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Seasonal and pandemic influenza H1N1 viruses induce differential expression of SOCS-1 and RIG-I genes and cytokine/chemokine production in macrophages

Gustavo Ramírez-Martínez [a,1], Alfredo Cruz-Lagunas [a,1], Luis Jiménez-Alvarez [a,1], Enrique Espinosa [a], Blanca Ortíz-Quintero [b], Teresa Santos-Mendoza [a], María Teresa Herrera [c], Elsy Canché-Pool [a], Criselda Mendoza [e], José L. Bañales [e], Sara A. García-Moreno [a], Juan Morán [a], Carlos Cabello [d,e], Lorena Orozco [f], Irma Aguilar-Delfín [f], Alfredo Hidalgo-Miranda [f], Sandra Romero [f], Benjamin T. Suratt [g], Moisés Selman [e], Joaquín Zúñiga [a,*]

[a] Department of Immunology, Instituto Nacional de Enfermedades Respiratorias Ismael Cosío Villegas, Mexico City, Mexico
[b] Department of Biochemistry, Instituto Nacional de Enfermedades Respiratorias Ismael Cosío Villegas, Mexico City, Mexico
[c] Department of Microbiology, Instituto Nacional de Enfermedades Respiratorias Ismael Cosío Villegas, Mexico City, Mexico
[d] Department of Virology, Instituto Nacional de Enfermedades Respiratorias Ismael Cosío Villegas, Mexico City, Mexico
[e] Research Unit, Instituto Nacional de Enfermedades Respiratorias Ismael Cosío Villegas, Mexico City, Mexico
[f] Laboratories of Multifactorial Diseases and Cancer Genomics, Instituto Nacional de Medicina Genómica, Mexico City, Mexico
[g] Department of Medicine, University of Vermont College of Medicine, Burlington, VT, USA

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ABSTRACT

Background: Infection with pandemic (pdm) A/H1N1 virus induces high levels of pro-inflammatory mediators in blood and lungs of experimental animals and humans.
Methods: To compare the involvement of seasonal A/PR/8/34 and pdm A/H1N1 virus strains in the regulation of inflammatory responses, we analyzed the changes in the whole-genome expression induced by these strains in macrophages and A549 epithelial cells. We also focused on the functional implications (cytokine production) of the differential induction of suppressors of cytokine signaling (SOCS)-1, SOCS-3, retinoid-inducible gene (RIG)-I and interferon receptor 1 (IFNAR1) genes by these viral strains in early stages of the infection.
Results: We identified 130 genes differentially expressed by pdm A/H1N1 and A/PR/8/34 infections in macrophages. mRNA levels of SOCS-1 and RIG-I were up-regulated in macrophages infected with the A/PR/8/34 but not with pdm A/H1N1 virus. mRNA levels of SOCS-3 and IFNAR1 induced by A/PR/8/34 and pdm A/H1N1 strains in macrophages, as well as in A549 cells were similar. We found higher levels of IL-6, TNF-α, IL-10, CCL3, CCL5, CCL4 and CXCL8 ($p < 0.05$) in supernatants from cultures of macrophages infected with the pdm A/H1N1 virus compared to those infected with the A/PR/8/34 strain, coincident with the lack of SOCS-1 and RIG-I expression. In contrast, levels of INF-α were higher in cultures of macrophages 48 h after infection with the A/PR/8/34 strain than with the pdm A/H1N1 virus.
Conclusions: These findings suggest that factors inherent to the pdm A/H1N1 viral strain may increase the production of inflammatory mediators by inhibiting SOCS-1 and modifying the expression of antiviral immunity-related genes, including RIG-I, in human macrophages.

