A tolerogenic peptide that induces suppressor of cytokine signaling (SOCS)-1 restores the aberrant control of IFN-\(\gamma\) signaling in lupus-affected (NZB×NZW)F1 mice

Amir Sharabi\(^a,1\), Zev M. Sthoeger\(^b,1\), Keren Mahlab\(^a,b\), Smadar Lapter\(^a\), Heidy Zinger\(^a\), Edna Mozes\(^a,*\)

\(^a\) Department of Immunology, The Weizmann Institute of Science, Rehovot, Israel
\(^b\) Department of Internal Medicine B, Kaplan Hospital, Rehovot, Israel

Received 24 June 2009; accepted with revision 29 June 2009
Available online 23 July 2009

KEYWORDS
Autoimmune diseases; Cytokine; Systemic lupus erythematosus (SLE); Peptide-based therapy; T cells; Suppressor of cytokine signaling-1 (SOCS-1); Signal transduction and activators of transcription-1 (STAT1); IFN-\(\gamma\) signaling pathway

Abstract
Interferon-\(\gamma\) (IFN-\(\gamma\)) plays a pathogenic role in systemic lupus erythematosus (SLE). Uncontrolled IFN-\(\gamma\) signaling may result from a deficiency in the negative regulator, namely, suppressor of cytokine signaling-1 (SOCS-1). We investigated the activation status of IFN-\(\gamma\) signaling pathway in SLE-affected (New-Zealand-Black×New-Zealand-White)F1 mice and determined its responsiveness when treating with a tolerogenic peptide, hCDR1, which ameliorates SLE. SOCS-1 was suppressed and pSTAT1 was enhanced in spleen-derived cells from SLE-affected mice as compared with healthy controls. Treatment with hCDR1 reversed the expression of these two molecules in association with clinical amelioration. In vitro stimulation with IFN-\(\gamma\) resulted in elevated levels of SOCS-1 in cells from both vehicle and hCDR1-treated mice but this effect reached significance only in cells of the latter group, which also exhibited reduced levels of pSTAT1. Thus, SOCS-1 is diminished in SLE-affected mice, and treatment with hCDR1 results in its up-regulation thereby restoring control of IFN-\(\gamma\) signaling pathway.

© 2009 Elsevier Inc. All rights reserved.

Introduction
Interferon-\(\gamma\) (IFN-\(\gamma\)) is a cytokine that has been implicated in playing a pathogenic role in patients with systemic lupus erythematosus (SLE) \([1,2]\), and in the development of serological and clinical manifestations of induced \([3]\) and spontaneous \([4,5]\) SLE in murine models. IFN-\(\gamma\) was reported to be involved in the induction of pathogenic autoantibodies \([6,7]\), and in the acceleration or progression of glomerulonephritis \([4,8]\), both of which are hallmarks of SLE.

The binding of IFN-\(\gamma\) to the IFN-\(\gamma\) receptor (IFN-\(\gamma\)R) induces the phosphorylation and dimerization of signal transduction and activators of transcription-1 (STAT1), which then translocates to the nucleolus to activate the transcription of IFN-\(\gamma\) inducible genes \([9]\). The magnitude and duration of this signaling pathway depend on the extent of IFN-\(\gamma\) produced, but more importantly, on the presence of an effective negative feedback mechanism in which the suppressor of
cytokine signaling-1 (SOCS-1) molecule plays a key role [10]. Upon stimulation with IFN-γ, SOCS-1 protein is rapidly induced, which inhibits STAT1 signaling [11]. Impaired expression of SOCS-1 results in uncontrolled IFN-γ signaling, and mice exhibiting the latter develop accelerated lymphocyte apoptosis, severe lymphopenia, hyperactivated T cells, and eventually they die within 3 weeks [12–14]. Notably, permitting the expression of SOCS-1 in T and B cells of mice on a SOCS-1−/− background led to a vast production of anti-dsDNA antibodies and the formation of glomerular immune complex deposits (ICD), consistent with an SLE-like disease [15].

