was performed under BSL4 facilities stored at 2–85 °C and maintained at 95% pure (CD11b+/CD11c+) as determined by flow cytometry with anti–CD11c, mouse CD4+ T cells were enriched from spleens of OTII mice by magnetic negative selection using a mouse CD4+ T cell purification kit II (Miltenyi, Auburn, CA) and labeled with 5 μM of the vital dye CFSE (Life Technologies). DCs (105 cells/well) plated in triplicate in a 96-well round-bottom plate were infected with LGTV for 24 h and stimulated as described earlier. At 24 h poststimulation, DCs were loaded with 1 μg/ml chicken OVA 323–339 peptide (Invivogen) for 2 h and washed to remove unbound peptide. Ag-loaded DCs were then cocultured with CFSE-labeled OVA-specific CD4+ T cells (5 × 105) in complete DC media at a DC/lymphocyte ratio of 1:5. After 4 d, cells were collected and stained with a combination of anti–CD11c-PE-Cy7 (clone HL3; BD Biosciences, San Diego, CA), anti–CD86-FITC (clone 16-10A1; BD Biosciences), anti–CD80-PerCP-Cy5.5 (clone 1B1.3a; BD Biosciences) or RTIgG (BD Biosciences) was added to the cultures 1 hpi and maintained throughout stimulation (29).

Flow cytometry

The surface phenotype of DCs was analyzed by flow cytometry using Abs against CD11c-PE-Cy7 (clone HL3; BD Biosciences, San Diego, CA), CD80–PerCP-Cy5.5 (clone GL1; eBiosciences, San Diego, CA), I-A/B-E-PE (MHC II; clone MS/115.15.2), and CD40-PE (clone 1C10; eBiosciences). For intracellular staining, cells were first stained with anti-CD11c, fixed, permeabilized, and then stained using Abs against TNF-α (clone MPX-XT22; eBiosciences) and IL-12p40 (clone C15.6; BD Biosciences). LGTV envelope protein was detected by intracellular staining using a mouse mAb (clone 1H121) kindly provided by Dr. C. Schmaljohn at the U.S. Army Medical Research Institute for Infectious Diseases. NS3 protein was also detected intracellularly using an anti-LGTG NS3 peptide Ab (17). Data were obtained on an LSRII flow cytometer (BD Biosciences) and analyzed using FlowJo version 8.3.3 (Tree Star, Ashland, OR). For all flow data analyses, debris and dead cells were excluded with initial gates based on forward and side scatter parameters, and positive staining with live/dead yellow fixable dye (Life Technologies). A CD11c+ gate was included in all analyses to confirm DC phenotype.

Cytokine analysis by Bio-Plex assay and quantitative RT-PCR

DC culture supernatants were collected at the indicated time points after stimulation, and cytokines were quantified using Bio-Plex Pro Assay according to the manufacturer’s instructions (Bio-Rad, Hercules, CA). Total RNA was isolated from cultured DCs using a Qiagen RNeasy kit (Qiagen, Valencia, CA) with DNase I digestion. RNA was reverse transcribed using a SuperScript VILO cDNA synthesis kit (Life Technologies). cDNA was then used as a template in TagMan-PCR according to the manufacturer’s instructions. The TaqMan primers and probes sets included IL-12p35 (assay ID: Mm00434165), IL-12p40 (assay ID: Mm99999067), IFN-1 (assay ID: Mm01288580), and HPRT (assay ID: Mm01545399; Life Technologies). Reactions were performed in triplicate and analyzed using Applied Biosystems 7900HT real-time PCR system (Life Technologies). The specificity of RT-PCR was confirmed by analysis of RNA samples that had not been reverse transcribed. Results were normalized to HPRT mRNA levels and expressed as fold change relative to RNA samples from mock-infected, unstimulated DCs using the comparative threshold cycle method.

T cell proliferation assays

OVA-specific CD4+ T cells were enriched from spleens of OTII mice by magnetic negative selection using the mouse CD4+ T cell purification kit II (Miltenyi, Auburn, CA) and labeled with 5 μM of the vital dye CFSE (Life Technologies). DCs (105 cells/well) plated in triplicate in a 96-well round-bottom plate were infected with LGTV for 24 h and stimulated as described earlier. At 24 h poststimulation, DCs were loaded with 1 μg/ml chicken OVA 323–339 peptide (Invigogen) for 2 h and washed to remove unbound peptide. Ag-loaded DCs were then cocultured with CFSE-labeled OVA-specific CD4+ T cells (5 × 105) in complete DC media at a DC/lymphocyte ratio of 1:5. After 4 d, cells were collected and stained with a combination of anti–CD11c-PE-Cy7 and anti–IL-12p40 (clone C15.6; BD Biosciences). Proliferation was determined by measuring CFSE dilution using an LSRII flow cytometer (BD Biosciences). Live/dead yellow fixable stain (Invitrogen) was used to exclude dead cells before subsequent flow analyses.

