The Establishment and Validation of the Human U937 Cell Line as a Cellular Model to Screen Immunomodulatory Agents Regulating Cytokine Release Induced by Influenza Virus Infection

Ge Liu¹,² · Si Chen¹,² · Ao Hu¹,² · Li Zhang¹,² · Wenyu Sun¹,² · Jungang Chen¹ · Wei Tang¹ · Haiwei Zhang¹ · Chunlan Liu¹ · Chang Ke³ · Xulin Chen¹,²

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Abstract
Severe influenza infections are often associated with the excessive induction of pro-inflammatory cytokines, which is also referred to as “cytokine storms”. Several studies have shown that cytokine storms are directly associated with influenza-induced fatal acute lung injury and acute respiratory distress syndrome. Due to the narrow administration window, current antiviral therapies are often inadequate. The efforts to use immunomodulatory agents alone or in combination with antiviral agents in the treatment of influenza in animal models have resulted in the achievement of protective effects accompanied with reduced cytokine production. Currently, there are no immunomodulatory drugs for influenza available for clinical use. Animal models, despite being ideal to study the anti-inflammatory responses to influenza virus infection, are very costly and time-consuming. Therefore, there is an urgent need to establish fast and economical screening methods using cell-based models to screen and develop novel immunomodulatory agents. In this study, we screened seven human cell lines and found that the human monocytic cell U937 supports the replication of different subtypes of influenza viruses as well as the production of the important pro-inflammatory cytokines and was selected to develop the cell-based model. The U937 cell model was validated by testing a panel of known antiviral and immunomodulatory agents and screening a drug library consisting of 1280 compounds comprised mostly of FDA-approved drugs. We demonstrated that the U937 cell model is robust and suitable for the high-throughput screening of immunomodulators and antivirals against influenza infection.

Keywords Influenza · Immunomodulatory agent · U937 cell · CCL2 · CXCL10

Introduction
Annual influenza epidemics cause approximately 290,000–650,000 deaths in addition to causing severe illness in 3–5 million individuals each year (WHO 2018). This threat poses a continuous challenge to the health care and public health system and is an economic burden to individuals and countries. The 1918 H1N1 Spanish flu, which was caused by the most virulent influenza virus to date, infected about 5% of the world’s population and had a mortality rate of 2% (Taubenberger and Morens 2006). In recent years, some of the most prevalent highly-pathogenic avian influenza viruses (such as H5N1 and H7N9) have crossed the species barrier, resulting in human infection and death (Liu et al. 2013). Although the pathogenesis of influenza is not fully understood, severe cytokine storm, with markedly higher levels of pro-inflammatory cytokines including interferons (IFNs), tumor necrosis factors

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Xulin Chen chenxl@wh.iov.cn

¹ State Key Laboratory of Virology, Wuhan Institute of Virology, Chinese Academy of Sciences, Wuhan 430071, China
² University of Chinese Academy of Sciences, Beijing 100049, China
³ Wuhan Virolead Biopharmaceutical Company, Wuhan 430075, China
infections (Beigel et al. 2005). This aggressive pro-
inflammatory response along with an insufficient anti-
inflammatory response is commonly referred to as the “cytokine storm” (Liu et al. 2016). Several studies have shown that cytokine storms are directly associated with influenza-induced fatal acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) (Tisoncik et al. 2012; Short et al. 2014). Antiviral treatments, which target viral components, are prone to develop drug-resistant viruses and have a narrow time window for their administration; they are also unable to control the excessive pro-
inflammatory response that occurs in the late stage of infection (Davidson 2018). Therefore, the attenuation of the host inflammatory response with immunomodulators may represent a good strategy for combating severe influenza. In addition, therapeutics designed to target host components are less likely to develop drug-resistant virus strains. In recent years, a few immunomodulatory agents, including proteinase-activated receptor 1 (PAR1) antagonist (Khoufache et al. 2013), sphingosine-1-phosphate (SIP1) receptor 1 agonists (Teijaro et al. 2011), and tyrosine protein kinase (TPK) inhibitors (Florence et al. 2018), have shown protective effects in animal models including alleviating the pulmonary inflammatory reaction and increasing survival rates. However, the lack of efficient cell-based screening models is a great impediment in the development of anti-inflammatory drugs targeting influenza-induced cytokine storms (Davidson 2018). Animal models, despite being ideal for studying potential anti-
inflammatory therapeutics, are very costly and time-con-
suming. Therefore, there is an urgent need to establish fast, economical and time-saving screening methods using cell-
based models that can be used to screen and develop more potent immunomodulatory agents.

Upon infection of host cells by an influenza virus, they can be subsequently recognized by pattern recognition receptors, such as TLR3/7 and RIG-I, which activate downstream pathways, including the NF-κB pathway and the IRF pathways, leading to the expression of multiple cytokines (Pulendran and Maddur 2015). Among the influenza-induced pro-inflammatory cytokines, some common and important pro-inflammatory factors including IFN-α/β, TNF-α, CCL2, CCL5, CXCL10, IL-1β, IL-6, and IL-8, have been detected in patients with severe influenza (Ramos and Fernandez-Sesma 2015). Based on their roles in influenza mediated inflammatory injuries in the lungs, IL-6, IL-8, TNF-α, CCL2, CCL3, and CCL5 are co-regu-
lated by the NF-κB pathway, and the interferons are regu-
lated by the IRF pathways, which can induce the expression of a panel of ISGs, e.g., CXCL9 and CXCL10, primarily through the STAT pathways and the NF-κB pathway (Iwasaki and Pillai 2014). Although the functions of these cytokines have not been fully elucidated, they can be further categorized into two classes according to their therapeutic effects in animal experiments when being tar-
gested: class one includes factors that do not improve or worsen the disease after gene knockout or antibody neutral-
ization, e.g., CCL5 (Wareing et al. 2004), IFN-β (Mordstein et al. 2008), and IL-1β (Kozak et al. 1995); class two includes factors that, along with gene knockout or antibody neutralization, can significantly increase the rate of survival and reduce inflammation in the lungs, e.g. CCL2 (Damjanovic et al. 2011; Hrincius et al. 2015), and CXCL10 (Ichikawa et al. 2013; Wang et al. 2013). Therefore, the second class of factors appears to be ideal therapeutic targets for influenza-induced inflammation.

