LETTER TO THE EDITOR

Statins reduce the expression of proinflammatory cytokines in influenza A virus infected CrFK cells

P. MEHRBOD1, M. EL ZOWALATY1,3, A. R. OMAR1,2, M. HAIR-BEJO1,2, A. IDERIS1,2,*

1Laboratory of Vaccines and Immunotherapeutics, Institute of Bioscience, Universiti Putra Malaysia, 43400 UPM, Serdang, Selangor Darul Ehsan, Malaysia; 2Faculty of Veterinary Medicine, Universiti Putra Malaysia, 43400 UPM, Serdang, Selangor Darul Ehsan, Malaysia; 3Department of Microbiology and Immunology, Faculty of Pharmacy, Zagazig University, Zagazig 44519, Egypt

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Influenza A viruses (IAVs) (the family Orthomyxoviridae, the genus Influenza virus A) account for respiratory infections with mild to severe symptoms in different species including avian, swine, equine, canine, feline, and human (1). Since 2009, a new recombinant A/H1N1 virus has been circulating worldwide among humans, causing morbidity and mortality and is now referred to as pandemic H1N1 (pH1N1) (2, 3). The spread of pH1N1 has been reported to cause sporadic infections in animal species including pigs (4), domestic pet species such as cats, ferrets, dogs and in cheetah (5, 6). The clinical manifestations and severity of illness following influenza infection are the result of immune dysregulation, which is characterized by the release of pro-inflammatory cytokines that cause serious risk factor for morbidity and mortality (7–9). The most important cytokines involved are tumor necrosis factor alpha (TNF-α) and interleukin 6 (IL-6), which induce innate immune response and lead to inflammation (10). Therefore, drugs other than antiviral agents have attracted attention, e.g. statins, by virtue of their anti-inflammatory effects that are independent of their lipid-lowering ability (11). Statins are clinically used to treat hypercholesterolemia, and, interestingly, they have pleiotropic effects on leukocyte-endothelial interaction, intra- and intercellular signaling, inflammatory gene transcription, hemoxygenase expression, and expression of MHC class II antigens (11, 12). The present study was conducted to investigate the effects of statins on TNF-α and IL-6 levels in an IAV-infected cell line. Crandell Feline Kidney (CrFK) cells were cultured in minimum essential medium supplemented with 10% fetal bovine serum and 1% Penicillin/Streptomycin. Cells were infected with influenza virus A/New Jersey/8/76 (H1N1) at 0.5 MOI. Virus titer was determined using hemagglutination test. Atorvastatin and simvastatin (Sigma-Aldrich, Missouri, USA) (10 mg) were dissolved in 1 ml of dimethylsulfoxide, while pravastatin (Sigma-Aldrich, Missouri, USA) (10 mg) was dissolved in 1 ml of distilled water. The final effective concentration of statins used in the treatments was 10 µmol. Control experiments were carried out using blank solvents to exclude the effects of these vehicles. Confluent monolayer of CrFK cells was exposed to 100 TCID50 of the virus sample in the presence and absence of statins. Untreated CrFK cells were considered as negative control. Cells were examined daily for 6 days post infection (p.i.) to detect the presence of cytopathic effect and cell-free supernatants were subsequently collected for cytokine analysis. The supernatants were stored at -80°C for further processing. The cultures were repeated on at least four separate occasions in duplicates. An ELISA was used to determine the effect of viral infection and statin treatment on the protein expression levels of

*Corresponding author. E-mail: aiini@upm.my; Phone: +6 03 8946 6024.

Abbreviations: CrFK = Crandell Feline Kidney cells; IAV(s) = influenza A virus(es); IL-6 = interleukin 6; p.i. = post infection; TNF-α = tumor necrosis factor alpha
The current study used a novel approach to directly examine the potential to control the expression levels of these cytokines. The intensity of the produced color was proportionally related to the amount of cytokines bound in the first step. Finally, values were read off the standard curve. Data presented as mean ± SD were collected and analyzed using SPSS 18.0. Statistical significance was analyzed using analysis of variance (ANOVA) post-hoc LSD test. \( P \leq 0.05 \) was considered significant.