Immunoblotting

Protein lysates were prepared from 4–6 × 107 DCs using lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 0.1% SDS, 1% NP-40, 0.5% Na-deoxycholate, and DNase I) containing proteinase inhibitor mixture (Roche, Indianapolis, IN). Total lysates from ∼1 × 109 cells were resolved on a 10% Tris-glycine gel (Life Technologies) and transferred to a nitrocellulose membrane (Bio-Rad). Membranes were probed with rabbit anti-mouse IRF-1 (clone D5E4; Cell Signaling Technology, Boston, MA), goat
anti-mouse IFN-γ (clone 10G10). Bound Abs were detected with HRP-conjugated species-specific anti-IgG Abs (Dako, Carpinteria, CA). Blots were developed using ECL Plus chemiluminescence reagents (GE Healthcare, Piscataway, NJ).

 Luciferase reporter assays

Human hepatoma cell line (HuH7) (30) was cotransfected with pTK-Renilla and either IFR-1 or guanylate-binding protein 2 (Gbp-2) firefly luciferase reporter plasmids (31). At 24 h posttransfection, cells were mock-infected or infected with LGTV (MOI 3) and either left untreated or treated with IFN-γ (100 ng/ml) at 24 hpi for 6 h. Cell extracts were prepared for measurement of luciferase activity using a Dual-Luciferase Reporter Assay System per the manufacturer’s instructions (Promega, Madison, WI). The reporter activity of each sample was normalized to constitutive luciferase activity of pTK-Renilla and expressed as fold change over the luciferase activity in the mock-infected, unstimulated control.

 Immunofluorescence assay

DCs (1–2 × 10^5 cells/well) were plated on eight-well Lab-Tek chamber slides (Thermo Scientific, Waltham, MA) in complete DC medium and infected with LGTV for 24 h. Cells were then stimulated with LPS (1 μg/ml) plus IFN-γ (20 ng/ml) for 3 h, fixed with 4% formaldehyde/PBS, and stored in PBS at 4°C. For detection of IFR-1 and viral protein, slides were incubated with 100% methanol for 8 min at −20°C, followed by incubation with blocking buffer (1% BSA [Sigma], 2% normal goat serum [Life Technologies], and 0.01 M glycine/Dulbecco’s PBS) for 1–2 h at room temperature. Slides were stained using rabbit anti-IRF-1 Ab (clone D5E4) and mouse anti-envelope protein (clone 11H12). Bound Abs were detected using Alexa Fluor 594–conjugated anti-rabbit IgG and Alexa Fluor 488–conjugated anti-mouse IgG, respectively (Invitrogen). Nuclei were stained using Prolong Gold + DAPI mounting media (Invitrogen). Immunofluorescent images were obtained using a Zeiss LSM710 confocal microscope.

 Statistical analyses

The Student t test was used for statistical analysis using Prism software (GraphPad Prism5, San Diego, CA).

 Results

 LGTV infection inhibits DC maturation in response to TLR ligand stimulation

Upon recognition of a pathogen, immature DCs in the periphery upregulate surface expression of MHC II and costimulatory molecules necessary for optimal development of adaptive immune responses. To investigate the effect of tick-borne flaviviruses on DC maturation, DCs were infected with LGTV and analyzed by flow cytometry for expression of the DC maturation markers CD40, CD86, and MHC II. Intracellular staining for viral envelope protein was used to distinguish viral Ag-positive cells (infected) from viral Ag-negative cells (bystander) in the same culture. The percentage of DCs expressing LGTV envelope protein at 24 hpi was 15–25% (Fig. 1A). A similar percentage of cells positive for LGTV were found by intracellular staining for the nonstructural protein, NS3, that is detectable only during virus replication, confirming that these cells contain replicating virus (Fig. 1B). As shown in Fig. 1C, LGTV* DCs showed negligible upregulation of CD40, CD86, and MHC II surface expression compared with the positive control of DCs treated with LPS plus IFN-γ, a strong stimulus for DC maturation (Fig. 1C). Thus, DCs fail to upregulate maturation markers post LGTV infection and, therefore, exhibit an immature phenotype.

