A Suppressor of Cytokine Signaling 1 Antagonist Enhances Antigen-Presenting Capacity and Tumor Cell Antigen-Specific Cytotoxic T Lymphocyte Responses by Human Monocyte-Derived Dendritic Cells

Yongjun Wang, a Shengyu Wang, b Yuan Ding, a Yanhua Ye, a Yingyi Xu, a Huixiang He, a Qiaozhen Li, a Yanjun Mi, a Chunhua Guo, a Zhicai Lin, a Tao Liu, a Yaya Zhang, a Yuqiang Chen, a Jianghua Yan b

Department of Oncology, 174th Hospital of the Chinese People’s Liberation Army, Affiliated Chongqing Hospital of Xiamen University, Xiamen, Fujian Province, People’s Republic of China; a Cancer Research Center, Medical College of Xiamen University, Xiamen, Fujian Province, People’s Republic of China

The suppressor of cytokine signaling 1 (SOCS1) has emerged as a critical inhibitory molecule for controlling the cytokine response and antigen presentation by dendritic cells (DCs), thereby regulating the magnitude of both innate and adaptive immunity. The aim of this study was to investigate whether the SOCS1 antagonist pJAK2(1001-1013) peptide can weaken or block the inhibition function of SOCS1 in DCs by evaluating the phenotype and cytokine production, antigen-presenting, and specific T-cell-activating capacities of DCs electroporated with human gastric cancer cell total RNA. Furthermore, STAT1 activation of the JAK/STAT signal pathway mediated by SOCS1 was analyzed by Western blotting. The results demonstrate that the SOCS1 antagonist pJAK2(1001-1013) peptide upregulated the expression of the maturation marker (CD83) and costimulatory molecule (CD86) of RNA-electroporated human monocyte-derived mature DCs (mDCs), potentiated the capacity of mDCs to induce T-cell proliferation, stimulated the secretion of proinflammatory cytokines, and enhanced the cytotoxicity of tumor cell antigen-specific CTLs activated by human gastric cancer cell total RNA-electroporated mDCs. Data from Western blot analysis indicate that STAT1 was further activated in pJAK2(1001-1013) peptide-loaded mDCs. These results imply that the SOCS1 antagonist pJAK2(1001-1013) peptide is an effective reagent for the enhancement of antigen-specific antitumor immunity by DCs.

Dendritic cells (DCs) are professional and potent antigen-presenting cells in the body that play pivotal roles in the maintenance of self-tolerance and in the activation of innate and adaptive immunity (1–3). In vivo, immature DCs (iDCs) constantly sample the microenvironment for “danger signals,” which include inflammation, damaged tissue, and pathogens. After uptake of antigen in the context of danger signals, iDCs begin the process of maturation. Maturing DCs actively migrate from the periphery to lymph nodes, where they present the processed antigens primarily as peptide-major histocompatibility complex (MHC) complexes. During this process, DCs express several costimulatory molecules (such as CD40, CD80, and CD86) and produce high levels of cytokines, including interleukin-12 (IL-12) and type 1 interferons (IFNs), to orchestrate the induction of CD4+ Th1 and CD8+ cytotoxic T lymphocyte (CTL) responses necessary for effective cell-mediated immunity (4–6).

In recent years, much work has been carried out to utilize the unique features of DCs in clinical applications, especially in cancer immunotherapy, as in vitro generation of DCs has become standard practice (7–9). Significant advances have also been made to focus on optimizing the pulsing of DCs with tumor-associated antigens in vitro and promoting DC maturation and costimulation as a means to enhance antigen-specific antitumor immunity of DC-based cancer vaccines (10–12). While strengthening of these positive regulators represents a promising approach, DCs remain susceptible to endogenous inhibitors that serve as negative feedback mechanisms to help maintain tolerance and prevent autoimmunity under normal circumstances. Since the major goal of DC-based cancer vaccines is to break self-tolerance to tumor antigens, these negative regulators are a key obstacle (6). Several types of inhibitors exist, including those expressed in the cytoplasm (e.g., suppressor of cytokine signaling 1 [SOCS1]) and on the cell surface (inhibitory receptors, e.g., PD-L1) and those secreted into extracellular spaces (soluble inhibitors, e.g., indoleamine-2,3-dioxygenase) (6). One reason for the failure of antitumor immunotherapy is believed to be the presence of such an immunosuppressive mechanism (13). Thus, sequestration of these suppressors could potentially lead to longer activation of DCs and might be beneficial for cancer immunotherapy.

