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Interferon-Induced Expression of MxA in the Respiratory Tract of Rhesus Macaques Is Suppressed by Influenza Virus Replication

Timothy D. Carroll,*† Shannon R. Matzinger,*† Merixtell Genescà,*† Linda Fritts,*† Roxana Colón,* Michael B. McChesney,*§ and Christopher J. Miller2*†§

To determine the relationship between influenza A virus replication and innate antiviral immune responses, rhesus monkeys were given oseltamivir before influenza A/Memphis/7/01 (H1N1) challenge. We found that oseltamivir treatment significantly reduced viral replication in the trachea (p < 0.029). Further, in the trachea of both treated and untreated monkeys the mRNA levels of most innate antiviral molecules in the IFN-αβ pathway were dramatically increased by 24 h postinfection. However, the mRNA level of a single IFN-stimulated gene, MxA (myxovirus resistance A), the IFN-stimulated gene known to be critical in blocking influenza virus replication, was significantly lower in the tracheal lavages of untreated monkeys than in the oseltamivir-treated monkeys (p = 0.05). These results demonstrate for the first time that uncontrolled influenza A virus replication actively suppresses MxA gene expression and emphasize the critical role of innate immunity in controlling influenza virus replication in vivo. The Journal of Immunology, 2008, 180: 2385–2395.

Seasonal influenza A virus is a highly contagious, acute respiratory tract infection that causes substantial morbidity and mortality, particularly among young, old, and immunocompromised individuals (1). Immune responses are especially critical for controlling influenza virus replication. IFN-stimulated genes (ISGs)3 play a central role in innate antiviral immunity (2). Among the ISGs, myxovirus resistance (Mx) proteins were originally identified as factors conferring resistance to lethal influenza A virus infections in mice (3). The human Mx homologue MxA is able to inhibit the replication of orthomyxoviruses and other RNA viruses (reviewed in Ref. 4). MxA expression and MxA-mediated resistance to the influenza virus is associated with IFN-αβ-dependent, IFN-αβ-stimulated response element (ISRE) induction (5), and MxA expression during influenza virus infection requires type I and type III IFN activity (5). Despite the clear and critical role of Mx1 in anti-influenza immunity in mice and in human cells in vitro, the role of MxA in controlling influenza in primates, including humans, is unknown.

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3 Abbreviations used in this paper: ISG, IFN-stimulated gene; CPE, cytopathic effect; HI, hemagglutinin inhibition; IP-10, IFN-inducible protein 10; ISRE, IFN-αβ-stimulated response element; MDCK, Madin-Darby canine kidney; MX, myxovirus-resistance protein; OAS, 2′-5′-oligoadenylate synthetase; PI, postinfection; TCD50, 50% tissue culture infectious dose; vRNA, viral RNA.
were significantly higher levels of MxA mRNA than in the tracheas of untreated monkeys. Thus, in primates, virus replication actively suppresses MxA expression at a major site of influenza A virus infection.

Materials and Methods

Animals

All animals used in this study were adult rhesus macaques (Macaca mulatta) that were housed at the California National Primate Research Center (Davis, CA) in accordance with the regulations of the Association for Assessment and Accreditation of Laboratory Animal Care International standards. When necessary, animals were anesthetized with ketamine hydrochloride (21 mg/kg body weight; Park Davis) or 0.7-9 mg/kg tiletamine (Telazol; Fort Dodge Animal Health) injected i.m. Animals with prechallenge hemagglutinin inhibition (HI) titers to A/Memphis/7/2001 greater than 1/8 were excluded from the study.

Virus stock production

The human influenza A virus isolate used in this study, A/Memphis/7/2001 (H1N1), was generously provided by R. Webby at St. Jude Children’s Research Hospital, Memphis, TN. This isolate was isolated on Madin-Darby canine kidney (MDCK) and was not passaged further before expansion in MDCK cells (American Type Culture Collection) to produce the virus stock used for animal inoculations. The virus stock had a titer of 10^8.5 TCID\textsubscript{50} /ml (where TCID\textsubscript{50} is 50% tissue culture infectious dose) on MDCK cells using the method of Reed and Muench (15). To produce the heat-killed virus used for inoculations, aliquots of the stock were heated at 56°C for 1 h just before animal inoculation. This procedure resulted in complete inactivation of the virus, as we were unable to isolate an infectious virus from treated aliquots of the stock (data not shown).

Animal inoculation groups and sampling

On day 0, animals were inoculated with either the live or heat-killed virus stock. The inoculum consisted of a either 1 or 6 ml of virus stock instilled into the trachea, 1 ml of virus stock dripped intranasally, and a drop of virus stock in each conjunctiva. On days -4, -1, 2, 3, 7, and 14 PI, tracheal washes were collected. Under ketamine anesthesia, either an 8- or a 5-French pediatric feeding tube (Kendall) was inserted into the trachea with the aid of a laryngoscope, and an effort was made to place the tip of the tube several centimeters cranial to the carina. Twelve milliliters of sterile PBS were instilled into the trachea, the maximum volume of sample was aspirated through the feeding tubes, and the samples was transferred to sample storage tubes on ice. On days -4, 0, 7, 14, and 28 PI, heparinized blood samples were collected from each animal.

