Research Article

Anti-Inflammatory Protein of *Schistosoma japonicum* Directs the Differentiation of the WEHI-3B JCS Cells and Mouse Bone Marrow Cells to Macrophages

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Sj16 is an anti-inflammatory protein identified from *Schistosoma japonicum*. Our previous studies showed that recombinant Sj16 (rSj16) could suppress host's inflammatory responses and inhibit macrophage maturation. In the present study, the effects of rSj16 on the differentiation of the murine myeloid leukemia WEHI-3B JCS cell line and on mouse hematopoiesis were investigated. Our data demonstrated that rSj16 expressed and purified from *Escherichia coli* could suppress the proliferation of the WEHI-3B JCS cells in a time- and concentration-dependent manner, while not affect the viability of the cells. Further studies indicated that rSj16 induced macrophage differentiation of the WEHI-3B JCS cells, and arrested the cell cycle in the G1/G0 and G2/M phases. The macrophage differentiation of the rSj16-treated WEHI-3B JCS cells was confirmed by their expression of macrophage specific antigen F4/80 and phagocytic activity. Furthermore, our results revealed that rSj16 biased the colony formation of mouse bone marrow cells towards macrophage lineage.

1. Introduction

Leukemia occurs when the normal hematopoietic process fails, and the leukemic cells retain the proliferative capacity of progenitor cells and unable to spontaneously undergo terminal maturation [1]. The cell line WEHI-3B JCS is a D-subclone isolated from the WEHI 3B myelomonocytic leukemia cell line [2]. Previous studies have reported that monocytic differentiation of this cell line could be induced by phorbol myristate acetate (PMA) [2], biochanin A [3], and conjugated linoleic acid [4], and that endogenous IL-1 α , IL-1 β , and TNF- α have been shown to play an important role during these differentiation-inducing processes.

Schistosome, the causative agent of schistosomiasis in the world, is a blood born fluke (trematode) of the genus *Schistosoma*. Plenty of studies have demonstrated that the schistosome-originated substances could modulate the host's

immune system [5, 6]. For example, the prostaglandin D2 from schistosome has been well known to block the migration of langerhans cells to the draining lymph nodes, thereby inhibiting a crucial step in the initiation of immunity [7]. An IL-4 inducing factor identified from *Schistosoma mansoni* (*S. mansoni*) egg has been demonstrated to skew the immune response to Th2 [8]. Another apoptosis inducing factor produced by skin-stage schistosomula of *S. mansoni* could cause apoptosis of T cells [9]. Trottein et al. have also reported that substances from *S. mansoni* suppressed host's inflammation by activating host microvascular endothelial cells [10]. However, up to now, little is reported about the immunomodulatory effects of schistosome-derived substances on leukemia and hematopoiesis.

Recently, we have cloned a gene named Sj16 from *Schistosoma japonicum* (*S. japonicum*) and demonstrated that the recombinant Sj16 (rSj16) expressed and purified

from *Escherichia coli* (*E. coli*) could suppress the recruitment of mature macrophages into the peritoneal cavity in thioglycollate-induced inflammation in BALB/c mice [11]. In the present study, we reported the effects of rSj16 on the proliferation and differentiation of the WEHI-3B JCS and on mouse hematopoiesis.

2. Materials and Methods

2.1. Cell Line. The murine myeloid leukemia cell line WEHI-3B JCS [2] was cultured in RPMI 1640 medium (Gibco-BRL, USA) supplemented with 10 mM L-glutamine, 10% fetal calf serum (FCS; HyClone, USA), and 1% antibiotics mixture (100 unit/ml penicillin, 100 µg/ml streptomycin, and 0.25 µg/ml amphotericin B; GibcoBRL, USA). The cultures were incubated under a humidified atmosphere of 95% air, 5% CO₂ at 37°C.

2.2. Animals. BALB/c mice (female, 4–6 week), bred and kept by the Laboratory Animal Services Centre of the Chinese University of Hong Kong, were used. All animal procedures were approved by the Animal Experimentation Ethics Committee (CUHK) in accordance with the Department of Health (Hong Kong) guidelines in Care and Use of Animals. All experiments were performed under licence from the Hong Kong Special Administrative Region Government.

2.3. Expression and Purification of Recombinant Proteins. The recombinant Sj16 (rSj16) and recombinant glutathione-S-transferase (rGST) were expressed and purified from *E. coli* as we have described before [11]. The purified rSj16 and rGST were passed through a Detoxi-Gel AffinityPak polymyxin B column (Pierce, USA) to eliminate endotoxins. The absence of endotoxins in the proteins was further confirmed by *Limulus* amebocyte lysate test (sensitivity 0.25 EU/ml, Associates of Cape Cod, USA) before the proteins were used in functional assays.