In this study, our goal was to establish a reliable cell-
based model for high-throughput anti-inflammatory drug screening, along with an efficacy study specific for inhibi-
tors of influenza-induced cytokine release. First, seven human cell lines that are reported to be involved in influenza-induced inflammation were screened to identify an appropriate cell line that can support influenza virus infection and the production of pro-inflammatory cyto-
kines. Using the protein levels of the two important chemokines CCL2 and CXCL10 as readouts, the human monocytc cell line U937 was identified as a suitable cell line and selected to establish a cell-based model. The U937 cell model was validated by testing a panel of agents with reported anti-inflammatory or antiviral activities in vivo and a drug library comprised of 1280 compounds, most of which are FDA-approved drugs. Our results indicate that the human U937 cell line can be used as a model to study the influenza-induced cytokine release and in high-
throughput screening of drugs that target components of the influenza-induced cytokine storm.

Materials and Methods

Cell Lines and Virus Strains

The Madin-Darby Canine Kidney cell line MDCK (CCL-
34), human lung adenocarcinoma cell line A549 (CCL-
185), human monocyte cell lines U973 (CRL-1593.2) and 
THP-1 (TIB-202), and human promyeloblast cell line HL-
60 (CCL-240) were purchased from the American Type 
Culture Collection (ATCC, Rockville, MD, USA). MDCK 
cells were cultured in Dulbecco’s modified Eagle’s me-
tedium (DMEM), and the other cells were maintained in 
RPMI 1640 medium. Both the DMEM and RPMI 1640 
medium were supplemented with 10% fetal bovine serum 
(FBS, Gibico) and 1% penicillin–streptomycin. The dif-
ferentiation of U937 and THP-1 cells into cells possessing
a macrophage-like phenotype was achieved by induction with 100 ng/mL of phorbol-12-myristate-13-acetate (PMA; Sigma-Aldrich) for 24 h (Garcia et al. 1999; Genin et al. 2015). To induce HL-60 cells to differentiate into neutrophil-like cells, HL-60 cells were cultured in growth media (RPMI1640 + 10% FBS) supplemented with 1.25% dimethyl sulfoxide (DMSO) for 5 days, as described previously (Millius and Weiner 2009). All cells were cultured at 37 °C in an incubator with 95% humidity and 5% CO₂.

The strains of influenza viruses used in this study, including A/PuertoRico/8/1934 (H1N1), A/Human/Hubei/3/2005 (H3N2), A/Duck/Hubei/216/1983 (H7N8), and B/Human/Hubei/1/2007 (IBV), were provided by the virus collection at the Wuhan Institute of Virology, Chinese Academy of Sciences, China. Virus stocks were prepared in 10-day-old embryonated chicken eggs. The virus titers were determined using the 50% tissue culture infective dose (TCID₅₀) assay in MDCK cells using the method developed by Reed and Muench (1938).

**Chemicals**

A library of compounds used for drug screening was purchased from MicroSource Discovery Systems, Inc. (Gaylordsville, CT, USA). The library consists of 1280 compounds with broad biological activities and structural diversity and was assigned the name FDA-drug library. This library includes: (1) more than 1000 drugs that have been approved by the FDA or have reached clinical trials in the United States; (2) drugs of international collections that are in clinical use in Europe and/or Asia, but have not yet received FDA approval; (3) a unique collection of approximately 100 pure and natural products and their derivatives. These compounds all have known biological activities with a purity of > 95% and are provided in a DMSO stock solution at a concentration of 10 mmol/L.

Compounds used in the validation of the U937 cell model, including the tyrosine protein kinase (TPK) inhibitor ibrutinib (S2680), protease-activated receptor 1 (PAR1) antagonist vorapaxar (S8067), sphingosine-1-phosphate receptor 1 (S1PR1) agonists ozanimod (S7952), macrolide antibiotics ascomycin (S7411), non-selective cyclooxygenase (COX) inhibitor ibuprofen (S1638), COX2 selective inhibitor etoricoxib (S4651), and peroxisome proliferator-activated receptor (PPAR)-γ agonist rosiglitazone (S2505), were purchased from Selleck Chemicals (Shanghai, China). Ribavirin was purchased from the Sigma Chemical Company (Sigma-Aldrich, MO, USA). Oseiltamivir (GS 4071) was purchased from Toronto Research Chemicals (Toronto, Canada). All compounds were initially dissolved in DMSO (Sigma-Aldrich, MO, USA).
384 well plates) of the treated U937 cells, a medium (RPMI 1640 + 10% FBS) containing 20% of the detection reagent was added and incubated at 37 °C for 1 h. After centrifugation of the test plate at 500 ×g for 3 min using a swing-out rotor, the optical density (OD) value at the specific wavelength of 490 nm (OD490) and the reference wavelength of 630 nm (OD630) was measured using the EnVision Multilabel Plate Reader. The final absorbance is equal to the value of OD490 minus the value of OD630 in order to rule out the effects of excess cell debris, fingerprints, and other non-specific absorption.

