Limited *In Vivo* Production of Type I or Type III Interferon After Infection of Macaques with Vaccine or Wild-Type Strains of Measles Virus

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The innate immune response to viral infections often includes induction of types I and III interferons (IFNs) and production of antiviral proteins. Measles is a severe virus-induced rash disease, but *in vitro* studies suggest that in the absence of defective interfering RNAs, neither wild-type (WT) nor vaccine strains of measles virus (MeV) induce IFN. To determine whether IFN is produced *in vivo*, we studied tissues from macaques infected with vaccine or WT strains of MeV using quantitative reverse transcriptase–polymerase chain reaction to assess levels of IFN and IFN-stimulated gene (ISG) mRNAs and a flow cytometry-based bioassay to assess levels of biologically active IFN. There was little to no induction of type I IFN, type III IFN, Mx, or ISG56 mRNAs in monkeys infected with vaccine or WT MeV and no IFN detection by bioassay. Therefore, the innate responses to infection with vaccine or WT strains of MeV are not dependent on IFN production.

Introduction

MEASLES IS A SEVERE rash disease caused by measles virus (MeV). Infection is initiated in the respiratory tract and spreads to draining lymph nodes (LNs) (Sakaguchi and others 1986; Lemon and others 2011). MeV-infected leukocytes then enter the blood, causing viremia (Forthal and others 1992) with spread to multiple tissues. In 2012, ~122,000 deaths were caused by measles (Perry and others 2014). Most of these deaths can be attributed to the immune suppression caused by MeV infection and the subsequent increased susceptibility to infections from other pathogens (Tamashiro and others 1987). In those that survive, there is life-long immunity to reinfection.

Protection from infection is also conferred by immunization with live-attenuated MeV vaccines that have significantly reduced infection and death due to measles (Wolfson and others 2007). Vaccination induces protective antibody and T-cell responses and does not cause disease or clinically significant immune suppression (Fireman and others 1969), but the basis of attenuation is not understood. Multiple mechanisms, including differences in induction of interferon (IFN) (Oldstone 2006), have been proposed to explain the

differences between wild-type (WT) and vaccine strains of MeV in their ability to cause disease (Griffin 2010).

Type I IFN (IFN α and β) and type III IFN (IFN λ , including IL-28a, IL-28b, and IL-29) can be induced as a part of the innate immune response when signaling pathways are activated after virus recognition by pathogen-recognition receptors such as toll-like receptors and RNA helicases (Kotenko and others 2003; Takeuchi and Akira 2010). Both types I and III IFNs activate a JAK-STAT signaling pathway, leading to induction of IFN-stimulated genes (ISGs) that code for proteins such as myxovirus resistance gene (MxA) and ISG56 (IFIT1) which can inhibit viral replication (Stetson and Medzhitov 2006).

Multiple *in vitro* studies have compared type I IFN induction by vaccine and WT strains (Naniche and others 2000; Shingai and others 2007; Pfaller and Conzelmann 2008) of MeV, and a few studies have also analyzed type III IFN (Naniche and others 2000; Shivakoti and others 2013). These studies have often shown more efficient induction of IFN by vaccine MeV, but many have been complicated by use of virus stocks that contain viral particles with defective interfering (DI) RNAs produced during MeV replication in tissue culture (Shingai and others 2007; Kessler and others

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2011; Shivakoti and others 2013). There is little evidence of type I IFN induction in humans with measles (Zilliox and others 2007; Devaux and others 2008), but this has been difficult to study because infection is not recognized until after the adaptive immune response (rash) with initiation of virus clearance that appears ~2 weeks after infection (Sato and others 2008).

In vivo studies of macaques show that MeV is detected in lymphocytes and respiratory tissues at about 6 days after infection, reaching peak virus levels at 7–11 days (De Swart and others 2007; de Vries and others 2010). To study the *in vivo* IFN response to respiratory infection with WT and vaccine strains of MeV, we have used the well-characterized and highly relevant macaque model of MeV infection that mimics the human disease. Specifically, we have compared the production of IFN (types I, II, and III) and expression of IFN, Mx, and ISG56 mRNAs in multiple tissues and body fluids from 2 different macaque species early (2–11 days) after respiratory infection with vaccine and WT strains of MeV before appearance of the adaptive immune response. No IFN α/β and only small amounts of IFN λ , Mx, and ISG56 mRNAs were induced after infection with either vaccine or WT strains of MeV.

Materials and Methods

Cells

Vero, Vero/hSLAM (Ono and others 2001), and BHK (ATCC) cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum, penicillin/streptomycin, and 2 mM L-glutamine (GIBCO) in 5% CO₂ at 37°C.

Viruses

Nine strains of WT and vaccine MeVs representing 4 different genotypes were used for infection of macaques. Vero cell-adapted Chicago-1, Edmonston WT, Moraten, Los Angeles USA/89 (LA-89), and St. Louis USA-91 (STL-91) strains of WT and vaccine-derived MeVs were obtained as previously described (Auwaerter and others 1999). Edmonston WT is the earliest available passage (7 times in primary human kidney and 6 times in Vero cells) of the original Edmonston isolate (Rota and others 1994). Moraten is a tissue culture-adapted attenuated vaccine strain derived

from the Edmonston WT virus (A genotype) (Enders and Peebles 1954). LA-89 and STL-91 were isolated from infants with severe combined immunodeficiency who developed progressive infection after vaccination with Moraten (Monafo and others 1994). Chicago-1 (D3 genotype) was isolated in Vero cells during a 1989 measles outbreak in Chicago (Smaron and others 1991; Rota and others 1992; World Health Organization 1998). The Edmonston-Zagreb (EZ) strain of MeV (A genotype) was manufactured by the Serum Institute of India and obtained as a dry powder from Aktiv-Dry LLC (Lin and others 2011). These viruses were grown and assayed by plaque formation in Vero cells.

