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Differential Induction of Type I Interferons in Macaques by Wild-type Measles Virus or Wild-type Measles Virus with the Hemagglutinin Protein of the Edmonston Vaccine strain

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Running title: Interferon induction by measles virus

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List of Abbreviations :

BAL, bronchoalveolar lavage ; DI, defective interfering ; EGFP, enhanced green fluorescent protein ; ELISA, enzyme-linked immunosorbent assay; H protein, hemagglutinin protein ; IFN, interferon ; ingLNs, inguinal lymph nodes ; MDA5, melanoma differentiation-associated gene 5; MV, measles virus ; PBMCs, peripheral blood mononuclear cells ; pDC, plasmacytoid DCs; PVRL4, poliovirus receptor-like protein 4 ; RIG-I, retinoic acid-inducible gene-I; RT-PCR, reverse transcriptase PCR ; SLAM, signaling lymphocyte activation molecule

ABSTRACT

Measles vaccines are highly effective and safe, but the mechanism underlying their attenuation has not been well understood. In this study, type I interferons (IFNs) (IFN-α and IFN-β) induction in macaques infected with measles virus (MV) strains was examined. Type I IFNs were not induced in macaques infected with wild-type MV. However, IFN-α was sharply induced in most of macaques infected with the recombinant wild-type MV bearing the hemagglutinin (H) protein of the Edmonston vaccine strain. These results indicate that the H protein of MV vaccine strains may have a role in MV attenuation.

Key words

interferon, hemagglutinin protein, macaque, measles virus

Measles is highly infectious and remains a major cause of childhood morbidity and mortality worldwide despite the availability of effective vaccines (1). Measles vaccines were generated by successive passages of field isolates of measles virus (MV) in cells from different origins (1). Measles vaccines are highly effective and safe, but the mechanism underlying their attenuation has not been well understood. Major difference between wild-type and vaccine strains of MV is receptor specificity of the hemagglutinin (H) protein in vitro. The H proteins of wild-type MV strains recognize the signaling lymphocyte activation molecule (SLAM), also known as CD150, which is expressed in certain immune system cells (2), and nectin-4, also known as poliovirus receptor-like protein 4 (PVRL4), which is expressed in epithelial cells in trachea, skin, lung, prostate and stomach, as cellular receptors (3, 4). On the other hand, the H proteins of vaccine MV strains recognize CD46, which is ubiquitously expressed on all nucleated human and monkey cells (5, 6), in addition to SLAM and nectin-4 as cellular receptors.

To examine the contribution of the H protein to the MV attenuation, an enhanced green fluorescent protein (EGFP)-expressing recombinant wild-type MV bearing the H protein of the Edmonston vaccine strain (EdH-EGFP$_2$) was generated using a reverse genetics system based on the pathogenic wild-type IC-B strain (7), and cynomolgus monkeys between 4 to 5 years old (three animals per each strain) were intranasally infected with wild-type MV (IC323-EGFP$_2$) or EdH-EGFP$_2$ (8). IC323-EGFP$_2$ and EdH-EGFP$_2$ have the EGFP gene between the N and P genes. All animal experiments were performed in compliance with the guidelines of National Institute of Infectious

67 Disease (permission number 510008). Interestingly, the replication of EdH-EGFP$_2$ in
68 tissues and lymphocytes of infected macaques were significantly lower than those of
69 the wild-type MV. From these results we speculated that type I interferons (IFNs) may
70 affect the growth of EdH-EGFP$_2$ in macaques, as type I IFNs are induced by many
71 viruses and play central roles in the host defense against viral infection (9).