2.4. Cell Proliferation Assessment. The colorimetric 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide (MTT) assay was employed to quantify the cell proliferation [12, 13]. Briefly, WEHI-3B JCS cells were incubated with RPMI 1640 medium containing serially diluted rSj16 or rGST in 96-well microplates (Nunc, Denmark) for 24 hours, 48 hours, 72 hours, and 96 hours. 20 µl MTT (5 mg/ml stock solution, Sigma, USA) were then added into each well and incubated for 4 hours, and the plates were centrifuged at 500×g for 10 minutes. The MTT solution was removed and replaced by 150 µl DMSO, and the plates were shaken until the formazans were thoroughly dissolved. The optical density was determined using a microplate reader (Dynatech Laboratories, USA) at a wavelength of 570 nm. Each assay was performed in triplicate.

2.5. Cell Viability. Cell viability was determined using trypan blue exclusion assay. Briefly, WEHI-3B JCS cells were incubated with RPMI 1640 medium in the presence or absence of 0.5 µg/ml rSj16 or 0.5 µg/ml rGST for 24 hours, 48 hours,

and 72 hours. The cells were then collected and mixed 1 : 1 with 0.4% trypan blue (Sigma, USA). The percentage of viable cells was recorded within 10 minutes with an inverted microscope. Greater than 300 cells per sample were examined and the data were expressed as percentage (%) viability. The experiment was performed in triplicate.

2.6. Cell Cycle Analysis. Cell cycle analysis was performed on WEHI-3B JCS cells incubated for 1–4 days with either rSj16 (0.5 µg/ml) or rGST (0.5 µg/ml). The cells were fixed in chilled ethanol overnight before staining with propidium iodide staining solution (3.8 mM sodium citrate, 50 µg/ml propidium iodide, 50 µg/ml RNase A in PBS) at room temperature for 30 minutes. Analysis was performed immediately after staining using a FACScan (Becton Dickinson, USA) and CELLFit program (Becton Dickinson, USA).

2.7. Analysis of WEHI-3B JCS Differentiation. WEHI-3B JCS cells were cultured with either rGST (0.5 µg/ml) or rSj16 (0.5 µg/ml) in the presence/absence of serially diluted neutralizing anti-IL-1α, anti-IL-1β, anti-TNF-α, or these three antibodies together (R&D Systems, USA) for up to 72 hours.

The adherent and suspension cells were collected, respectively, and counted with a hemacytometer (Bright-line, Sigma, USA) 24, 48, and 72 hours after treatment. The percentage of adherent cells was calculated. The culture supernatants after 24, 48, and 72 hours treatment were collected, stored at –80°C until cytokine measurement.

The morphology of the WEHI-3B JCS cells was studied up to 72 hours of treatment using a phase-contrast microscope (Nikon, Japan). Monocytic differentiation of the treated cells was also examined using modified Wright-Giemsa stain (Sigma, USA) after cytospin smear preparation. All experiments were independently done at least 3 times.

Expression of cell surface antigens was determined by flow cytometry 72 hours after treatment. After twice washing with FACS medium (2% heat-inactivated FCS and 0.05% sodium azide in PBS), cells were incubated at 4°C for 30 minutes with antimouse FcγR antibody (CD16/CD32, eBioscience, USA). The cells were then washed twice and incubated at 4°C for 30 minutes with affinity purified antimouse F4/80 or antimouse Gr-1 antibodies (both from eBioscience, USA). Isotype-matched control antibodies (eBioscience, USA) were used as negative controls. Unbound antibodies were removed by washing the cells twice with FACS medium. The cells were then stained with FITC-conjugated sheep antirat IgG (BD PharMingen, USA) at 4°C for 30 minutes and then washed and fixed with 1% (w/v) paraformaldehyde in PBS. Cells were analyzed by a FACScan (Becton Dickinson, USA).

Phagocytic activity of WEHI-3B JCS was determined by the modified yeast phagocytosis assay as described previously [2, 3]. Briefly, opsonized yeasts were prepared by incubating heat-inactivated yeast cells (*Saccharomyces cerevisiae*) with fresh mouse serum at 37°C for 30 minutes. 2 × 10⁷ opsonized yeasts were added to untreated, 72 hours post rSj16 or rGST treated WEHI-3B JCS cell cultures in 6-well

microplates (Nunc, Denmark). The mixtures were incubated at 37°C for 16 hours, and the percentages of phagocytic cells were counted under microscope. Cells with a minimum of three ingested yeasts were considered positive. A total of 500 cells were counted in each sample.