WT strains of MeV included Bilthoven (Bilt) (C2 genotype) isolated during a 1991 outbreak in The Netherlands (van Binnendijk and others 1994) and grown in Vero/hSLAM cells; Khartoum Sudan (KS) (B3 genotype) isolated during a 1997 outbreak in Khartoum and grown in CD150⁺ human B-LCL cells (El Mubarak and others 2007); and IC(Ichinose)-B (D3 genotype) isolated in 1984 in Japan. Recombinant KS (rMV^{KS}) and IC323-EGFP were generated as previously described (Hashimoto and others 2002).

Vesicular stomatitis virus expressing green fluorescent protein (VSV-GFP) (gift of Sean Whelan, Harvard Medical School) was grown and assayed in BHK cells.

Animals

Tissue samples from 32 seronegative macaques infected with WT or vaccine strains of MeV and 2 uninfected macaques were obtained from 4 different studies (Table 1):

Group 1: Lung, LN, spleen, peripheral blood mononuclear cells (PBMCs), plasma, bronchoalveolar lavage (BAL), and oropharynx samples were obtained from necropsies performed at 7 days after infection as a part of a measles vaccine dry powder toxicology study performed at Avanza Laboratories (Lin, unpublished data). In this study, rhesus macaques (*Macaca mulatta*) were infected by inhalation with a dry-powder formulation of the EZ vaccine virus (6.5×10^4 pfu) ($n=6$) or dry powder without MeV ($n=2$) as previously described (Lin and others 2011).

Group 2: PBMCs, nasal and throat swabs were collected from 3 Indian-origin male juvenile rhesus macaques from the Johns Hopkins University breeding colony at 7 days after intra-tracheal infection with the Bilthoven strain of WT MeV (10^4 pfu).

TABLE 1. THE 4 DIFFERENT GROUPS OF MONKEYS STUDIED

Monkey group	No. of monkeys	Virus strain	Type of MeV	Samples	Assay
1	8 (6 infected + 2 control)	EZ	Vaccine	PBMCs, plasma, spleen, lung, lymph nodes, BAL, oropharynx	qRT-PCR IFN bioassay DI RNA test
2	3	Bilt	WT	PBMCs, plasma, nasal swabs, throat swabs	qRT-PCR IFN bioassay
3	3	IC323 KS	WT	Lymph nodes	qRT-PCR
4	20 (4 per virus)	Chi, Edm, Mor, LA-89 and STL-91	Vaccine	Plasma, NP, CS and tracheal fluids	IFN bioassay

The monkey group number, the total number of monkeys in each group, the viruses used for infection, the samples collected, and assays that were run on those samples are identified for each group.

BAL, bronchoalveolar lavage; Bilt, Bilthoven; CS, conjunctival swab; DI, defective interfering; EZ, Edmonston-Zagreb; IFN, interferon; KS, Khartoum Sudan; MeV, measles virus; NP, nasopharyngeal; PBMC, peripheral blood mononuclear cell; qRT-PCR, quantitative reverse transcriptase-polymerase chain reaction; WT, wild-type.

Group 3: Tracheobronchial and/or axillary LNs were collected from 3 cynomolgus macaques (*Macaca fascicularis*) infected intra-tracheally with rMV^{KS} (KS) or IC323 WT strains of MeV (10^4 pfu). Animals were necropsied at day 9 (IC323) (De Swart and others 2007) or day 11 (KS) (de Vries and others 2012) after infection.

Group 4: Nasopharyngeal (NP), conjunctival swab (CS), and tracheal fluids and aspirates were collected from 20 rhesus macaques from the Caribbean Primate Research Facility in Puerto Rico after intratracheal and intranasal infection (10^4 TCID₅₀) with 5 different strains of MeV (Edmonston WT, Moraten, STL-91, LA-89 and Chicago-1) (Auwaerter and others 1999).

Animals were maintained, and studies were performed in accordance with experimental protocols approved by the Johns Hopkins Animal Care and Use Committee, the independent animal experimentation ethical review committee DCC in Driebergen, The Netherlands, the National Institutes of Health, or Avanza laboratories.

Sample preparation

PBMCs and plasma were isolated from Lympholyte Mammal (Cedarlane Laboratories) gradients of heparinized whole blood. For group 2, nasal and throat swabs were immersed in 1 mL phosphate-buffered saline (PBS) followed by centrifugation. PBMCs (groups 1 and 2) and cells from nasal and throat swabs (group 2) were stored at -80°C until further use. RNA was isolated using the RNeasy Plus Micro Kit (Qiagen).

For tissues from group 1, fresh tissues were transported in PBS, snap frozen, and stored at -80°C . Before RNA isolation, QIAzol lysis reagent (Qiagen) was added to each tissue in a lysing matrix D tube and homogenized in a Fast-prep 24 machine (MP Biomedicals). RNA isolated from homogenates using the RNeasy lipid tissue mini kit (Qiagen) was used for quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) and DI RNA detection. LNs from group 3 were collected in RNeasy lysis buffer (Qiagen) and stored at -80°C . For RNA isolation, tissues were homogenized in TRIzol LS reagent (Ambion) and RNA isolated for qRT-PCR using TRIzol LS (Invitrogen) according to the manufacturer's directions.