1. Introduction

The 2009 outbreak of swine-origin influenza A/H1N1 [1,2] continues to affect many countries, having caused over 18,000 deaths worldwide [3]. A growing body of evidence supports the hypothesis that the development of severe pneumonia in patients with pandemic (pdm) A/H1N1 infection is associated with increased immune activation and immune complex deposition [4,5]. Therefore, it is of great importance to understand the factors that determine the development of severe disease. In this regard, high levels of pro-inflammatory cytokines and chemokines have been detected in peripheral blood and lung tissue from patients with severe pneumonia associated to the pdm A/H1N1 infection [4,6]. Even though cytokines, chemokines, and growth factors are required to control influenza virus infection, their overproduction in an

* Corresponding author. Address: Laboratory of Immunobiology and Genetics, Department of Immunology, Instituto Nacional de Enfermedades Respiratorias Ismael Cosío Villegas, Tlalpan 4502, Sección XVI, 14080 Mexico City, Mexico. Tel.: +1 5255 54871700x5327.
E-mail address: joazu@yahoo.com (J. Zúñiga).
[1] These authors contributed equally to this work.

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uncontrolled inflammatory response can lead to lung tissue damage [4,6,7]. The suppressors of cytokine signaling (SOCS) are a family of proteins that down-regulate cytokine signaling [8–11] by negatively regulating JAK/STAT-mediated signal transduction [9–11]. Experimental infection of A549 epithelial cells with seasonal influenza A virus up-regulates the expression of SOCS-1 and SOCS-3. These proteins regulate the immune response against influenza A viruses through a retinoid-inducible gene (RIG)-I/mitochondrial antiviral signaling protein (MAVS)/interferon (alpha and beta) receptor 1 (IFNAR1)-dependent pathway [12].

On the other hand, the combination of gene segments from North American and Eurasian swine lineages of the pdm A/H1N1 virus [13] create unique molecular structures [14,15] that could regulate host immune responses differently than seasonal influenza strains, and contribute to the particular clinical presentation of pandemic influenza infections.

We therefore hypothesized that immune feedback or regulatory mechanisms that normally control host inflammatory responses to pathogens may be absent or impaired in severe pdm A/H1N1 infection, due to the virus itself.

To establish if particular patterns of gene expression and alterations of immune regulation are attributable to specific viral factors, we analyzed the whole genome expression patterns induced by the pdm A/H1N1 and A/PR/8/34 strains through microarray analysis of infected human macrophages and A549 cells. In addition, we performed *in vitro* assays of macrophages and A549 cells in order to evaluate the differences between the pdm A/H1N1 and A/PR/8/34 in their capacity to induce SOCS-1, SOCS-3, and the antiviral response molecule RIG-I, as well as the production of pro-inflammatory cytokines, chemokines and growth factors.

2. Materials and methods

2.1. Ethics statement

The Institutional Review Board of the National Institute of Respiratory Diseases (INER) reviewed and approved this protocol (protocol number B27-10), under which all subjects were recruited. All subjects provided written informed consent, and authorized the storage of their samples at INER repositories for this and future studies.

2.2. Seasonal and pandemic A/H1N1 influenza virus isolation, identification, and propagation

Influenza pdm A/H1N1 virus isolates were obtained from patients with severe pneumonia, who signed an informed consent letter, during the 2009 outbreak in Mexico City, at the National Institute for Respiratory Diseases. Detection of pdm A/H1N1 viral RNA from the respiratory specimens was assessed by real time RT-PCR according with CDC and WHO guidelines. Live influenza pdm A/H1N1 and seasonal A/PR/8/34 viruses were isolated in Madin–Darby canine kidney cells (MDCK). Virus infectivity was assessed by determination of tissue culture infection dose 50% ($TCID_{50}$) in MDCK cells. The titers of virus stocks were adjusted to 1×10^6 $TCID_{50}$/mL. The H1N1 strain (A/PR/8/34) was obtained from the American Type Culture Collection (ATCC) and titrated to the same concentration as pdm A/H1N1.