2.8. Cytokine Determination. Levels of IL-1 α , IL-1 β , and TNF- α in the culture supernatants were measured using DuoSet ELISA Development kit (R&D Systems, USA) according to the manufacturer's protocol. Briefly, 96-well Maxisorp multititer plates (Nunc, Denmark) were coated overnight at 4°C with appropriate capture antibodies (2 μ g/ml for anti-IL-1 α , 4 μ g/ml for anti-IL-1 β , and 0.8 μ g/ml for anti-TNF- α) in PBS at pH 7.4 and then washed three times with PBS containing 0.05% Tween 20. The plates were blocked for 2 hours at room temperature with 1% BSA in PBS, followed with three washes as described above. Serially diluted murine recombinant cytokine standards IL-1 α (1.95–1,000 pg/ml), IL-1 β (1.95–1,000 pg/ml), or TNF- α (3.91–1,000 pg/ml) and the culture supernatants were then added in duplicate and incubated for 2 hours at room temperature. After three washes, the plates were incubated for 2 hours at room temperature with appropriate biotinylated detection antibodies and then washed again. To detect biotinylated antibodies, streptavidin-linked horseradish peroxidase (1/1,000 dilution) was added and incubated for 20 minutes in the dark at room temperature. After a final washing of five times, 3, 3', 5, 5'-tetramethylbenzidine liquid substrate (Sigma, USA) was added to each well. The absorbance was read at 450 nm with the wavelength correction at 570 nm using a microplate reader (Dynatech Laboratories, USA), and the concentration of the samples was calculated using the standard curve.

2.9. IL-1 α , IL-1 β , and TNF- α Neutralization Assay (by MTT Assay). For neutralization assay, WEHI-3B JCS cells were incubated in 96-well microplates (Nunc) with RPMI 1640 medium containing 0.5 μ g/ml rSj16 in the presence or absence of serially diluted neutralizing anti-IL-1 α , anti-IL-1 β , anti-TNF- α , or these three antibodies together (R&D Systems) for 72 hours. To test whether anti-IL-1 α , anti-IL-1 β , or anti-TNF- α antibody itself affects the WEHI-3B JCS proliferation, WEHI-3B JCS cells were also incubated in 96-well microplates (Nunc) with RPMI 1640 medium containing serially diluted anti-IL-1 α , anti-IL-1 β or anti-TNF- α antibody (R&D Systems). 20 μ l MTT (5 mg/ml stock solution, Sigma, USA) were then added into each well and incubated for 4 h, and the proliferation was assessed as mentioned above. Each assay was performed in triplicate. The results of neutralization assay were expressed as proliferation index which was calculated as following: *proliferation index* = mean O.D.570 of triplicate wells containing 0.5 μ g/ml rSj16 in the presence of neutralizing anti-IL-1 α , anti-IL-1 β , or anti-TNF- α antibodies/mean O.D.570 of triplicate wells containing 0.5 μ g/ml rSj16 in the absence of neutralizing anti-IL-1 α , anti-IL-1 β , or anti-TNF- α antibodies.

2.10. Mouse Bone Marrow Cell Colony-Forming Unit Assay. Female BALB/c mice were sacrificed and wet thoroughly with

70% ethanol. The femurs were isolated, the bone marrows were flushed out, and the single cell suspension was then prepared. To measure the number of nucleated cells, 10 μ l of the single cell suspension was first diluted in 490 μ l of 3% acetic acid and then counted using a hemacytometer (Bright-line, Sigma, USA). The cell suspension was adjusted to 5×10^6 nucleated cells per ml with cold RPMI-1640 medium.

The bone marrow cell suspension was added into prewarmed RPMI-1640 medium containing 20% FCS (HyClone), 2% antibiotics mixture (GibcoBRL), and 0.33% agar (cell culture tested; sigma, USA), and mixed well (60 μ l of cell suspension per 6 ml of medium). The mixture was transferred into 24-well plates (Nunc), 0.4 ml per well, and then kept at room temperature for 15 minutes to allow the agar solidification. After solidification, 25 μ l of PBS alone, 25 μ l of PBS containing 0.5 μ g/ml of rSj16 or 0.5 μ g/ml rGST was added into corresponding wells and incubated in a humidified 37°C incubator for one hour, followed by adding 25 μ l of PBS, 25 μ l of PBS containing 0.5 ng/ml of IL-3, 25 μ l of PBS containing 36 ng/ml of M-CSF, or 25 μ l of PBS containing 12 ng/ml of G-CSF into corresponding wells and incubated for further seven days. Each semisolid agar gel was then transferred onto a cleaning glass slide, covered with 3 layers of Whatman filter papers, and dried overnight at 37°C oven. The slides were stained with hematoxylin solution (Sigma, USA), and the cell colonies of greater than 40 cells were counted. All samples were run in triplicate.