Plasma (groups 1, 2, and 4) and the supernatant fluids from respiratory secretions (group 4) and swabs (group 2) were stored at -20°C until they were used for IFN bioassay. To obtain tissue homogenates, 100 mg of lung, LN, spleen, and oropharynx from group 1 monkeys were mixed with PBS, added to lysing matrix A tubes (MP Biomedicals), and homogenized in a Fast-prep 24 machine. Samples were stored at -80°C .

IFN bioassay

Plasma, respiratory fluids, and tissue homogenates were tested for IFN activity in an IFN bioassay (Shivakoti and others 2013). Residual MeV in samples was inactivated by UV radiation for 75 s at 250 mJ at least 24 h before analysis. Samples to be tested for IFN activity were incubated with confluent Vero cells in 96-well plates ($50\ \mu\text{L}/\text{well}$) for 24 h. Cells were washed, and VSV-GFP was added at a MOI of 1. After 24 h, the percentage of GFP-positive cells was determined by flow cytometry. Controls included VSV-GFP alone, and negative controls lacked sample or VSV-GFP.

Standard curves were constructed using recombinant human IFN α , IFN β , IFN γ (PBL Interferon Source), and IFN λ (eBioscience) at 0.1–10,000 U/mL.

Quantitative RT-PCR

TaqMan gene expression assays were used with TaqMan RNA to CTTM 1-Step Kit following the manufacturer's instructions (Applied Biosystems). Input mRNA varied by tissue and cell type from 30 to 100 ng per well. Relative real-time qRT-PCR was performed for the following rhesus macaque gene mRNAs: GAPDH, IFN β , IL28b, IFN γ , ISG56 (IFIT1), and Mx1. Amplification of GAPDH mRNA was used as the endogenous control in all experiments. The mRNA levels for the infected group 1 and 3 monkeys were compared with mRNA levels from control group 1 monkeys. The mRNA levels for group 2 monkeys were compared with the baseline levels of the same monkeys before infection (day 0). Data are presented on a logarithmic scale (Figs. 4 and 5) to visualize the large tissue-dependent variability in gene expression.

MeV RNA detection used the following primers and probe for the nucleocapsid (N) gene (Pan and others 2005): primers (MeV 608-629 F: 5'-GGGTACCATCCTAGCCCCAAATT-3' and MeV 680-662 R: 5'-CGAATCAGCTGCCGTGTCT-3') and probe (MeV 639-659 F: FAM-CTCGCAAAGGCGGTTACGGCC-TAMRA). Power SYBR green RNA to CT 1-Step Kit (ABI) was used with primers for the relative quantification of pan-IFN α that identify 12 out of 13 macaque IFN α subtypes (Integrated DNA Technologies) (Alammar and others 2011).

Detection of DI RNA

MeV stocks used for infection of macaques were tested for a common species of MeV DI RNA using RT-PCR as previously described in detail (Shingai and others 2007; Shivakoti and others 2013). Briefly, Superscript III First-Strand Synthesis System (Invitrogen) was used for complementary DNA synthesis of RNA extracted from virus stocks. The primer sequences were as follows: standard genome primer 1: MeV 15591-15613 F: 5'-TTTATCCAGAATCTCAARTCCGG-3'; standard genome primer 2: *Hind*III MeV 15894-15866 R: 5'-TATAAGCTTACCAGACAAAGCTGGGAATAGAACTTCG-3'; and DI RNA primer: MeV 15648-15622 R: 5'-CGAAGATATTCTGCTGTAA GTCTAGTA-3' (Integrated DNA Technologies). Standard genome primers 1 and 2 were used to detect the genome, whereas standard genome primer 2 and the DI RNA primer were used to detect DI RNA. Agarose gel electrophoresis was used to detect these PCR products. This does not rule out the presence of other DI RNA species (Calain and others 1992).

Results

Identification and characterization of vaccine and WT MeV in tissues

Infection of tissues was determined by qRT-PCR of the N gene at 7–11 days after respiratory infection of macaques by DI-free MeV (Fig. 1A), a period that covers both the early detection of virus and peak virus replication in tissue (De Swart and others 2007; de Vries and others 2010). Vaccine strain EZ (group 1) N gene RNA was detected in lung

FIG. 1. Detection and characterization of measles virus (MeV) RNA. (A) Edmonston-Zagreb (EZ) vaccine and Biltoven (Bilt) wild-type (WT) viruses used for infection were tested by reverse transcriptase-polymerase chain reaction (RT-PCR) for the presence of defective interfering (DI) RNA (DI genome) using 1 primer that detects the standard genome (MeV genome) and another primer which detects a common species of DI RNA (DI genome). (B) Tissues from monkeys at 7–11 days after infection with vaccine (EZ) and WT (Bilt) strains of MeV were analyzed for presence of MeV N gene RNA by qRT-PCR. N gene RNA was assessed in lung, lymph node (LN), peripheral blood mononuclear cells (PBMCs), bronchoalveolar lavage (BAL), oropharynx, and spleen from EZ-vaccinated monkeys and in LNs, PBMCs, nasal swabs, and throat swabs from WT (Bilt)-infected monkeys. Graphs show the mean and individual levels of N RNA for each monkey. One-way ANOVA with Bonferroni multiple-comparison tests was used to compare the N mRNA levels from infected and control monkeys. * $P < 0.05$; ** $P < 0.005$.

and BAL but not in LNs, PBMCs, spleen, or oropharynx (Fig. 1B) (Lin and others, unpublished). WT MeV (groups 2 and 3) N gene RNA was detected in PBMCs and LN but not in cells from nasal and throat swabs at 7–11 days after infection (Fig. 1B).