SOCS1, a member of the SOCS and cytokine-induced Src homology 2 (SH2) protein (CIS) family of intracellular proteins, has emerged as a critical inhibitory molecule for controlling the cytokine response and antigen presentation by DCs, thereby regulating the magnitude of adaptive immunity (14–19). It has been reported that DCs from SOCS1 knockout mice (SOCS1−/−) were hypersensitive to lipopolysaccharide (LPS) stimulation and exhibited a more mature phenotype than DCs from their wild-type littermates (14). Small interfering RNA (siRNA)-mediated silencing of SOCS1 can break high-dose DC immunotherapy-induced immune tolerance and enhance antigen presentation by DCs and antigen-specific antitumor immunity (15, 19). There-
fore, blocking of SOCS1 in DCs may be a potentially useful strategy to enhance DC vaccine-induced immune responses. SOCS1 suppresses cytokine signaling by binding to Janus kinase/signal transducer and activator of transcription (JAK/STAT) to prevent downstream signal transduction (20, 21). A previous study has demonstrated that SOCS1 specifically recognizes the autophosphorylation sequence 1001 to 1013 containing the phosphoryrosine residue (pY1007) in the activation loop of JAK2 and that the phosphorylation of Y1007 is required for activation (22). On the basis of these findings, Waiboci and colleagues developed a small peptide antagonist of SOCS1, pJAK2(1001-1013), that corresponds to the activation loop of JAK2. Research results demonstrated that the pJAK2(1001-1013) peptide can block SOCS1-induced inhibition of STAT3 phosphorylation in IL-6-treated prostate cancer cells and enhance antigen-specific splenocyte proliferiation (23). Later, they reported that, in addition to a direct antiviral effect and synergism with IFN-γ, the pJAK2(1001-1013) peptide exhibits adjuvant effects on humoral and cellular immunity, as well as an enhancement of polyinosinic-poly(C) activation of Toll-like receptor 3 (TLR3) (24). However, the effect of the SOCS1 antagonist pJAK2(1001-1013) peptide on DCs is still unknown. Spurred on by these promising results, we hypothesized that the SOCS1 antagonist pJAK2(1001-1013) peptide would be a novel and effective reagent for the enhancement of antigen-specific antitumor immunity by DCs. Therefore, in this study, we investigated whether the SOCS1 antagonist pJAK2(1001-1013) peptide can weaken or block the inhibition function of SOCS1 in DCs by evaluating the phenotype and cytokine production, antigen-presenting, and specific T-cell-activating capacities of DCs electroporated with human gastric cancer cell total RNA. Furthermore, molecular signaling events mediated by SOCS1, such as STAT activation of the JAK/STAT signal pathway, were analyzed.

MATERIALS AND METHODS

RNA extraction. Human gastric cancer MGC-803 cells (Cancer Research Center, Medical College of Xiamen University, Xiamen, China) were routinely cultured in RPMI 1640 medium (Sigma-Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS; PAA Laboratories), 100 U/ml penicillin (PAA Laboratories), and 100 μg/ml streptomycin (PAA Laboratories) at 37°C in a humidified atmosphere containing 5% CO₂. The total RNA of MGc-803 cells was then extracted with TRIzol Reagent (Life Technologies Corporation, Grand Island, NY) according to the manufacturer’s protocol and stored at −20°C. RNA integrity was confirmed by electrophoresis on agarose gel. RNA concentration and purity were estimated by spectrophotometry at 260 and 280 nm.

Peptide synthesis. The amino acid sequence of the SOCS1 antagonist pJAK2(1001-1013) peptide is 1001LPQDKEKYVKVE1013, which is based on the sequence of human JAK2 with phosphorylated Y1007 (underlined). In addition, the JAK2(1001-1013)2A alanine-substituted peptide, whose sequence is 1001LPQDEKAACKVE1013 (the alanines introduced are underlined), was used as a control peptide. Both of the peptides were synthesized by GL Biochem (Shanghai) Ltd. A lipophilic group (palmitoyl-lysine) was added to the N terminus of the synthetic peptide to facilitate entry into cells as a last step by a semiautomated protocol. The lipo-pJAK2(1001-1013) and lipo-JAK2(1001-1013)2A peptides were characterized by mass spectrometry and purified by high-performance liquid chromatography. Peptides were dissolved in water or dimethyl sulfoxide (Sigma-Aldrich) (24).

In vitro generation of monocyte-derived DCs. DCs were generated as previously described (25, 26), with slight modifications. Briefly, peripheral blood mononuclear cells (PBMC) were isolated from 50 ml heparinized whole blood from 18 healthy volunteers by Ficoll-Hypaque (Amer-
treated mDCs (stimulator [S] cells) were added to a 96-well plate (100 µl/well) with different numbers (ranging from 2 × 10⁴/well to 1.25 × 10⁵/well) and cocultured with 1 × 10⁶ T lymphocytes in 100 µl of RPMI 1640 medium. After 5 days of coculture, 1.0 µCi [³H]thymidine was added to each well for the last 16 h. Then cells were harvested on glass filter paper, and the levels of [³H]thymidine incorporation into the cellular DNA were calculated as counts per minute (cpm) by liquid scintillation counting. The background proliferation of allogeneic T cells in the absence of mDCs was subtracted for data analysis (25, 27).

**Induction of antigen-specific CTLs and cytotoxicity assay in vitro.** Antigen-specific CTL induction was performed as described previously (28), with some modifications. Briefly, autologous T cells were enriched from PBMC with a human CD3⁺ T cell isolation kit and suspended in RPMI 1640 with 10% FBS, 12.5 mM HEPES, 4 mM L-glutamine, 100 U/ml penicillin, and 100 U/ml streptomycin. Meanwhile, RNA-electroporated and/or peptide-loaded mDCs were treated with MMC (25 µg/10⁶ mDCs) for 30 min at 37°C and washed with RPMI 1640 medium. On day 1, 1 × 10⁵ T cells were plated onto a 24-well plate and cocultured with 1 × 10⁵ MMC-treated mDCs in 2 ml of RPMI 1640 medium supplemented with IL-7 (10 ng/ml) at 37°C for 7 days. IL-2 at a concentration of 50 U/ml was added to each well on day 3 and supplemented every 3 days. After 7 days, the effectors were harvested, washed, counted, and restimulated with newly RNA-electroporated and/or peptide-loaded mDCs. With IL-2 and IL-7 added to the culture at the same concentrations, the cells were incubated for another 7 days. The same process was repeated every 7 days another two times. On day 28, antigen-specific CTLs were successfully induced. In order to detect the cytotoxic activity of the CTLs against target cells, the antigen-specific CTLs and MGC-803 cells served as effector (E) and target (T) cells, respectively. Cytotoxicity was determined by measuring lactate dehydrogenase (LDH) release at the indicated E/T cell ratios (10:1, 20:1, and 40:1) after incubation for 4 h. Subsequently, the supernatants were collected and analyzed for LDH release with a CyTox 96 nonradioactive cytotoxicity assay kit (Promega). The percentage of specific LDH release, which is proportional to the cytotoxicity of antigen-specific CTLs for target cells, was calculated by the following equation: % Cytotoxicity = [(Experimental LDH release – Effector spontaneous – Target spontaneous)/(Target maximum LDH release – Target spontaneous)] × 100.

