

# Sulfated Polymannuroguluronate, a Novel Anti-AIDS Drug Candidate, Inhibits T Cell Apoptosis by Combating Oxidative Damage of Mitochondria

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## ABSTRACT

Sulfated polymannuroguluronate (SPMG) has entered the phase II clinical trial as the first anti-AIDS drug candidate in China. Herein, we report that SPMG was effective at protecting T lymphocytes against apoptosis. Further studies indicated that SPMG significantly elevated mitochondrial membrane potential (MMP) of T cells; inhibited mitochondrial release of cytochrome c (cyto c) in T cells; enhanced the activities of mitochondrial enzyme complex I, III, and V; and subsequently increased ATP level and ATP/ADP ratio. In addition, SPMG potently suppressed reactive oxygen

species (ROS) generation in mitochondria at cellular level and scavenged free radicals in cell-free system. The molecular mechanism underlying the ATP-involved and ROS-dependent anti-apoptosis of SPMG is characterized as having been caused by its engagement with mitochondrial import receptor and ADP/ATP carrier in T-cell outer and inner mitochondrial membrane, respectively. All these might shed new light on the understanding of anti-AIDS functions of SPMG by protecting T cells of persons infected with human immunodeficiency virus.

During human immunodeficiency virus (HIV) infection, oxidative damage and ATP level depletion threaten T-cell homeostasis and integrity and subsequently lead to apoptosis of T cells, which seems to have an adverse effect on the immune system (Pace and Leaf, 1995; Olinski et al., 2002). Apoptosis-mediated physiological depletion of T lymphocytes in the course of viral infection can silence the immune response and induce immunodeficiency (Wattre et al., 1996; Galati et al., 2002). In AIDS patients, apoptotic T-cell death is thought to play a major role in T-cell loss and HIV pathogenesis (Gougeon et al., 1991; Ng et al., 1997). The use of antioxidant drugs in the therapy of HIV-infected patients may offer protection against oxidative stress and apoptotic cell death and slow down AIDS progression (Schreck et al., 1992; Jaruga et al., 2002). Many studies have shown that polysaccharides were capable of scavenging free radicals and protecting cells from death, which became a crucial

mechanistic explanation of their antiapoptotic actions (Liu et al., 1997; Sun et al., 2004).

Sulfated polymannuroguluronate (SPMG) is a new form of sulfated polysaccharide extracted from brown alga with an average molecular mass at 8.0 kDa. It is characterized by a rich amount of 1,4-linked  $\beta$ -D-mannuronate with 1.5 sulfate and 1.0 carboxyl groups averaging each sugar residue (Fig. 1A). SPMG has entered the phase II clinical trial in China as the first marine sulfated polysaccharide with the potential of becoming an anti-AIDS drug. SPMG has manifested potential antiviral and immunomodulating efficacy in AIDS patients in clinical trials. It is noteworthy that our previous investigations have also verified that SPMG exerted significant immunopotentiating actions particularly on T lymphocytes (Xia et al., 2005). The goals of the present study are to investigate the antiapoptotic and protective effects of SPMG on T cells and to elucidate the underlying mechanisms.

## Materials and Methods

### Drugs and Reagents

SPMG, SPMG-Sepharose, and SPMG-fluorescein-5-isothiocyanate (FITC) were provided by Marine Drug and Food Institute