72 In this study, we examined type I IFNs induction in those macaques to investigate
73 the mechanism for the growth attenuation of EdH-EGFP$_2$ in macaques. For this
74 purpose, we first examined the presence of defective interfering (DI) RNA in virus
75 stocks, because it is known that DI RNAs, especially the 5' copy-back DI RNAs, in
76 virus stocks of MV is able to induce type I IFN through interaction with the RNA
77 helicases retinoic acid-inducible gene-I (RIG-I)/ melanoma differentiation-associated
78 gene 5 (MDA5) (10, 11, 12, 13). Viral RNA was extracted from virus stocks using
79 QIAamp Viral RNA Mini kit (QIAGEN), and DI RNA was detected using reverse
80 transcriptase PCR (RT-PCR) as previously described (10). DI RNAs were detected in
81 the Edmonston (laboratory strain) and IC-V (wild-type strain isolated in Vero cells)
82 stocks as reported (10), while no DI RNA was detected in IC323-EGFP$_2$ and
83 EdH-EGFP$_2$ virus stocks used in this experiment (Fig. 1).

84 To examine the interferon responses elicited by IC323-EGFP$_2$ and EdH-EGFP$_2$ in
85 macaques, we compared the transcription of IFN-α and IFN-β genes in peripheral
86 blood mononuclear cells (PBMCs) of infected monkeys as previously reported (14).
87 PBMCs were collected at 0, 3, 7, and 10 days post infection (dpi), and stored in
88 RNAprotect Animal Blood Tubes (QIAGEN, Hilden, Germany) at -30˚C. Tissues of

inguinal lymph nodes (IngLN) were collected at 7 days prior infection and at 10 dpi, and stored in RNAlater solution (QIAGEN) at -30˚C. Tissues of lung were collected at 10 dpi and stored in RNAlater solution at -30˚C. Plasma was collected at 7 days prior infection, and at 0, 3, 7, and 10 dpi, and stored at -80˚C. Bronchoalveolar lavage (BAL) were collected at 10 dpi and stored at -80˚C. Total RNA was isolated from PBMCs and tissues by using RNeasy mini kit and RNase-free DNase (QIAGEN) according to the manufacturer's protocol, reverse transcribed using oligo (dT) primer and PCR amplified with a Thermal Cycler Dice TP800 (Takara, Tokyo, Japan) by using FastStart SYBR Green Master (Roche, Mannheim, Germany). In macaques infected with IC323-EGFP$_2$ (no. 5058, 5062, and 5069), IFN-α and IFN-β transcription was transiently down-regulated at day 3 (Fig. 2A and B). Then, the levels of IFN-α and IFN-β transcription were returned to the baseline at day 7. In macaques infected with EdH-EGFP$_2$ (no. 5056, 5057, and 5068), IFN-α and IFN-β transcription was gradually induced from day 0 to day 7. At day 10, the levels of IFN-α and IFN-β transcription were decreased in all monkeys infected with both strains.

Type I IFN responses elicited by IC323-EGFP$_2$ and EdH-EGFP$_2$ were also examined in several tissues of infected monkeys. However, the levels of IFN-α and IFN-β transcription in inguinal lymph nodes were not significantly changed between at 7 days before infection and at day 10 for all monkeys (Fig. 2C and D). Day 10 may be too late to detect the change of the levels of IFN-α and IFN-β transcription. Similar levels of IFN-α and IFN-β transcription were detected in lung of monkeys infected with both strains (Fig. 2C and D).