Biologically active IFN after infection with vaccine and WT strains of MeV

Production of IFN 3–10 days after MeV infection was assessed using a VSV-GFP-based IFN bioassay that measures the inhibition of VSV replication in Vero cells and was previously shown to have a sensitivity of 1–10 U IFN β , 1–10 U IFN λ , 100–1,000 U IFN α 2a, and >1,000 U IFN γ (Fig. 2A) (Shivakoti and others 2013). Homogenates of EZ-infected (group 1) lung, LN, spleen, and oropharynx had no detectable IFN activity at 7 days after MeV infection (Fig. 2B). Similarly, IFN activity was not detected in the plasma of EZ-infected (group 1) or WT MeV-infected (group 2) monkeys (Fig. 2C). Supernatant fluids from nasal (Fig. 2D) and throat (Fig. 2E) swabs from WT MeV-infected monkeys (group 2) also had no detectable IFN activity at 3, 7, or 10 days after MeV infection.

IFN activity was also assessed for respiratory samples collected from monkeys (group 4) infected with 4 strains of Edmonston vaccine virus (Edmonston, LA-89, Moraten and STL-91) or Chicago-1, an attenuated WT MeV that replicates in Vero cells. No IFN activity was detected in nasopharyngeal fluids at 2, 9, or 11 days after MeV infection; conjunctival fluids at 9, 11, 14, or 16 days after MeV infection; or tracheal aspirates at 11 or 14 days after MeV infection (data not shown). In summary, biologically active IFN was not detected in plasma or lung, LN, spleen, or oropharynx tissue homogenates or in nasopharyngeal, tracheal, or conjunctival fluids from monkeys

infected with vaccine or WT strains of MeV by the respiratory route.

Baseline IFN mRNA levels in uninfected control monkeys

To determine constitutive expression of genes relevant to innate IFN-mediated antiviral responses, we assessed mRNA levels for IFN α , β , λ , γ , Mx, and ISG56 of control uninfected monkeys (lung, LN, spleen, BAL, PBMCs, and oropharynx) from group 1 and cells (PBMCs, nasal and throat swab) collected before WT infection from group 2. The cycle threshold (Ct) values are inversely proportional to the amount of RNA. A Ct value of 50 was assigned to genes that were not detected as the qRT-PCR was run for 50 cycles. GAPDH mRNA Ct values for the samples were similar (Ct 17–21) except for cells from nasal (Ct 26) and throat (Ct 31) swabs that had lower levels of input RNA (Fig. 3).

In lung homogenates (group 1), there was baseline expression of IFN α , β , and γ mRNAs (Ct 29–32) but not IL28b (IFN λ) mRNA (Fig. 3A). Mx and ISG56 mRNAs were more readily detected at baseline (Ct 20–23) than any of the IFNs (Fig. 3A). A pattern of constitutive gene expression, similar to that of the lung, was present in LN (Fig. 3B), BAL (Fig. 3C), oropharynx (Fig. 3D), spleen (Fig. 3E), and PBMCs (Fig. 3F, G). IFN α , β , and γ mRNAs were detectable (Ct 26–36), but IL28b mRNA was not (Fig. 3B–G). Similarly, expression of Mx and ISG56 ISG mRNAs was higher in these tissues (Ct 20–30) than IFN mRNAs.

Due to the limited amount of RNA extracted from nasal and throat swab samples, baseline Ct values for all genes, including GAPDH, were lower than for other tissues. Both nasal and throat swab cells had detectable IFN α mRNA (Ct 33–34), and nasal cells also had detectable IFN β mRNA (Ct 37) (Fig. 3H, I). However, cells from nasal swabs had no

FIG. 2. Assay for biologically active interferon (IFN). Samples were added to Vero cells overnight. Vero cells were then infected with vesicular stomatitis virus-expressing green fluorescent protein (VSV-GFP), and the percentage of GFP-positive cells was determined by flow cytometry. (A) Recombinant IFN α , IFN β , and IFN λ (IL28b) were used as positive controls for the IFN bioassay. (B–E) Analysis of fluids and tissue homogenates (B), plasma (C), and respiratory fluids (D, E) collected at 3–10 days after infection with vaccine (EZ) and WT (Bilt) strains of MeV. Tissue homogenates (B) were collected from lung, LN, spleen, and oropharynx (oro) after vaccine virus infection of monkeys. Plasma (C) was collected from monkeys infected with either vaccine or WT virus. Respiratory fluids were collected from nasal swabs (D) and throat swabs (E) from WT-infected monkeys. Graphs show the mean $\pm$ SEM. *t*-Tests or 1-way ANOVA test with Bonferroni multiple-comparison tests were used to compare percent of VSV-infected cells. No significant differences between any of the groups compared with the control (mock infected or day 0) were present.

detectable IFN γ or IL28b mRNAs, and cells from throat swabs had no detectable IFN β , IFN γ , or IL28b mRNAs (Fig. 3H, I). Baseline levels of ISG mRNAs (Ct 33–37) were detectable in both nasal and throat swab cells (Fig. 3H, I).

