Differential Expression of SOCS1/SOCS3 Ratios in Virus-Infected Macrophage Cell Lines

Nancy J. Bigley, Ph.D.
Microbiology and Immunology Program, and Department of Neuroscience, Cell Biology, and Physiology, College of Science and Mathematics and Boonshoft School of Medicine, Wright State University, Dayton, Ohio

2014
We previously noted that murine keratinocyte cell lines (HEL-301 7 PAM-212) produced large amounts of SOCS1 mRNA and protein following infection with SV-1 or treatment with interferon-gamma (IFN-γ). In contrast, murine fibroblasts 929) exhibited minimal increase in SOCS1 levels when treated with IFN-γ following infection with HSV-1 (Frey et al. 2009).

An antiviral state was induced in fibroblasts but not in keratinocytes. This resistance of keratinocytes to IFN-γ corresponded to the hyperinduction of SOCS1 in these cells.

The goal of the present study was to determine the effects of HSV-1 infection on morphology, CD14-CD86 expression, cell viability, and SOCS protein levels in polarized M1 and M2 macrophage cell lines (J774A.1 and RAW 264.7) during the first 24 hour of infection. For comparison we examined these responses against the monocyte-macrophage trophic Dengue virus (DENV2) in the RAW 264.7).
**Viruses**

**Herpes Simplex Virus-1 strain Syn 17+ (HSV-1)** initially obtained from Dr. Nancy Sawtell, Children’s Hospital Medical Center, Cincinnati, OH was propagated on confluent monolayers of Vero cells. After 4-5 days post infection or when CPE was evident, the cells were spun down, supernatant was aliquoted and stored at -80°C. Virus was quantified by infecting Vero cell monolayers with different dilutions of virus and plaque forming units were counted to calculate volume required for 0.1 multiplicity of infection (MOI).

**Dengue Virus DENV serotype 2 (DENV-2)** was provided by Dr. Eric M. Vela, Battelle Memorial Institute research Center. DENV2 was propagated on Vero 76 cells. Briefly, Vero 76 cells grown in 100 mm petri dishes to a confluence of approximately 85% at 37°C, were infected with DENV-2 for 5-6 days or until CPE was evident. Cells were then scraped and centrifuged at 1500 rpm to eliminate cell debris. The supernatant was aliquoted and stored at −80°C until use. Dengue virus titers were determined by plaque assay on confluent monolayers of Vero 76 cells grown in 6-well plates.

**Murine Macrophage Cell Lines**

**264.7 (ATCC TIB-71)** and **J774A.1 (TIB-67) cells lines** were obtained from the American Type Culture Collection (ATCC) Manassas, VA.
DENV2 infection of RAW 264.7 macrophages at 3 days post infection
Macrophage Polarization Treatment

- M0: No treatment
- M1: LPS (100 ng/mL) + IFN-γ (20 ng/mL) for 12-24 hours
- M2: IL-4 (20 ng/mL) for 12-24 hours

Markers:
- CD86
- SOCS1
- SOCS3
J774A.1 Macrophages at 24 hours after polarization

LPS & IFN-γ

IL-4
Vacuolated M1 Macrophages

M1 J774A.1

M1 RAW 264.7

Vacuolated M1 Macrophages
J774A.1 Macrophages

Uninfected  HSV-1-infected

Control  Control

M1  M1

M2  M2
RAW 264.7 Macrophages
Virus Treatment of Macrophages

1. Release cells/centrifuge

2. Count

3. Count

4. Add virus (0.1 MOI)/cytokines

In order to accurately determine cell number and calculate MOI accurately:

- Cells were released from the culture plate using enzymatic dissociation reagent.
- Cell count was taken using the TPP PCV cell counter, making it possible to calculate MOI accurately.
<table>
<thead>
<tr>
<th>Monocyte/Macrophage Markers</th>
<th>Scavenger Receptors</th>
<th>Membrane Glycoprotein</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD14- LPS receptors</td>
<td>CD206 macrophage mannose receptor (MMR)</td>
<td>CD200R- expressed mainly on monocytes and neutrophils. Interaction between CD200R and CD200 limit and suppress macrophage-induced inflammatory damage.</td>
</tr>
<tr>
<td>CD80 (B7.1) co-receptor on antigen-presenting cells (APCs)</td>
<td>CD163- hemoglobin-haptoglobin receptor; expressed on both monocytes and macrophages</td>
<td></td>
</tr>
<tr>
<td>CD86 (B7.2) co-receptor on APSs</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
J774.1 macrophages polarized to M1 phenotype

- Stained with FITC-labeled anti-mouse CD14
- Stained with brilliant violet 421-labeled anti-mouse CD86
CD14/CD86 Expression in Non-Virus and Virus Treated Polarized Macrophages (24 hours)

Percentage of CD14+/CD86+ Cells

Control, Control w/ Virus, M1, M1 w/ Virus, M2, M2 w/ Virus

J774a.1 Murine Macrophages
Jaguin and colleagues recently used monocytes purified from the buffy coats of human peripheral blood cells to characterize phenotypic and genomic markers.