To determine whether the lack of DC maturation was due to a virus-mediated block in signal transduction, or simply an inability of DCs to recognize LGTV infection, the response of infected DCs to stimulation with LPS/IFN-γ was tested. At 24 hpi, DCs were left untreated or treated with LPS/IFN-γ for 24 h before staining for viral Ag and DC maturation markers. Bystander cells responded to LPS/IFN-γ stimulation by upregulating CD40 and CD86 to similar levels as those observed in stimulated, mock-infected DCs (compare Fig. 1D with 1C). In contrast, LGTV* DCs in the same culture maintained low expression of maturation markers except for MHC II that was comparably upregulated in both LGTV-infected and bystander DCs (Fig. 1D, 1E). Similarly, LGTV-infected DCs did not upregulate expression of CD40 and CD86 surface expression in response to LPS alone (Fig. 1E). Although treatment with polyriboinosinic-polyribocytidylic acid, a TLR3 ligand, did not induce CD40 or MHC II expression, upregulation of CD86 by this stimulus was also suppressed in LGTV-infected DCs (Fig. 1E). Incubation of DCs with UV-inactivated virus did not result in suppression of LPS/IFN-γ–stimulated CD40 and CD86 expression (Fig. 1F). Taken together, these results indicate that LGTV replication inhibits DC maturation induced by multiple TLR signaling pathways.

 LGTV suppresses production of IL-12 and induction of T cell proliferation by DCs

In addition to maturation phenotype, activated DCs produce cytokines required for optimal immune responses. To determine the effect of LGTV infection on cytokine production, DCs were stained for intracellular IL-12p40/p70, TNF-α, or IL-6, in conjunction with viral envelope protein, and analyzed by flow cytometry. Cytokine expression was notably weak in LGTV-infected DCs with a similar low percentage (5–10%) of both infected and bystander DCs expressing TNF-α (Fig. 2A), IL-12 (Fig. 2B), and IL-6 (data not shown). To determine whether infection actively inhibited cytokine expression, we left DCs untreated or treated with LPS, LPS/IFN-γ, or loxoribine (a TLR7 ligand) at 24 hpi and analyzed at 8 h post-stimulation. A high percentage (~80%) of infected, bystander, or mock-infected DCs expressing TNF-α (Fig. 2A, 2C), indicating that infection had no effect on induction of this cytokine. However, a lower percentage of infected DCs produced IL-12p40/p70 upon stimulation with LPS or LPS/IFN-γ compared with bystander or mock-infected DCs (Fig. 2B, 2D). LGTV infection did not affect IL-12 production after stimulation with loxoribine, suggesting that a lack of IL-12p40/p70 was due to a specific block in signal transduction and not to a general inability of infected cells to produce this cytokine. The suppressive effect also required virus replication because DCs exposed to UV-irradiated virus showed no defect in IL-12 expression after stimulation (Fig. 2E).

To further investigate these findings, we measured IL-12p40, IL-12p70, TNF-α, IL-6, and IL-10 in culture supernatants using a multiplex cytokine bead assay (Fig. 3A). LPS/IFN-γ–stimulated high levels of IL-12p40 and IL-12p70 in mock-infected DC cultures that were significantly reduced in LGTV-infected cultures. This effect of infection was not observed after stimulation with loxoribine (Fig. 3A). Furthermore, LGTV infection resulted in similar IL-6 production throughout the time course and enhanced TNF-α secretion at early time points (3 h) after LPS/IFN-γ or loxoribine treatment compared with mock-infected cultures. Thus, LGTV-infected DCs exhibit a specific defect in IL-12 production that is not associated with a general inability to produce and secrete cytokines.

A possible explanation for suppressed IL-12 expression is that LGTV infection induced IL-10, a well-characterized immunosuppressive cytokine that inhibits DC production of IL-12 (reviewed in Ref. 32). Indeed, approximately a 3-fold greater concentration of IL-10 was detected in infected cultures compared with mock-infected cultures after stimulation with LPS/IFN-γ (Fig. 3A). To determine whether suppression of IL-12 was mediated by IL-10, we repeated experiments in the presence of IL-10R blocking Ab. Blocking the IL-10R did not recover IL-12 expression in infected DCs at 8 h poststimulation as measured by flow cytometry (Fig. 3B, upper graph). Blocking the IL-10R did increase the overall

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levels of IL-12p40/p70 in supernatants at 24 h poststimulation, confirming that IL-10R signaling was blocked. However, it did not recover suppression of IL-12p40/p70 by LGTV infection (Fig. 3B, lower graph). Thus, LGTV-mediated suppression of IL-12 secretion is not due to the inhibitory effects of IL-10 but rather appears to be a direct effect of virus replication.