Assessment of body temperature

Two approaches were used to determine body temperature in animals in different experiments and temperatures were collected from all animals at approximately the same time of day to account for changes in temperature due to circadian rhythm. In the experiment to determine the effect of virus inoculum size on viral replication, body temperature was determined using a real-time RT-PCR assay on a Prism 7900 sequence detector system (Applied Biosystems) was used for quantitation of influenza RNA in serum. A previously described influenza A virus matrix gene-specific PCR primer set was used for PCR amplification (16). The forward primer was 5’-AGATGAGTCTTCTAACCAGGATGC-3’; the reverse primer was 5’-TCAGGCCCCCTCAAGCGCGA-3’, and the probe was 5’-TCAGGCCCCCTCAAGCGCGA-3’, producing a 100-bp amplicon. The nominal influenza A matrix copy numbers were determined by a modification of a method previously described (17). Briefly, the copy number of matrix gene was determined by interpolation of the average measured threshold cycle number onto a standard curve produced with a purified plasmid containing a fragment of the M1 gene cloned from the A/Memphis/7/2001 stock. Quantiﬁcation of the purified plasmid was based on A\textsubscript{260} measurements.
was conducted in a 96-well optical plate (Applied Biosystems) in a 25 μl reaction volume containing 5 μl of cDNA plus 20 μl of Master Mix (Applied Biosystems). All sequences were amplified using the 7900 default amplification program: 2 min at 50°C, 10 min at 95°C, followed by 40 to 45 cycles of 15 s at 95°C and 1 min at 60°C. The results were analyzed with the SDS 7900 system software, version 2.1 (Applied Biosystems). Cytokine mRNA expression levels were calculated from normalized ΔΔCT values. ΔCT values correspond to the cycle number at which the fluorescence, due to enrichment of the PCR product, reaches significant levels above the background fluorescence (threshold). In this analysis, the Ct value for the housekeeping gene (GAPDH) is subtracted from the Ct value of the target (cytokine) gene (ΔCT). In general, the ΔCT value for the influenza A-infected sample is then subtracted from the pre-infection ΔCT value (ΔΔCT). Assuming that the target gene (cytokine) and the reference gene (GAPDH) are amplified with the same efficiency (data not shown), the increase in cytokine mRNA levels in test samples is then calculated as follows: increase = 2 ^-ΔΔCT (user bulletin no. 2 for the ABI Prism 7700 sequence detection system from Applied Biosystems). Cytokine mRNA levels are expressed as the increase relative to the level for that cytokine in the individual monkey’s pretreatment lavage sample. Because the mRNA expression level of housekeeping genes such as GAPDH can change under activating conditions, we were careful to use the same input amount of RNA for untreated and experimental samples in the PCR. Samples with the same input amount of RNA consistently resulted in similar PCR amplification (Ct) values for GAPDH. Therefore, it seems that GAPDH was not significantly up-regulated in response to the treatments used in this study.

Influenza Ab ELISA

All plasma samples were initially tested for anti-influenza Abs in a 1/800 dilution-screening assay. Results of the screening assay were calculated using the following ratio: change in OD (ΔOD)/cutoff, where ΔOD is defined as the difference between the mean OD of a diluted sample tested in two influenza Ag-coated wells and the mean OD of the same diluted sample tested in two egg Ag-coated wells. The cutoff value is the mean ΔOD of two preinfection time points of a sample plus 3 SD values. If the ΔOD/cutoff ratio for a sample was >1.0, the sample was considered to be positive and the titer of anti-influenza Abs was determined.

To determine anti-influenza A Ab titers in plasma samples that were positive in the screening assay, 96-well plates (Nunc-Immuno Maxisorp plate II) were coated with either detergent-disrupted A/New Caledonia/20/99 influenza A (Biodesign International) or whole egg protein (Charles River Laboratory) at 5 μg/ml in 0.1 M Na2CO3/NaHCO3 buffer (pH 9.6) and blocked with 4% nonfat powdered milk. Samples were serially diluted (1/2) in duplicate and the plates were incubated overnight at 4°C. The initial dilution of serum tested was 1/4,000. Ab binding was detected using a 1/2,000 dilution of peroxidase-conjugated goat anti-monkey IgG(Fc) (Accurate Chemicals) for 1 h at 37°C. Plates were developed with o-phenylenediamine dihydrochloride (Sigma-Aldrich) for 5 min and...
stopped with H₂SO₄ before reading the OD at 490 nm with a background subtraction of 540 nm. For each serum sample, the endpoint titer of anti-influenza A Abs was defined as the last dilution giving a ∆OD value >0.1, where ∆OD was defined as the difference between med OD values in two-influenza Ag-coated and two-egg Ag coated wells.