2.11. Statistics. Comparisons of data within experiments were tested for significance using the Student's *t*-test.

3. Results

3.1. rSj16 Inhibited WEHI-3B JCS Cell Proliferation. In this study, rSj16 was first expressed and purified as a rGST-rSj16 fusion protein using the prokaryotic expression vector pGEX-4T-1, and the rGST tag was then removed by thrombin digestion to release free rSj16 [11]. However, during purification of free rSj16, traces of rGST may not be absolutely excluded from the rSj16 solution. On the other hand, the rGST tag in vector pGEX-4T-1 is also *S. japonicum* originated [14]. These made rGST an ideal control protein during the rSj16 functionary assays. Thus, the rGST was purified and used as a control protein throughout the study.

The antiproliferative effect of rSj16 was evaluated by MTT assay. Data were expressed as the percentage inhibition by the ratio of each absorbance relative to the absorbance of the nontreated control, multiplied by 100%. A concentration (Figure 1(a)) and time (Figure 1(b)) dependent inhibition of rSj16 on WEHI-3B JCS cell growth was observed at 48 hours (more than 10% inhibition) and persisted until 96 h. rSj16 inhibited the proliferation of WEHI-3B JCS cells at a maximal effect of more than 40% at 72 hours, and reached this maximal effect at the concentration of 0.5 μ g/ml or higher (Figure 1(a)). Thus, in the following part of this study, 0.5 μ g/ml of rSj16 was used. rGST did not show significant effect on the proliferation of WEHI-3B JCS cells when being used as high as 5 μ g/ml (data not shown).

FIGURE 1: Antiproliferative effect of rSj16 on WEHI-3B JCS cells. WEHI-3B JCS cells were incubated with 0.2 ml of medium containing different doses of rSj16 for 72 hours (a) or containing 0.5 $\mu\text{g/ml}$ of rSj16 for up to 96 hours (b). The proliferation was quantified by MTT assay.

In order to test whether the suppression of WEHI-3B JCS proliferation by rSj16 is due to its cytotoxic effect on the cells, WEHI-3B JCS cells were incubated with rSj16 for up to 72 hours and the cell viability was assessed by trypan blue exclusion assay. The results indicated that the viability of WEHI-3B JCS cells was not affected after 0.5 $\mu\text{g/ml}$ of rSj16 treatment for 24 hours ($98.51 \pm 0.14\%$ versus $98.86 \pm 0.28\%$ viability, $P > .2$; rSj16 treatment versus untreated; $n = 4$), 48 hours ($97.80\% \pm 0.35\%$ versus $97.36 \pm 0.43\%$, $P > .2$; $n = 4$) and 72 hours ($96.48\% \pm 0.57\%$ versus $97.43\% \pm 0.51\%$, $P > .2$; $n = 4$). rGST did not show any effects on WEHI-3B JCS viability (data not shown).

3.2. Effect of rSj16 on the WEHI-3B JCS Cell Cycle. Because rSj16 exerted significant effect on WEHI-3B JCS cells proliferation (Figure 1), while it did not affect the viability of the cells (see above), we were interested in studying the pattern of cell cycle distribution of rSj16-treated cells. The cell cycle distribution was assessed by flow cytometry after treatment with rSj16 for various times.

A significant accumulation of WEHI-3B JCS cells in the G1/G0 phases of the cell cycle occurred at 24 hours (Figure 2(a)), reached the peak at 72 hours (Figure 2(c)), and retained a high level at 96 hours (Figure 2(d)), after treatment with 0.5 $\mu\text{g/ml}$ of rSj16, accompanied with a concomitant decrease in the proportion of those in S phase (Figures 2(c) and 2(d)). A significant accumulation of WEHI-3B JCS cells in the G2/M phase of the cell cycle also occurred at 48 hours (Figure 2(b)), and reached the peak at 72 hours (Figure 2(c)) post 0.5 $\mu\text{g/ml}$ of rSj16 treatment. After 72 h, the accumulation of G1/G0 and G2/M population did not increase anymore even exposure for longer time (Figure 2(d)). rGST had no effect on the cell cycle distribution of WEHI-3B JCS cells (Figures 2(a)–2(d)).

3.3. Morphological Study of rSj16-Treated WEHI-3B JCS Cells. The effect of rSj16 on morphology of WEHI-3B JCS cells

was studied in a time course of 72 h. The WEHI-3B JCS cells became adherent and developed pseudopodia in the presence of 0.5 $\mu\text{g/ml}$ of rSj16 (Figures 3(a) and 3(b)). Aggregation of the WEHI-3B JCS cells to each other followed by the adherence of the cells to the plate was observed within 24 hours of rSj16 exposure (Figures 3(a) and 3(b)). By the time of 48 h, more than 50% of the cells attached to the plate and began to form pseudopodia (Figures 3(a) and 3(b)). After 72 hours of rSj16 exposure, 71.28% $\pm$ 3.15% of the cells were adherent to the plate, and over 50% of the cells had pseudopodia and cytoplasmic vacuoles (Figures 3(a) and 3(b)). All of these morphological changes indicated that rSj16 may induce macrophage differentiation of WEHI-3B JCS cells, as the abilities to develop prominent pseudopodia and adhere to charged surface are typical features of normal tissue macrophages [15, 16]. In contrast, cells exposed with rGST (0.5 $\mu\text{g/ml}$) did not developed significant adhesion (only 0.02% $\pm$ 0.007% of the cells were adherent at 72 h) and pseudopodia formation (Figures 3(a) and 3(b)).