A critical measure of DC function is its capacity to induce proliferation of CD4+ T cells. To examine this, we treated mock-infected, infected with LGTV strain TP21, or exposed to UV-inactivated LGTV. At 40 hpi, cells were stained for surface CD11c and intracellular viral protein, and analyzed by flow cytometry. (A and B) Representative dot plots of CD11c and viral envelope protein (A) or NS3 protein (B) show the gating and percentage of LGTV+ and LGTV bystander populations. (C) Expression of CD40, CD86, and MHC II on mock-infected (filled gray lines) and gated LGTV+ cells (black line) are shown. DCs treated with LPS/IFN-γ (gray dashed line) for 24 h served as a positive control for maturation. (D) At 28 hpi, mock- and LGTV-infected DCs were cultured in the presence of various activation stimuli, and changes in surface phenotype were assessed. Expression of CD40, CD86, and MHC II on LGTV bystander (gray line) and LGTV+ DCs (black line) after stimulation with LPS/IFN-γ compared with mock-infected, untreated (filled gray lines) cells. (E) Fold change in the geometric mean fluorescence intensity (MFI) of surface markers relative to isotype controls after no treatment or stimulation with LPS, LPS/IFN-γ, or polyriboinosinic-polyribocytidylic acid (PIC). Results are presented as the mean ± SD of three independent experiments. *p < 0.05. (F) The fold change in MFI of CD40, CD86, and MHC II after LPS/IFN-γ stimulation of DCs exposed to UV-inactivated virus and mock-infected controls. Results from one of two independent experiments are presented as the mean ± SD of triplicate samples.
or LGTV-infected DCs with various maturation stimuli as described earlier. At 24 h poststimulation, DCs were loaded with OVA323 peptide and cocultured with CFSE-labeled OVA-specific CD4+ T cells. LGTV- and mock-infected DCs induced similar low levels of T cell proliferation as demonstrated by CFSE dilution (Fig. 3B). Although LPS/IFN-γ stimulation increased the T cell stimulatory capacity of both DC cultures, the degree of T cell proliferation was significantly lower in LGTV-infected DCs. This suppressed T cell proliferative capacity of infected DCs was also observed with LPS stimulation, but not with loxoribine (Fig. 3B).

Thus, impairment of costimulatory marker upregulation and IL-12 production in DCs by LGTV is associated with a compromised ability to drive Ag-specific T cell proliferation.

**LGTV-mediated suppression of DC function involves both IFN-dependent and -independent pathways**

Optimal production of IL-12 by DCs requires cooperation between TLR and IFN-1 signaling (29), suggesting that IFN-1 antagonism by LGTV may be responsible for the loss of IL-12 expression. To examine this, we removed the influence of IFN-1 by repeating our

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**FIGURE 2.** Production of IL-12p40/p70, but not TNF-α, is suppressed in LGTV-infected DCs after TLR stimulation. DCs were mock-infected, or infected with LGTV for 28 h and then stimulated with LPS, LPS/IFN-γ, or loxoribine for 8 h and analyzed by flow cytometry. Representative dot plots for viral protein and TNF-α (A) or IL-12p40/p70 (B) in CD11c+ cells are shown. The percentage of cells expressing TNF-α (C) or IL-12p40/p70 (D) for each population as indicated is shown. *p < 0.05. (E) The percentage of DCs, mock-infected or exposed to UV-inactivated virus, that express IL-12p40/p70 after stimulation with LPS or LPS/IFN-γ. Results are presented as mean ± SD of three independent experiments.

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or LGTV-infected DCs with various maturation stimuli as described earlier. At 24 h poststimulation, DCs were loaded with OVA323 peptide and cocultured with CFSE-labeled OVA-specific CD4+ T cells. LGTV- and mock-infected DCs induced similar low levels of T cell proliferation as demonstrated by CFSE dilution (Fig. 3B). Although LPS/IFN-γ stimulation increased the T cell stimulatory capacity of both DC cultures, the degree of T cell proliferation was significantly lower in LGTV-infected DCs. This suppressed T cell proliferative capacity of infected DCs was also observed with LPS stimulation, but not with loxoribine (Fig. 3B). Thus, impairment of costimulatory marker upregulation and IL-12 production in DCs by LGTV is associated with a compromised ability to drive Ag-specific T cell proliferation.