Next, we examined plasma levels of IFN-α using an enzyme-linked immunosorbent assay (ELISA) kit. IFN-α levels in the plasma and BAL were measured by VeriKine^{TM} cynomolgus/rhesus IFN-α serum ELISA kit (PBL, Piscataway, USA) according to the manufacturer's protocol. Plasma levels of IFN-α were not significantly changed in wild-type IC323-EGFP$_2$-infected macaques (no. 5058, 5062, and 5069), although slight induction was observed in one macaque (no. 5062) at day 7 (Fig. 3A). On the other hand, plasma levels of IFN-α were sharply elevated by 4- to 5-fold in two (no. 5056 and 5057) out of three macaques infected with EdH-EGFP$_2$ at day 7, and then declined by day 10. To confirm the IFN-α induction in EdH-EGFP$_2$-infected macaques, we examined plasma collected in former experiments in which we infected macaques with recombinant MV strains. In the first group, two old (10 years old) macaques (no. 4568 and 4569) were used. One macaque (no. 4568) was infected with wild-type MV (IC323-EGFP) having the EGFP gene between the leader sequence and the N gene (8), and the other (no. 4569) was infected with IC323-EGFP$_2$. In the second group, seven juvenile (1 year old) macaques (no. 4848, 4849, 4850, 4858, 4859, 4860, and 4865) were used. Three of them (no. 4850, 4860, and 4865) were infected with IC323-EGFP$_2$, and the other four (no. 4848, 4849, 4858, and 4859) were infected with EdH-EGFP$_2$. Again, plasma levels of IFN-α were sharply elevated in two EdH-EGFP$_2$-infected macaques (no. 4848 and 4849) at day 7 but not in IC323-EGFP- and IC323-EGFP$_2$-infected macaques (no. 4568, 4569, 4850, 4860 and 4865) (Fig. 3B). Macaques (no. 4860, 4865, 4858 and 4859) and macaques (no. 4850, 4848 and 4849) were sacrificed at day 3 and 7, respectively. Therefore, their

133 samples were not available hereafter. Induction of IFN-α was not observed in BAL of
134 all infected monkeys at 10 days (Fig. 3A).

135 Although many studies indicated the IFNs production in vitro by MV, little is
136 known about the IFNs production in measles patients. Previous clinical study using a
137 sensitive radioimmunoassay indicated that IFN-α was not induced in plasma of
138 measles patients (15). In another clinical study, Yu et al. found that IFN-α expression
139 was suppressed in PBMCs of measles patients (16). In vivo study using macaques,
140 Devaux et al. reported that expression of type I IFN genes were well regulated (14). In
141 addition, Shivakoti et al. recently found that type I IFNs were not induced in macaques
142 infected by wild-type MV (17). We found that IFN-α was not induced in macaques
143 infected with wild-type MV (Fig. 3A and B). Our result is consistent with previous
144 clinical studies (15, 16) and in vivo studies using monkeys (14, 17). These results
145 suggest that MV has way to circumvent the host IFNs production possibly by the C
146 and V proteins (18). Likewise, little is known about the IFNs production in measles
147 vaccinee. In a previous clinical study, IFN was induced after measles vaccination (19).
148 We found that IFN-α was sharply induced in plasma of macaques infected with
149 EdH-EGFP$_2$ (Fig. 3). Interestingly, it was shown that large amounts of IFN-α were
150 rapidly produced from plasmacytoid DCs (pDCs) after infection of the Edmonston
151 strain mostly independent of the viral infection cycles (20). As pDCs express CD46 but
152 not SLAM (20), pDCs in macaques would be infected with EdH-EGFP$_2$ by
153 CD46-mediated pathway and may produce large amounts of IFN-α in plasma. In
154 summary, we found that IFN-α was induced in macaques infected with wild-type MV

bearing the H protein of the Edmonston vaccine strain but not with wild-type MV. Our results suggest that the H protein of vaccine strains of MV may have a role in the MV attenuation.

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DISCLOSURE

The authors have no conflicts of interest associated with this study.

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FIGURE LEGENDS

Fig. 1. Absence of 5' copy back DI RNA in MV stocks. Stocks of IC323-EGFP$_2$ and EdH-EGFP$_2$ used for infection were tested for the absence of 5' copy back DI RNA. 5' copy back DI genomes were detected with primers (JM396; 5'-TATAAGCTTACCAGACAAAGCTGGGAATAGAAACTTCG-3' and JM403; 5'-CGAAGATATTCTGGTGTAAGTCTAGTA-3'). MV standard genomes were detected using primers (JM396 and JM402; 5'-TTTATCCAGAATCTCAARTCCGG-3'). The Edmonston and IC-V strains, which are known to contain 5' copy back DI RNA, were used for positive control.