To further characterize the morphological changes, cytospin smear of WEHI-3B JCS cells treated with rSj16 or rGST was prepared and stained with Modified Wright-Giemsa stain (Sigma, USA). The result showed that after 72 hours exposure to 0.5 $\mu\text{g/ml}$ of rSj16, the nuclear: cytoplasmic ratio of the cells reduced, the nuclear chromatin became condensed and eccentrically placed, the cytoplasmic borders were protruded, and cytoplasmic vacuoles were often present (Figure 3(e)). These morphological features were similar to those described for mature tissue macrophages [15–17]. WEHI-3B JCS cells treated with rGST did not exhibit significant morphological changes (Figure 3(d)).

3.4. Expression of Macrophage Marker on rSj16 Induced WEHI-3B JCS Cells. To confirm the macrophage differentiation-inducing activity of rSj16 on WEHI-3B JCS cells, flow cytometric analysis was carried out to examine for the expression of differentiation antigens by

FIGURE 2: *Modulation of WEHI-3B JCS cell cycle by rSj16.* WEHI-3B JCS cells were cultured for one day (a), two days (b), three days (c), and four days (d) in the presence of 0.5 $\mu\text{g/ml}$ of rSj16 (rSj16), 0.5 $\mu\text{g/ml}$ of rGST (rGST), or medium alone (untreated), and the cell cycle status was analyzed by flow cytometry. Data indicate mean $\pm$ SEM of three independent experiments. * $P < .1$; ** $P < .01$; *** $P < .001$ as compared with that of untreated group.

rSj16-treated cells. Untreated WEHI-3B JCS cells expressed little F4/80+ and Gr-1+ (Figure 4). 72 hours exposure of WEHI-3B JCS cells to rSj16 (0.5 $\mu\text{g/ml}$) resulted in nearly 40% increase in the expression of the antigen F4/80+, while there were no significant changes on the expression of Gr-1+ (Figure 4). rGST did not show significant effect on the expression of these antigens (Figure 4).

3.5. rSj16 Induced WEHI-3B JCS Cells Showed Phagocytic Activity. A key function of normal mononuclear phagocytes is ingestion of foreign materials. The biological function of rSj16-treated WEHI-3B JCS cells was therefore measured by their capacity of yeast phagocytosis. The results indicated

that WEHI-3B JCS cells developed the ability to efficiently phagocytose *Saccharomyces cerevisiae* after rSj16 exposure (Figures 5(a)–5(c)). More than 50% of the WEHI-3B JCS cells ingested three or more yeast cells after cultured in the presence of 0.5 $\mu\text{g/ml}$ rSj16 for 3 days (Figure 5(d)). Less than 2% of the untreated or rGST-treated WEHI-3B JCS cells phagocytized yeast cells.

3.6. Up-Regulation of IL-1 α , IL-1 β , and TNF- α Expression in rSj16-Treated WEHI-3B JCS Cells. Previous studies reported that TNF- α could induce monocytic differentiation of WEHI-3B JCS cells [2], and that IL-1 α and IL-1 β were involved in the differentiation-inducing activity of TNF- α

FIGURE 3: Effect of rSj16 on adherence and morphology of WEHI-3B JCS cells. WEHI-3B JCS cells were treated with 0.5 $\mu\text{g/ml}$ rSj16 (rSj16), 0.5 $\mu\text{g/ml}$ rGST (rGST), or medium alone (untreated) for various time periods, and morphological changes were studied using a light microscope (Nikon). (a) Time course morphological changes of WEHI-3B JCS cells (magnification, $\times 200$). Black bars represent 50 μm . (b) Induction of adherence of WEHI-3B JCS cells after exposure to rSj16. The data were expressed as mean $\pm$ SEM, $n = 3$. ** $P < .01$; *** $P < .001$ as compared with that of untreated group. (c)–(e) Modified Wright Giemsa staining of WEHI-3B JCS cells 72 hours post treatment (magnification, $\times 400$). Black bars represent 25 μm . Data are representative of three individual experiments.