HI assay
Titers of anti-H1 Abs were calculated using the HI test of hemagglutinin inhibition. The HI test, originally described by Hirst (20) and later modified by Salk (21), was performed using the microtiter plate protocol revised by the World Health Organization (22). Briefly, monkey plasma samples were treated with receptor-deactivating enzyme (RDE) II (WVR International) according to the manufacturer’s instructions. Treated plasma were then serially diluted 1/2 in PBS. Twenty-five microliters of standardized whole virus solution containing four hemagglutinin units/25 μl were added to each well. After a 10-min incubation at room temperature, 50 μl of 0.5% chicken RBC (Colorado Serum) were added to each well. Results were read after a 1-h incubation. The viral Ag used was the A/Memphis/7/2001 stock virus grown in 10-day-old embryonated chicken eggs.

Intracellular cytokine staining for assessing anti-influenza T cell responses
For intracellular staining to detect influenza-specific T cells in PBMCs, a modification of a previously reported protocol was used (23). Briefly, frozen samples were thawed and rested overnight at 37°C in 5% CO₂ atmosphere in complete RPMI 1640 medium containing 10% FCS. The next day, cells were adjusted to 1 × 10⁷/200 μl and incubated with anti-CD28 and anti-CD49d Abs (1 μg/ml final concentration; BD Biosciences) as costimulatory molecules in a total volume of 200 μl of complete RPMI 1640 and 10% FCS. In all experiments the following samples were prepared: sucrose-purified (A/New Caledonia/20/1999) H1N1 influenza virus at 5 μg/ml, background controls containing costimulatory molecules, and a positive control stimulated with staphylococcal enterotoxin B (0.2 μg/ml, Sigma-Aldrich). A mixture of anti-CD107a and anti-CD107b-FITC Abs (clones H4A3 and H4B4, respectively; BD Pharmingen) was added at a pretitrated volume. Cells were incubated for 16 h at 37°C and brefeldin A (Sigma-Aldrich) was added to the pretitrated volume. Cells were incubated for 16 h at 37°C and brefeldin A (Sigma-Aldrich) and monensin (GolgStop; BD Biosciences) were added for the last 10 h. Following incubation, cells were washed and surface-stained with anti-CD3-Pacific Blue, anti-CD4-PerCP-Cy5.5, or anti-CD8-allophycocyanin-Cy7. A marker to exclude dead cells (7-aminoactinomycin D; Molecular Probes) was added and then samples were fixed (1% paraformaldehyde), permeabilized (0.5% saponin), and stained intracellularly with IFN-γ-allophycocyanin (clone B27), TNF-α-PE-Cy7 (clone Mab11), or IL-2-PE (clone MQ1-17H12) for 20 min at room temperature. All mAbs were from BD Pharmingen unless specified. After washing with permeabilizing buffer, cells were fixed in PBS containing 1% paraformaldehyde.

Data were acquired using a FACSAria flow cytometer (BD Biosciences) and analyzed using FlowJo software (Tree Star) and a Macintosh G5 computer (Apple). At least 100,000 events in the forward/side scatter lymphocyte gate were acquired. The background level of cytokine staining varied from sample to sample but was typically <0.05% of the unstimulated CD8+ T lymphocytes. The only samples considered positive were those in which, after subtracting the background control, there were at least five positive events for a single functional marker, three positive events for two or more simultaneous functional markers, and the sum of the different combinations of responses represented at least 10 events. In addition, a sample was not considered positive for a particular combination of functions if the frequency of responding T cells responding with that particular combination of functions was lower than 0.02%. All data are reported after subtraction of the medium control cultures.

Statistical analysis
To compare the mean TCID₅₀ in tracheal washes of the treated vs untreated animal groups, a one-way Mann-Whitney U test was used. A one-way ANOVA test and Bonferroni’s multiple comparison post hoc test were applied to compare the mean levels of vRNA and host gene mRNA in tracheal washes in the treated, untreated, and heat-inactivated virus naive animal groups. GraphPad Prism version 4.0a for Apple OSX10.4 (GraphPad Software) and Macintosh G5 computers (Apple) were used for all analyses.

Results
The effect of influenza virus inoculum size on virus replication, body temperature, and anti-influenza Ab responses
In order to determine the effect of inoculum size (viral dose) on virus replication in rhesus monkeys, three monkeys were inoculated intratracheally with 6 ml and intranasally with 1 ml of the A/Memphis/7/2001 (H1N1) stock; this is designated as the high-dose group. A second group of three monkeys was inoculated intratracheally with 1 ml and intranasally with 1 ml of the same virus stock; this is designated as the low-dose group. Tracheal lavages were obtained daily for 5 days PI.