Fig. 2. IFN-α/β mRNA expression in PBMCs, inguinal lymph nodes and lungs. IFN-α (A) and IFN-β (B) mRNA expression in PBMCs from monkeys infected with IC323-EGFP$_2$ or EdH-EGFP$_2$ were measured by RT-qPCR. PBMCs were collected at 0, 3, 7, and 10 days post infection (dpi). IFN-α (C) and IFN-β (D) mRNA expression in inguinal lymph nodes (IngLN) and lungs from monkeys infected with IC323-EGFP$_2$ or EdH-EGFP$_2$ were measured by RT-qPCR. IngLN were excised at 7 days prior to infection and at 10 dpi, and lungs were excised at 10 dpi from monkeys. Three

266 monkeys (no. 5058, 5062, and 5069) were infected with IC323-EGFP$_2$, and three
267 monkeys (no. 5056, 5057, and 5068) were infected with EdH-EGFP$_2$. For
268 amplification of the IFN-α mRNA, IFN-α F primer
269 5'-GCCTGAAGGACAGACATGACTTT-3' and IFN-α R primer
270 5'-GGATGGTTTGAGCCTTTTGG-3' were used. For amplification of the IFN-β
271 mRNA, IFN-β F primer 5'-TGCCTCAAGGACAGGATGAA-3' and IFN-β R primer
272 5'-ATGGTCCAGGCACAGTGACT-3' were used. For amplification of the 18S rRNA
273 segment, the 18S sense primer 5'-TCAAGAACGAAAGTCGGAGG-3' and 18S
274 antisense primer 5'-GGACATCTAAGGGCATCACA-3' were used. For determining
275 the relative amounts of IFN-α/β mRNA, the amounts of IFN-α/β mRNA in
276 cynomolgus monkey PBMCs infected with Sendai virus (Cantell strain), which is
277 commonly used to induce IFN-α/β, in vitro were set to 10^1.
278
279 **Fig. 3. Plasma and BAL levels of IFN-α**
280 (A) Plasma and BAL levels of IFN-α in monkeys infected with IC323-EGFP$_2$ or
281 EdH-EGFP$_2$ were measured by ELISA. Plasma was collected at 7 days prior
282 infection, and at 0, 3, 7, and 10 dpi. BAL was collected at 10 dpi. Three monkeys
283 (no. 5058, 5062, and 5069) were infected with IC323-EGFP$_2$, and three monkeys (no.
284 5056, 5057, and 5068) were infected with EdH-EGFP$_2$. (B) Plasma levels of IFN-α in
285 monkeys infected with IC323-EGFP, IC323-EGFP$_2$ or EdH-EGFP$_2$ were measured by
286 ELISA. Plasma was collected at 0, 3, 7, and 10 dpi. One monkey (no. 4568) were
287 infected with IC323-EGFP. Four monkeys (no. 4569, 4850, 4860, and 4865) were

infected with IC323-EGFP$_2$, and four monkeys (no. 4848, 4849, 4858, and 4859) were infected with EdH-EGFP$_2$. Sensitivity of this assay is 0.30 pg/ml. nd, not done.