[18]. In addition, TNF- α has been identified as the active substance in PMA-induced macrophage differentiation of WEHI-3B JCS cells [2]. Thus, we investigated the effects of rSj16 on the expression of IL-1 α , IL-1 β , and TNF- α of WEHI-3B JCS cells. Untreated WEHI-3B JCS cells expressed little IL-1 α and IL-1 β (both less than 1 pg/ml in the culture media, Figures 6(b) and 6(c)) and expressed low level of TNF- α (Figure 6(a)). Treatment of WEHI-3B JCS cells with rSj16, but not rGST, caused marked time-dependent up-regulation of the expression of these three cytokines (Figures 6(a)–6(c)). The levels of IL-1 α , IL-1 β , and TNF- α in the culture supernatants increased to 60.77 pg/ml, 8.57 pg/ml, and 97.15 pg/ml, respectively, after 3 days exposure to rSj16 (Figures 6(a)–6(c)).

3.7. Differentiation-Inducing Effect of rSj16 on WEHI-3B JCS Cells is not Attributable to Endogenous Production of IL-1 α , IL-1 β , and TNF- α . To investigate the role of endogenous IL-1 α , IL-1 β , and TNF- α in the rSj16-induced WEHI-3B JCS cells differentiation, specific antibodies were adopted to neutralize each cytokine, respectively. The neutralizing ability of the antibodies was verified using D10.G4.1 cell proliferation assay for anti-IL-1 α and anti-IL-1 β , and using L929 cell cytolytic assay for anti-TNF- α (data not shown). Before evaluating the blocking activity of the antibodies on rSj16-mediated differentiation, an initial experiment was performed to test the effect of each antibody on the proliferation (by MTT assay) and morphological changes of WEHI-3B JCS cells, and the results indicated that the effect

(a)

(b)

FIGURE 4: Induction of macrophage marker expression on WEHI-3B JCS cells by rSj16. WEHI-3B JCS cells were treated for 72 hours in the presence of 0.5 $\mu\text{g/ml}$ rSj16 (rSj16), 0.5 $\mu\text{g/ml}$ rGST (rGST), or medium alone (untreated) and then analyzed for expression of F4/80 and Gr-1 using flow cytometry. (a) Red line indicates isotype-matched control antibody; black line, F4/80 or Gr-1. (b) Column indicates mean $\pm$ SEM of four independent experiments. * $P < .05$ as compared with that of untreated group.

FIGURE 5: *rSj16 induced WEHI-3B JCS cells showed phagocytic activity.* WEHI-3B JCS cells were treated for 72 hours with 0.5 $\mu\text{g/ml}$ rSj16 (rSj16, (c)), 0.5 $\mu\text{g/ml}$ rGST (rGST, (b)), or medium alone (untreated, (a)) and then analyzed for the capacity of yeast-ingesting. (a)–(c) Arrows indicate the yeast-ingesting cells. White bars represent 50 μm (magnification, $\times 400$). (d) Percentage of phagocytic cells after 72 hours treatment. The results are the mean $\pm$ SEM of three independent experiments. *** $P < .001$ as compared with that of untreated group.

of each of them neither inhibited the proliferation nor affected morphological changes of WEHI-3B JCS cells when being used as high as 10 $\mu\text{g/ml}$ (data not shown). The blocking effect of the antibodies on rSj16-induced WEHI-3B JCS differentiation was evaluated by both MTT assay and morphological studies as described in Section 2. The results showed that none of them, no matter be used separately or combined, could block rSj16-mediated morphological changes and antiproliferation (data not shown) of WEHI-3B JCS cells at the concentration being used as high as 10 $\mu\text{g/ml}$. Therefore, it is suggested that rSj16-induced WEHI-3B JCS differentiation does not depend on the endogenous production of IL-1 α , IL-1 β , and TNF- α .

3.8. Effect of rSj16 on IL-3, M-CSF, or G-CSF Induced Proliferation and Differentiation of Mouse Bone Marrow Cells. As mentioned above, rSj16 could induce macrophage differentiation of WEHI-3B JCS cells (Figures 3–5). Since the WEHI-3B JCS cells are derived from bone marrow progenitors [3], it raised a question whether rSj16 can also affect hematopoiesis of bone marrow cells. It is well

characterized that hematopoietic growth factors such as G-CSF, M-CSF, and IL-3 can regulate the proliferation and differentiation of bone marrow cells and stimulate related colony formation [19, 20]. To test the effects of rSj16 on hematopoiesis, mouse bone marrow cells were cultured in semisolid agar cultures containing optimal G-CSF, M-CSF, or IL-3 in the presence or absence of 0.5 $\mu\text{g/ml}$ of rSj16.

After treated with G-CSF or M-CSF, significant increases of CFU-G colonies (Figure 7(a)) or CFU-M colonies (Figure 7(b)) were found in the corresponding plates. Addition of rSj16 into the culture before the treatment of G-CSF greatly reduced the colony counts of G-CSF-induced CFU-G (Figure 7(a)). It is interesting that the presence of rSj16 in the culture did not affect the number of M-CSF-induced CFU-M colonies (Figure 7(b)) but only increased the compactness of the cells within the colonies (Figure 8(b)).