Our laboratory has developed a peptide [16], namely hCDR1, which is based on the sequence of the first complementarity-determining region (CDR1) of an autoimmune antibody [17]. Administration of hCDR1 to mice with SLE resulted in the amelioration of the disease, as manifested by reduced production of autoantibodies, reduced levels of proteinuria, and decreased glomerular ICD [18]. The beneficial effects of the tolerogenic peptide hCDR1 were associated with the down-regulation of the pathogenic cytokines TNF-α, IL-1β, IFN-γ, and IL-10, and the up-regulation of the immunosuppressive cytokine TGF-β [18].

The induction of CD4 and CD8 regulatory T cells plays a key role in the mechanism of action of hCDR1 because these inhibitory cells mediate the down-regulated production of the pathogenic cytokines and improve the disease manifestations [19–21]. Further, hCDR1 was shown to decrease the rates of T-cell apoptosis [22–24]. In addition, treatment with hCDR1 diminished the rate of maturation and differentiation of B cells by reducing the levels of B-cell activating factor (BAFF, also known as BlyS) [25] and increased B-cell apoptosis in the spleen and bone marrow of the treated mice [25,26]. hCDR1 inhibited the maturation of dendritic cells (DCs) as well [27]. Moreover, treatment with hCDR1 up-regulated the negative regulators Foxj1, Foxo3a [28], and early growth response factors (Egr) 2 and 3 [29], resulting in the inhibition of IFN-γ secretion. Thus, the ability of hCDR1 to down-regulate the production of IFN-γ, which plays a key role in many of the pathogenic processes associated with SLE, contributed to ameliorating the disease manifestations.

In the present study, we assessed the extent of activation of the IFN-γ signaling pathway in SLE-affected (NZB × NZW)F1 (BWF1) mice. Concomitant analysis of the expression of SOCS-1 and the phosphorylated forms of STAT1 was performed on spleen-derived cells of the diseased mice. The ability of treatment with hCDR1 to manipulate the expression of these molecules and its capacity to restore the control of IFN-γ signaling were investigated.

Materials and methods

Mice

Female BWF1 mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). This study was approved by the Animal Care and Use Committee of the Weizmann Institute of Science.

Synthetic peptides

A peptide [16], GYYWSWIRQPPGKGEEWIG (hCDR1), based on the CDR1 of the human anti-DNA monoclonal antibody, bearing the 16/6Id [17], was synthesized by Polypeptide Laboratories (CA, USA) and was used in this study. A peptide containing the same amino acids as hCDR1, in a scrambled order (control peptide), SKGIPQYGWPEGWRYEI, was used as a control.

Monoclonal antibodies

The following antibodies that were used: anti-CD4-allophycocyanin (clone L3T4), anti-CD25-FITC (clone 7D4), and their matched isotype controls were obtained from Southern Biotechnology Associates (Birmingham, AL). Anti-pSTAT1-PE (clone A-2) and its isotype control, anti-SOCS-1 (clone N-18) and anti-STAT1 (clone E-23) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phospho-STAT1 (Tyr701) was obtained from Cell Signaling Technology (Danvers, MA). Anti-tubulin was purchased from Sigma-Aldrich (St. Louis, MO).

Treatment of SLE-inflicted BWF1 mice with hCDR1

Eight-month-old BWF1 female mice with established lupus were given 10 weekly s.c. injections of hCDR1 (50 μg/mouse) or the vehicle alone (Captisol®, sulfobutylether beta cyclodextrin, CyDex, Inc., KS, USA). In the experiments 10–12 mice per group were used. In some experiments analyses were performed on pools of cells from each group and in other experiments evaluations were performed using cells of individual mice.

Measurement of dsDNA-specific antibodies

Anti-dsDNA antibodies were detected using λ phage dsDNA, as previously described [18].

Proteinuria

Proteinuria was measured by a standard semi-quantitative test, using an Albustix kit (Bayer Diagnostic, Newbury, UK).