It was found that the expression levels of TNF-\( \alpha \) and IL-6 in supernatants of CrFK cells following statin treatment were higher in influenza-infected cells compared to the statin combination treatment and mock-treated cells in a time-dependent manner. Virus infection resulted in higher expression levels of these cytokines, which was significantly different from statin-treated supernatants (\( P \leq 0.001 \)). The expression of cytokines after treatment with different statins showed similar significant decrements, especially the TNF-\( \alpha \)-expression after the simvastatin treatment and the IL-6 expression after atorvastatin treatment. These showed the lowest expression levels in all treatment combinations as shown in the Table. It is hypothesized that the hyper-induction or dysregulation of pro-inflammatory cytokines, which is caused by the immune system overreaction, plays an important role in the pathogenesis of influenza, leading to lung inflammation as well as other serious inflammatory reactions causing mortality (13, 14). Therefore, anti-inflammatory agents could be protective and of clinical benefits against influenza infections. Statins are frequently used to treat hypercholesterolemia and are cardio-protective agents (9, 15). Additionally, their effect on modulating TNF-\( \alpha \) induced adhesion molecule expression in human endothelial cells was investigated (16).

Furthermore, the efficacy of statins has been tested in case studies against infections with IAV subtypes H5N1, H3N2 and H1N1 (17). The anti-inflammatory and immunomodulatory properties of statins in lung infections have also been reported in other studies (18, 19). The most important pro-inflammatory cytokines that have been suggested to be involved in the pathogenesis of influenza virus subtypes H1N1 and H5N1 are TNF-\( \alpha \) and IL-6, cytokines secreted from macrophages (9). The current study evaluated the anti-inflammatory activity of three available statins against IAV infection in CrFK cells. We reported the inhibitory effects of statins on the induction of pro-inflammatory cytokines TNF-\( \alpha \) and IL-6 in IAV-infected cells. We investigated whether these cells are potentially able to produce pro-inflammatory cytokines as an indicator for the stimulation of the innate immune system. CrFK cells are known to support the production of cytokines in response to infectious agents (20). The ability of CrFK cells to respond to infectious agents and to produce inflammatory cytokines was clearly demonstrated by highly significant up-regulation of the pro-inflammatory cytokines TNF-\( \alpha \) and IL-6 as shown in the Table. We demonstrated the induction of TNF-\( \alpha \) and IL-6 protein expression in CrFK cells following IAV infection, and the combination treatment of statins and virus sample showed the inhibitory effects of statins on the secretion of these cytokines. The current results thus indicated the potential of IAV to induce the secretion of pro-inflammatory cytokines at high levels in CrFK cells. Anti-inflammatory drugs, such as statins, are by virtue of their beneficial effects superior therapeutics with a clinical potential to control the expression levels of these cytokines. The current study used a novel approach to directly examine the effects of statin treatment in vitro to diminish the cellular inflammatory response to influenza infection, the potential

### Table. The effect of different statins on TNF-\( \alpha \) and IL-6 levels in influenza A virus (H1N1) infected Crandell Feline Kidney cells