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studies using DCs from IFN-I receptor–deficient (Ifnar$^{-/-}$) mice. The percentage of mock-infected and bystander cells expressing detectable levels of IL-12 was reduced in Ifnar$^{-/-}$ DCs compared with WT DCs (compare Fig. 4A with 2D) by $\sim$25%, indicating

![Graphs showing IL-12 production and T cell proliferative capacity](image_url)

**FIGURE 3.** LGTV infection of DCs inhibits IL-12 production and T cell proliferative capacity. DCs were mock-infected or infected with LGTV for 28 h and then treated with medium alone, or medium containing LPS/IFN-γ or loxoribine. (A) Culture supernatants were harvested at 0, 3, 8, and 24 h after stimulation, and levels of IL-12p40, IL-12p70, TNF-α, IL-6, and IL-10 were determined by Bio-Plex immunoassay. Data are compiled from three independent experiments and expressed as the mean $\pm$ SD. (B) DC infection and LPS/IFN-γ stimulation were performed in the presence of anti–IL-10R or RtlgG control Abs. Cells were stained for intracellular IL-12p40 at 8 h poststimulation and analyzed by flow cytometry (upper graph), or supernatants were collected at 24 h poststimulation and secreted cytokines were measured by Bio-Plex (lower graph). The graphs represent the mean $\pm$ SD of cells expressing IL-12p40 in each population or of IL-12p40 production. Data are representative of two independent experiments performed in triplicate. (C) At 24 h poststimulation, DCs were loaded with Ova323 peptide and cocultured with CFSE-labeled, Ova-specific CD4+ T cells at a ratio of 1:5 (DCs/T cells). After 4 d, cells were harvested and analyzed by flow cytometry. The dot plots (CFSE versus CD3) show the loss of CFSE fluorescence in T cells (identified by CD3$^+$ staining) cocultured with untreated or LPS/IFN-γ–stimulated DCs. Representative dot blots show the percentage of proliferating cells, as indicated by the boxed gate, among total gated live T cells. The graph represents the mean $\pm$ SD of proliferating cells from three independent experiments. *p < 0.05.
that maximum expression of IL-12 requires IFN-I signaling. However, this effect of IFN-I signaling on IL-12 expression was not observed in LGTV-infected DCs, confirming that viral inhibition of IFN-I signaling contributes to IL-12 suppression. Nevertheless, a significant inhibition of IL-12 expression (Fig. 4A) and an impaired capacity to stimulate OVA-specific T cell proliferation after stimulation (Fig. 4B) remained evident in LGTV-infected Ifnar$^{-/-}$ DCs (Fig. 4B). Thus, LGTV must encode a second mechanism that synergizes with IFN-I antagonism to inhibit IL-12 production and T cell proliferative capacity of infected DCs.

**LGTV suppresses IL-12p40 mRNA expression by inhibiting IFN-I signaling and IRF-1**

To determine the second mechanism of antagonism, we concentrated on IL-12 as a critical cytokine for T cell activation (reviewed in Ref. 32). Biologically active IL-12 (IL-12p70) is composed of two subunits, IL-12p35 and IL-12p40. Examination of the kinetics of IL-12p35 and IL-12p40 mRNA expression in DC cultures revealed that LPS/IFN-γ-induced transcription of IL-12p40 mRNA, but not that of IL-12p35, was inhibited in LGTV-infected WT DCs (Fig. 5A, 5B). The inhibitory effect of LGTV infection on IL-12p40 mRNA expression was nearly eliminated in Ifnar$^{-/-}$ DCs at 8 and 16 h poststimulation (Fig. 5C), suggesting a contribution of IFN-I antagonism at these time points. However, an IFN-I-independent block in IL-12p40 expression remained evident at 3 h poststimulation. Expression of IL-12p40 mRNA is regulated by multiple transcription factors, predominantly NF-κB, IRF1, IRF2, and IRF8 (33–37). A block associated with NF-κB could be ruled out because flaviviruses are known to activate this transcription factor (38), and LGTV infection of DCs had no inhibitory effect on the expression of cytokines driven by NF-κB, such as TNF-α and IL-6. Therefore, we examined the role of IRF-1 (34, 35) by using DCs derived from Ifnr$^{-/-}$ mice. LPS/IFN-γ-stimulated IL-
12p40 mRNA expression was not different between LGTV- and mock-infected *Irf-1*−/− DCs (Fig. 5D). Thus, LGTV suppression of IL-12p40 mRNA is dependent on IRF-1, particularly during early gene induction, identifying IRF-1 as a potential target of viral antagonism.

**LGTV infection reduces IRF-1 nuclear localization and transcriptional activity**

To examine whether LGTV directly affects IRF-1 transcriptional activity, we performed luciferase reporter assays in Huh7 cells known to express IRF-1 (39) using two plasmids: one containing an IRF-1–responsive Gbp-2 promoter and another containing the *Irf-1* gene promoter (31, 40). IFN-γ stimulation resulted in 60- to 80-fold induction of luciferase expression driven by the *Irf-1* gene promoter in both mock- and LGTV-infected cells (Fig. 6). However, LGTV infection reduced reporter activity driven by the IRF-1–responsive Gbp-2 promoter compared with mock-infected cells (Fig. 6), suggesting that LGTV can antagonize IRF-1 function.