**ELISA of IL-12 secretion.** The supernatants of 5-day iDC and 7-day mDC cultures were harvested by centrifugation and stored at −80°C for the assessment of secreted cytokines. The levels of IL-12/p70 secretion in the supernatants were evaluated by enzyme-linked immunosorbent assay (ELISA) with a human IL-12p70 high-sensitivity ELISA kit (eBioscience) according to the manufacturer’s protocol.

**ELISPOT assay of IFN-γ release.** A mixture of 2 × 10⁴ autologous T cells and 2 × 10⁵ MMC-treated mDCs in a total volume of 200 µl RPMI 1640 medium containing 10% FBS was added to an anti-human IFN-γ antibody precoated 96-well plate (200 µl/well). Each experimental condition was plated in triplicate, and all of the cells were incubated at 37°C in a humidified atmosphere containing 5% CO₂ for 24 h. Then an enzyme-linked immunospot (ELISPOT) assay of IFN-γ release was performed with a human IFN-γ ELISPOT assay kit (R&D Systems) according to the instruction manual, and the developed microplate was analyzed by counting spots with an automatic BD ELISPOT Reader. The frequency of spot-forming cells (SFC) was then estimated from the number of spots in the well and the cell input (26, 29).

**Western blot analysis.** Western blot analysis was performed as previously described (17, 30), with a slight modification. Briefly, iDCs and mDCs were lysed in lysis buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1% NP-40, 1 mM EDTA, 0.25% sodium deoxycholate, 1 mM NaF, 1 mM sodium orthovanadate, 5 µg/ml leupeptin, 5 µg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride) for 30 min on ice and then centrifuged for 20 min at 13,000 rpm. The supernatants were transferred to new tubes and stored at −80°C. The protein concentration was quantified with the Bio-Rad Bradford protein assay (Bio-Rad) with bovine serum albumin (BSA) as the standard. Equal amounts of protein were heated for 10 min at 95°C in 1× sample buffer, electrophoresed by 10% SDS-PAGE, and then transferred to the nitrocellulose membrane. After blocking with 5% BSA, the membrane was probed with antibodies specific for STAT1 and tyrosine-phosphorylated STAT1 (pSTAT1; Cell Signaling Technology, Danvers, MA). Subsequently, signals were detected with horseradish peroxidase-conjugated secondary antibodies by the ECL system (Amersham Pharmacia Biotech). In addition, β-actin was used in parallel as a loading control.

**Statistical analysis.** Statistical analysis was performed with SPSS 18.0 software. All experiments were repeated at least three times, and the data were expressed as means ± standard deviations (SDs) or as means and medians. Differences among groups were analyzed by two-way analysis of variance (ANOVA) in combination with Tukey’s multiple-comparison test. A P value of <0.05 was considered statistically significant.

**RESULTS**

The pJAK2(1001-1013) peptide upregulates the expression of the maturation markers and costimulatory molecules on human monocyte-derived DCs. To exert effective immune responses, DCs must mature and express high levels of MHC-antigen complexes and costimulatory molecules that enhance interactions with T cells. Therefore, we first assessed their respective phenotypes, as well as their capacity to differentiate into mDCs by flow cytometric analysis. In addition to determination of the percentage of positive cells compared to an isotype control, the MFI was analyzed. The data obtained (Table 1; see Fig. S1 in the supplemental material) demonstrated that after 30 µM pJAK2(1001-1013) peptide loading, RNA-electroporated mDCs showed a more mature phenotype. For instance, CD83, the characteristic functional marker of maturation, showed an increase in MFI (73.35 ± 8.91) of the RNA-electroporated plus pJAK2(1001-1013) peptide-loaded mDC group compared with the RNA-electroporated mDC group. However, a control peptide that consisted of alanine pJAK2(1001-1013)2A, had no such effect, as there were no significant differences in phenotypic expression (HLA-DR, CD83 and CD86) when the RNA-electroporated plus JAK2(1001-1013)2A peptide-
The pJAK2(1001-1013) peptide potentiates the capacity of human monocyte-derived DCs to induce T-cell proliferation. MLRs were performed to test the ability of DCs to stimulate allogeneic T lymphocytes (31). As shown in Fig. 1, RNA-electroporated plus pJAK2(1001-1013) peptide-loaded mDCs induced enhanced T-cell proliferation in comparison to all other mDCs. For example, the mean value reached 117.19 ± 500 cpm in the RNA-electroporated plus pJAK2(1001-1013) peptide-loaded mDC group, when the S/R ratio was 1:4. However, the control peptide JAK2(1001-1013)2A had little or no stimulating effect on T-cell proliferation, as there were no significant differences in counts per minute between the RNA-electroporated plus JAK2(1001-1013)2A peptide-loaded mDC group and the RNA-electroporated mDC group. In addition, electroporation did not obviously alter the ability of DCs to stimulate T-cell proliferation, since mock-electroporated and nonelectroporated mDCs had nearly equivalent counts per minute. Thus, these data indicate that the pJAK2(1001-1013), but not the JAK2(1001-1013)2A peptide, can potentiate the capacity of mDCs to induce T-cell proliferation.