**High-Throughput Screening (HTS) of FDA-Drug Library**

In the primary screen, 1280 compounds in the FDA-drug library were dissolved in DMSO at a concentration of 10 mmol/L and added to four 384-well source plates (Labcyte, LP-0200) with 320 compounds per plate. Subsequently, 320 μL of each compound, positive control drugs, or DMSO were transferred to four sterile, clear-bottom view 384-well plates (PerkinElmer, 6007460) using an acoustic droplet ejection (ADE) system (Echo 550, Labcyte, CA, USA). Forty μL of complete medium (RPMI 1640 + 10% FBS + 1% penicillin–streptomycin) was added to each well to dilute the drug to a final concentration of 40 μmol/L. To prepare for viral infection, U937 cells were resuspended at a density of 1 × 10⁶ cells/mL in complete medium and infected with 0.05 multiplicity of infection (MOI) of the A/PuertoRico/8/1934 (H1N1) virus. Immediately after mixing, 40 μL of the cell-virus mixture was added to each well in the compound-containing 384-well plates with a cell density of 40,000 cells/well; 40 μL of uninfected cells were also added to the negative control wells. After incubating at 37 °C/5% CO₂/95% relative humidity for 48 h, the cell culture plates were centrifuged at 500 ×g for 3 min using a swing-out rotor, and 70 μL of supernatant per well were taken for the detection of NA activity and cytokine levels. The remaining cells were used to test for cell viability.

In the confirmation screen, the serially diluted hit compounds (0.04–90 μmol/L) were added to 384-well plates. The U937 cells and influenza virus were added and incubated for 48 h in the presence or absence of the drugs as described previously to confirm the inhibitory effect of the drug and to study the kinetics of the drug response. In parallel, the cytotoxicities of hit compounds were determined in the same conditions but without viral infection. Finally, the half maximal inhibitory concentration (IC₅₀), half maximal toxicity concentration (CC₅₀) and selective index (SI) of the hit compounds were determined.

**Measurement of Virus Titer**

Viral titers were determined using a TCID₅₀ assay. Briefly, MDCK cells were seeded into 96-well cell culture plates at a density of 20,000 per well. 24 h later, tenfold serial dilutions of the virus solution were inoculated on an MDCK monolayer at 37 °C for 72 h, and the cytopathic effects (CPE) were examined. The virus titers were calculated using the method developed by Reed and Muench (Reed and Muench 1938).

**Total RNA Extraction and Real-Time PCR**

U937 cells were collected 12 h post-infection with the H1N1 PR8 virus at 0.1 MOI, and the total RNA was extracted according to the protocol in the E.Z.N.A Micro Elute Total RNA kit (OMEGA, R6831). Reverse transcription was performed using the M-MLV Reverse Transcriptase (Promega, M170A) and random hexamer primer. Quantitative mRNA expression analysis was performed using the SYBR green method (iTaq™ Universal SYBR® Green Supermix, Bio-Rad, 1725121) according to the manufacturer’s instructions. The PCR Conditions were as follows: denaturing at 95 °C for 30 s; 40 cycles of 94 °C for 5 s and 60 °C for 30 s. To avoid intra-plate errors, all experiments were performed in a 96-well plate in triplicate per sample. Primer sequences of the cytokines and chemokines used in the experiments were all taken from published literature (Giulietti et al. 2001).

**Statistical Analysis**

The IC₅₀ and CC₅₀ of the compounds were determined by non-linear regression using the Graph Pad Prism 5.0 software. The data in the figures are presented as the mean ± SEM. The Z’ value is statistically calculated using a previously reported method (Zhang et al. 1999). Specifically, the Z’ value is calculated as follows:

\[
Z’ = 1 - \frac{[(3 \times SD of sample + 3 \times SD of control)/\text{average of sample} - \text{average of control}]}{\text{SD of sample}}
\]

where the SD value represents the standard deviation of the fluorescence signal.

**Results**

**Identification of the U937 Cell Line as a Cell-Based Model for Drug Discovery Against Cytokines Induced by Influenza Virus Infection**

Lung epithelial cells, neutrophils, and monocytes/macrophages all play important roles in the cytokine storm induced by influenza virus infection and the
subsequent effects of ALI and ARDS (Short et al. 2014). In order to identify a human cell line that can support influenza virus infection and the production of important pro-inflammatory cytokines, while also being suitable for screening anti-influenza drugs suppressing cytokine release, we examined seven human cell lines, including the epithelial lung A549 cells, promyelocytic HL-60 cells, neutrophil-like iHL-60 cells, monocytic TPH-1 cells, macrophage-like iTPH-1 cells, monocyctic U937 cells, and macrophage-like iU937 cells, for their abilities in supporting influenza virus infection and the production of pro-inflammatory cytokines based on signal-to-background ratios (SBRs) during virus replication and CCL2/CXCL10/IL-8/IL-6 expression levels (Table 1). Among the cell lines tested, A549 has lower SBRs in viral replication and pro-inflammatory cytokine secretion when infected with H1N1 PR8 influenza virus at MOI of 0.1 and cultured for 48 h, while HL-60 and iHL-60 can support influenza virus replication under the same conditions, but they only induce specific cytokine expression (CCL2 for HL-60, IL-6 for iHL-60). Therefore, these cell lines are not suitable for developing cell models for screening of anti-influenza drugs suppressing cytokine release.