To examine this possibility further, we assessed nuclear localization of IRF-1 in infected WT DCs by IFA using Abs specific for IRF-1 and viral envelope protein. IRF-1 was clearly accumulated in the cell nucleus of LPS/IFN-γ-stimulated mock-infected DCs compared with unstimulated controls (Fig. 7, upper panels). In stimulated LGTV-infected cultures, strong nuclear localization of IRF-1 was observed in bystander cells (Fig. 7, lower panels) but was obviously diminished in LGTV+ DCs. This was associated with reduced levels of IRF-1 protein in LGTV+ cells as shown by flow cytometry (Fig. 8A) and Western blot (Fig. 8B). LGTV infection alone induced low levels of IRF-1 in WT DCs (Fig. 8B), but this occurred only in bystander cells as indicated by flow cytometry. Loss of IRF-1 protein was independent of viral IFN-I antagonism because it occurred in both WT and *Iftar*−/− DCs, but dependent on virus replication, as it was not evident in cells incubated with UV-inactivated virus (Fig. 8A). Expression of IRF-8, another positive regulator of IL-12–p40 (41), was unaffected by LGTV infection (Fig. 8B). Furthermore, consistent with the luciferase reporter experiments, LGTV had no effect on the induction of *Irf-1* mRNA expression by LPS/IFN-γ in either WT or *Iftar*−/− DCs (Fig. 8C). Thus, LGTV infection specifically depletes cellular levels of IRF-1 protein without affecting *Irf-1* mRNA expression. Finally, we extended our analysis to include the highly pathogenic Sofjin strain of TBEV. WT or *Iftar*−/− DCs infected with TBEV and stimulated with LPS or LPS/IFN-γ for 3 h had reduced IRF-1 protein compared with mock-infected controls, whereas IRF-8 expression was not different (Fig. 8D). Taken together, these studies suggest that both LGTV and TBEV antagonize IRF-1 by reducing IRF-1 protein levels and nuclear localization.

**Discussion**

IRF-1 is emerging as an important regulator of early cellular responses to virus infection, responsible for induction of antiviral effector genes that partly overlap with, but do not require, IFN-1 (25, 26). Hence IRF-1 may provide cellular resistance to viral infection at a crucial time postinfection before IFN-1 can be expressed. In this study, we demonstrate that tick-borne flaviviruses inhibit IRF-1 independently of their ability to antagonize IFN-I signaling. One outcome of IRF-1 antagonism in virus-infected DCs was a failure in early IL-12p40 gene induction resulting in reduced IL-12p40 and IL-12p70 protein expression. This suppressive effect was compounded by a requirement for signaling through IFNAR to amplify and sustain IL-12p40 expression, which is potently inhibited in LGTV- or TBEV-infected cells (19, 22). The cumulative effect was a disruption of DC function in driving T cell proliferation. Thus, our studies have revealed that the tick-borne flaviviruses, LGTV and TBEV, coordinate suppression of signals through both IRF-1 and IFNAR in a multifaceted strategy to avoid early antiviral immunity.

DCs are critical for adaptive immunity against flavivirus infections, particularly for generation of CTL responses (42). Post virus infection, innate responses control the magnitude and quality of the adaptive immune response by modulating the function of DCs (reviewed in Ref. 5). Specifically, both signaling through PRR and secreted IFN are important for phenotypic and functional maturation of DCs. LGTV infection inhibited upregulation of DC costimulatory molecules, thereby resulting in an immature and potentially tolerogenic phenotype. Furthermore, we previously showed that LGTV infection inhibits IFN-induced STAT1 phosphorylation in both human monocyte-derived DCs and mouse bone marrow–derived DCs (19). Therefore, we can speculate that the lack of DC maturation post LGTV infection involves IFN-I antagonism. Consistent with this hypothesis, inhibition of IFN-I signaling using an anti-IFNAR mAb during WNV infection of mice resulted in decreased expression of CD86 on APCs, lower levels of IL-12p40 production, and elevated levels of IL-10, all factors that likely diminish optimal Ag presentation to CD8+ T cells (43). WNV also antagonizes IFN-I–dependent signaling (23, 44). Collectively, these data suggest that, similar to other models of virus infection (45, 46), a significant link exists between flavivirus IFN-I antagonism and efficient priming and development of adaptive immune responses. However, it is also possible that WNV suppresses IRF-1, because Scherbik et al. (47) reported delayed *Irf1* mRNA transcription and decreased IRF-1 protein levels in WNV-infected mouse embryonic fibroblasts, although they did not explore this finding any further. Thus, it is possible that suppression of IRF-1 and IFN-I is a common feature of multiple flaviviruses.