The pJAK2(1001-1013) peptide stimulates the secretion of proinflammatory cytokine IL-12. The ability to produce IL-12, which drives the T-helper type 1 (Th1) response, is considered to be one of the most important functions of DCs, as IL-12 secretion appears to correlate with therapeutic efficacy in clinical trials. Our results showed that all mDCs secreted levels of IL-12p70 significantly higher than those of iDCs (P < 0.01) (Fig. 2). Among the different combinations of treatments of mDCs, RNA electroporation contributed to the secretion of IL-12p70 and pJAK2(1001-1013) peptide loading further increased the level of IL-12p70 secretion. However, mock electroporation and addition of the JAK2(1001-1013)2A peptide to RNA-electroporated mDCs did not improve this capability. Evaluation of IL-10 secretion showed that secretion of large amounts of IL-12p70 is accompanied by low levels of IL-10 secretion (see Fig. S2 in the supplemental material), indicating an obvious Th1-biased cytokine profile for pJAK2(1001-1013) peptide-loaded mDCs.

The pJAK2(1001-1013) peptide enhances the cytotoxicity of tumor antigen–specific CTLs activated by human gastric cancer cell total RNA-electroporated human monocyte-derived DCs. It is known that IL-12 is of special importance for Th1 polarization. In fact, IL-12 promotes the differentiation of naive CD4+ T cells into Th1 cells that produce IFN-γ and aid in cell-mediated immunity. Furthermore, IFN-γ facilitates the development of CD8+ CTLs, which are responsible for the cell-mediated immune response to viruses and tumor cells (32, 33). In this study, we detected the release of IFN-γ with an ELISPOT assay to determine whether these IL-12-secreting mDCs have the capacity to induce Th1 polarization and subsequent CTL activation. Figure 3 shows that RNA electroporation treatment and pJAK2(1001-1013) peptide loading promoted the release of IFN-γ compared to the corresponding controls (nonelectroporated mDCs and RNA-electroporated mDCs, respectively). In particular, there was a significant increase in IFN-γ secretion in the RNA plus pJAK2(1001-1013) mDC group (P < 0.01 versus the RNA-electroporated mDC group), which clearly indicated that the most potent Th1 polarization and CTL activation were induced when RNA-electroporated mDCs were loaded with pJAK2(1001-1013). Next, in order to evaluate the cytotoxicity of tumor cell antigen–specific CTLs for target cells, LDH release was measured with a CytoTox 96 nonradioactive cytotoxicity assay kit. As shown in Fig. 4, both CTLs primed with nonelectroporated mDCs and mock-electroporated mDCs showed weak cytolytic activity (the cytotoxicity levels...
A SOCS1 Antagonist and Dendritic Cells

DISCUSSION

DCs are found in different populations within the body, including bone marrow, blood, interstitial, Langerhans, mucosal surface-associated, afferent lymphatic, lymph node, tonsil, and splenic DCs. DCs from the blood are the population most commonly used in ex vivo studies because they are easily isolated (34). However, DCs constitute only approximately 1% of the PBMC, which renders the isolation of sufficient numbers for clinical use difficult. As a result, alternative methods of generating functional human DCs in vitro have been established. In recent years, major advances have been made in the identification of DC precursors and methods to expand and manipulate these cells ex vivo. Monocyte-derived DCs, which are morphologically and phenotypically similar to blood DCs, are the DC preparations most widely used for experimental and clinical purposes (11, 35–38). In addition, significant efforts have been made to use cultured DCs pulsed with tumor antigens to induce antitumor immunity (31, 39). However, it is still unclear what the appropriate conditions for antigen loading and stimulation to achieve DC maturation are. The use of RNA from a defined tumor antigen or isolated from tumor cells has several unique advantages that may overcome the challenges of other antigen sources. For example, RNA has efficient cytoplas-

FIG 3 Antigen-specific T-cell ELISPOT assay responses. (A) A mixture of 2 × 10⁶ autologous T cells and 2 × 10⁶ mDCs was incubated in the wells of a 96-well cell culture plate for 24 h, and IFN-γ spots were developed with a human IFN-γ ELISPOT assay kit. Shown are results representative of the original ELISPOT assay. 1, nonelectroporated mDCs; 2, mock-electroporated mDCs; 3, RNA-electroporated mDCs; 4, RNA plus JAK2(1001-1013)2A mDCs; 5, RNA plus pJAK2(1001-1013) mDCs. (B) Spots corresponding to IFN-γ-secreting T cells were enumerated, and the frequency of SFC was estimated from the number of spots in the well and expressed as the mean number of SFC per 10⁴ T cells. The data shown are means of three independent experiments with 18 healthy volunteers, *, P < 0.01 for the RNA plus pJAK2(1001-1013) mDC group versus the RNA-electroporated mDC group.

FIG 4 Cytotoxicity of tumor antigen-specific CTLs induced by human gastric cancer cell total RNA-electroporated mDCs. Human gastric cancer MGC-803 cells were cocultured with CTLs at the E/T ratios indicated to determine antigen-presenting efficiency. Data are expressed as means ± SDs of three independent experiments with 18 healthy volunteers. *, P < 0.05 for the RNA plus pJAK2(1001-1013) mDC group versus the RNA-electroporated mDC group.

ranged from 4.69 to 7.82% at the E/T ratios indicated), which indicated no specific recognition of human gastric cancer MGC-803 cells, whereas CTLs activated by RNA-electroporated mDCs exerted greater cytotoxicity (17.70 to 33.30% at the E/T ratios indicated). Moreover, the cytotoxicity was further enhanced in the RNA plus pJAK2(1001-1013) mDC group. However, control peptide JAK2(1001-1013)2A treatment did not enhance the cytolytic activity of tumor cell antigen-specific CTLs.