To confirm whether the U937 cell line is capable of supporting influenza virus replication and the secretion of pro-inflammatory cytokines, they were infected with the influenza virus PR8 strain at different MOIs. Viral replication levels were determined according to neuraminidase (NA) levels and viral titers in a time course assay. As shown in Fig. 1A, NA activities in the supernatants increased with increasing MOIs of the viruses and reached a peak level at 48 hpi (hours post infection). Interestingly, both the culture time and the MOI can affect the viral titers of the supernatants of infected U937 cells (Fig. 1B). We found that the viral growth curves in U937 cells determined by the TCID_{50} endpoint dilution assay were consistent with what was determined using the NA activities when the MOIs were equal to or less than 0.1, suggesting that under such infection MOI conditions, the NA activities correlate well with the influenza virus titers. The TCID_{50} growth curves at high MOIs are partially inconsistent with the NA

Table 1

| Name | Cell-type | Signal to background ratio (SBR)<sup>a</sup> |
|------|-----------|---------------------------------------------|
|      |           | NA<sup>b</sup> | CCL2<sup>c</sup> | CXCL10<sup>c</sup> | IL-8<sup>c</sup> | IL-6<sup>c</sup> |
| A549 | Lung epithelial cell line | 2.3 ± 0.5 | 1.6 ± 0.1 | 1.5 ± 0.3 | 1.9 ± 0.1 | 3.6 ± 0.1 |
| THP-1 | Monocyte cell line | 5.8 ± 2.1 | 3.2 ± 0.7 | 1.7 ± 0.2 | 2.0 ± 0.2 | 1.2 ± 0.1 |
| iTPH-1<sup>d</sup> | Macrophage-like cell | 1.5 ± 0.6 | 1.4 ± 0.2 | 3.4 ± 0.2 | 1.2 ± 0.1 | 1.1 ± 0.1 |
| U937 | Monocyte cell line | 7.3 ± 1.1 | 16.2 ± 2.5 | 23.6 ± 3.3 | 17.9 ± 2.3 | 13 ± 3.7 |
| iTU937<sup>d</sup> | Macrophage-like cell | 2.4 ± 0.7 | 1.1 ± 0.1 | 2.5 ± 0.2 | 1.2 ± 0.2 | 1.6 ± 0.2 |
| HL-60 | Promyeloblast | 6.1 ± 0.6 | 13.0 ± 2.7 | 1.2 ± 0.2 | 4.4 ± 0.1 | 1.3 ± 0.3 |
| iHL-60<sup>e</sup> | Neutrophil-like cell | 4.1 ± 0.4 | 1.4 ± 0.3 | 1.0 ± 0.1 | 2.2 ± 0.3 | 13.2 ± 2.6 |

<sup>a</sup>Cells were incubated with 0.1 MOI of A/PuertoRico/8/1934 (H1N1) influenza virus and tested for the signal to background ratios (SBRs) at 48 h post infection. The SBR was calculated from the fluorescence value of the infected wells (signal) divided by the uninfected control wells (background) for each assay.

<sup>b</sup>NA (neuraminidase), measured by neuraminidase activity assay, represents the replication level of the influenza virus.

<sup>c</sup>Cytokine expression was measured by AlphaLISA. U937 and iTPH-1 cells were prepared from U937 and THP-1 cells induced by 100 ng/mL TPA for 24 h. iHL-60 was prepared from HL-60 induced by 1.25% DMSO for 5 days.

**U937 Cells Support the Replication of Influenza Virus H1N1 and Produce Multiple Cytokines upon Influenza Virus Infection**

Although the total expression levels of inflammatory cytokines in iTU937 and iTTHP-1 were significantly increased (data not shown), the amount of cytokine expression in the uninfected group was also greatly increased, which resulted in the lower SBRs of the three pro-inflammatory cytokines. These results indicate that the un-induced monocyte cell line U937 has the greatest potential to be a cell model for screening anti-inflammatory agents among the seven cell types tested.
growth curves, in that the TCID₅₀ reached peak levels at 24 hpi, possibly due to the cell death caused by virus-induced cytopathic effects with longer culture times (Fig. 1A–1C). Next, we tested the mRNA levels of the seven major pro-inflammatory cytokines induced post-infection in the influenza-infected U937 cells. As shown in Fig. 1D, at 12 hpi, the mRNA levels of CCL2, CCL5, CXCL10, IL-6, IL-8, IFN-β and TNF-α in U937 cells increased by 26.3, 1.7, 318.3, 27.5, 7.5, 344.2 and 2.15 times, respectively, indicating that PR8 infection can stimulate the transcription of pro-inflammatory cytokines in U937 cells. To study the correlation between the production of cytokines and virus MOIs, we then measured the protein levels of CCL2 and CXCL10 in the supernatants of U937 cells infected with the PR8 virus at different MOIs. The protein levels of CCL2 (E) and CXCL10 (F) in the supernatants of U937 cells infected with the PR8 virus at different MOIs were determined using AlphaLISA. Each data point in the figure was repeated three times and the values were presented as mean ± SEM. NC represents cell control.

U937 cells can support the replication of the influenza virus PR8 as well as the production of multiple cytokines. More importantly, infection of U937 cells with a relatively low MOI is critical for the production of the influenza-induced cytokine storm; infection with a high MOI can cause severe CPE and a low MOI can result in a low level of viral replication, both conditions can attenuate influenza-induced cytokine expression.