They generated macrophages from these primary human cells by treatment with M-CSF and polarized them using the same inducers as used in the present study, LPS and IFN-γ to induce M1 phenotype and IL-4 to induce the M2 phenotype.

The cell membrane marker unique to M1 cells was CD80 (B7.1) and CD200R expression was unique to the M2 polarized human macrophages.

As did we using M1 and M2 polarized murine macrophage cell lines (data not shown), they found that the mannose receptor CD 206 did not distinguish between M1 and M2 phenotypes of human macrophages.
Flow cytometry summary of SOCS1 and SOCS3 expression by uninfected and infected J774A.1 macrophage subpopulations.

Left Panel. Note Uninfected cells at 24 h after polarization. M1 cells expressed higher levels of SOCS1 than SOCS3 with a SOCS1/SOCS3 ratio of 7:1.

Right Panel. Virus-infected M1 cells expressed a SOCS1/SOCS3 ratio of 1:1 while M2-infected cells exhibited a SOCS1/SOCS3 ratio of 1:2.
CD14/CD86 Expression in RAW 264.7 Murine Macrophages
### SOCS1:SOCS3 Ratios in DENV2-infected Cells Determined by Flow Cytometry and Western Blot

<table>
<thead>
<tr>
<th></th>
<th>J774.A</th>
<th>RAW 264.7</th>
</tr>
</thead>
<tbody>
<tr>
<td>M0</td>
<td>1:2</td>
<td>1:2</td>
</tr>
<tr>
<td>M0-HSV-1</td>
<td>1:2</td>
<td>1:2</td>
</tr>
<tr>
<td>M1*</td>
<td>7:1</td>
<td>1:3</td>
</tr>
<tr>
<td>M1-HSV-1</td>
<td>1:1</td>
<td>1:1</td>
</tr>
<tr>
<td>M2</td>
<td>1:2</td>
<td>1:2</td>
</tr>
<tr>
<td>M2-HSV-1</td>
<td>1:2</td>
<td>1:2</td>
</tr>
</tbody>
</table>

* Difference because of cell line or detection method. Western Blot detects denatured antigenic fragments; Flow cytometry detects native protein conformation. SOCS1:SOCS3 ratios in all DENV2-infected cells was 1:1.
Summation of Observations

- HSV-1 infection led to morphological differences in all 3 experimental groups.
- HSV-1 infection decreased CD14/CD86 expression in all 3 experimental groups.
- M1 macrophages did not show an up regulation of SOCS1 following virus challenge, however, SOCS3 levels were increased.
- HSV-1-infected unpolarized (M0) J774A.1 cells exhibited significant increases in expression levels of native SOCS1.
At 24 h after infection, M0 control and M2 cells showed greater virus yield than did the M1 cells, presumably reflecting the loss of viable M1 cells.

**Table 1. HSV-1 Titers in HSV-Infected Unpolarized and Polarized Macrophage Cell Lines**

<table>
<thead>
<tr>
<th>Cell line/treatment</th>
<th>pfu/mL</th>
<th>Fold decrease from M0</th>
</tr>
</thead>
<tbody>
<tr>
<td>J774A.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>M0</td>
<td>$10.2 \times 10^2$</td>
<td>—</td>
</tr>
<tr>
<td>M1</td>
<td>$2.5 \times 10^2$</td>
<td>4</td>
</tr>
<tr>
<td>M2</td>
<td>$6.3 \times 10^2$</td>
<td>$1.4^a$</td>
</tr>
<tr>
<td>RAW264.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>M0</td>
<td>$30 \times 10^2$</td>
<td>—</td>
</tr>
<tr>
<td>M1</td>
<td>$10 \times 10^2$</td>
<td>3</td>
</tr>
<tr>
<td>M2</td>
<td>$35 \times 10^2$</td>
<td>—</td>
</tr>
</tbody>
</table>

---

$^a$M1 cells demonstrated a 2.5-fold reduction in virus pfu by comparison with infected M2 cells.

HSV, Herpes simplex virus; pfu, plaque forming unit.
up regulation of SOCS3 expression in HSV-1-infected M1 macrophages over that seen in uninfected M1 cells reflect the effects of M1 polarization or suggest the cell’s attempt to counteract effects of proinflammatory molecules?