To uncover flavivirus antagonism of IRF-1, our studies used LPS/IFN-γ as a classic, potent stimulus of DC maturation, cytokine production, and driver of T cell proliferation (48–50). Aspects of inhibition were also seen with TLR3 stimulation (polyriboinosinic-
polyribocytidylic acid–stimulated CD86 expression) or with IFN-γ alone (IRF-1–dependent luciferase expression), suggesting that viral antagonism is not restricted to TLR4 signaling. Interestingly, suppression was not observed after TLR7 stimulation (loxoribine), which may reflect the relative importance of IRF-1 and IFN-I in each pathway. Alternatively, TLR7 may not be a strong enough stimulus to reveal the block in IRF-1 signaling.

The regulation of biologically active IL-12 production is complex and involves the coordinated expression of each of its subunits, IL-12p35 and IL-12p40. We observed suppression of IL-12p40 expression, but not IL-12p35, suggesting that signaling events proximal to TLRs are not affected by infection. Because IRF-1 can regulate the expression of both IL-12p40 and IL-12p35, it was surprising that IL-12p35 was not negatively affected by LGTV. However, because of the complexity of the two promoters, it is possible that, at least in the context of murine DCs, IL-12p35 is less dependent on IRF-1 or its expression is compensated by other transcription factors.

IL-12p40 forms additional complexes with IL-23p19 to produce IL-23, also expressed by macrophages and DCs (51). IL-23 is an important stimulator of memory T cell proliferation and can promote CD4+ T cells to differentiate into Th17 cells that have central roles in inflammation (51). Interestingly, IL-12p40 was critical for resistance to WNV-induced disease, necessary for IL-23–dependent homing of macrophages to the CNS and control of virus replication (52). Thus, it is possible that flavivirus suppression of IL-12p40 expression promotes virus pathogenesis through suppression of both IL-12 and IL-23.

IL-12p40 is likely to be only one of many gene products affected by viral degradation of IRF-1. In support of this, IRF-1 restricts WNV replication in macrophages independently of IFN-I levels, suggesting that IRF-1 transcriptional targets include ISGs that directly inhibit virus replication (27). The relative role of IRF-1 in induction of specific genes is complex and depends on the cell type, the availability of other transcription factors (e.g., IRF-2), and the gene itself. For example, transcription of the gene encoding CIITA, the major regulator of MHC II expression, is controlled by three independent promoters that have differential cell-type–specific usage and requirements for IRF-1 (53). Nonhematopoietic cells (endothelial, fibroblasts, and astrocytes, etc.) use a promoter that contains an IRF-1 binding site and involves IFN-γ–induced expression of IRF-1, whereas professional APCs, such as DCs, use a distinct promoter for constitutive CIITA expression that does not require IRF-1. Thus, virus modulation of IRF-1 may have specific and unique consequences to primary cellular targets of flavivirus infection, including DCs, endothelia, fibroblasts, and neurons, as has been suggested for WNV infection (27).

Our findings suggest that LGTV and TBEV interfere with IRF-1 function in a cell-type– and species-independent manner. Virus infection had no effect on the expression of *Ifi1* mRNA in infected mouse DCs, or on the amplitude of reporter gene expression driven by the *Ifi1* promoter in human Huh7 cells after stimulation with IFN-γ. This was surprising considering the ability of many flaviviruses including LGTV to inhibit IFN-γ–dependent STAT1 phosphorylation (19). It is possible that virus antagonism of IFN-γ signaling is cell type dependent or that alternative transcription factors drive *Ifi1* expression. In reference to the latter, NF-κB can function as an alternative regulator of IRF-1 transcription, particularly in the absence of STAT1 (31). Nevertheless, IRF-1–dependent gene expression was suppressed in LGTV-infected Huh7...
cells, suggesting that IRF-1 function was compromised. Diminished levels of IRF-1 were observed in LGTV- or TBEV-infected DCs, although only apparent after stimulation. This observation, together with the fact that IRF-1 degradation was incomplete, raises the intriguing possibility that only the activated form of IRF-1 is modulated by flavivirus infection. An incomplete loss of IRF-1 may be sufficient to suppress gene expression because, in the absence of high levels of IRF-1, IRF-2 competes with IRF-1 for promoter binding and represses target gene transcription (54).