**U937 Cells Support the Replication of Multiple Subtypes of Influenza Viruses and the Expression of Pro-Inflammatory Cytokines**

Influenza viruses are classified into types A, B, C and D and make up four of the seven genera of orthomyxoviridae. The type A viruses are the most virulent pathogens and can also be subdivided into different serotypes based on the antibody response to the viral components HA and NA. Influenza B viruses almost exclusively infect humans and can cause severe disease; they have only one serotype. Each of these influenza viruses may replicate and induce a cytokine storm in host cells differently (Hay et al. 2001). To investigate whether U937 cells can support the replication of different subtypes of influenza viruses and as well as the expression of the major pro-inflammatory cytokines in addition to the IAV
H1N1 strain we initially used, three other subtypes of influenza virus strains from type A and one strain from type B, including H3N2, H7N8, and IBV, were selected and used to infect U937 cells. All four virus strains were able to replicate in U937 cells, although IBV was shown to replicate much less efficiently, as shown in Fig. 2A. Next, we examined the expression of CCL2, CXCL10, and IL-8 in the supernatants of the infected U937 cells. H1N1, H3N2, and H7N8 virus strains were shown to induce the expression of CCL2, CXCL10, and IL-8 (Fig. 2B–2D). Interestingly, IBV, which was shown to replicate inefficiently in U937 cells, activates the expression of the three cytokines as efficiently as the other three IAV strains (Fig. 2). These results indicate that U937 cells can support the replication of different types and subtypes of influenza viruses and induce the expression of multiple pro-inflammatory cytokines in response to these virus infections. In addition, the infection of a relatively low MOI of influenza virus in U937 cells is critical and sufficient for the production of multiple cytokines.

The U937 Cell-Based Model was Validated Using a Panel of Immunomodulatory and Antiviral Agents with Known Activities In Vivo

To validate the U937 cell model, we tested a panel of immunomodulatory and antiviral agents with known *in vivo* activities (Table 2). These immunomodulatory agents, including the TPK inhibitor ibrutinib, PAR1 antagonist vorapaxar, S1PR1 agonist ozanimod, PPARγ agonist rosiglitazone, and macrolides antibiotics have been reported to improve the survival rate and pathology in influenza-infected mice. As expected, most of these immunomodulatory agents, with the exception of rosiglitazone, showed anti-inflammatory activities in the U937 cell model, as determined by their inhibition of CCL2 and CXCL10 expression. The antiviral agents, including the macrolide antibiotic ascomycin and the antiviral drugs ribavirin and Oseltamivir, were shown to have antiviral effects in the U937 cell model, as determined by the neuraminidase activity assay (Table 2). In contrast, some of
the anti-inflammatory agents, including the COX non-selective inhibitor ibuprofen and the COX2-specific inhibitor etoricoxib, which have been reported to have no inhibitory effect on the production of CCL2 and CXCL10 in U937 cell model (Table 2). Though the diversity and numbers of agents tested here were limited, these results suggest that, when using NA as an antiviral readout and CCL2/CXCL10 expression as anti-inflammatory readouts, the U937 cell model can be used to screen antiviral and immunomodulatory agents against influenza infection.

**U937 Cell-Based HTS Model was Established and Validated by Screening an FDA-Drug Library**

After the optimization of several parameters, including the number of cells per well, the MOI, and culture time, a U937 cell-based HTS model was established. Briefly, U937 cells were cultured in 384-well plates at a density of $1 \times 10^6$/mL, followed by infection with 0.05 MOI of influenza virus. The plates were cultured for 48 h at 37°C. Viral replication and the expression levels of cytokines were determined using a neuraminidase assay and AlphaLISA, respectively. The performance of our U937 cell-based HTS platform was evaluated by screening the FDA-approved drug library to identify agents that can inhibit the induction of the expression of CCL2 and CXCL10 by influenza infection (Fig. 3A). At a concentration of 20 μmol/L, 1280 compounds were screened on four 384-well plates. The SBRs of NA in the four plates were between 7.5 and 15, as shown in Fig. 3B. The SBRs of CCL2 and CXCL10 were between 17.6 and 21.5, and 20.8 and 28.3, respectively. The average Z' factors were $0.74, 0.75$ and $0.84$, respectively; all of the Z' factors were greater than 0.65, suggesting that the U937 cell model is robust and desirable for the HTS of immunomodulatory agents against the production of pro-inflammatory cytokines induced by influenza virus infection.

In the screen of the FDA-approved drug library, a hit was defined as a compound with cell viability > 75%, and an inhibition rate for CCL2 or CXCL10 > 70%, and an NA inhibition rate < 75%. We identified 60 hits with an anti-CCL2 effect with a hit rate of 4.7%, and 33 hits with an anti-CXCL10 effect with a hit rate of 2.6%. Interestingly, when analyzing the composition of the hit compounds with anti-CCL2 and anti-CXCL10 effects, listed in Supplementary Table S1, we found that most of the traditional anti-inflammatory drugs, such as steroidal anti-inflammatory drugs, cox inhibitors, prostaglandin synthetase inhibitor, histamine antagonist, 5-HT2 receptor antagonist, β-adrenergic blocker, and cholinergic receptor inhibitor, were identified as hits with anti-CCL2 effects, but not as hits with anti-CXCL10 effects. In addition to traditional anti-inflammatory drugs, many agents with new targets were identified as well, which include the majority of the 16 hits found in both the anti-CCL2 and anti-CXCL10 hits, including HMG-CoA reductase inhibitors, DNA