IFN mRNA in tissues after vaccine and WT MeV infection

We assessed the mRNA levels of IFN from tissues of monkeys at 7–11 days after infection with both vaccine (EZ) and WT (Bilt, KS, or IC323) strains of MeV compared with mRNA levels in control monkeys to calculate a fold change.

IFN α mRNA levels in LN and oropharynx after EZ infection were similar to those of control monkeys, and the

small increases (3- to 7-fold) in lung, PBMCs, BAL, and spleen were not significant (Fig. 4A). After WT MeV infection, PBMCs, throat swab cells, and nasal cells, but not LN, also had modest (5- to 21-fold) but insignificant increases in IFN α mRNA (Fig. 4A).

IFN β mRNA levels were very similar between control monkeys and monkeys infected with either EZ or WT virus with little (2- to 3-fold) to no increase after infection in all tissues studied (Fig. 4B). IFN γ mRNA levels in infected monkeys were also not significantly different from control monkeys for any of the tissues assessed (Fig. 4D).

IL28b (IFN λ) mRNA increased to detectable levels in the lungs (250-fold, mean Ct=41) and LN (5,000-fold, mean Ct=42, $P<0.005$) of EZ-infected monkeys and LN

FIG. 3. Constitutive expression of IFN and IFN-stimulated gene (ISG) mRNAs in uninfected tissues. Quantitative RT-PCR was performed on RNA extracted from the following tissues and cells from uninfected monkeys: lung (A), LN (B), BAL (C), oropharynx (D), spleen (E), PBMC (F, G) nasal cells (H), and throat swab cells (I). Cycle threshold (Ct) values for GAPDH, IFN α , IFN β , IL28b, IFN γ , Mx, and ISG56 mRNAs are shown. Lung, LN, PBMC-Grp1, BAL, oropharynx, and spleen were obtained from mock-infected monkeys that were a part of the EZ infection studies (group 1) and were used as controls for qRT-PCR of EZ and group 3 monkeys (for LN). PBMC-Grp2 and cells from nasal and throat swabs were obtained before infection (day 0) and used as controls for the WT infection studies (group 2). Graphs show the mean $\pm$ SEM Ct values.

(1,000-fold, mean Ct=40, $P<0.0005$) of WT-infected monkeys. IL28b mRNA remained undetectable in the other tissues from EZ- and WT MeV-infected monkeys (Fig. 4C).

ISGs induced in both EZ and WT MeV-infected monkeys

ISG56 and Mx are ISGs induced by type I and type III IFN that are also viral stress-inducible genes which can be induced directly by virus infection in the absence of IFN (Sarkar and Sen 2004). In EZ-infected monkeys, there were modest increases (3- to 9-fold) in ISG56 mRNA in lung, LN, oropharynx, and spleen and greater increases in BAL and PBMCs (24- to 34-fold) (Fig. 5A). WT-infected monkey PBMCs had ISG56 mRNA increases (31-fold) comparable to

EZ-infected monkeys (25-fold) (Fig. 5A) that were significantly higher than control PBMCs ($P<0.0005$). In WT-infected monkeys, there were significant increases of ISG56 mRNA in nasal cells (259-fold, $P<0.0005$), throat swab cells (23-fold, $P<0.0005$), and LN (15-fold, $P<0.005$) (Fig. 5A).

Patterns of change for levels of Mx mRNA were very similar to those for ISG56 mRNA. All of the tissues from EZ-infected monkeys except for the oropharynx had higher Mx mRNA levels than control monkeys with the greatest increase (24-fold, $P<0.0005$) in BAL (Fig. 5B). The increases compared with the control in Mx mRNA in PBMCs from both EZ (9-fold, $P<0.0005$) and WT-infected (11-fold, $P<0.0005$) monkeys were similar (Fig. 5B). The nasal, throat swab, and LN Mx mRNAs from WT MeV-infected monkeys were also significantly higher than the control (Fig. 5B).

FIG. 4. IFN gene induction in monkeys at 7–11 days after infection with vaccine and WT strains of MeV. RNA was isolated from monkeys infected with vaccine (EZ) or WT strains of MeV and compared with control monkeys. qRT-PCR was performed for the following genes: IFN α (A), IFN β (B), IL28b (C), and IFN γ (D). IFN gene expression was assessed for lung, LN, PBMCs, BAL, oropharynx, and spleen from EZ-infected monkeys and in LN and cells from PBMCs, nasal swabs, and throat swabs from WT-infected monkeys. Graphs show the mean and individual fold change compared with uninfected tissue for each monkey. One-way ANOVA with Bonferroni multiple-comparison tests were used to determine significance. ** $P < 0.005$; *** $P < 0.0005$.

FIG. 5. ISG induction in monkeys at 7–11 days after infection with vaccine and WT strains of MeV. qRT-PCR was performed for ISG56 (A) and Mx (B) genes compared with control monkeys. RNA was isolated from monkeys infected with vaccine (EZ) or WT strains. The ISG expression was assessed in lung, LN, PBMCs, BAL, oropharynx, and spleen from EZ-infected monkeys and in LN and cells from PBMCs, nasal swabs, and throat swabs from WT-infected monkeys. Graphs show the mean and individual fold change for each monkey. One-way ANOVA with Bonferroni multiple-comparison tests were used to determine significance. * $P < 0.05$; ** $P < 0.005$; and *** $P < 0.0005$.