Table 1. Effect of prophylactic oseltamivir on influenza virus replication in trachea

<table>
<thead>
<tr>
<th>Animal Groups</th>
<th>TCID₅₀</th>
<th>Peak Day vRNA</th>
<th>Peak Day</th>
</tr>
</thead>
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<td>Untreated</td>
<td>33073</td>
<td>4.67</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>33178</td>
<td>3.50</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>34421</td>
<td>4.50</td>
<td>1</td>
</tr>
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<td>Mean ± SEM</td>
<td>4.22 ± 0.62a</td>
<td>5.88 ± 0.48b</td>
<td></td>
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<tr>
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<td>31657</td>
<td>1.50</td>
<td>2</td>
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<tr>
<td></td>
<td>31981</td>
<td>1.50</td>
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<tr>
<td></td>
<td>32410</td>
<td>2.33</td>
<td>2</td>
</tr>
<tr>
<td>Mean ± SEM</td>
<td>1.92 ± 0.048a</td>
<td>4.96 ± 0.27b</td>
<td></td>
</tr>
<tr>
<td>Heat-inactivated virus</td>
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<td>1</td>
</tr>
<tr>
<td></td>
<td>31625</td>
<td>0</td>
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</tr>
<tr>
<td></td>
<td>31705</td>
<td>0</td>
<td>2.43</td>
</tr>
<tr>
<td>Mean ± SEM</td>
<td>2.97 ± 0.45e, f</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a Mean values are significantly different between treated and untreated monkeys (p = 0.029; two-tailed unpaired t-test).

b Mean values are significantly different between groups that were treated, untreated, and inoculated with heat-inactivated virus (p < 0.001; one-way ANOVA test).

FIGURE 2. The effect of oseltamivir treatment on A/Memphis/7/2001 (H1N1) replication in the trachea. A. Average infectious viral titers (TCID₅₀) in tracheal lavages. B. Average vRNA copy number (log₁₀ copies/ml) in tracheal lavages. Inset, Graph of one-way ANOVA with Bonferroni’s multiple comparison post hoc test comparing the average peak of tracheal lavage vRNA of untreated, oseltamivir-treated, and heat-inactivated influenza virus animals. ■, Untreated (n = 3); ○, oseltamivir-treated (n = 4); and ▲, heat-inactivated virus (n = 4).
before inoculation and on days 1, 2, 3, 7, and 14 PI. The levels of virion-associated influenza virus RNA detected by PCR in the tracheal lavages of the high-dose and low-dose monkey groups were indistinguishable. Tracheal lavage vRNA levels ($>10^6$ copies/ml) peaked at 24 h PI followed by a rapid 3 log$_{10}$ decline by day 7 PI. Very little vRNA was detected in tracheal lavages of any animals on day 14 PI (Fig. 1).

Similarly, the titers of infectious virus isolated from tracheal samples of the high-dose and low-dose groups were very similar (Fig. 1). In addition, at 12 h PI both groups had similar spikes in body temperature that resolved by 96 h PI (Fig. 1). Finally HI titers and anti-whole virus IgG Ab titers in the high- and low-dose groups were indistinguishable. All animals had preinoculation HI titers of 1:4 that dramatically increased by day 14 PI. The IgG Ab responses to whole influenza virus were identical, with strong responses in all animals by day 14 PI (Fig. 1).

Oseltamivir effectively blocks influenza virus replication in rhesus monkeys

To determine the effect of prophylactic oseltamivir on influenza virus replication in rhesus monkeys, four monkeys were treated with oseltamivir twice daily for 5 days beginning 24 h before influenza virus inoculation. The monkeys were inoculated intratraeally with 6 ml and intranasally with 1 ml of the A/Memphis/7/2001 (H1N1) stock; the concentration of virus was $10^6.5$ TCID$_{50}$/ml. The levels of infectious virus and vRNA in tracheal lavages of the four treated and three untreated monkeys were compared.

In the untreated and oseltamivir-treated animals, influenza RNA was detectable in the tracheal lavage samples on days 1, 2, and 3 PI (Fig. 2). By day 7 PI, vRNA was still detectable in the samples from oseltamivir-treated and untreated animals. However, the levels of vRNA in the trachea were different among the groups. Thus, the mean peak vRNA level in the oseltamivir-treated monkeys was significantly lower ($p < 0.05$) than the mean peak vRNA level in the untreated monkeys (Fig. 2 and Table I).

![FIGURE 3.](image) Tracheal lavage cytospins during A/Memphis/7/2001 (H1N1) infection of an untreated and an oseltamivir-treated rhesus monkey. **A**, **C**, **E**, and **G** are from an untreated monkey. **B**, **D**, **F**, and **H** are from an oseltamivir-treated monkey. Samples from days 4, 1, 2, and 3 are shown for each animal. Note that healthy ciliated epithelial cells are much more common in the oseltamivir-treated monkey at all time points. Intracytoplasmic inclusion bodies in influenza A virus-infected epithelial cells and inflammatory cells (neutrophil and macrophage) are common in the untreated monkey after virus inoculation. Wright-Giemsa stain was used. Bar equals 20 μm.

![FIGURE 4.](image) Body temperature and Ab response during A/Memphis/7/2001 (H1N1) infection in untreated-, oseltamivir-, and inactivated virus-inoculated rhesus monkeys. **A**, Average whole anti-($\alpha$)-A/New Caledonia/20/1999 (H1N1) plasma IgG Ab titer. **B**, Average anti-($\alpha$)-A/Memphis/7/2001 plasma HI Ab titer. **C**, Average change in body temperature (°F) compared with day 0. Temperatures were collected every 24 h by rectal thermometer. ■, Untreated ($n = 3$); ○, oseltamivir-treated ($n = 4$); and ▲, heat-inactivated virus ($n = 4$) monkeys.
The mean titer of infectious virus isolated from tracheal samples of untreated animals was 10^4 TCID<sub>50</sub>/ml, significantly higher than the mean virus titer in the oseltamivir-treated animals (p = 0.029; Fig. 2 and Table I). Taken together, these data demonstrate that oseltamivir is effective at blocking viral replication in the lower respiratory tract of the rhesus monkey.