**Table 2 Validation of U937 cell model with a panel of antiviral and immunomodulatory agents.**

| Chemical name | CC50 | IC50 | SI | CC50 | IC50 | SI | Target and possible mechanism | References |
|---------------|------|------|----|------|------|----|--------------------------------|------------|
| Ibrutinib     | 26.5 | > 80 | -  | 0.62 | 9.6  | 4.2 | TPK inhibitor                 | +          |
| Vorapaxar     | > 80 | –    | –  | 5.2  | 13.3 | > 15.4 | PAR1 antagonist               | +          |
| Ozanimod      | 26   | –    | –  | 7.7  | 10.7 | 3.4 | S1PR1 agonist                 | +          |
| Ascomycin     | 62   | 21.3 | 2.9 | 8.1  | 9.9  | 7.7 | Macrolides antibiotics        | +          |
| Ribavirin     | > 160| 26.2 | > 6.1| –    | –    | –  | Anti-influenza                 | +          |
| Oseltamivir   | > 100| 0.58 | > 172.4| –    | –    | –  | Anti-influenza                 | +          |
| Rosiglitazone | > 100| –    | –  | –    | –    | –  | PPARG agonist                 | +          |
| Etoricoxib    | > 50 | –    | –  | –    | –    | –  | COX-2 inhibitor               | ±          |
| Ibuprofen     | > 50 | –    | –  | –    | –    | –  | COX non-selective inhibitor    | ±          |

The anti-CCL2 and anti-CXCL10 hits, identified as well, which include the majority of the 16 hits found in both the anti-CCL2 and anti-CXCL10 hits, including HMG-CoA reductase inhibitors, DNA

| Chemical name | CC50 | IC50 | SI | CC50 | IC50 | SI | Target and possible mechanism | References |
|---------------|------|------|----|------|------|----|--------------------------------|------------|

IC50: 50% inhibition concentration (μmol/L) determined by NA activity assay. NA IC50: 50% inhibition concentration (μmol/L) determined by AlphaLISA. S1 (Selective Index): a ratio of CC50/IC50. CC50: 50% cytotoxic concentration (μmol/L) determined by MTS assay. SI (Selective Index): a ratio of CC50/IC50. 

References:

- Florence et al. (2018)
- Khoufache et al. (2013)
- Teijaro et al. (2011)
- Sato et al. (1998)
- Smee et al. (2008)
- Tsai et al. (2015)
- Moseley et al. (2010)
- Zheng et al. (2008)
- Lauder et al. (2011)
gyrase/topoisomerase IV inhibitors, ion channel inhibitors, and tyrosine kinase inhibitors. It appears that the target distributions of CCL2 and CXCL10 are different but related to some extent, suggesting that they are co-regulated by certain signaling pathways, and specifically regulated by their own signaling pathways.

**Six Hit Compounds were Confirmed to Inhibit the Production of Multiple Pro-Inflammatory Cytokines on U937 Cell Model**

In the primary single dose (20 μmol/L) screen using the influenza virus infected U937 cell model on 384-well plates, we identified 16 hit compounds with an anti-inflammatory effect (inhibiting both CCL2 and CXCL10) without an antiviral effect. To further confirm and evaluate their efficacies in repressing cytokine expression, we tested all 16 hit compounds for their kinetic inhibition of CCL2 and CXCL10 and cell viabilities in U937 cells. Their IC_{50s} and CC_{50s} were determined. Compounds with an IC_{50} > 10 μmol/L or shown to have no effect were considered to be inactive, 10 out of 16 hit compounds met this criterion (data not shown). Compounds with an IC_{50} < 10 μmol/L were considered to be active; 6 out of the 16 primary hit compounds met this criterion, they are dasatinib, pitavastatin, simvastatin,
fluvastatin, protriptyline, and levofloxacin (Fig. 4A–4F). The cytotoxicity of the six hit compounds on uninfected U937 cells was determined, as shown in Fig. 4 (right panel). The CC50s were all greater than 90 μmol/L, except for simvastatin which had a CC50 of 66.8 μmol/L. To exclude compounds with antiviral activities that may lead to a reduction in CCL2 and CXCL10 levels, we examined the antiviral effects of the six hit compounds in the same confirmation experiments simultaneously (Fig. 4 right panels). Surprisingly, all 6 of the hits were shown to

Fig. 4 Six hit compounds were confirmed to inhibit the production of CCL2 and CXCL10 in the U937 cell model. The kinetics of the inhibition of CCL2 and CXCL10 expression of the primary hit compounds was tested in the U937 cell model for Dasatinib (A), Pitavastatin (B), Simvastatin (C), Fluvastatin (D), Protriptyline (E), Levofloxacin (F). The structures of the six compounds are shown in the left panel. The inhibitory effects on CCL2 and CXCL10 are shown in the middle panels. The compound toxicity (green) and antiviral activity (red) expressed as a histogram are shown in the right panel. Each data point represents the mean ± SEM. The IC50 values for inflammatory factors are listed in the figure and were calculated using Graph Pad Prism 5.0 software.
promote viral amplification to varying degrees, and viral replication was shown to increase with increasing drug concentrations, suggesting that these compounds may also inhibit the expression or functions of certain host factors associated with the innate antiviral response that correlated to the influenza-induced cytokine storm. Based on the kinetic inhibition of the two important cytokines CCL2 and CXCL10, and the IC$_{50}$s and SIs (Fig. 4 and Table 3), we identified 6 compounds with potent inhibitory effects on influenza-induced expression of cytokines in U937 cells. Taken together, our results suggest that the U937 cell model is suitable for high throughput screening of anti-inflammatory drugs against influenza-induced cytokine release.