2.3. PBMC isolation, monocyte isolation and macrophage differentiation

Buffy coats from five healthy blood donors, who signed an informed consent letter, were obtained from the Blood Bank of the INER. Total peripheral blood mononuclear cells (PBMCs) were obtained by density gradient centrifugation using Lymphoprep (*Axis-Shield*, *Oslo*, *Norway*). $CD14^+$ monocytes were purified using magnetic beads (*Miltenyi*, *Auburn*, *CA*, *USA*). Purity of isolated monocytes was assessed by flow cytometry using anti-human monoclonal antibodies: CD14-FITC and CD3-PE (*BioLegend*, *San Diego*, *CA*, *USA*), obtaining a 99% purity. Isolated monocytes were seeded at a concentration of $5x10^5$ cells per well onto 24-well low-adherence culture plates (*Corning Life Sciences*, *Corning*, *NY*) in 10% FBS, 1% L-glutamine (*Gibco BRL*, *Life Technologies*, *Gaithersburg*, *MD*) supplemented RPMI-1640 culture medium (*Sigma Chemical Co.*, *St. Louis*, *MO*, *USA*) with penicillin (0.6 mg/mL), and streptomycin (60 mg/mL) (*Gibco BRL*, *Life Technologies*), and were incubated at 37 °C and 5% CO_2 during 14 days. At day 14, 98% of macrophage differentiation was obtained, as assessed by flow cytometric analysis of CD11b, HLA-DR and CD14 expression (*BD Biosciences*, *San José*, *CA*, *USA*).

2.4. In vitro infection of macrophages and epithelial cells with seasonal A/PR/8/34 or pdm A/H1N1 influenza viruses

Macrophages were infected with 5×10^5 $TCID_{50}$ of the pdm A/H1N1 or seasonal A/PR/8/34 strains. Mock-treated cells received virus-free culture medium. Culture supernatants were collected 30 min, 1 h, 2 h, 5 h, 10 h, 15 h, 24 h, and 48 h later for cytokine, chemokine, and growth factor measurements. Macrophages were harvested for RNA isolation. All assays were performed by triplicate. A549 epithelial cells were infected with pandemic or A/PR/8/34 virus under the same conditions used for human macrophages. The infection of macrophages was confirmed using monoclonal antibody anti-influenza A virus hemagglutinin (HA) (*Light Diagnostics*, *Millipore*, *Billerica*, *MA*, *USA*), after 6 and 48 h of infection (Supplementary Fig. 1A and B). In addition, we analyzed the viral titers using the haemagglutination inhibition (HAI) assay. Briefly, two fold dilutions of supernatants from infected macrophages or A549 cells were prepared and mixed with chicken red blood cells and incubated at 37 °C during 90 min. A significant rise of the viral titers after 5 h of infection of macrophages and A549 cells was detected. However, higher titers of pdm A/H1N1 in cultures of macrophages were detected earlier (Supplementary Fig. 1C).

2.5. Microarray gene expression analysis

Total RNA was obtained from macrophages and A549 epithelial cell cultures 10 h after infection with either the A/PR/8/34 or pdm A/H1N1 strains and from uninfected cells (Mock). Equimolar concentrations of total RNA from five independent *in vitro* experiments were pooled for microarray gene expression analysis. Each RNA pool was processed in duplicate. cDNA synthesis, amplification, and gene expression profiling were done according to the manufacturers instructions (Affymetrix WT Sense Target labeling assay manual). Labeled DNA was added to hybridization cocktail and the sample was injected into the array, (GeneChip Human Gene 1.0 ST Array). Wash and stain processes were performed in the GeneChip Fluidics Station 450. The probe arrays were scanned using The GeneChip® Scanner 3000 7G (*Affmetrix*, *Santa Clara CA*, *USA*).

2.6. SOCS-1, SOCS-3, RIG-I and IFNAR1 mRNA expression

Total RNA was isolated from macrophages and A549 epithelial cells using the RNA easy isolation Kit (*Qiagen*, *Valencia CA*, *USA*). SOCS-1, SOCS-3, RIG-I and, IFNAR1 mRNA expression levels were measured by real time RT-PCR using validated TaqMan assays from Applied Biosystems (SOCS-1: hs00864158_g1, SOCS-3: hs01000485_g1, RIG-I: hs00204833 and IFNAR1: hs01066116). β-