<table>
<thead>
<tr>
<th>CrFK cells</th>
<th>Statin</th>
<th>Days p.i.</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF-( \alpha ) (pg/ml)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Infected</td>
<td>–</td>
<td>92.86 ± 2.02</td>
<td>94.11 ± 2.27</td>
<td>128.75 ± 0.76</td>
<td>130.89 ± 0.76</td>
<td>164.29 ± 1.52</td>
<td>190.36 ± 2.02</td>
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</tr>
<tr>
<td></td>
<td>Atorvastatin</td>
<td>48.04 ± 2.27</td>
<td>48.39 ± 7.83</td>
<td>53.57 ± 3.03</td>
<td>64.46 ± 4.29</td>
<td>57.68 ± 5.30</td>
<td>66.07 ± 3.54</td>
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</tr>
<tr>
<td></td>
<td>Simvastatin</td>
<td>46.25 ± 5.30</td>
<td>44.11 ± 0.25</td>
<td>45.71 ± 0.00</td>
<td>45.18 ± 0.25</td>
<td>56.43 ± 4.04</td>
<td>56.25 ± 3.79</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Pravastatin</td>
<td>51.79 ± 4.04</td>
<td>45.54 ± 0.76</td>
<td>52.14 ± 1.01</td>
<td>53.39 ± 0.76</td>
<td>53.57 ± 0.51</td>
<td>60.18 ± 8.33</td>
<td></td>
</tr>
<tr>
<td>Non-infected</td>
<td>–</td>
<td>35.71 ± 1.52</td>
<td>38.39 ± 0.76</td>
<td>39.46 ± 0.25</td>
<td>40.18 ± 3.28</td>
<td>44.29 ± 0.51</td>
<td>47.50 ± 4.55</td>
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<tr>
<td>IL-6 (pg/ml)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Infected</td>
<td>–</td>
<td>410.83 ± 4.71</td>
<td>420.83 ± 11.79</td>
<td>447.50 ± 2.36</td>
<td>592.50 ± 2.36</td>
<td>602.50 ± 0.00</td>
<td>619.17 ± 4.71</td>
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<tr>
<td></td>
<td>Atorvastatin</td>
<td>107.50 ± 0.00</td>
<td>97.50 ± 0.00</td>
<td>95.00 ± 3.54</td>
<td>219.17 ± 0.00</td>
<td>163.33 ± 1.18</td>
<td>210.00 ± 1.18</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Simvastatin</td>
<td>120.00 ± 1.18</td>
<td>160.83 ± 0.00</td>
<td>148.33 ± 1.18</td>
<td>237.50 ± 7.07</td>
<td>185.83 ± 2.36</td>
<td>290.83 ± 0.00</td>
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<tr>
<td></td>
<td>Pravastatin</td>
<td>151.67 ± 3.54</td>
<td>143.33 ± 1.18</td>
<td>222.50 ± 7.07</td>
<td>180.83 ± 2.36</td>
<td>225.83 ± 0.00</td>
<td>212.50 ± 4.71</td>
<td></td>
</tr>
<tr>
<td>Non-infected</td>
<td>–</td>
<td>81.67 ± 1.18</td>
<td>80.00 ± 22.39</td>
<td>64.17 ± 7.07</td>
<td>57.50 ± 0.00</td>
<td>92.50 ± 0.00</td>
<td>63.33 ± 1.18</td>
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</tbody>
</table>

The values were expressed as percentage decrement in TNF-\( \alpha \) and IL-6 levels.
clinical applications of these findings suggest that, due to their immunomodulatory effect, statins might be promising therapeutic agents for future influenza pandemics afflicting different animal species as well as humans. In addition, the role of statins in lowering cholesterol levels required for IAV entry might contribute to their protective role during influenza infection (21). The present study highlights the potential benefits of statins in preventing the immunopathology associated with IAV infection. In conclusion, statins, being inexpensive and widely accepted pharmaceutical treatment, may represent enforcement in the battle against influenza complications and future outbreaks, especially in developing and non-wealthy nations. By diminishing the cytokine over-expression and modulating the intense inflammatory response, the use of statins may contribute to the protection against inflammatory responses in the pulmonary system and other inflammatory reactions associated with severe influenza illness (22). The availability of statins may reduce influenza-related inflammatory outcomes and could be used as supplementary or prophylactic medications to alleviate the severity of the disease as well as potential preventive treatment in case of unexpected pandemics.

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