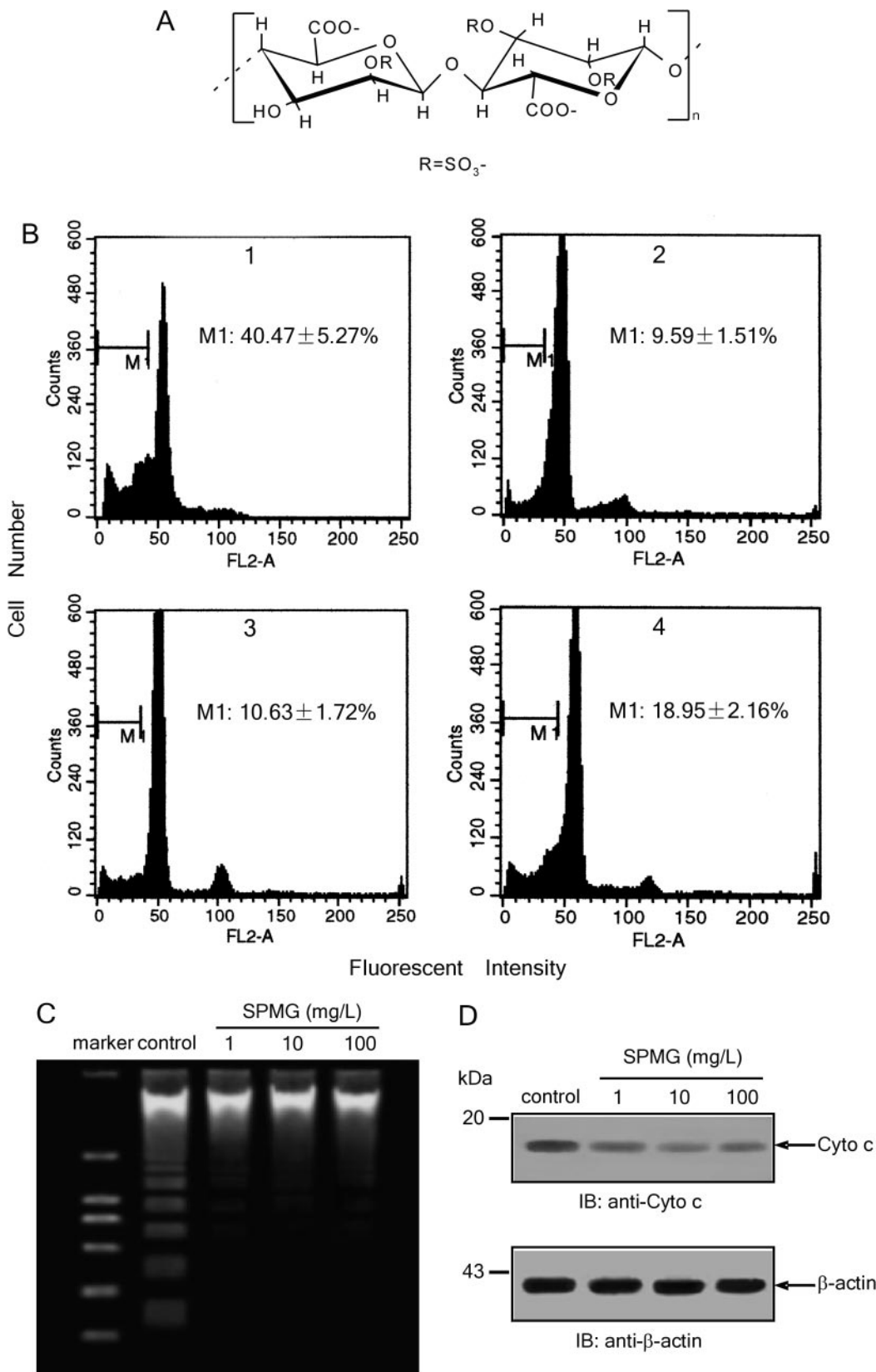
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**ABBREVIATIONS:** HIV, human immunodeficiency virus; SPMG, sulfated polymannuroguluronate; FITC, fluorescein-5-isothiocyanate; PMSF, phenylmethylsulfonyl fluoride; cyto c, cytochrome c; PI, propidium iodide; DHR, dihydrorhodamine; LC-MS/MS, liquid chromatography-tandem mass spectrometry; FCM, flow cytometry; PBS, phosphate-buffered saline; HPLC, high-performance liquid chromatography; MMP, mitochondrial membrane potential; CoQ1, ubiquinone; ROS, reactive oxygen species; au, arbitrary unit; AAC, ADP/ATP carrier.