To determine how much of the vRNA detected in tracheal lavages represented the input inoculum rather than de novo virus replication, four additional animals were inoculated with the same dose of heat-killed A/Memphis/7/2001. As expected, the mean peak vRNA level of the killed virus group was dramatically (~1,000-fold) and significantly lower than those of both the oseltamivir-treated and untreated animal groups (Fig. 2 and Table I). Infectious virus was not isolated from either tracheal samples collected from the heat-killed-virus-inoculated animals (Fig. 2 and Table I). Thus, in untreated monkeys the input viral inoculum contributed <0.1% to the peak vRNA levels after inoculation of live virus.

Oseltamivir treatment reduces inflammatory cell infiltrates associated with influenza infection

Before influenza challenge, tracheal wash cytospins from the oseltamivir-treated and untreated monkeys contained ciliated epithelial and rare inflammatory cells (Fig. 3). At 24 and 48 h PI, the number of epithelial cells was increased in the tracheal washes of both treated and untreated monkeys and the CPEs (cellular swelling, cytoplasmic vacuolization, and inclusion bodies) in epithelial cells induced by influenza virus infection were common in samples from untreated monkeys (Fig. 3). By 72 h PI, regenerating epithelial cells were common, viral CPEs were rare, and lymphocytes and neutrophils were common (Fig. 3). CPEs in epithelial cells and all inflammatory cells were much less numerous in the tracheal wash samples of the oseltamivir-treated monkeys (Fig. 3).

Oseltamivir blunts the body temperature spike associated with acute influenza infection but has no effect on the generation of anti-influenza Ab responses

The monkeys inoculated with heat-killed virus had no change in body temperature after inoculation but both the untreated and oseltamivir-treated monkeys had a spike in body temperature at 24 h PI that resolved by 96 h PI (Fig. 4). Although the difference was not statistically significant, the increase in the peak body temperature was more marked in the untreated monkeys than in the oseltamivir-treated monkeys. Despite the decreased viral replication in oseltamivir-treated monkeys, HI titers and anti-whole virus IgG Ab titers were not affected by oseltamivir treatment (Fig. 4 and Table II). The monkeys inoculated intratracheally and intranasally with heat-killed virus made HI and anti-whole virus IgG Ab responses in plasma, although they were relatively weak and delayed compared with the animals inoculated with live virus (Fig. 4).

Anti-influenza T cell responses in untreated and oseltamivir-treated influenza infection

To assess influenza virus-specific T cell responses, PBMCs were restimulated with inactivated influenza virions for 16 h and then permeabilized and stained to detect cytokine production and degranulation. On the day of the experimental inoculation, influenza-specific CD4<sup>+</sup> T cell responses were found in seven of 13 animals and four of 13 animals had influenza-specific CD8<sup>+</sup> T cell responses on day 0 (Figs. 5 and 6). These responses were easily detected as the mean frequency of 855 (±371 SEM) influenza-specific CD4<sup>+</sup> T cells per 10<sup>6</sup> CD3<sup>+</sup> T cells and 603 (±344 SEM) influenza-specific CD8<sup>+</sup> T cells per 10<sup>6</sup> CD3<sup>+</sup> T cells. These day-0 T cell responses were characterized by a large fraction of TNF-α-secreting T cells in both subsets, with more IL-2 and less IFN-γ secretion in CD4<sup>+</sup> T cells compared with CD8<sup>+</sup> T cells (Figs. 5 and 6A). None of the T cells degranulated upon stimulation. Note that although all four of the oseltamivir-treated animals were negative for influenza-specific T cell responses on day 0 (Fig. 5), differences in cell viability or the oseltamivir treatment do not explain the differences in responses, as the day 0 T cells from all animals made similar responses to staphylococcal enterotoxin B stimulation (data not shown). Furthermore, three of the four oseltamivir-treated animals with no influenza-specific T cells had HI titers against the challenge virus of 1:8 (Table II), suggesting that these animals had prior exposure. The influenza-specific memory T cells and Ab responses found at day 0 were presumably induced by prior exposure to an antigenically similar influenza A virus circulating in the humans and introduced by the animal care staff. The strength of the T cell responses, relative to the HI titers, suggests that they are directed against relatively conserved influenza Ags such as matrix and nucleoprotein (NP).