Discussion

This study of IFN and ISG induction in the early stages of MeV infection of macaques has shown that neither vaccine nor WT strains of MeV elicit a substantial IFN response during virus replication before the onset of the adaptive immune response (rash). Biologically active IFN was not detectable after infection, despite evidence that the tissues were infected (Fig. 1B) (Auwaerter and others 1999; De Swart and others 2007; de Vries and others 2012). There was little to no induction of IFN α , IFN β , or IFN γ mRNAs above background in either EZ or WT-infected monkeys. However, there was an increase in IFN λ mRNA in LN and lungs. Increases in Mx and ISG56 gene expression in multiple tissues after infection by either strain may reflect IFN λ activity not detected by bioassay or the direct effects of virus replication. Therefore, MeV induces no increase in type I IFN and a modest increase in type III IFN gene expression *in vivo*. The absence of a difference by strain suggests that differential modulation of the IFN response is not important for attenuation.

There was a clear difference in the sites of detectable WT and vaccine MeV replication. MeV RNA was detected in PBMCs and LN of WT but not EZ-infected monkeys. Other studies have suggested that there is an important difference in the ability of WT and vaccine strains of MeV to replicate in cells of the immune system, despite the ability of both viruses to use CD150 as an immune cell receptor (Sakaguchi and others 1986; Ono and others 2001; de Vries and others 2010; Lin and others 2011, 2012).

Despite differences in infection levels by tissues and strain, the levels of type I IFN(α , β) induction elicited by the 2 strains were similar. IL28b levels (IFN λ) were increased but comparable in LN of EZ and WT-infected monkeys, although no viral RNA was detected in LN of EZ-vaccinated monkeys. Despite analysis of samples from multiple experimental conditions (strains of virus, species of macaques, tissues, and time after infection), little to no induction of IFN could be detected. Absence of IFN induction by MeV is compatible with the long incubation phase of measles.

Undetectable levels of biologically active IFN suggest that these amounts of types I and III IFN mRNAs measured were insufficient to induce production of sufficient amounts of IFN proteins for detection in a bioassay. However, there were consistent and comparable increases in ISG expression after infection by both strains. This may be explained by the presence of IFN protein below the level of detection or by the direct induction of Mx and ISG56 by viral infection, independent of IFN signaling (Sarkar and Sen 2004).

It is not yet fully understood why infection with MeV does not efficiently induce IFN similar to other negative-strand RNA viruses such as influenza virus (Coccia and others 2004). MeV encodes 2 nonstructural proteins, C and V, that regulate IFN induction and have similar sequences between vaccine and WT MeV strains (Fontana and others 2008; Seya 2011). The V protein interacts with RNA helicases MDA5 and LGP2 and I κ B kinase α to interfere with IFN induction activities (Pfaffer and Conzelmann 2008; Parisien and others 2009), and the C protein downregulates viral RNA synthesis to decrease intracellular detection (Ito and others 2013). Other mechanisms for low IFN production by MeV-infected cells might include inhibition of IFN signaling by an increase in proteins such as suppressors of

cytokine signaling that block IFN signaling or induction of mechanisms which decrease detection of viral RNAs during replication (Parks and Alexander-Miller 2013). A primary role for V and C expression in suppression of IFN induction is supported by the observation that WT MeVs lacking V or C are attenuated in macaques and somewhat more likely than WT MeV to induce PBMC expression of IFN α , but not Mx, mRNA at 7 days after infection (Devaux and others 2008). A role for P and V is further suggested by decreased virulence of a virus unable to inhibit STAT1 activation (Devaux and others 2011). Although encapsidation of the MeV genome and anti-genome by nucleoprotein might result in low IFN induction through reduced intracellular detection of naked single-stranded or double-stranded RNA, this is a property shared with other negative-strand viruses and likely plays a less important role than the V and C proteins.

In conclusion, we have shown that monkeys infected with EZ and WT strains of MeV have few differences in IFN mRNA levels and no detectable production of biologically active IFN. Understanding the mechanisms of suppression of the IFN response by MeV as well as the roles of other innate immunity factors during infection and other differences between WT and vaccine strains of MeV will provide important insights into the effects of MeV on the immune system.

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Author Disclosure Statement

The authors have no commercial or other associations that might pose a conflict of interest.

References

- Alammar L, Gama L, Clements JE. 2011. Simian immunodeficiency virus infection in the brain and lung leads to differential type I IFN signaling during acute infection. *J Immunol* 186(7):4008–4018.
- Auwaerter PG, Rota PA, Elkins WR, Adams RJ, DeLozier T, Shi Y, Bellini WJ, Murphy BR, Griffin DE. 1999. Measles virus infection in rhesus macaques: altered immune responses and comparison of the virulence of six different virus strains. *J Infect Dis* 180(4):950–958.
- Calain P, Curran J, Kolakofsky D, Roux L. 1992. Molecular cloning of natural paramyxovirus copy-back defective interfering RNAs and their expression from DNA. *Virology* 191:62–71.
- Coccia EM, Severa M, Giacomini E, Monneron D, Remoli ME, Julkunen I, Cella M, Lande R, Uzé G. 2004. Viral infection and toll-like receptor agonists induce a differential expression of type I and lambda interferons in human plasmacytoid and monocyte-derived dendritic cells. *Eur J Immunol* 34(3):796–805.