(Ocean University of China, Qingdao, China). Aprotinin, pepstatin A, leupeptin, phenylmethylsulfonyl fluoride (PMSF), ATP, ADP, cytochrome *c* (cyto *c*), coenzyme Q1, NADH, reductive glutathione, propidium iodide (PI), rhodamine 123, dihydrorhodamine (DHR) 123, bovine serum albumin, dithiothreitol, and iodoacetamide were

purchased from Sigma-Aldrich (St. Louis, MO). Rabbit anti-rat cyto *c* antibody (IgG) and goat anti-rabbit IgG conjugated to horseradish peroxidase were purchased from Boster Biotechnology Company (Wuhan, Hubei, China). The ECL Western blotting kit was supplied by GE Healthcare (Little Chalfont, Buckinghamshire, UK). RNase



**Fig. 1.** Effects of SPMG on apoptosis of T cells and mitochondrial release of cyto *c*. Thymocytes were incubated with SPMG (1, 10, and 100 mg/l) for 12 h at 37°C and then stained with PI (5 mg/l) and analyzed by FCM. B, proportion of T cells in the sub- $G_0$  fraction representing apoptotic cells is shown. C, for DNA fragmentation analysis, T lymphocytes treated with 1, 10, and 100 mg/l SPMG were lysed in ice-cold lysis buffer, and cellular debris was spun down. DNA was isolated and detected by 1% agarose gel electrophoresis to screen for the presence of a DNA ladder pattern. D, to determine the influence of SPMG on mitochondrial release of cyto *c*, cytosolic fractions were extracted from SPMG (1, 10, and 100 mg/l)-treated T cells and subjected to Western blotting analysis using anti-cyto *c* antibody. As a control for equal loading of proteins, filter was also probed with anti- $\beta$ -actin antibody. Four parallel samples were prepared in each group, and the result shown is a representative of three separate experiments with similar results. A, structure of SPMG; B1, control; B2, 1 mg/l SPMG; B3, 10 mg/l SPMG; and B4, 100 mg/l SPMG.

was obtained from Hyclone Laboratories (Logan, UT), RPMI 1640 medium was from Invitrogen (Carlsbad, CA), and trypsin (sequencing grade) was from Roche Diagnostics (Mannheim, Germany).

### Cell Culture and Preparation

Rat thymus lymphocytes were obtained from male Wistar rats (180–200 g) for binding and liquid chromatography-tandem mass spectrometric (LC-MS/MS) analysis, or from male Wistar rats (~700 g; 24 months old) for antiapoptotic and antioxidative studies following published methods (Chen et al., 1994). Cells were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum, 100 U/ml penicillin, and 0.1 g/l streptomycin at 37°C in humidified 5% CO<sub>2</sub> incubator.

### Apoptosis Assays

**PI Staining.** T cells prepared as described above were incubated with or without SPMG at final concentrations of 1, 10, and 100 mg/l for 12 h. Next, the T cells were collected, washed, and stained with a mixture of RNase (1 g/l) and PI (5 mg/l) in 1 g/l sodium citrate containing 0.5% Triton X-100 (v/v) for 30 min. T lymphocytes were then harvested for apoptosis analysis using flow cytometry (FCM) (BD Biosciences, San Jose, CA), and the percentage of hypodiploidy was analyzed with CellQuest and ModFIT LT software (BD Biosciences).

**DNA Fragmentation.** Thymocytes were seeded, and SPMG was added at 1, 10, and 100 mg/l in culture medium. After a 12-h incubation, T lymphocytes ( $5 \times 10^6$ ) were collected and washed in phosphate-buffered saline (PBS) and subsequently lysed in ice-cold lysis buffer [142.5 mM KCl, 5 mM MgCl<sub>2</sub>, 10 mM HEPES, pH 7.3, 1 mM EGTA, 1% Triton X-100 (v/v), 0.5% Nonidet P-40 (v/v), 2.7 μM aprotinin, 0.3 μM pepstatin A, 10 μM leupeptin, and 0.2 mM PMSF] for 45 min. Cellular debris was spun down, and DNA was isolated from the supernatant by phenol extraction. DNA fragmentation was subsequently detected by 1% agarose gel electrophoresis to screen for the presence of a DNA ladder pattern.