To assess the effect of viral replication on T cell responses to experimental influenza virus inoculation, the influenza-specific T cell responses of the four oseltamivir-treated animals and the three matched untreated animals (Table I) on days 15 and 30 PI were compared (Fig. 6B). All seven animals had influenza-specific CD4<sup>+</sup> T cell responses on day 15 PI, but one animal in each group did not have detectable influenza-specific CD8<sup>+</sup> T cells. By day 30 PI all seven animals had easily detected antiviral CD4<sup>+</sup> T and CD8<sup>+</sup> T cells in blood. The mean frequency of influenza-specific CD4<sup>+</sup> and CD8<sup>+</sup> T cells was lower in the oseltamivir-treated animals (Fig. 6B) at both time points. It is important to note that on day 0 all three of the control animals, but none of the oseltamivir-treated animals, had pre-existing T cell responses. However, efficient expansion of a memory influenza-specific T cell pool in the untreated animals after challenge does not explain the results, as the frequencies of influenza-specific T cells

Table II. Effect of oseltamivir on antibody responses to influenza A virus infection

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<th>ELISA Titters</th>
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<td>Post&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>Mean</td>
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</tbody>
</table>

<sup>a</sup> Pre represents a time point prior to the day of challenge and Post represents day 28 postchallenge.
FIGURE 5. Antiviral T cell responses in PBMCs before and after A/Memphis/7/2001 (H1N1) influenza virus infection in untreated and oseltamivir-treated rhesus monkeys. The response to purified inactivated H1N1 influenza virus stimulation is shown. The pie charts in the top panels summarize the CD4⁺ T cell responses on days 0, 15, and 30 PI and the extent to which the CD4⁺ T cell response was polyfunctional. The pie charts in the bottom panels summarize the T cell responses on days 0, 15 and 30 PI and the extent to which the CD8⁺ T cell response was polyfunctional. Empty circles indicate that there was no positive response in the indicated T cell subset in that sample. Each portion of a pie chart indicates the percentage of influenza-specific T cells that responded with one, two, three, or four functions, and the arcs around the pie show the function or combination of functions to which the specific response corresponds (see color legend). “ID sample” indicates the animal number.
in these animals declined after challenge. The quality of the influenza-specific CD4+ and CD8+ T cell responses was variable among animals within groups (Fig. 5).

**The decreased viral replication mediated by oseltamivir does not affect IFN-α, 2′,5′-oligoadenylate synthetase (OAS), IFN-γ, or IFN-inducible protein 10 (IP-10) mRNA expression, but MxA mRNA expression is increased**

To determine the effect of the oseltamivir-mediated reduction in viral replication on innate antiviral immune responses, the levels of mRNA for anti-viral molecules; IFN-α, IFN-γ, OAS, IP-10, and MxA in tracheal secretions of the treated and untreated animals were compared. To control for the possibility that oseltamivir directly affects gene expression, the levels of the same innate immune molecules in the tracheal lavages of five animals treated with same dose and schedule of oseltamivir but never inoculated with influenza virus were also determined. Finally, to control for the possibility that the input vRNA in the inoculum and not viral replication was responsible for inducing gene expression, the levels of the same innate immune molecules in the tracheal lavages of the four monkeys inoculated with the heat-inactivated influenza virus were determined.

At 24 h PI, there was a 10^3- to 10^4-fold-increase in IFN-α mRNA levels in tracheal washes of oseltamivir-treated and untreated monkeys relative to the preinoculation levels. Although the peak (24 h) IFN-α mRNA levels tended to be marginally higher in the untreated monkeys, the difference was not significant. In the oseltamivir-treated and untreated groups of animals, IFN-α mRNA levels declined to baseline by day 7 PI (Fig. 7). Furthermore, the mRNA expression levels for three of the four IFN-inducible genes, OAS, IFN-γ, and IP-10, were not significantly different in the oseltamivir-treated and untreated groups, although a trend toward increased expression of IP-10 in the untreated animals compared with the oseltamivir-treated animals was apparent (Fig. 7). Thus, despite the significant reduction in viral replication in oseltamivir-treated monkeys, both the treated and untreated monkeys demonstrated a similar gene expression pattern for IFN-α, IFN-γ, and most ISGs (Fig. 7).
Although the mRNA levels of the genes assessed were much lower than those in animals inoculated with live influenza virus, there was a low level induction of IFN-α, OAS, IP-10, and MxA in the animals inoculated with heat-inactivated virus (Fig. 7). There was no change in gene expression in the tracheas of the oseltamivir-only treatment group (Fig. 7).

In marked contrast to the other ISGs measured, the pattern of MxA mRNA expression in the trachea was very different. The level of MxA mRNA was significantly higher in the oseltamivir-treated animals than in the untreated animals \((p < 0.05)\), the heat-killed virus animals \((p < 0.01)\), or the oseltamivir sham animals \((p < 0.001)\) (Fig. 7E). Thus, IFN-α levels were relatively similar in treated and untreated animals, but MxA mRNA expression was significantly increased in the tracheas of animals in which viral replication was suppressed by oseltamivir treatment. Taken together, these results suggest that there is active suppression of MxA by a viral protein that is produced during influenza virus replication.