Fig. 1

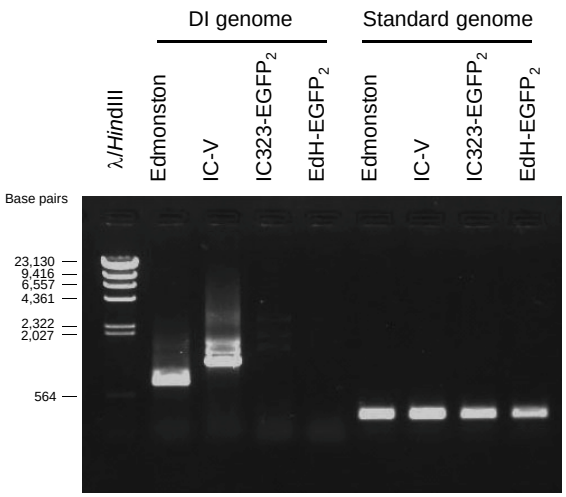

DI genome
Standard genome
λ/HindIII
Edmonston
IC-V
IC323-EGFP2
EdH-EGFP2
Edmonston
IC-V
IC323-EGFP2
EdH-EGFP2
Base pairs
23,130
9,416
6,557
4,361
2,322
2,027
564

Fig. 2

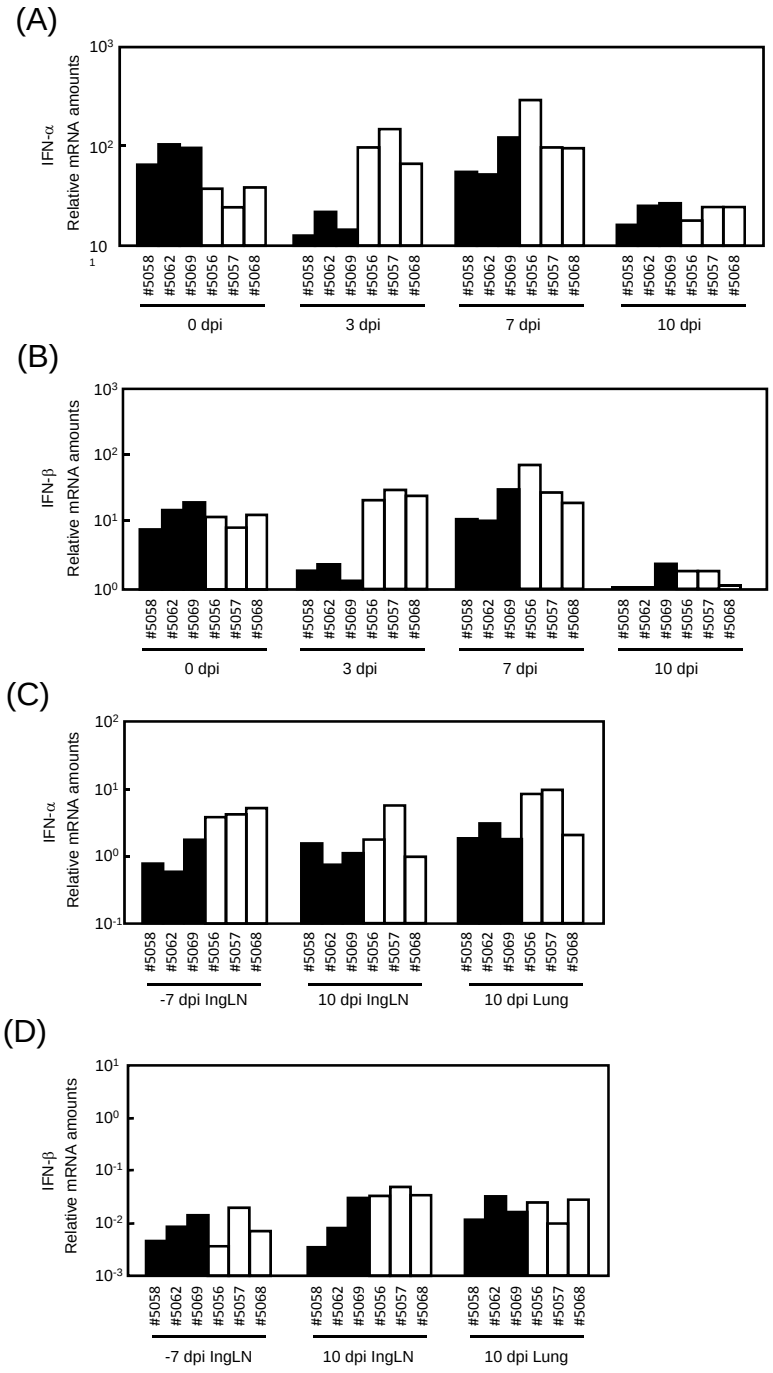
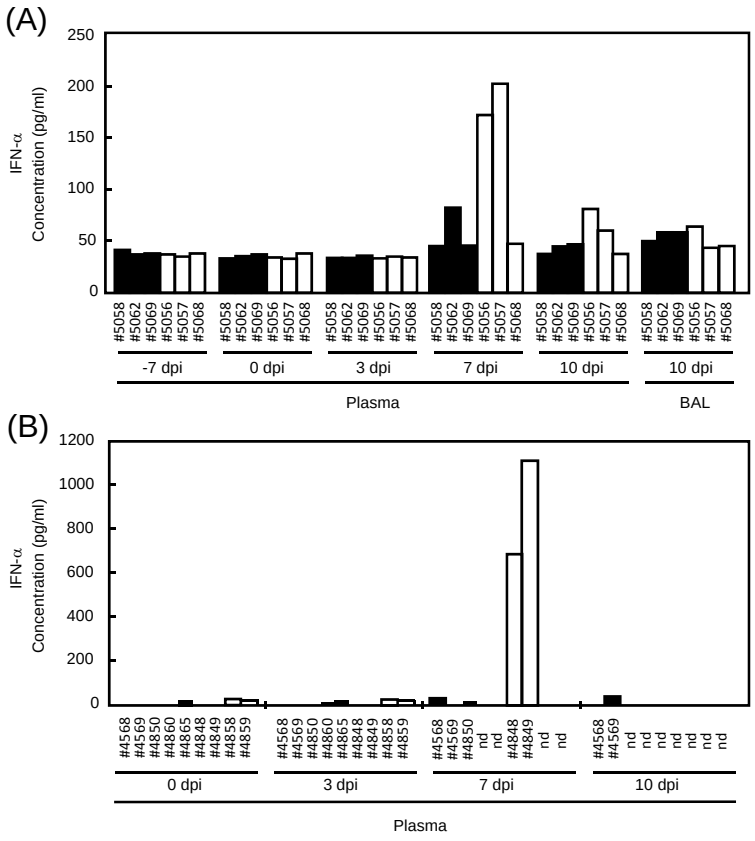