- De Swart RL, Ludlow M, De Witte L, Yanagi Y, Van Amerongen G, McQuaid S, Yüksel S, Geijtenbeek TBH, Duprex WP, Osterhaus ADME. 2007. Predominant infection of CD150+ lymphocytes and dendritic cells during measles virus infection of macaques. *PLoS Pathog* 3(11):e178.
- Devaux P, Hodge G, McChesney MB, Cattaneo R. 2008. Attenuation of V- or C-defective measles viruses: infection control by the inflammatory and interferon responses of rhesus monkeys. *J Virol* 82(11):5359–5367.
- Devaux P, Hudacek AW, Hodge G, Reyes-del Valle J, McChesney MB and Cattaneo R. 2011. A recombinant measles virus unable to antagonize STAT1 function cannot control inflammation and is attenuated in rhesus monkeys. *J Virol* 85:348–356.
- De Vries RD, Lemon K, Ludlow M, McQuaid S, Yüksel S, van Amerongen G, Rennick LJ, Rima BK, Osterhaus ADME, de Swart RL, Duprex WP. 2010. *In vivo* tropism of attenuated and pathogenic measles virus expressing green fluorescent protein in macaques. *J Virol* 84(9):4714–4724.
- De Vries RD, McQuaid S, van Amerongen G, Yüksel S, Verburgh RJ, Osterhaus ADME, Duprex WP, de Swart RL. 2012. Measles immune suppression: Lessons from the macaque model. *PLoS Pathog* 8:e1002885.
- El Mubarak HS, Yüksel S, van Amerongen G, Mulder PGH, Mukhtar MM, Osterhaus ADME, de Swart RL. 2007. Infection of cynomolgus macaques (*Macaca fascicularis*) and rhesus macaques (*Macaca mulatta*) with different wild-type measles viruses. *J Gen Virol* 88:2028–2034.
- Enders J, Peebles T. 1954. Propagation in tissue cultures of cytopathogenic agents from patients with measles. *Proc Soc Exp Biol Med* 86(2):277–286.
- Fireman P, Friday G, Kumate J. 1969. Effect of measles vaccine on immunologic responsiveness. *Pediatrics* 43(2):264–272.
- Fontana JM, Bankamp B, Bellini WJ, Rota PA. 2008. Regulation of interferon signaling by the C and V proteins from attenuated and wild-type strains of measles virus. *Virology* 374(1):71–81.
- Forthal DN, Aarnaes S, Blanding J, de la Maza L, Tilles JG. 1992. Degree and length of viremia in adults with measles. *J Infect Dis* 166(2):421–424.
- Griffin DE. 2010. Measles virus-induced suppression of immune responses. *Immunol Rev* 236:176–189.
- Hashimoto K, Ono N, Tatsuo H, Minagawa H, Takeda M, Takeuchi K, Yanagi Y. 2002. SLAM (CD150)-independent measles virus entry as revealed by recombinant virus expressing green fluorescent protein. *J Virol* 76:6743–6749.
- Ito M, Iwasaki M, Takeda M, Nakamura T, Yanagi Y, Ohno S. 2013. Measles virus nonstructural C protein modulates viral RNA polymerase activity by interacting with host protein SHCBP1. *J Virol* 87:9633–9642.
- Kessler JR, Kremer JR, Muller CP. 2011. Interplay of measles virus with early induced cytokines reveals different wild type phenotypes. *Virus Res* 155(1):195–202.
- Kotenko SV, Gallagher G, Baurin VV, Lewis-Antes A, Shen M, Shah NK, Langer JA, Sheikh F, Dickensheets H, Donnelly RP. 2003. IFN-lambdas mediate antiviral protection through a distinct class II cytokine receptor complex. *Nat Immunol* 4(1):69–77.
- Lemon K, de Vries RD, Mesman AW, McQuaid S, van Amerongen G, Yüksel S, Ludlow M, Rennick LJ, Kuiken T, Rima BK, Geijtenbeek TBH, Osterhaus ADME, Duprex WP, de Swart RL. 2011. Early target cells of measles virus after aerosol infection of non-human primates. *PLoS Pathog* 7(1):e1001263.
- Lin WH, Griffin DE, Rota PA, Papania M, Cape SP, Bennett D, Quinn B, Sievers RE, Shermer C, Powell K, Adams RJ, Godin S, Winston S. 2011. Successful respiratory immunization with dry powder live-attenuated measles virus vaccine in rhesus macaques. *Proc Natl Acad Sci U S A* 108(7):2987–2992.
- Lin WHW, Kouyos RD, Adams RJ, Grenfell BT, Griffin DE. 2012. Prolonged persistence of measles virus RNA is characteristic of primary infection dynamics. *Proc Natl Acad Sci U S A* 109(37):14989–14994.
- Monafo WJ, Haslam DB, Roberts RL, Zaki SR, Bellini WJ, Coffin CM. 1994. Disseminated measles infection after vaccination in a child with a congenital immunodeficiency. *J Pediatr* 124(2):273–276.
- Naniche D, Yeh A, Eto D, Manchester M, Friedman RM, Oldstone MB. 2000. Evasion of host defenses by measles virus: wild-type measles virus infection interferes with induction of alpha/beta interferon production. *J Virol* 74(16):7478–7484.
- Oldstone MBA. 2006. Viral persistence: parameters, mechanisms and future predictions. *Virology* 344(1):111–118.
- Ono N, Tatsuo H, Hidaka Y, Aoki T, Minagawa H, Yanagi Y. 2001. Measles viruses on throat swabs from measles patients use signaling lymphocytic activation molecule (CDw150) but not CD46 as a cellular receptor. *J Virol* 75(9):4399–4401.
- Pan C, Valsamakis A, Colella T, Nair N, Adams RJ, Polack FP, Greer CE, Perri S, Polo JM, Griffin DE. 2005. Modulation of disease, T cell responses, and measles virus clearance in monkeys vaccinated with H-encoding alphavirus replicon particles. *Proc Natl Acad Sci U S A* 102(33):1581–1588.
- Parisien JP, Bamming D, Komuro A, Ramachandran A, Rodriguez JJ, Barber G, Wojahn RD, Horvath CM. 2009. A shared interface mediates paramyxovirus interference with antiviral RNA helicases MDA5 and LGP2. *J Virol* 83(14):7252–7260.
- Parks GD, Alexander-Miller MA. 2013. Paramyxovirus activation and inhibition of innate immune responses. *J Mol Biol* 425:4872–4892.
- Perry RT, Gacic-Dobo M, Dabbagh A, Mulders MN, Strebel PM, Okwo-Bele JM, Rota PA, Goodson JL. 2014. Global control and regional elimination of measles, 2000–2012. *MMWR Morb Mortal Wkly Rep* 63:103–107.
- Pfaller CK, Conzelmann KK. 2008. Measles virus V protein is a decoy substrate for IkappaB kinase alpha and prevents toll-like receptor 7/9-mediated interferon induction. *J Virol* 82(24):12365–12373.
- Rota JS, Hummel KB, Rota PA, Bellini WJ. 1992. Genetic variability of the glycoprotein genes of current wild-type measles isolates. *Virology* 188(1):135–142.
- Rota JS, Wang ZD, Rota PA, Bellini WJ. 1994. Comparison of sequences of the H, F, and N coding genes of measles virus vaccine strains. *Virus Res* 31:317–330.
- Sakaguchi M, Yoshikawa Y, Yamanouchi K, Sata T, Nagashima K, Takeda K. 1986. Growth of measles virus in epithelial and lymphoid tissues of cynomolgus monkeys. *Microbiol Immunol* 30(10):1067–1073.
- Sarkar SN, Sen GC. 2004. Novel functions of proteins encoded by viral stress-inducible genes. *Pharm Ther* 103(3):245–259.
- Sato H, Kobune F, Ami Y, Yoneda M, Kai C. 2008. Immune responses against measles virus in cynomolgus monkeys. *Comp Immunol Microbiol Infect Dis* 31(1):25–35.
- Seya T. 2011. Addendum to “Strain-to-strain difference of V protein of measles virus affects MDA5-mediated IFN- β -inducing potential” [Mol. Immunol. 48(4) (2011) 497–504]. *Mol Immunol* 48(12–13):1589–1590.
- Shingai M, Ebihara T, Begum NA, Kato A, Honma T, Matsumoto K, Saito H, Ogura H, Matsumoto M, Seya T. 2007. Differential