The finding that LGTV infection diminished IRF-1 protein levels without affecting Irf-1 mRNA expression suggests that the protein may be targeted for enhanced degradation. IRF-1 has a short half-life (≈30 min) and is normally turned over in the cell via the ubiquitin-proteosome degradative pathway. Interestingly, treatment of LGTV-infected DCs with inhibitors of the proteasome or lysosome, another major cellular pathway of protein degradation, failed to recover IRF-1 protein levels in LGTV-infected DCs (S. Robertson and S. Best, unpublished observations). It is possible that IRF-1 is more directly targeted as a substrate for flavivirus-encoded proteases (i.e., NS2B/3) or a cytosolic cellular protease. Alternatively, tick-borne flaviviruses may have evolved a strategy to inhibit nuclear export or translation of Irf-1 mRNA. These possibilities will be the subject of future studies to define the molecular mechanism by which tick-borne flaviviruses inhibit IRF-1.

Other viral and bacterial pathogens also antagonize IRF-1 as a means of dampening host innate immunity. In contrast with LGTV and TBEV, suppression of IRF-1 by hepatitis C virus, a member of the Flaviviridae family, occurs at the level of transcription and is associated with capsid expression (55). Hepatitis C virus is also able to prevent IRF-1 activation in response to dsRNA stimulation via NS5A-mediated suppression of protein kinase R activation (39). Vaccinia virus, as well as other members of the poxvirus family, encodes two host-range genes (K1L and C7L) that suppress IRF-1–dependent restriction of virus replication (56). In this case, vaccinia virus appears to inhibit a critical IRF-1–inducible factor rather than directly targeting IRF-1. In bacteria, adenylate cyclase toxin (CyaA) of Bordetella pertussis suppresses LPS/IFN-γ–induced expression of IRF-1 and IRF-8 mRNA expression (57). In addition, lipids isolated from virulent Francisella tularensis strain (SchuS4) prevent binding of IRF-1 and IRF-8 to the human IL-12p40 promoter, thereby inhibiting IL-12p40 production in primary human DCs (58). Thus, the broad spectrum of pathogens shown to interfere with IRF-1 function underscores the importance of this factor in the host–pathogen interface.

**FIGURE 8.** Flavivirus infection of DCs diminishes IRF-1 protein levels without affecting Irf-1 mRNA expression. WT or Ifnar−/− DCs were mock-infected, infected with LGTV, or exposed to UV-inactivated virus for 28 h. Cells were treated with medium alone or medium containing LPS/IFN-γ. (A) Histograms for intracellular IRF-1 expression are shown for mock-infected DC cultures, gated infected cells, or bystander cells from infected cultures and DCs exposed to UV-inactivated virus as indicated. Most DCs (70–90%) were infected in Ifnar−/− cultures; therefore, IRF-1 expression in bystander cells was not determined. MFI values of IRF-1 expression in untreated and stimulated samples are shown. (B) Total protein lysates were assayed for IRF-1 and IRF-8 by immunoblot. Duplicate blots were probed for β-actin as a loading control. (C) RNA was isolated at various times poststimulation, and Irf-1 mRNA expression was determined by quantitative RT-PCR. Data shown are representative of three independent experiments. (D) WT and Ifnar−/− DCs were infected with TBEV (Sofjin strain) for 28 h and left untreated, or stimulated with LPS or LPS/IFN-γ for 3 h. IRF-1 and IRF-8 were detected by immunoblot. Blots were stripped and reprobed for β-actin. A representative immunoblot of three independent experiments for both LGTV and TBEV is shown.
Because DCs are early cellular targets of flavivirus infection, virus modulation of IFN-1 and IFN-I signaling in DCs offers an opportunity to manipulate innate and adaptive immune responses. Important insight into the pathogenic role of flavivirus antagonism of IFN-1 will come from identifying the viral protein responsible and its host cell interacting partner(s). The pathogenesis of recombiant viruses that can no longer antagonize IFN-1 can then be studied. Elucidation of the IFN-1–specific transcriptional response as it relates to restriction of flavivirus replication will also be important for a complete understanding of both the antiviral flavivirus innate immune response and the consequence of IFN-1 antagonism to viral pathogenesis.

Acknowledgments

We thank Anita Mora for assistance with graphics and Heinz Feldmann, Jeff Shannon, and Catherine Bosio for critical review of the manuscript. The work with TBEV was conducted in compliance with regulation 42 CFR 73 under the Division of Select Agents and Toxins (Centers for Disease Control and Prevention).

Disclosures

The authors have no financial conflicts of interest.

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