STAT1 is further activated in pJAK2(1001-1013) peptide-loaded human monocyte-derived DCs. Considering the positive effects of the pJAK2(1001-1013) peptide on DC function, we examined the signal transduction pathways that underlie their effects. As we know, DC maturation serves as the critical switch from the maintenance of self-tolerance to the induction of immunity. In order to induce effective antigen-specific T cell responses, DCs must mature and express high levels of MHC-antigen complexes and costimulatory molecules that enhance interactions with T cells (15, 31). In a previous report, it was demonstrated that DC maturation requires STAT1 and is under feedback regulation by SOCS (17). However, it was unknown whether the SOCS1 antagonist pJAK2(1001-1013) peptide has an effect on STAT1 activation in mDCs. Therefore, we performed a Western blot analysis with whole-cell extracts from freshly generated mDCs and antibodies specific for STAT1 and pSTAT1 to examine the STAT1 levels in differentiating DCs and mDCs. As shown in Fig. 5A, pSTAT1 was obviously detected in all mDCs but not in iDCs. In addition, RNA electroporation contributed to the activation of STAT1, as the band intensity of pSTAT1 in the RNA-electroporated mDC group was stronger than that in the mock-electroporated mDC group. Furthermore, the pJAK2(1001-1013) peptide can activate STAT1 in a concentration-dependent manner. pJAK2(1001-1013) peptide loading at 30 µM obviously increased the pSTAT1 level by more than 2-fold over that in RNA-electroporated mDCs. In contrast, the level of pSTAT1 in the JAK2(1001-1013)2A peptide-loaded mDC group was comparable to that of the RNA-electroporated mDC group, suggesting that the control peptide JAK2(1001-1013)2A failed to help the activation of STAT1 (Fig. 5B).
mic expression, allowing the use of the total tumor antigen repertoire with no MHC restriction, and is considered to be safe because of its transient expression and lack of integration into the host genome (40–42). Besides, RNA is easily replicated by the PCR technique and is absolutely free of tumor cell contamination (43). Hence, RNA transfer is becoming one of the most attractive methods of antigen loading. Meanwhile, a large number of studies have evaluated three main methods of RNA delivery into DCs, including passive pulsing, lipofection, and electroporation. Electroporation was proved to be a superior RNA-DC loading strategy (44–46). On the other hand, accumulating evidence has revealed that various signals can induce DC maturation, including (i) components derived from pathogenic agents, such as LPS, lipoteichoic acid, bacterial DNA, cytidine-phosphate-guanosine oligonucleotides, viral or synthetic double-stranded RNA poly(I·C); (ii) inflammatory cytokines, such as TNF-α, IL-1β, and IL-6; (iii) T cell-dependent signals like CD40L and IFN-γ; and (iv) signals resulting from tissue damage, such as heparin sulfate (38). Thus, these stimuli were used singly or in combinations to induce DC maturation in experimental and clinical studies, such as IL-1β plus IL-6 plus TNF-α plus prostaglandin E2 (47), IL-1β plus TNF-α (48), TNF-α plus poly(I·C) (38), IL-1β plus TNF-α plus poly(I·C) plus IFN-α plus IFN-γ (49), and IL-1β plus IL-6 plus TNF-α plus IFN-α plus CD40 ligand (50). Alternatively, we used RNA-electroporated human monocyte-derived DCs and maturation stimulation with LPS and TNF-α in the present study. Our results demonstrated that prepared DCs expressed high levels of MHC and costimulatory molecules and secreted a certain amount of IL-12. Moreover, these mDCs induced tumor cell antigen-specific CTLs to exert effective cytolytic activity in vitro. In brief, we concluded that prepared DCs were fully mature.

SOCS family members are thought to act as classic negative feedback inhibitors, being induced by cytokines and subsequently inhibiting their function. There are four major ways that SOCS proteins inhibit cytokine signaling (51), (i) by blocking STAT recruitment to the cytokine receptor, (ii) by targeting the receptor for degradation by the proteasome, (iii) by binding to JAKs and directly inhibiting their kinase activity, and (iv) by targeting JAKs for degradation by the proteasome. The SOCS family consists of eight members (SOCS1 to SOCS7 and CIS) that contain a conserved SOCS box, a central SH2 domain, and an N terminus of variable length and organization (52–54). Two of the family members, SOCS1 and SOCS3, contain a kinase inhibitory region (KIR) that serves as a pseudosubstrate for JAKs, blocking JAK kinase activity even in the absence of the SOCS box (55). Interestingly, knockout of one of the only KIR-containing SOCS members, SOCS1 or SOCS3, results in a fatal phenotype, which has not been observed with any other SOCS member. These findings might highlight the biological importance of the KIR. The fact that the KIR of SOCS1 can bind directly to pJAK2(1001-1013) raises the possibility that pJAK2(1001-1013) can function as an antagonist of SOCS1. pJAK2(1001-1013) was able to antagonize SOCS1 by four different approaches. First, pJAK2(1001-1013) enhanced suboptimal IFN-γ activity. Second, LNCaP prostate cancer cells transfected for constitutive production of SOCS1 protein had reduced activation of STAT3 by IL-6 treatment, pJAK2(1001-1013) reversed the SOCS1 effect. Third, pJAK2(1001-1013) enhanced IFN-γ activation of the luciferase reporter gene via the IFN-γ-activated sequence promoter element. Fourth, pJAK2(1001-1013) enhanced antigen-specific splenocyte proliferation (23). Subsequently, pJAK2(1001-1013) was reported to enhance antiviral immunity (24). In this study, the SOCS1 antagonist pJAK2(1001-1013) peptide upregulated the expression of the maturation marker (CD83) and costimulatory molecule (CD86) of RNA-electroporated mDCs, potentiated the capacity of mDCs to induce T-cell proliferation, stimulated the secretion of proinflammatory cytokine IL-12, and enhanced the cytotoxicity of tumor cell antigen-specific CTLs activated by human gastric cancer cell total RNA-electroporated mDCs. These results prove that the SOCS1 antagonist pJAK2(1001-1013) peptide is indeed an effective agent to enhance antigen-specific antitumor immunity by DCs, as expected.