- type I IFN-inducing abilities of wild-type versus vaccine strains of measles virus. *J Immunol* 179(9):6123–6133.
- Shivakoti R, Siwek M, Hauer D, Schultz KLW, Griffin DE. 2013. Induction of dendritic cell production of type I and type III interferons by wild-type and vaccine strains of measles virus: role of defective interfering RNAs. *J Virol* 87:7816–7827.
- Smaron MF, Saxon E, Wood L, McCarthy C, Morello JA. 1991. Diagnosis of measles by fluorescent antibody and culture of nasopharyngeal secretions. *J Virol Methods* 33(1–2):223–229.
- Stetson DB, Medzhitov R. 2006. Type I interferons in host defense. *Immunity* 25(3):373–381.
- Takeuchi O, Akira S. 2010. Pattern recognition receptors and inflammation. *Cell* 140(6):805–820.
- Tamashiro VG, Perez HH, Griffin DE. 1987. Prospective study of the magnitude and duration of changes in tuberculin reactivity during uncomplicated and complicated measles. *Pediatr Infect Dis J* 6(5):451–454.
- Van Binnendijk RS, van der Heijden RW, van Amerongen G, UytdeHaag FG, Osterhaus AD. 1994. Viral replication and development of specific immunity in macaques after infection with different measles virus strains. *J Infect Dis* 170(2):443–448.
- Wolfson LJ, Strebel PM, Gacic-Dobo M, Hoekstra EJ, McFarland JW, Hersh BS. 2007. Has the 2005 measles mortality reduction goal been achieved? A natural history modelling study. *Lancet* 369(9557):191–200.
- World Health Organization. 1998. Expanded Programme on Immunization (EPI). Standardization of the nomenclature for describing the genetic characteristics of wild-type measles viruses. *Wkly Epidemiol Rec* 73(35):265–269.
- Zilliox MJ, Moss WJ, Griffin DE. 2007. Gene expression changes in peripheral blood mononuclear cells during measles virus infection. *Clin Vaccine Immunol* 14(7):918–923.

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