### Western Blot Analysis

After incubation with SPMG (1, 10, and 100 mg/l) for 12 h at 37°C, T lymphocytes were harvested, and the mitochondria were removed as described below. The cytosolic extracts were separated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. After being blocked in Tris-buffered saline containing 0.1% Tween 20 (v/v) and 5% bovine serum albumin (w/v) at room temperature for 2 h, the membranes were rinsed and incubated at 4°C overnight with 2 mg/l anti-rat cyto c antibody (IgG) or anti-β-actin antibody as control. The membranes were then incubated with goat anti-rabbit IgG conjugated to horseradish peroxidase (1:2000 dilution) at room temperature for 1 h, developed with chemiluminescence substrate, and exposed to Hyperfilm MP (GE Healthcare).

### Measurement of Intralymphocyte ATP and ADP

T cells were seeded, and SPMG was added at 1, 10, and 100 mg/l, respectively. After a 12-h incubation at 37°C, T lymphocytes were harvested, and the viability of cells was monitored by trypan blue exclusion method. Adenine nucleotides were extracted from samples of  $1 \times 10^8$  viable T cells in 0.5 M perchloric acid on ice for 10 min. After a centrifugation at 25,000g for 15 min at 2°C, the supernatant was obtained and neutralized to pH 6.5–6.8. Potassium perchlorate was removed, and the supernatant was stored at –80°C. The concentrations of ATP and ADP were examined by high-performance liquid chromatography (HPLC) on a CAPCELL PAK C18 SG column (4.6 × 150 mm; SHISEIDO, Tokyo, Japan). The absorbance of the eluents was monitored at 260 nm, and the detector signals were recorded and integrated by ChemStation HP software (Agilent Technologies, Palo Alto, CA) (Smolenski et al., 1998).

### Measurement of Mitochondrial Membrane Potential

Alteration of the MMP of T lymphocytes after treatment with SPMG was assessed by the retention of rhodamine 123. After T lymphocytes were treated with 1, 10, and 100 mg/l SPMG for 12 h, rhodamine 123 was added at a final concentration of 1 μM and incubated for 30 min to stain the mitochondria. Next, T lymphocytes were harvested and washed with PBS three times. The changes in rhodamine 123 fluorescence were evaluated by FCM with a 488-nm laser excitation and a 530-nm emission filter.

### Assays for Mitochondrial Enzyme Activities

**NADH Dehydrogenase (Complex I) Assay.** The activity of mitochondrial complex I was measured using a modification of the method of Ragan et al. (1987) determining the decrease in NADH absorbance at 340 nm, which leads to the reduction of ubiquinone (CoQ1) to ubiquinol. After a 12-h incubation with 1, 10, and 100 mg/l SPMG, T lymphocytes were harvested, washed, and mitochondria were isolated as described below, followed by three cycles of freeze/thawing. The reaction was initiated by the addition of 50 μM CoQ1 to the reaction mixture containing 20 mM potassium phosphate, pH 7.2, 10 mM MgCl<sub>2</sub>, 0.15 mM NADH, 1 mM KCN, and the mitochondrial sample. The changes of absorbance at 340 nm were examined (Cardoso et al., 1999).

**Cytochrome c Reductase (Complex III) Assay.** The activity of mitochondrial complex III was measured following the method of Ragan et al. This enzyme donates electrons from ubiquinol (UQ<sub>1</sub>H<sub>2</sub>) to cyto c, leading to the reduction of cyto c that was monitored at 550 nm. The reaction mixture contained 35 mM potassium phosphate, pH 7.2, 1 mM EDTA, 5 mM MgCl<sub>2</sub>, 1 mM KCN, 5 μM rotenone, 15 μM cyto c, and the mitochondrial sample obtained as mentioned below. The reaction was initiated by addition of substrate ubiquinol (15 μM). The changes of absorbance at 550 nm were examined (Cardoso et al., 1999).