**Discussion**

Due to the narrow therapeutic window for the administration of anti-flu drugs, typically within 2 days of the onset of symptoms, and the association of excessive inflammatory responses with severe influenza pneumonia, the treatment of influenza pneumonia cannot rely solely on antiviral drugs. Combination therapies that include both antiviral agents and anti-inflammatory agents represent a potential solution in curing influenza pneumonia. Immunomodulators that can inhibit the excessive inflammatory response in the lungs of patients infected by influenza viruses are urgently needed. However, the use of traditional anti-inflammatory drugs, including corticosteroids, during an influenza infection has been found to have either no effect or even detrimental effects in clinical retrospective studies (Hui and Lee 2013; Ramos and Fernandez-Sesma 2015). Therefore, the establishment of a cell-based influenza virus infection model that can be used for both antiviral and anti-inflammatory drug discovery is very valuable and urgently needed.

Cell models are quite different from animal models in that they do not show indications of viral pneumonia found in the lungs of animals. To establish a useful cell model, the right cell type must be chosen; one that plays an important role in influenza-induced cytokine storms and has the appropriate readouts (e.g. induces the expression of the most important cytokines upon influenza infection). Lung epithelial cells, neutrophils, and monocytes/macrophages all play important roles in the influenza-induced cytokine storm and the subsequent ALI and ARDS (Short et al. 2014). In order to identify a human cell line that can support multi-types and -subtypes of influenza virus infection and the production of important pro-inflammatory cytokines, we examined seven human cell lines, including epithelial cell, neutrophils, and monocytes/macrophages, that are important for the pathogenesis of severe influenza and found that monocytic U937 cells can support the infection and replication of the three IAV and one IBV strains tested. Next, we measured the expression of 7 cytokines, including CCL2, CCL5, CXCL10, IL-6, IL-8, IFN-β, and TNF-α, by quantifying their mRNA levels. A strong response was observed for five of the seven cytokines. For the purpose of using U937 cells as a model for the influenza-induced cytokine production, we measured the correlation between the kinetic expression of CCL2 and CXCL10 at different time points and the MOIs of the viruses used for infection and found that the expression of CCL2 and CXCL10 reached their

| Chemical name | IC$_{50}$ | IC$_{50}$ | CC$_{50}$ | SI | Approved of intended use | Possible mechanism |
|---------------|---------|---------|---------|----|--------------------------|-------------------|
|               | CCL2    | CXCL10  | CCL2    | CXCL10 |                           |                   |
| Dasatinib     | 0.63    | 0.71    | > 90    | > 142.9 | > 126.8 | Antineoplastic           | Tyrosine-protein kinases inhibitor |
| Pitavastatin  | 0.85    | 0.87    | > 90    | > 105.9 | > 103.4 | Antlipemic               | HMG-CoA reductase inhibitor |
| Simvastatin   | 1.16    | 1.75    | 66.8    | 57.6    | 38.2    | Antilipemic              | HMG-CoA reductase inhibitor |
| Fluvoxetin    | 2.43    | 2.95    | > 90    | > 37.0  | > 30.5  | Antilipemic              | HMG-CoA reductase inhibitor |
| Protriptyline | 2.23    | 3.15    | > 90    | > 40.4  | > 28.6  | Antidepressant           | Serotonin reuptake inhibitor |
| Levofloxacin  | 8.94    | 3.12    | > 90    | > 10.1  | > 28.8  | Antibiotic               | DNA gyrase and topoisomerase IV inhibitor |
peak levels when the U937 cells were infected with an influenza virus at an MOI between 0.025 and 0.05. Furthermore, the U937 cell model was demonstrated to support complete viral replication and the expression of CCL2, CXCL10, and IL8 when infected by all of the four subtypes of influenza viruses tested. Therefore, the U937 cell model exhibits great potential for drug discovery of anti-inflammatory and antiviral agents against influenza. However, we think other human cell lines of epithelial, neutrophil, monocyte, and macrophage origins may also be useful for the study of influenza mediated cytokine responses; however, their applications are not as robust for HTS as the U937 cell line and need to be carefully tested and optimized.

CCL2 and CXCL10 were chosen as the readouts for anti-inflammatory drug screening against influenza in the U937 cell model for the following reasons: (1) these two factors are closely related to the immunopathology caused by influenza. In the fatal cases caused by an influenza virus infection, the levels of these two cytokines were significantly higher in patients than in healthy controls (de Jong et al. 2006; Deng et al. 2008; To et al. 2010; Gao et al. 2013). (2) Functional studies have shown that knocking out the CCL2 or CXCL10 genes or using neutralizing antibodies to deplete their protein levels and subsequently reducing their functional activity, can significantly alleviate the inflammatory response to influenza infection and improve the survival rate in experimental animal models of influenza infection (Damjanovic et al. 2011; Ichikawa et al. 2013; Wang et al. 2013; Hrincius et al. 2015). (3) CCL2 and CXCL10 are two of the major cytokines/chemokines that recruit monocytes/macrophages and neutrophils, both of which play important roles in influenza-induced inflammation (Ichikawa et al. 2013; Tavares et al. 2017). The reduction of the levels of these two cytokines may decrease the infiltration of inflammatory cells. (4) CCL2 and CXCL10 are regulated by different signaling pathways. CCL2 is primarily regulated by the NF-κB pathway whereas CXCL10 is predominantly regulated by the interferon pathway, both of which are key pathways for controlling inflammatory cytokine responses (Chen et al. 2018). (5) Although the inclusion of more cytokines and chemokines may be a better representation of the influenza-induced cytokine storm, too many readouts may unnecessarily complicate the screen. Based on these reasons and concerns, we decided to start with two of the most important pro-inflammatory cytokines, CCL2 and CXCL10.