### Discussion

In mice, an intact \(Mx\) gene confers resistance to experimental influenza virus infections. Haller et al. demonstrated that in the presence of IFN, mice with a functional \(Mx1\) gene, the murine homologue of the human \(MxA\) gene, were more likely to survive a lethal influenza virus challenge than mice without a functional \(Mx1\) gene (3). IFN-α is required to induce the \(Mx1\)-mediated influenza virus resistance, because treatment of mice with an anti-IFN Ab eliminates the beneficial effect of the intact \(Mx1\) gene (3, 24, 25). However, the role of IFN-induced MxA in controlling influenza virus replication in humans and other animals is unclear. Treatment with oseltamivir before influenza inoculation reduces peak viral replication in animal models (26, 27) and humans (11) and, thus, oseltamivir provides a straightforward approach to explore the effect of viral replication on antiviral immunity. In the current study we found that reduced viral replication in the tracheas of oseltamivir-treated animals did not affect the expression levels of most molecules in the IFN-α/β pathway. As in influenza virus infection of humans and other animal models (28–30), the untreated monkeys had dramatically increased mRNA levels for these gene products by 24 h PI. However, the mRNA level of MxA was significantly lower in the tracheal lavages of untreated monkeys compared with those of the oseltamivir-treated monkeys. The simplest explanation for these observations is that high-level virus replication specifically blocks transcription of the MxA gene. This inverse in vivo relationship between the level of influenza virus replication and the level of MxA mRNA expression has not been previously described. Such a relationship suggests that to achieve maximal replication in rhesus monkeys and presumably humans, influenza virus suppresses MxA expression.

Although there was more cytopathology in the sloughed cells in the tracheal lavages from untreated monkeys than from treated monkeys, for almost all genes tested the mRNA expression patterns in treated and untreated monkeys were very similar. Thus, in both untreated and treated monkeys all ISGs measured were markedly elevated, with the significant exception of the key anti-influenza effector molecule MxA. Thus, in the tracheas of oseltamivir-treated animals, viral replication was significantly reduced and MxA expression was significantly increased in comparison to the MxA expression levels in untreated monkeys. Of note, while IFN-α expression was apparently required for MxA expression, viral replication was not required for IFN-α to be produced, as the heat-killed influenza virus induced weak IFN-α and MxA responses in tracheas. Finally, oseltamivir treatment did not directly induce MxA expression in rhesus monkeys, ruling out the pharmacologic induction of MxA.

Although the above observations suggest that unsuppressed MxA expression could significantly blunt influenza virus replication in primates, the mechanism by which MxA controls influenza virus replication in human cells is unknown. In mouse cells, Mx1 is expressed in the nucleus and specifically blocks the replication of orthomyxoviruses (24). Because of its location, Mx1 inhibits the primary transcription of influenza virus mRNA in the host cell nucleus (31). In contrast, human MxA localizes to the smooth endoplasmic reticulum in the cytoplasm of human cells (32) and, thus, MxA would not be expected to affect the primary transcription of influenza virus mRNA. When murine 3T3 cells line are engineered to constitutively express high levels of MxA and are infected with influenza virus, viral mRNAs are transcribed normally by the virion-associated RNA polymerase in the nucleus. Viral protein synthesis and genome amplification is strongly inhibited in these cells, although viral mRNAs direct viral protein synthesis in vitro and appear to be efficiently transported to the cell cytoplasm. These results suggest that, in mouse 3T3 cells, MxA interferes with either intracytoplasmic transport of viral mRNAs, viral protein synthesis, or translocation of newly synthesized viral proteins to the cell nucleus (33). More recent studies have shown that if MxA is artificially made to translocate into the nucleus of 3T3 cells, it binds newly synthesized influenza virus nucleoprotein, preventing viral transcription (4, 31, 33). There is only one report of MxA-mediated antiviral activity in human cells; it has been demonstrated that MxA inhibits cytoplasmic LaCrosse virus genome replication (34). However, to our knowledge IFN-induced MxA-mediated suppression of influenza virus replication in human cells has never been reported.

The transcription of MxA has been studied and it is known that the regulation of MxA expression is tightly controlled and, in human cell lines, is dependent on the expression of IFN-α/β or IFN-γ (5). Induction of IFN-α/β occurs after TLRs, the retinoic acid-inducible gene 1 (RIG-I), or the melanoma differentiation-associated gene 5 (MDA-5) bind dsRNA or ssRNA, leading to the activation of IFN-regulatory factors 3 and 7 (IRF-3 and IRF-7), NF-κB, and AP-1 (35–37). Secreted IFN-α/β binding to the IFN-α receptor activates ISG factor 3 (ISGF3), which binds the ISRE (3, 24, 35). ISGF3 binding to ISRE activates the transcription of MxA. Although activated IRF-3 and IRF-7 can form complexes with p300 and CREB-binding protein (CBP) that induce transcription of a subset of ISREs independently of IFN-α/β (38), MxA transcription does not occur by this mechanism. Further, in vivo MxA mRNA expression appears to lack a strong IFN-α/β independent response; thus, this mode of gene regulation does not seem to play a significant role in inducing MxA expression (39). In fact, MxA expression and MxA-mediated resistance to influenza virus is associated with IFN-α/β -dependent ISRE induction (5). It has recently been shown that, in influenza-infected human cells, type I and type III IFN activity is required for MxA expression (5). Thus, the suppression of MxA in the setting of increased IFN-α expression, as seen in the influenza-infected macaques, must be mediated through inhibition of the specific transcriptional regulators that drive MxA expression.