As expected, colonies CFU-M, CFU-G, and CFU-GM were significantly induced following seven days culture with IL-3 (Figure 7(c)). However, when rSj16 was preadded into the culture one hour before the IL-3 treatment, the total colonies and the colony counts of CFU-G and CFU-GM were

FIGURE 6: Effect of rSj16 on IL-1 α , IL-1 β , and TNF- α production from WEHI-3B JCS cells. WEHI-3B JCS cells were treated with 0.5 μ g/ml rSj16 (rSj16), 0.5 μ g/ml rGST (rGST), or medium alone (untreated), and then the culture supernatants were collected at different time as indicated for the detection of TNF- α (a), IL-1 α (b), and IL-1 β (c) by Sandwich ELISA. Values represent mean $\pm$ SEM of three independent experiments. *** $P < .001$; ** $P < .01$, as compared with that of untreated group.

significantly decreased, while the colony counts of CFU-M were increased (Figure 7(c)). Besides, just as similar as shown in Figure 8, the cells within CFU-M colonies in the presence of rSj16 also became more compact than those treated with IL-3 alone (data not shown).

There were no colonies induced when the mouse bone marrow cells were treated with PBS alone 0.5 μ g/ml of rSj16 alone, or 0.5 μ g/ml of rGST alone (data not shown).

4. Discussion

Sj16 is a secretory protein produced by *S. japonicum*. It has 100% identity with the protein sequence of the anti-inflammatory protein Sm16 which is abundantly present in the excretory secretions of schistosomulae of *S. mansoni* [21, 22]. Previous studies reported that Sm16 could suppress the host immune responses by inhibiting the antigen-induced

FIGURE 7: Effect of rSj16 on IL-3, M-CSF, or G-CSF induced colony formation of mouse bone marrow cells. Mouse bone marrow cells were plated in 0.33% agar cultures containing G-CSF (a), M-CSF (b), or IL-3 (c) in the presence of rSj16, rGST, or PBS in 24-well plates. The agar gels were dried and stained with hematoxylin solution following seven days of incubation. Differential colony counts were performed based on the morphology of the cells, and only colonies of greater than 40 cells were counted. Colony counts are indicated as number of colonies per well. Data are mean $\pm$ SEM of triplicate wells and are representatives of three individual experiments. * $P < .05$; ** $P < .01$; NS, not significant versus the corresponding data in the PBS + G-CSF/M-CSF/IL-3 group.

lymphoproliferation and suppressing the IL-2 production from lymphocytes [22]. Sm16 could also suppress LPS-induced neutrophil infiltration and downregulate production of the proinflammatory cytokines [23]. In addition, a single intradermal injection of a full-length cDNA of Sm16 resulted in a significant suppression of cutaneous inflammation [24]. By using recombinant protein obtained from

E. coli, we also demonstrated that rSj16 dramatically suppressed the thioglycollate-mediated recruitment of leukocytes to the peritoneal cavity of BALB/c mice and suppressed thioglycollate-induced peritoneal macrophages maturation, accompanied by marked upregulation of IL-10 and IL-1 receptor antagonist transcripts, and down-regulation of IL-12p35, IL-1 β , and MIP-2 transcripts in peritoneal cells [11].

FIGURE 8: Effect of rSj16 on morphology of M-CSF-induced CFU-M colonies after hematoxylin staining. Mouse bone marrow cells were treated with M-CSF in the presence of rSj16 (b), rGST (c), or PBS (a) as described in Figure 7. The black bars represent 50 μ m (magnification, $\times$ 100). The photos are representatives of three individual experiments.

Further analysis revealed that rSj16 also inhibited both humoral and cellular immunity to heterologous antigens using BALB/c mouse model (Hu et al., unpublished data). Thus, it is evident that rSj16 retain an immunomodulatory function.

In this study, to extend our knowledge of the immunomodulatory function of rSj16, we first investigated the effects of rSj16 on the proliferation and differentiation of the leukemia cell line WEHI-3B JCS. Our data indicated that rSj16 could inhibit the growth of WEHI-3B JCS cells in a time- and concentration-dependent manner (Figure 1). Interestingly, it is obvious that the rSj16-mediated inhibition of WEHI-3B JCS cell proliferation is not attributable to its cytotoxic effect on the cell, since the viability of the WEHI-3B JCS cells was not affected by the rSj16 treatment. Further studies indicated that rSj16 also induced macrophage morphological changes of the WEHI-3B JCS cells (Figure 3), companied with markedly decrease of the cells in the S phases and concomitant accumulation in the proportion of those in G1/G0 and G2/M phase (Figure 2). These results may suggest that rSj16 induces macrophage differentiation of the WEHI-3B JCS cells.