(A)
IFN-α
Relative mRNA amounts
10^3
10^2
10^1
#5058 #5062 #5069 #5056 #5057 #5068
0 dpi
3 dpi
7 dpi
10 dpi
(B)
IFN-β
Relative mRNA amounts
10^3
10^2
10^1
10^0
#5058 #5062 #5069 #5056 #5057 #5068
0 dpi
3 dpi
7 dpi
10 dpi
(C)
IFN-α
Relative mRNA amounts
10^2
10^1
10^0
10^-1
#5058 #5062 #5069 #5056 #5057 #5068
-7 dpi IngLN
10 dpi IngLN
10 dpi Lung
(D)
IFN-β
Relative mRNA amounts
10^1
10^0
10^-1
10^-2
10^-3
#5058 #5062 #5069 #5056 #5057 #5068
-7 dpi IngLN
10 dpi IngLN
10 dpi Lung

Fig. 3


(A)
IFN-α Concentration (pg/ml)
250
200
150
100
50
0
#5058 #5062 #5069 #5056 #5057 #5068
-7 dpi
0 dpi
3 dpi
7 dpi
10 dpi
10 dpi
Plasma
BAL
(B)
IFN-α Concentration (pg/ml)
1200
1000
800
600
400
200
0
#4568 #4569 #4850 #4860 #4865 #4848 #4849 #4858 #4859
nd
0 dpi
3 dpi
7 dpi
10 dpi
Plasma