**Mitochondrial ATP-Synthase (Complex V) Assay.** The enzymatic activity of ATPase in the mitochondrial inner membrane was monitored following the method of Taussky and Shorr (1953). The mitochondrial samples were incubated in 20 mM Tris-HCl, pH 7.2, containing 150 mM NaCl at 37°C for 30 min. The reaction was initiated by the addition of 10 mM ATP and 10 mM MgCl<sub>2</sub>, and the synaptosomes were incubated for further 20 min at 37°C. The reaction was stopped by the addition of 5% ice-cold perchloric acid. The supernatant was obtained, and the absorbance was measured at 660 nm, 15 min after the addition of molybdate reagent. The difference of the absorbance corresponds to the ATP-synthase activity (Cardoso et al., 1999; Sudo et al., 2000).

### Dihydrorhodamine 123 Conversion Assay

The level of intramitochondrial reactive oxygen species (ROS) was measured with DHR 123, which can enter mitochondria of living cells and react with ROS, yielding membrane-impermeable fluorescent products. Thus, the fluorescent intensity is indicative of ROS levels within the mitochondria (Kooy et al., 1994; Dugan et al., 1995). T cells were seeded, and SPMG at final concentrations of 1, 10, and 100 mg/l were added, respectively. After a 12-h incubation at 37°C, T lymphocytes were harvested, washed, and loaded with DHR 123 at 10 μM in PBS containing 2 mM NaN<sub>3</sub> for 30 min. Then, T cells were washed with PBS, and cellular fluorescence was acquired using FCM with excitation at 488 nm and emission at 530 nm.

### Chemiluminescence Analysis

The scavenging ability of SPMG on superoxide radical (O<sub>2</sub><sup>•</sup>) in pyrogallol-luminol system was evaluated as follows: 100 μl of PBS containing SPMG at final concentrations of 1, 10, 100, 200, 400, 600, 800, or 1000 mg/l or PBS alone was added into a rigid plastic tube (55 × 10 mm), followed by the addition of 50 μl of pyrogallol (0.625 M). The background intensity of chemiluminescence was first tested for 10 s using a chemiluminescence analysis instrument. Then, 850



$\mu$ l of luminol (1 mM) was added into the tube, and the intensity of chemiluminescence was measured, from which the background intensity was subtracted. The scavenging activity of SPMG on hydroxyl radical ( $\cdot$ OH) in  $V_C$ - $Cu^{2+}$ -yeast suspension-luminol- $H_2O_2$  system was evaluated as follows: 0.2 ml of  $V_C$  (2 mM), 0.4 ml of  $CuSO_4$  (2 mM), 50  $\mu$ l of luminol (0.1 mM), 0.2 ml of yeast suspension (75 g/l), and 0.6 ml of SPMG solution at the same concentrations as mentioned above or PBS was added into a rigid plastic tube and mixed thoroughly. After a 30-min incubation at 37°C, The background intensity of chemiluminescence was measured. Then, 0.6 ml of  $H_2O_2$  (68 mM) was added into the tube to start the reaction. The chemiluminescence intensity was measured, and the background intensity was subtracted from it. Reductive glutathione at the same concentrations was used as control.

### Flow Cytometry and Confocal Microscope Analysis

To detect the binding and entrance of SPMG to T cells, thymic lymphocytes were seeded, and SPMG-FITC was added at 100 mg/l or not as control. After an incubation at 37°C for 6 h, the T cells were harvested, washed three times with PBS, gated, and analyzed by FCM with a 488-nm laser excitation and a 530-nm emission filter. Data were analyzed with CellQuest software. An aliquot of each sample was spotted on a slide, analyzed, and photographed under a confocal laser scanning microscope (Carl Zeiss, Jena, Germany). Optical section series were collected with a spacing of 1  $\mu$ m in the z-axis through T cells.