Membrane antigens serve as excellent markers of murine macrophage differentiation in vivo and in vitro [25]. To confirm macrophage differentiation of the WEHI-3B JCS cells, we analyzed the membrane antigens by flow cytometry. Among the two antigens being analyzed, the F4/80 is expressed by a majority of mature macrophages and is the best marker for this population of cells [26], while Gr-1 is expressed by bone marrow granulocytes as well as on peripheral neutrophils [27]. As expected, our data showed that rSj16 could stimulate the expression of F4/80 on the WEHI-3B JCS cells surface but not Gr-1 (Figure 4), thus supporting the macrophage differentiation of the cells. Subsequently, this differentiated WEHI-3B JCS cells have been verified to be functional, as they acquired the yeast-phagocytizing activity after rSj16 treatment (Figure 5).

It is well known that LPS could induce macrophage differentiation of certain leukemia cells [28, 29]. In order to exclude the possible contamination of endotoxins during preparation of rSj16, the purified proteins were passed

through a Detoxi-Gel AffinityPak polymyxin B column (Pierce) to eliminate endotoxins. The absence of endotoxins in the protein samples was further confirmed by *Limulus* ameobocyte lysate test (sensitivity 0.25 EU/ml, Associates of Cape Cod). Besides, the rGST prepared as the same way as rSj16 was served as negative control in the whole study. Thus, in this study, the rSj16-induced macrophage differentiation should not due to the endotoxins contamination.

Cytokines, such as IL-1, leukemia inhibitory factory, TNF- α , and IL-4, have been shown to regulate the growth and differentiation of hematopoietic cell [30, 31]. Previous studies reported that PMA-induced macrophage differentiation of WEHI-3B JCS cells was mediated by endogenous production of TNF- α , as the differentiation-inducing effect of PMA could be prevented by neutralizing anti-TNF- α antibodies [2]. Further studies showed that macrophage differentiation of WEHI-3B JCS cells could be also induced by TNF- α , IL-1 α , or IL-1 β . We were thereby interested in whether rSj16-induced WEHI-3B JCS cells differentiation was results of endogenous production of TNF- α , IL-1 α or IL-1 β . Unexpectedly, although the production of these three cytokines has been upregulated after rSj16 treatment (Figure 6), they were obviously not involved in the rSj16-induced WEHI-3B JCS differentiation, as none of the anti-IL-1 α , anti-IL-1 β , anti-TNF- α , or these three antibodies together could prevented the activity of rSj16 (data not shown). Thus, these results indicated that rSj16 induce WEHI-3B JCS cells differentiation through some other mechanisms. However, currently we don't have information about the mechanisms. This will be addressed in our following studies.

The WEHI-3B JCS cell line is a subclone isolated from the murine myelomonocytic leukemia WEHI-3B cell line and therefore possesses some certain features of myelomonocytic progenitor cells [2]. This cell line has been widely used to be a useful model in the study of hematopoietic cell differentiation [2–4, 32, 33]. In the present study, we have demonstrated that rSj16 could induce macrophage differentiation of the WEHI-3B JCS cell (Figures 3–5). It is thus reasonable to investigate the regulatory effect of rSj16 on the mouse hematopoiesis. Our results revealed that rSj16

could suppress G-CSF-induced CFU-G colony formation (Figure 7(a)) and suppress IL-3-induced CFU-G and CFU-GM colony formation (Figure 7(c)). On the other hand, although rSj16 alone did not induce any colony formation of the mouse bone marrow cells, we demonstrated that rSj16 could increase the number of IL-3-induced CFU-M colony formation (Figure 7(c)). This result corresponded with our previous observation that rSj16 induces macrophage differentiation of WEHI-3B JCS cell (Figures 3–5). However, unexpectedly, the addition of rSj16 into the culture did not affect the number of M-CSF-induced CFU-M colonies (Figure 7(b)). A possible interpretation is that M-CSF is a potent inducer of macrophage differentiation which may mask the inducing effect of rSj16.

Furthermore, it is very interesting that the macrophages within the CFU-M colonies induced by IL-3 along with rSj16 (data not shown) and M-CSF along with rSj16 (Figure 8(b)) became more compact than those induced with IL-3 (data not shown) or M-CSF (Figure 8(a)) alone, which suggested that the differentiated macrophages after rSj16 treatment may lose the ability of migration. This result may indicate that although rSj16 induced macrophage differentiation of WEHI-3B JCS cell and increased the number of IL-3-induced CFU-M colony formation of mouse bone marrow cells, the differentiated macrophage cells may not display full functions as normal mature macrophages. As the inhibition of antigen-presenting cell migration has been proved to be a strategy for the schistosomes to escape host immune system [7], our results may further suggest that Sj16 helps the parasite to survive from host immune attack. However, further studies are needed to support this postulation and explore the potential mechanisms.

Conflict of Interest

The authors have no financial or commercial conflict of interest.

Acknowledgments

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