Qasimi and colleagues showed that different domains of SOCS3 protein are used to mediate interleukin-10 (IL-10) inhibition of TNF-α and nitric oxide production by this same macrophage cell line (Qasimi and others 2006). In this same macrophage cell line (J774A.1), IL-10 was responsible for the anti-inflammatory response to Borrelia burgdorferi (Dennis and others 2006).

SOCS1/SOCS3 expression levels appeared relatively unchanged in virus-infected M2 macrophages when compared to their uninfected counterparts, suggesting microenvironment signals such as IL-4 play a greater role in SOCS expression levels than does HSV-1 infection.

We then hypothesized that the HSV-1-infected J774A.1 M1 macrophages were attempting to counteract the effects of inflammatory molecules induced by polarization.
Note that only SOCS1 contains a nuclear localization sequence (NLS).

Structural domains of SOCS molecules
Overview of SOCS1 inhibition of cytokine-induced STATs
Pro-inflammatory and anti-inflammatory effects of SOCS3
- LPS (100 ng/ml)
- IFN-γ (20 ng/ml)
- 0.1 MOI HSV-1
- SOCS3 peptide or SOCS1 Inhibitor

Incubate 24 hours

The effects of SOCS1 and SOCS3 peptide mimetics and SOCS1 inhibitor (pJAK2) on polarized J774A.1 macrophages were compared with the controls and pJAK2 groups. p<0.001 when the SOCS groups were compared with the unmodified and pJAK2 groups.
Jo et al. (2005) used a recombinant cell-penetrating form of SOCS3 (CP-SCS3) to protect mice (C3H/HeJ) from the lethal effects of SEB and LPS by reducing production of inflammatory cytokines and attenuating apoptosis and hemorrhagic necrosis. Within 2 hours after injection, CP-SOCS3 was distributed in multiple organs and persisted for at least 8 hours.

The membrane-translocating motif (MTM) was composed of 12 amino acids from a hydrophobic signal sequence from fibroblast growth factor 4. The MTM was attached to either the N-terminal or C-terminal of SOCS3. Only these forms were capable of penetrating RAW cells.

Based on these observations, we tested whether the SOCS3 peptide mimetic could modify the cytotoxicity of the M1 polarization treatment or virus infection. The peptide mimetics in this present study were provided by Dr. H.M. Johnson and his colleagues, University of Florida at Gainesville. These peptides contain an addition of a lipophilic group (palmitoyl-lysine) to the N terminus of the synthetic peptide which provides them with the ability to penetrate cells.
SOC3 Peptide mimetic protects macrophages (RAW 264.7) from the lytic effect of HSV-1 and from the lytic effect of M1 polarization.

- SOC3 peptide (35μM/ml) or TC medium for 30 minutes prior to LPS (100 ng/ml)
- IFN-γ (20 ng/ml)
- 0.1 MOI HSV-1

Incubate 24 hours

Cell Viabilities of RAW 264.7 macrophages 24 hours after M1 polarization
CONCLUSIONS

SOCS3 peptide mimetic and the SOCS1 inhibitor (pJAK2) increased the viability of polarized M1 cells over SOCS1-treated M1 J774A.1 macrophages similar to the observations in comparable cell groups infected with HSV-1 (p<0.001)
Prediction: The anti-inflammatory effect in these cells will be characterized by increased levels of IL-10

SOCS1 peptide mimetic decreases the viability of polarized M1 cells and HSV-1-infected M1 J774A.1 macrophages (p<0.001)
Prediction: The inflammatory effect in these cells will be characterized by increased levels of TNF-α.

SOCS3 Peptide Mimetic protects macrophages (RAW 264.7) from the lytic effect of HSV-1 and from the lytic effect of M1 polarization

These characterization are in progress at present.
Significance

- Benefits of SOCS3 Peptide Mimetic
  Neuro inflammation - already shown in microglial cells by Benveniste’s group (Qin et al, 2012).

  Anti-inflammatory effects in inflammatory diseases including viral diseases such as Dengue fever and autoimmune tissue destruction.

- Benefits of SOCS1 Peptide Mimetic
  Convert the M2-type macrophage in solid tumors to an inflammatory M1 phenotype
References


Reichard AC, Cheemarla NR, Bigley NJ. SOCS1/3 Expression Levels in HSV-1-Infected, Cytokine-Polarized and -Unpolarized Macrophages on Cytokine Res. 2014 Jun 23. [Epub ahead of print]


Acknowledgements

Graduate Students
Adam C. Reichard
Nagarjuna Reddy Cheemarla
Sarah Al Sharif
Hind Albershi
Kelley J. Williams

University of Florida Colleagues contributing the SOCS peptides and inhibitor
Drs. Howard M. Johnson, Chulbul M.I. Ahmed, and Joseph Larkin

Dr. Barbara E. Hull, Director of the Microbiology and Immunology Program, Wright State University