The use of the U937 cell model to study the anti-inflammatory effects on influenza-induced cytokine responses was validated by testing a panel of known antiviral and immunomodulatory agents, in addition to screening a drug library with 1280 compounds comprised mostly of FDA-approved drugs. Almost all of the immunomodulatory and antiviral agents with known activities in vivo (Table 2) showed expected anti-cytokine and antiviral effects. The screen of the FDA- drug library led to the identification of 16 hit compounds that were shown to inhibit the production of both cytokines, and many hits were shown to inhibit either CCL2 or CXCL10. The average Z' factor for NA, CCL2, and CXCL10 were 0.74, 0.75 and 0.84, respectively, suggesting that the U937 cell model is robust and desirable for HTS of immunomodulatory agents against influenza infection. Interestingly, most of the traditional anti-inflammatory drugs, i.e. steroidal and non-steroid anti-inflammatory drugs, are identified as CCL2 inhibitors. Many novel immunomodulatory agents with new targets were identified in both the anti-CCL2 and anti-CXCL10 hits, covering diverse host targets, including histamine receptors, 5-HT2 receptors, β-adrenergic and cholinergic receptors, HMG-CoA reductase inhibitors, DNA gyrase/topoisomerase, ion channels, and tyrosine kinases. It appears that the target distribution of CCL2 and CXCL10 inhibitors are different but related to some extent, suggesting that the expression of CCL2 and CXCL10 are co-regulated by certain signaling pathways, and specifically regulated by their own signaling pathways.

Using the U937 cell model, a secondary screen of the 16 hit compounds identified as inhibiting the expression of both CCL2 and CXCL10 in the primary screen led to the discovery of 6 compounds with IC50 s lower than 10 μmol/L and SIs greater than 20 for anti-cytokine activity. It is worth noting that the 6 re-confirmed hit compounds can be categorized into four classes: (A) TPK inhibitors, (B) Quinolone antibiotics, (C) Statins, and (D) Serotonin reuptake inhibitors. Interestingly, inhibitors targeting these four classes of targets have been previously reported in the literature to reduce inflammation and improve lung pathology caused by influenza infection. The TPK inhibitors, ibrutinib, and imatinib have been reported to protect mice from lethal influenza infection and improve acute lung injury caused by influenza (Hrincius et al. 2015; Florence et al. 2018). The quinolone antibiotic levofloxacin, which has antioxidant effects, increased the survival rate in mice infected with the H1N1 PR8 virus and was shown to inhibit excessive superoxide production in the lungs of mice (Enoki et al. 2015). Statins block the inflammatory signaling pathway and inhibit the production of pro-inflammatory factors in cells. Their use in animal experiments has shown to reduce the mortality of H1N1 and H3N2-infected mice. More importantly, in the retrospective study of human drug use, the use of statins is associated with a reduction in influenza-induced lethality (Liu et al. 2009; Mehrbod et al. 2014; Davidson 2018). Serotonin reuptake inhibitors can alleviate lung inflammation in mice induced by the H1N1 influenza virus and reduce mortality in mice (Sharma et al. 2013). These results indicate that using CCL2 and CXCL10 as readouts...
in the U937 cell-based model for anti-inflammatory drug screening have predictive value for in vivo efficacy. Of note, many hits that inhibit only CCL2 or CXCL10, with new targets covering HMG-CoA reductase inhibitors, DNA gyrase/topoisomerase IV inhibitors, ion channel inhibitors, and tyrosine kinase inhibitors, may be potentially effective immunomodulators. However, their efficacies need to be further validated.

Conventional cell-based anti-influenza drug screening models typically focus on only one activity, such as anti-inflammatory or antiviral activity. Screening anti-inflammatory activity without screening for antiviral effects can be misleading, since antiviral activity itself may lead to a reduction in inflammation (i.e., anti-inflammatory activity). In this study, we have established the U937 cell model to detect both anti-inflammatory and antiviral effects in one assay. This model can be very useful in the identification of the following three types of agents: (1) Agents that only have antiviral effects (2) Agents that only have anti-inflammatory effects. (3) Agents that have both anti-inflammatory and antiviral effects.

In summary, we have established a human monocytic cell-based model for high-throughput anti-influenza drug discovery. We have demonstrated that the U937 cell model supports the replication of influenza viruses as well as the production of pro-inflammatory cytokines. Using only three readouts, cytokine CCL2, and CXCL10 expression, the U937 cell model can be used to screen immunomodulators and antiviral agents for the treatment of influenza. Because it is cheap and easy to use, the U937 cell model is an important addition to the existing cellular model and the expensive and time-consuming treatment of influenza. Because it is cheap and easy to use, to screen immunomodulators and antiviral agents for the and virus NA expression, the U937 cell model can be used three readouts, cytokine CCL2, and CXCL10 expression, the production of pro-inflammatory cytokines. Using only supports the replication of influenza viruses as well as the discovery. We have demonstrated that the U937 cell model cell-based model for high-throughput anti-influenza drug inflammatory and antiviral effects.

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