Previous reports have demonstrated that inhibition of SOCS1 function enhances the immune response of DCs. SOCS1 silencing in human myeloid-derived DCs resulted in enhanced cytokine responses to LPS and a strong mixed-lymphocyte reaction. Moreover, DCs treated with SOCS1 siRNA elicited strong primary in vitro responses to well-characterized HLA-A*0201-restricted Melan-A/MART-1 and human immunodeficiency virus (HIV) Gag epitopes in naive CD8+ T cells from healthy donors. In addition, stimulation of CD8+ T cells from HIV-seropositive subjects with SOCS1-silenced DCs resulted in an augmented polyfunctional CTL response (20). Shen and colleagues reported that DCs transfected with SOCS1 siRNA showed a slightly more mature phenotype than did siRNA-DC mutants either before or after IFN-γ and LPS stimulation. In addition, both transfected DCs
were more mature than mock-transfected DCs. They explained that these results may reflect the effect of nonspecific activation of IFN genes by siRNA. However, maturation marker expression and cytokine production were not significantly different between SOCS1-silenced DCs and control DCs after strong stimulation with LPS. Surprisingly, SOCS1-silenced mDCs exhibited stronger CTL induction activity than normal mDCs. They speculated that SOCS1 gene downregulation might be able to maintain the mature state of DCs for a much longer period in vitro or SOCS1-silenced DCs might turn off the activity of CD47, CD25 regulatory T cells by producing larger amounts of IL-6. Nevertheless, what happens inside pJAK2(1001–1013) peptide-treated DCs and the precise cellular mechanism of enhanced CTL induction by SOCS1-blocked DCs remain to be clarified.

SOCS1 is deeply involved in the development, maturation, and activation of DCs. GM-CSF is an essential factor in the generation of DCs. Although signaling through TLRs induced the maturation of DCs from iDCs, it suppressed GM-CSF-mediated DC development from CD14+ human monocytes. SOCS1 was shown to be involved in this process, as SOCS1 was highly upregulated by TLR signals, thereby suppressing GM-CSF signals. SOCS1 also negatively regulated LPS- and IL-4-induced DC maturation. In addition, the STAT6 signaling pathway was constitutively activated in iDCs and declined as these cells differentiated into mDCs, whereas STAT1 was upregulated during this process. SOCS1 expression was also upregulated during maturation, and therefore, SOCS1 was proposed to be involved in the switch from STAT6 to STAT1 expression in DCs. In SOCS1-deficient DCs, STAT1 was hyperactivated and many IFN-regulated genes are upregulated. Our Western blot assay results revealed that STAT1 was further activated in pJAK2(1001–1013) peptide-loaded mDCs.

In summary, the use of the pJAK2(1001–1013) peptide strongly enhanced the antigen-presenting capacity and tumor cell antigen-specific CTL responses of tumor cell total RNA-electroporated human monocyte-derived DCs in vitro. This study has implications not only for understanding the quantitative and qualitative regulation of antigen presentation and adaptive immunity but also for the development of effective vaccines against cancer by enhancing the stimulatory potential of DCs.

ACKNOWLEDGMENTS

This work was in part supported by grants from the Science and Technology Innovation Fund of Xiamen City (350220104014) and the Medical Science and Technology Innovation Fund of the Nanjing Military Region of the People’s Liberation Army (10MA068).

We have no conflict of interest to declare.