Immunohistology

Detection of glomerular ICD was performed as described earlier [18]. The intensity of ICD was graded as follows: 0 — no immune complex deposits, 1 — low intensity, 2 — moderate intensity, and 3 — high intensity of immune complexes. ICD analysis was performed by two persons blinded to whether the mice belonged to control or experimental groups.

FACS analysis

Cells were incubated with the relevant antibody and analyzed by FACS, with forward and side scatter gates adjusted to include all cells and to exclude debris (Becton Dickinson, Franklin Lakes, NY). For intracellular staining, the cells were incubated with a fixation solution, washed, and resuspended in permeabilization solution (Serotec; Oxford, UK).

Western blot analysis

Whole cell lysates were prepared as described [24]. Lysates were applied to SDS-PAGE by using 10% SDS-polyacrylamide
and transferred to nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA). The membranes were blocked and then incubated with the relevant antibody. Immunoreactive protein bands were visualized using labeled secondary antibodies and an enhanced chemiluminescent (ECL) system. The results were calculated as densitometric units using the NIH Image program and are presented as percentages of total levels of tubulin.

**In vitro assays**

Spleen-derived cells (5×10⁶/well) from the vehicle or hCDR1-treated, SLE-affected BWF1 mice were incubated in enriched medium in the presence (2 ng/ml) of IFN-γ (BD Biosciences, San Diego, CA) for 15 and 150 min.

**Real-time RT-PCR**

Total RNA was isolated from spleen-derived lymphocytes of the experimental mice and then RNA was reverse-transcribed to prepare cDNA using M-MLV reverse transcriptase (Promega, Madison, WI). The resulting cDNA was subjected to real-time RT-PCR using LightCycler (Roche, Mannheim, Germany) according to the manufacturer’s instructions. Primer sequences (forward and reversed, respectively) were as follows: SOCS-1 (5′-CGAGACCTTGGACTGC-3′, 5′-ACGGAGTACCGGGTTA-3′) and β-actin (5′-gacgttgacatccgtaaag-3′). β-actin levels were used for normalizing the expression levels of the SOCS-1 gene.

**Statistical analysis**

The Mann–Whitney U test was used. Values of \( P < 0.05 \) were considered significant.

**Results**

**SOCS-1 is reduced and pSTAT1 is increased in spleen-derived lymphocytes of BWF1 mice with SLE**

mRNA expression of SOCS-1 was determined in spleen-derived cells from young, healthy BWF1 mice in comparison with cells from old, SLE-inflicted mice. The mRNA expression of SOCS-1 was determined in pools of spleen-derived cells from all mice within a group. The mean (±SE) results of 3 independent experiments are presented in Figure 1A. As shown, the mRNA levels of SOCS-1 in the cells from SLE-affected mice were significantly reduced by >50% in comparison with the mRNA levels of healthy controls. mRNA was prepared from individual mice from the two groups as well. Figure 1A also shows a similar reduction in SOCS-1 expression in the diseased mice. The levels of SOCS-1 in BWF1 mice with SLE were further determined at the protein level by Western blotting of the cell lysates. The results of a representative blot as well as the mean (±SE) levels of SOCS-1 in 3 experiments are presented in Figure 1B. It can be seen that similarly to the mRNA expression, the protein levels of SOCS-1 in the spleen-derived cells from SLE-affected mice were significantly reduced in comparison with healthy controls.

The correlation of SOCS-1 expression with that of pSTAT1 was determined. A representative blot and the mean levels of pSTAT1 in 3 experiments are presented in Figure 1C. As shown, the levels of pSTAT1 in the cells from mice with SLE were significantly elevated (by 2-fold) in comparison with the levels determined in the cells from the healthy controls.