Although we do not know the mechanism by which high level influenza virus replication interacts with the transcriptional regulators of MxA expression in vivo, there are examples of viral proteins that directly bind to ISREs and regulate the expression of specific ISGs. Thus, the core protein of hepatitis B virus can directly activate OAS expression, presumably to moderate viral replication (40), and the precore and core proteins of hepatitis C virus directly down-regulate MxA expression by binding to both of the
ISREs that control IFN-induced expression of MxA (41). Hepatitis B virus-mediated regulation of MxA expression appears to be at work in vivo as well, because chronically infected hepatitis B virus patients with high levels of viral replication have low levels of IFN-induced MxA, while other ISGs such as OAS are expressed at high levels (41). Thus, the viral core or capsid proteins of two hepatitis viruses control ISG expression by binding to ISREs and either enhance or suppress ISG expression, depending on the specific interaction. Based on the above precedents with two hepatitis viruses, it is likely that high-level influenza replication suppresses MxA expression by a specific viral protein, such as the nucleocapsid protein, that binds to one or more of the ISREs. However, the viral molecule and mechanism responsible for influenza A virus-mediated suppression of MxA mRNA expression remains to be determined.

Nonhuman primates were chosen for these studies because profound differences exist between the immune systems of mice and humans (42, 43) and the laboratory mouse strains commonly used in influenza research do not express Mx1. Because Mx1 is a key mediator of the innate immune responses to influenza in wild mice, the lack of Mx1 expression in laboratory mice fundamentally alters the host virus relationship in ways that affect the adaptation of influenza virus to mice, the levels of viral replication, influenza virus pathogenesis, and the nature of the anti-influenza virus immune response. Further, the influenza virus responsible for human seasonal influenza are highly adapted to the human host and, in general, are not easily transmitted to and have aberrant pathogenicity in nonhuman hosts (44). Serial passage of some human influenza virus isolates in eggs and small animals can result in adaptation of the virus to replicate in naturally nonsusceptible hosts. Thus, the nature of transmission and pathogenesis unique to human influenza virus infection are lost in animal models that use host-adapted viruses (45–50). These serial passage strategies exert extreme selection pressure on human influenza virus isolates to adapt to replication in an abnormal environment producing altered pathogenesis. Thus, the mouse-adapted strain of influenza A virus (PR8) is highly virulent and often fatal in mice, but seasonal influenza is rarely fatal in humans. This feature of influenza virus pathogenesis was also used to reduce the virulence of human influenza strains and produce a safe live influenza vaccine. The currently licensed, live-attenuated, cold-adapted influenza vaccine uses a master donor virus that is adapted for replication in primary chicken kidney cells at low temperature, producing a virus capable of only limited nonpathogenic infection of humans and limited transmission among humans (51). Ferrets are more susceptible to infection with human influenza isolates than mice and develop rhinitis that is similar to that in humans. However, the ability of human isolates to readily infect ferrets varies with influenza strains (50). In addition, differences in the immune systems of primates and carnivores and the lack of reagents to study immune responses in ferrets limit their role as models of human anti-influenza immunity (52–54). Macaque monkeys are naturally infected with human strains of influenza virus as evidenced by the presence of HI Abs to circulating human isolates in captive animals (Table II and Ref. 55), and macaques can be experimentally infected with human influenza virus isolates with varying degrees of virulence (56–58). Furthermore, as previously reported and confirmed here, the level and kinetics of viral replication and the timing and nature of the antiviral immune response in experimentally infected humans (28, 59, 60) and macaques (56–58) are very similar. The relatively close phylogenetic relationship between humans and macaques provides a high degree of homology between the immune systems of these species, and many of the reagents used to study human immune responses also work in rhesus macaques. Thus, macaques are well suited for the detailed studies of influenza virus pathogenesis and immunity (13, 14) that are needed to produce novel and clinically useful interventions for seasonal and pandemic influenza.

As in humans (11), oseltamivir prophylaxis did not alter the timing or strength of HI Ab responses in rhesus monkeys, but there was a trend toward fewer influenza-specific T cells in the PBMCs of oseltamivir-treated animals compared with untreated animals. Of note, even the monkeys inoculated mucosally with heat-killed virus made innate antiviral and anti-influenza plasma Ab responses. The Ab responses were relatively weak and delayed compared with the responses in animals inoculated with live virus, but it is clear that mucosal exposure to moderate amounts of inactivated virus can elicit adaptive anti-influenza immune responses. The relatively low expression levels of IFN-α and the ISGs in response to the killed virus exposure may contribute to these weak Ab responses.

This is the first report showing that high level influenza replication interferes with the innate antiviral immune responses in primates by actively suppressing expression of MxA. The most direct explanation for these findings is that high-level influenza virus replication results in production of a viral protein or proteins that block MxA expression and that this may permit the virus replication to continue despite an otherwise strong innate antiviral immune response. Additional experiments are needed to determine which influenza protein depresses MxA transcription in the respiratory epithelia where viral replication is occurring. However, the results reported here highlight the complex and dynamic relationship between influenza A virus replication and the innate antiviral immune response of primates.

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Disclosures

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