REFERENCES

1. Steinman RM, Hawiger D, Nussenzweig MC. 2003. Tolerogenic dendritic cells. Annu. Rev. Immunol. 21:685–711.
2. Steinman RM. 2010. Some active areas of DC research and their medical potential. Eur. J. Immunol. 40:2085–2088.
3. Steinman RM. 2008. Dendritic cells in vivo: a key target for a new vaccine science. Immunobiol 29:319–324.
4. Steinman RM. 2008. Dendritic cells and vaccines. Proc. (Baylor Univ. Med. Cent.) 21:3–8.
5. Steinman RM. 2007. Dendritic cells: understanding immunogenicity. Eur. J. Immunol. 37(Suppl 1):S53–S60.
6. Turnis ME, Rooney CM. 2010. Enhancement of dendritic cell vaccines for cancer. Immunotherapy 2:847–862.
7. Gilboa E. 2007. DC-based cancer vaccines. J. Clin. Invest. 117:1195–1203.
8. Aarnitzen EH, Fidgor CG, Adema GJ, Punt CJ, de Vries JH. 2008. Dendritic cell vaccination and immune monitoring. Cancer Immunol. Immunother. 57:1559–1568.
9. Kalantari T, Kamali-Sarvestani E, Girci B, Karimi MH, Kalantari M, Faridar A, Xu H, Rostami A. 2011. Generation of immunogenic and tolerogenic clinical-grade dendritic cells. Immunol. Res. 51:153–160.
10. Houghton HH, Kim HO, Lee K, Baek EJ, Kim HS. 2010. Two-step maturation of immature DCs with proinflammatory cytokine cocktail and poly(I:C) enhances migratory and T cell stimulatory capacity. Vaccine 28:2877–2886.
11. Hovden AO, Karlsen M, Jonsson R, Aastrad HJ, Appel S. 2011. Maturation of monocyte derived dendritic cells with OK3432 boosts IL-12p70 secretion and conveys strong T-cell responses. BMC Immunol. 12:20. doi: 10.1186/1471-2172-12-20.
12. Ten Brinke A, Karsten ML, Dieter MC, Zwagginga JJ, van Ham SM. 2007. The clinical grade maturation cocktail monophosphoryl lipid A plus IFN gamma generates monocyte-derived dendritic cells with the capacity to migrate and induce Th1 polarization. Vaccine 25:7145–7152.
13. Pardoll D. 2003. Does the immune system see tumors as foreign or self? Annu. Rev. Immunol. 21:807–839.
14. Hanada T, Yoshida H, Kato S, Tanaka K, Masutani T, Tsukada J, Nomura Y, Mimata H, Kubo M, Yoshimura A. 2003. Suppressor of cytokine-signaling-1 is essential for suppressing dendritic cell activation and systemic autoimmunity. Immunity 19:437–450.
15. Shen L, Evel-Kabler K, Strube R, Chen SY. 2004. Silencing of SOCS1 enhances antigen presentation by dendritic cells and antigen-specific anti-tumor immunity. Nat. Biotechnol. 22:1546–1555.
16. Kang B, Ren W, Song XT, Evel-Kabler K, Chen SY, Huang XP. 2009. Human suppressor of cytokine signaling 1 controls immunostimulatory activity of monocyte-derived dendritic cells. Cancer Res. 69:8076–8084.
17. Jackson SH, Yu CR, Mahdi RM, Ebonq S, Egwuagu CE. 2004. Dendritic cell maturation requires STAT1 and is under feedback regulation by suppressors of cytokine signaling. J. Immunol. 172:2307–2315.
18. Bartz H, Avalos NM, Baetz A, Heeg K, Dalpek AH. 2006. Involvement of suppressors of cytokine signaling in Toll-like receptor-mediated blockade of dendritic cell differentiation. Blood 108:4102–4108.
19. Hu Q, Qin X, Qian G, Jiang S, Li H, Jiang M, Li X, Chen SY, Zang YQ. 2008. SOCS1 silencing can break high-dose dendritic cell immunotherapy-induced immune tolerance. Mol. Med. Rep. 1:61–70.
20. Subramanya S, Armant M, Salkowitch JR, Nyakeriga AM, Haridas V, Hasan M, Bansal A, Goepfert PA, Wynn KK, Ladell K, Price DA, N M, Kan-Mitchell J, Shankar P. 2010. Enhanced induction of HIV-specific cytotoxic T lymphocytes by dendritic cell-targeted delivery of SOCS-1 siRNA. Mol. Ther. 18:2028–2037.
21. Cooney RN. 2002. Supressors of cytokine signaling (SOCS): inhibitors of the JAK/STAT pathway. Shock 17:833–90.
22. Yasukawa H, Misawa H, Sakamoto H, Masuhara M, Sasaki A, Wakioka T, Obata H, Inoue S, Imaiizumi T, Matsuda T, Ihle JN, Yoshimura A. 1999. The JAK-binding protein JAB inhibits Janus tyrosine kinase activity through binding in the activation loop. EMBO J. 18:1309–1320.
23. Waiboci LW, Ahmed CM, Mujtaba MG, Flowers LO, Martin JP, Haider MI, Johnson HM. 2007. Both the suppressor of cytokine signaling 1 (SOCS-1) kinase inhibitory region and SOCS-1 mimetic binding to JAK2 phosphatase/kinase inhibition: implications for the development of a SOCS-1 antagonist. J. Immunol. 178:5058–5068.
24. Ahmed CM, Dabicel R, Martin JP, Jager LD, Haider SM, Johnson HM. 2010. Enhancement of antiviral immunity by small molecule antagonist of suppressor of cytokine signaling. J. Immunol. 185:1103–1113.
25. Michiels A, Tuyaerts S, Bonehill A, Corthals J, Breckpot K, Heirman C, Van Meirvenne S, Dullaers M, Allard S, Brasseur F, van der Bruggen P, Thielemans K. 2005. Electroformation of immature and mature dendritic cells: implications for dendritic cell-based vaccines. Gene Ther. 12:772–782.
26. Gholamin M, Moaven O, Farschian M, Mahmoudi M, Sankian M, Memar B, Forghani MN, Malekhzadeh R, Rajabi-Mashhad MT, Abbaszadegan MR. 2010. Induction of cytotoxic T lymphocytes primed with tumor RNA-loaded dendritic cells in esophageal squamous cell carcinoma: preliminary step for DC vaccine design. BMC Cancer 10:2621. doi: 10.1186/1471-2407-10-261.
27. Milano F, van Baal JW, Rygiel AM, Bergman J, Van Deventer SJ, Kapsenberg ML, Peppelenbosch MP, Krishnadath KK. 2007. An improved protocol for generation of immuno-potent dendritic cells through direct electroporation of CD14+ monocytes. J. Immunol. Methods 321:94–106.
28. Tanaka F, Yamaguchi H, Haraguchi N, Mashino K, Ohta M, Inoue H, Mori M. 2006. Efficient induction of specific cytotoxic T lymphocytes to tumor rejection peptide with functional matured 2-day-cultured dendritic cells derived from human monocytes. Int. J. Oncol. 29:1263–1268.

29. Kim S, Park M, Kim C, Sohn H, Kim H, Park J, Kim H, Oh S, Kim T. 2008. Modification of CEA with both CRT and TAT PTD induces potent anti-tumor immune responses in RNA-pulsed DC vaccination. Vaccine 26:6433–6440.

30. Wu N, Wang Y, Wang S, Chen Y, Yan J. 23 September 2012. Recombinant human leptin induces growth inhibition and apoptosis in human gastric cancer MGC-803 cells. Clin. Exp. Med. (Epub ahead of print.) doi: 10.1007/s10238-012-0211-8.

31. Ghanekar SA, Bhatia S, Ruitenberg JJ, DeLa Rosa C, Disis ML, Maino ML, Gaudernack G, Saebøe-Larssen S, Hammerstad H, Tierens A, O'Neill DW, Adams S, Bhardwaj N.

32. Lichtneger FS, Mueller K, Otte B, Hiddemann W, Schendel Lichtenegger FS, Mueller K, Et al. 2005. The biology of IL-12: coordinating innate and adaptive immune responses. Cytokine Growth Factor Rev. 14:361–368.

33. Hart DN. 1997. Dendritic cells: unique leukocyte populations which control the primary immune response. Blood 90:3245–3287.