**The beneficial effects of hCDR1 on manifestations of SLE are associated with up-regulation of SOCS-1 and down-regulation of pSTAT1**

We wanted to determine whether treatment with hCDR1 affected the IFN-γ-signaling pathway in correlation with clinical
amelioration. Therefore, BWF1 mice with SLE were treated with 10 weekly s.c. injections of hCDR1 (50 μg/mouse), a control (scrambled) peptide (50 μg/mouse), or the vehicle only. The clinical data from a representative of 3 treatment experiments are summarized in Table 1. As shown, treatment with hCDR1 resulted in reduced production of anti-dsDNA autoantibodies, reduced levels of proteinuria, and diminished glomerular ICD, in comparison with mice that received the vehicle only. Injection of mice with the control (scrambled) peptide did not significantly affect the disease manifestations.

We determined the mRNA expression of SOCS-1 in the spleen-derived cells that were pooled from all mice within a treatment group. The mean (±SE) levels of expression based on 4 independent experiments are presented in Figure 2A. As shown, mRNA levels of SOCS-1 from hCDR1-treated mice were significantly up-regulated and those from the control peptide-treated mice were insignificantly changed, in comparison with the effect of the injected vehicle. The expression of the SOCS-1 gene was also determined for mRNA prepared from the effector CD4 cells but more profoundly in the hCDR1-derived cells. These results were reproduced in 2 independent experiments and the means of response to stimulation with IFN-γ in the two experiments are shown in Figure 3C.

The effects of IFN-γ challenge on pSTAT1 expression in CD4 effector cells were also studied utilizing FACS analysis. As shown in Figure 4, prior to the addition of IFN-γ to the cultures, the expression of pSTAT1 in effector CD4 cells from hCDR1-treated, SLE-inflicted mice was decreased by 33% in comparison with that detected in vehicle-injected mice (Fig. 4A). However, a 15-minute stimulation of the cells with IFN-γ resulted in comparable levels of expression of pSTAT1 in the effector CD4 cells from both treatment groups (Fig. 4B).

Figure 4 also presents the mean (±SE) absolute numbers of pSTAT1-expressing CD4 effector cells determined in two experiments with similar and significant effects.

### Discussion

The main findings of the present study are that BWF1 mice with SLE display an aberrant IFN-γ signaling pathway. The mRNA and protein levels of this pathway’s negative regulator, namely, SOCS-1, are diminished, whereas the levels of the IFN-γR-downstream molecule, pSTAT1, are elevated. Further, the responsiveness of the spleen-derived cells from the diseased mice to a challenge with IFN-γ is compromised. Moreover, treatment of the SLE-inflicted BWF1 mice with the tolerogenic peptide hCDR1, which ameliorated the disease manifestations, resulted in a significant elevation in the levels of SOCS-1 mRNA and protein associated with a significant reduction in the levels of pSTAT1. In addition, the compliance of spleen-derived cells from hCDR1-treated mice to an IFN-γ challenge is better controlled because SOCS-1 is up-regulated and pSTAT1 is down-regulated over time. To the best of our knowledge, we demonstrated here for the first time a decreased production of SOCS-1 in BWF1 mice with SLE, and that the administration of a tolerogenic peptide up-

### Table 1  Effects of treatment with hCDR1 on SLE manifestations.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>dsDNA Abs (OD)</th>
<th>P</th>
<th>Proteinuria (g/L)</th>
<th>P</th>
<th>ICD</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>1.58 ± 0.26</td>
<td>–</td>
<td>9.3 ± 2.0</td>
<td>–</td>
<td>2.8 ± 0.2</td>
<td>–</td>
</tr>
<tr>
<td>Control</td>
<td>1.34 ± 0.18</td>
<td>NS</td>
<td>7.5 ± 2.4</td>
<td>NS</td>
<td>2.5 ± 0.2</td>
<td>NS</td>
</tr>
<tr>
<td>hCDR1</td>
<td>0.53 ± 0.07</td>
<td>0.03</td>
<td>2.5 ± 1.0</td>
<td>0.01</td>
<td>1.3 ± 0.2</td>
<td>0.0004</td>
</tr>
</tbody>
</table>

2 SLE-afflicted (NZB×NZW)F1 mice were treated once a week with weekly subcutaneous injections of the vehicle, hCDR1, or a control (scrambled) peptide for 10 weeks.