34. Fong I, Engleman EG. 2000. Dendritic cells in cancer immunotherapy. Annu. Rev. Immunol. 18:245–273.

35. Conti L, Gessani S. 2008. GM-CSF in the generation of dendritic cells from human blood monocyte precursors: recent advances. Immunobiology 213:859–870.

36. Mu LJ, Gaudernack G, Saebøe-Larssen S, Hammerstad H, Tiere A, Kvalheim G. 2003. A protocol for generation of clinical grade mRNA-transfected monocyte-derived dendritic cells for cancer vaccines. Scand. J. Immunol. 58:575–586.

37. Spisek R, Brelaudeau L, Barbier X, Mella K, Gregoire M. 2001. Standardized generation of fully mature p70 IL-12 secreting monocyte-derived dendritic cells for clinical use. Cancer Immunol. Immunother. 50:417–427.

38. O'Neill DW, Adams S, Bhardwaj N. 2004. Manipulating dendritic cell biology for the active immunotheraphy of cancer. Blood 104:2235–2246.

39. Van Tendeloo VF, Ponsaerts P, Berneman ZN. 2007. mRNA-based gene transfer as a tool for gene and cell therapy. Curr. Opin. Mol. Ther. 9:423–431.

40. Bringmann A, Held S, Heine A, Brossart P. 2010. RNA vaccines in cancer treatment. I. Biomed. Biotechnol. 2010:23687.

41. Sousa-Canavez JM, Canavez FC, Leite KR, Camara-Lopes LH. 2008. Therapeutic dendritic cell vaccine preparation with tumor RNA transfection: a promising approach for the treatment of prostate cancer. Genet. Vaccines Ther. 6:2. doi:10.1186/1479-0586-6-2.

42. Miura S, Kagamu H, Tanaka H, Yoshizawa H, Gejyo F. 2008. Appropriate timing of CD40 ligation for RNA-pulsed DCs to induce antitumor immunity. Scand. J. Immunol. 67:385–391.

43. Kalady MF, Onaitis MW, Padilla KM, Emanu S, Tyler DS, Pruitt SK. 2002. Enhanced dendritic cell antigen presentation in RNA-based immunotherapy. J. Surg. Res. 105:17–24.

44. Saebøe-Larssen S, Fossberg E, Gaudernack G. 2002. mRNA-based electrotansfection of human dendritic cells and induction of cytotoxic T lymphocyte responses against the telomerase catalytic subunit (hTERT). J. Immunol. Methods 259:191–203.

45. Van Tendeloo VF, Ponsaerts P, Lardon F, Nijs G, Lenjou M, Van Broeckhoven C, Van Boekstaele DR, Berneman ZN. 2001. Highly efficient gene delivery by mRNA electroporation in human hematopoietic cells: superiority to lipofection and passive pulsing of mRNA and to electroporation of plasmid cDNA for tumor antigen loading of dendritic cells. Blood 98:49–56.

46. Jonuleit H, Kühn U, Müller G, Steinbrink K, Paragnik L, Schmitt E, Knop J, Enk AH. 1997. Pro-inflammatory cytokines and prostaglandins induce maturation of potent immunostimulatory dendritic cells under fetal calf serum-free conditions. Eur. J. Immunol. 27:3135–3142.

47. Kalinski P, Schuitemaker JH, Hilkens CM, Wierenga EA, Kapsenberg ML. 1999. Final maturation of dendritic cells is associated with impaired responsiveness to IFN-gamma and to bacterial IL-12 inducers: decreased ability of mature dendritic cells to produce IL-12 during the interaction with Th cells. J. Immunol. 162:3231–3236.

48. Maillard RB, Wankowicz-Kalinsa A, Cai Q, Wesa A, Hilkens CM, Kapsenberg ML, Kirkwood JM, Storkus WJ, Kalinski P. 2004. Alphatype-1 polarized dendritic cells: a novel immunization tool with optimized CTL-inducing activity. Cancer Res. 64:5934–5937.

49. Kaka AS, Foster AE, Weiss HL, Rooney CM, Leen AM. 2008. With dendritic cell maturation and IL-12 producing capacity as markers of function: a cautionary tale. J. Immunother. 31:359–369.

50. Palmer DC, Restifo NP. 2009. Suppressors of cytokine signaling (SOCS) in T cell differentiation, maturation, and function. Trends Immunol. 30:592–602.

51. Croker BA, Kiu H, Nicholson SE. 2008. SOCS regulation of the JAK/STAT signalling pathway. Semin. Cell Dev. Biol. 19:414–422.

52. Yoshimura A, Naka T, Kubo M. 2007. SOCS proteins, cytokine signalling and immune regulation. Nat. Rev. Immunol. 7:454–465.

53. Larsen L, Röpke C. 2002. Suppressors of cytokine signaling: SOCS. APMIS 110:833–844.

54. Zhang JG, Metcalf D, Rakar S, Asimakis M, Greenhalgh CJ, Willson TA, Starr R, Nicholson SE, Carter W, Alexander WS, Hilton DJ, Nicola NA. 2001. The SOCS box of suppressor of cytokine signaling-1 is important for inhibition of cytokine action in vivo. Proc. Natl. Acad. Sci. U. S. A. 98: 13261–13265.

55. Kobayashi T, Yoshimura A. 2005. Keeping DCs awake by putting SOCS1 to sleep. Trends Immunol. 26:177–179.

56. Hanada T, Tanaka K, Matsumura Y, Yamauchi M, Nishihakamura H, Abaratani H, Mmashima R, Kubo M, Kobayashi T, Yoshimura A. 2005. Induction of hyper Th1 cell-type immune responses by dendritic cells lacking the suppressor of cytokine signaling-1 gene. J. Immunol. 174:4325–4332.