3 Results are of sera from mice that were bled after the end of treatment. Dilution of sera 1:50.

4 Statistical evaluation was based on the Mann–Whitney U test to compare post-treatment effects between the vehicle-treated groups and the remaining treatment groups.

5 Proteinuria was always measured at about the same time of day and all mice in an experimental cohort were tested together.

6 Immune complex deposits (ICD) were assessed at sacrifice.

Incubation for 150 min with IFN-γ resulted in elevated levels of SOCS-1 in cells from vehicle-treated mice and in a more prominent increase in the cells from hCDR1-treated mice. As shown in Figure 3B, the levels of pSTAT1 following a 15-minute incubation with IFN-γ were similarly high in the cells of vehicle and hCDR1-treated mice. At 150 min of incubation, the levels of pSTAT1 were down-regulated in the vehicle-derived cells but more profoundly in the hCDR1-derived cells. These results were reproduced in 2 independent experiments and the means of response to stimulation with IFN-γ in the two experiments are shown in Figure 3C.

The effects of IFN-γ challenge on pSTAT1 expression in CD4 effector cells were also studied utilizing FACS analysis. As shown in Figure 4, prior to the addition of IFN-γ to the cultures, the expression of pSTAT1 in effector CD4 cells from hCDR1-treated, SLE-inflicted mice was decreased by 33% in comparison with that detected in vehicle-injected mice (Fig. 4A). However, a 15-minute stimulation of the cells with IFN-γ resulted in comparable levels of expression of pSTAT1 in the effector CD4 cells from both treatment groups (Fig. 4B). Yet, following a 150-minute incubation with IFN-γ, pSTAT1 was reduced by 38% in effector CD4 cells from hCDR1-treated mice as compared with cells from the vehicle-treated mice. Figure 4 also presents the mean (±SE) absolute numbers of pSTAT1-expressing CD4 effector cells determined in two experiments with similar and significant effects.

IFN-γ signaling in cells challenged with IFN-γ is better controlled in BWF1 mice with SLE following treatment with hCDR1

Pools of spleen-derived cells (5×10^6/ml) from SLE-afflicted BWF1 mice that were treated with hCDR1 or the vehicle were incubated in the presence of IFN-γ (2 ng/ml) for 15 and 150 min. As shown in Figure 3A, after 15-minute incubation with IFN-γ, the levels of SOCS-1 were higher in the lysates from hCDR1-treated mice as compared with the vehicle.
regulated SOCS-1 thereby restoring the control of the IFN-γ signaling pathway in association with disease amelioration.

The production of IFN-γ is up-regulated in patients and mice with SLE, where it plays a pathogenic role [1–8]. The various pathways that underlie the mechanisms by which hCDR1 ameliorate SLE manifestations result in the down-regulation of IFN-γ production [19–21,23,24,27–29]. In the present study, we demonstrated for the first time that, in addition to the increased production of IFN-γ, aberrant control of the IFN-γ signaling pathway was implicated in spleen-derived cells from BWF1 mice with SLE. The cells from the diseased mice exhibited reduced mRNA and protein levels of SOCS-1 expression in comparison with healthy controls (Figs. 1A and B), along with increased phosphorylated forms of the IFN-γR-downstream signaling molecule, STAT1 (Fig. 1C). In accordance, elevated levels of pSTAT1 could also be detected in PBMC of SLE patients, which correlated with clinical disease activity, though SOCS-1 levels were not determined in the latter study [30]. SOCS-1 is a critical negative regulator of the IFN-γ signaling pathway [14], which is present at the highest levels in the thymus and spleen [11,13]. The reasons for the reduced levels of SOCS-1 in SLE-affected BWF1 mice have not been elucidated. It can be speculated that the combination of genetic and environ-
that the expression level of SOCS-1 protein was controlled by modulation of its protein stability [33]. In addition, it is possible that levels of SOCS-1 were reduced in the diseased mice because of an accelerated degradation process of the JAK kinases to which the SOCS proteins bind. Indeed, a binding partner of the SOCS box, namely, Elongin BC, targets SOCS proteins for proteasomal degradation [34].

Under normal conditions, the induction of SOCS-1 occurs immediately upon IFN-γ stimulation [10,11]. However, despite the up-regulated production of IFN-γ in the SLE-inflicted BWF1 mice [4,5,7,8], we demonstrated here a significant reduction in the levels of SOCS-1 (mRNA and protein) in comparison with those in healthy controls (Figs. 1A and B). Furthermore,

mental factors predisposes for SOCS-1 down-regulation. For instance, in SOCS-1−/− mice, signaling through prolactin, which is capable of augmenting autoimmunity [31], was reported to be predominant [32]. Further, it was suggested
treatment of the diseased mice with hCDR1, which resulted in down-regulated production of IFN-γ [18–21,23–29], elevated the levels of SOCS-1 by 2-fold comparable to SOCS-1 levels found in healthy controls (Figs. 2A and B). Thus, it is possible that the decrease in IFN-γ in hCDR1-treated, SLE-affected mice, contributed to the restoration of baseline levels of SOCS-1. Indeed, in support of this notion, the responsiveness of spleen-derived cells from BWF1 mice with SLE to a challenge with IFN-γ yielded insignificant changes in SOCS-1 levels (Fig. 3A). As demonstrated here, treatment with hCDR1 improved the compliance of the cells to stimulation with IFN-γ, as manifested by the significant up-regulation of SOCS-1 and by the significant reduction of pSTAT1 levels (Fig. 3).

The importance of SOCS-1 was especially emphasized in studies utilizing SOCS1−/− mice. These mice were found to exhibit a severe inflammatory disease owing to elevated levels of IFN-γ, hypersensitivity of tissues to IFN-γ [14], and aberrant activation of T and NKT cells [13,35]. Moreover, these mice were found to exhibit a failure in negative selection, which supports the notion that the severe inflammation observed in these mice occurred because of the development of an autoimmune disease [36]. Therefore, the ability of treatment with hCDR1 to augment the expression of SOCS-1 in the SLE-affected mice is likely to contribute to the clinical amelioration of the treated mice.

An additional important consequence of diminished expression of SOCS-1 is the status of DCs. Deficient expression of SOCS-1 specifically in DCs resulted in elevated levels of B7 and CD40 molecules, and in the production of large amounts of IFN-γ [37,38] and BAFF by the DCs [15]. The latter events led to the production of autoantibodies by the B cells and to the development of an SLE-like disease [15]. We previously reported that the amelioration of SLE manifestations by hCDR1 were associated with a decrease in the production of BAFF [25], and with the down-regulation of B7 and MHC class II molecules on DCs [27]. It is therefore possible that part of the beneficial effects of hCDR1 are due to the induction of SOCS-1 in DCs of the hCDR1-treated mice. Our preliminary results indicated elevated mRNA expression of SOCS-1 following incubation of peripheral blood lymphocytes of SLE patients with hCDR1, suggesting that the control of IFN-γ signaling plays an important role in SLE patients as well.

It appears that SOCS-1 does not play a substantial role only in SLE. More specifically, SOCS-1 was also identified as an important negative regulator of acute arthritis, as indicated in the mBSA/IL-1 arthritis model where the severity of synovial inflammation and joint destruction was greater in the absence of SOCS-1 [39]. The association between up-regulated expression of SOCS-1 and the modulation of inflammatory-mediated diseases such as SLE and rheumatoid arthritis emphasizes the importance of a negative feedback mechanism. Therefore, agents and peptides designed to mimic the function of SOCS-1 [40], or to up-regulate the expression of SOCS-1 (exemplified by hCDR1) may offer a novel therapeutic strategy for treating SLE and other inflammatory diseases in humans.

References

[18] D. Lugher, M. Dayan, H. Zinger, J.P. Liu, E. Mozes, A peptide based on the complementarity determining region 1 of a human monoclonal autoantibody ameliorates spontaneous and induced