### Determination of Intramitochondrial Sulfated Polymannuroguluronate

After treated with SPMG-FITC (1, 10, and 100 mg/l) for 12 h at 37°C, T lymphocytes were harvested and washed three times with ice-cold PBS. In another experiment, T cells were incubated with 100 mg/l SPMG-FITC at 37°C for 3, 6, or 12 h. Then, T lymphocytes were placed in cold isolation buffer (210 mM mannitol, 70 mM sucrose, 10 mM HEPES, pH 7.4, 1 mM EDTA, 1 mM EGTA, 10 mM KCl, 1 mM  $MgCl_2$ , and 1 mM PMSF) and hand homogenized. The homogenate was centrifuged at 1300g for 5 min at 4°C to remove nuclei and cellular debris. The supernatant was pooled and centrifuged at 10,000g for 10 min at 4°C. The pellet was saved and washed three times in isolation buffer. Then, precooled redistilled water was added to lyse mitochondria by homogenizing on ice. The supernatant was measured by Spectrofluorometer (Jasco, Tokyo, Japan) with a 488-nm laser excitation and a 530-nm emission filter.

### Purification of T-Cell Membrane Proteins

T-cell membrane fraction was isolated from rat thymus lymphocytes. In brief, the thymuses were separated, minced, ground, and filtrated. T lymphocytes were harvested, counted, and centrifuged at 500g for 5 min in a refrigerated centrifuge. Next, they were suspended in lysing buffer [10 mM Tris-HCl, pH 7.4, 1% Triton X-100 (v/v), 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, and 75 units/ml aprotinin] and left for 10 min at 4°C. The T cells were Dounce-homogenized, and the lysate was centrifuged for 5 min at 1300g. Then, the supernatant was collected and subjected to sucrose density gradient centrifugation at 7000g for 1 h. The membrane fraction located at 37 to 41% (w/v) sucrose was harvested and washed for three times with 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, and 1 mM PMSF (Maeda and Kashiwabara, 1996; Geetha and Deshpande, 1999).

The membrane fractions were then resuspended in solubilization buffer [20 mM Tris-HCl, pH 7.4, 2% Triton X-100 (v/v), 150 mM NaCl, 1 mM  $CaCl_2$ , 1 mM  $MgCl_2$ , 1 mM EDTA, and 1 mM PMSF] and stirred at 4°C for 2 h, followed by centrifugation at 100,000g for 30 min. The supernatant was collected and applied to a SPMG-Sepharose affinity column (1  $\times$  6 cm). The column was eluted with linear gradient of 0.15 to 2 M NaCl in Tris-HCl, pH 7.4, containing 0.1% Triton X-100 (v/v) (Maeda and Kashiwabara, 1996; Geetha and

Deshpande, 1999). The protein content was estimated with 0.01% Coomassie Brilliant Blue G-250 (w/v) in ethanol, phosphoric acid, and water [1:2:20 (v/v)], and the absorbance was measured at 595 nm with a microplate reader (Bradford, 1976). Fractions were pooled, dialyzed, and lyophilized.

### Liquid Chromatography-Tandem Mass Spectrometric Analysis

The above-mentioned purified proteins were dissolved in 200  $\mu$ l of 6 M hydrochloric carbamidine, pH 8.3, and subsequently reduced with 1 M DTT and alkylated with 1 M iodoacetamide, followed by addition of 100 mM  $NH_4HCO_3$  and ultrafiltration at 12,000 rpm for 2 h at 4°C. A 100- $\mu$ l sample was obtained, and tryptic digestion was carried out at 37°C for 20 h. After ultrafiltration at 12,000 rpm for 90 min at 4°C, the sample was separated on a reverse-phase (C18) capillary column (0.15  $\times$  120 mm; Thermo Electron Corporation, Waltham, MA) and then analyzed with electrospray ionization-MS/MS system. The mass spectrometer was set up to take one full-scan MS from the mass range of 400 to 2000  $m/z$  followed by three MS/MS spectra of the three most intense peaks. All MS/MS spectra were analyzed by SEQUEST (Thermo Electron Corporation) against International Protein Index rat protein database (Bodnar et al., 2003; Li et al., 2004).

### Statistics

Student's *t* test and analysis of variance were performed using StatView (SAS Institute, Cary, NC). *P* < 0.05 was accepted as significant and *P* < 0.01 was regarded as highly significant. All the experiments were replicated at least for three times.

## Results

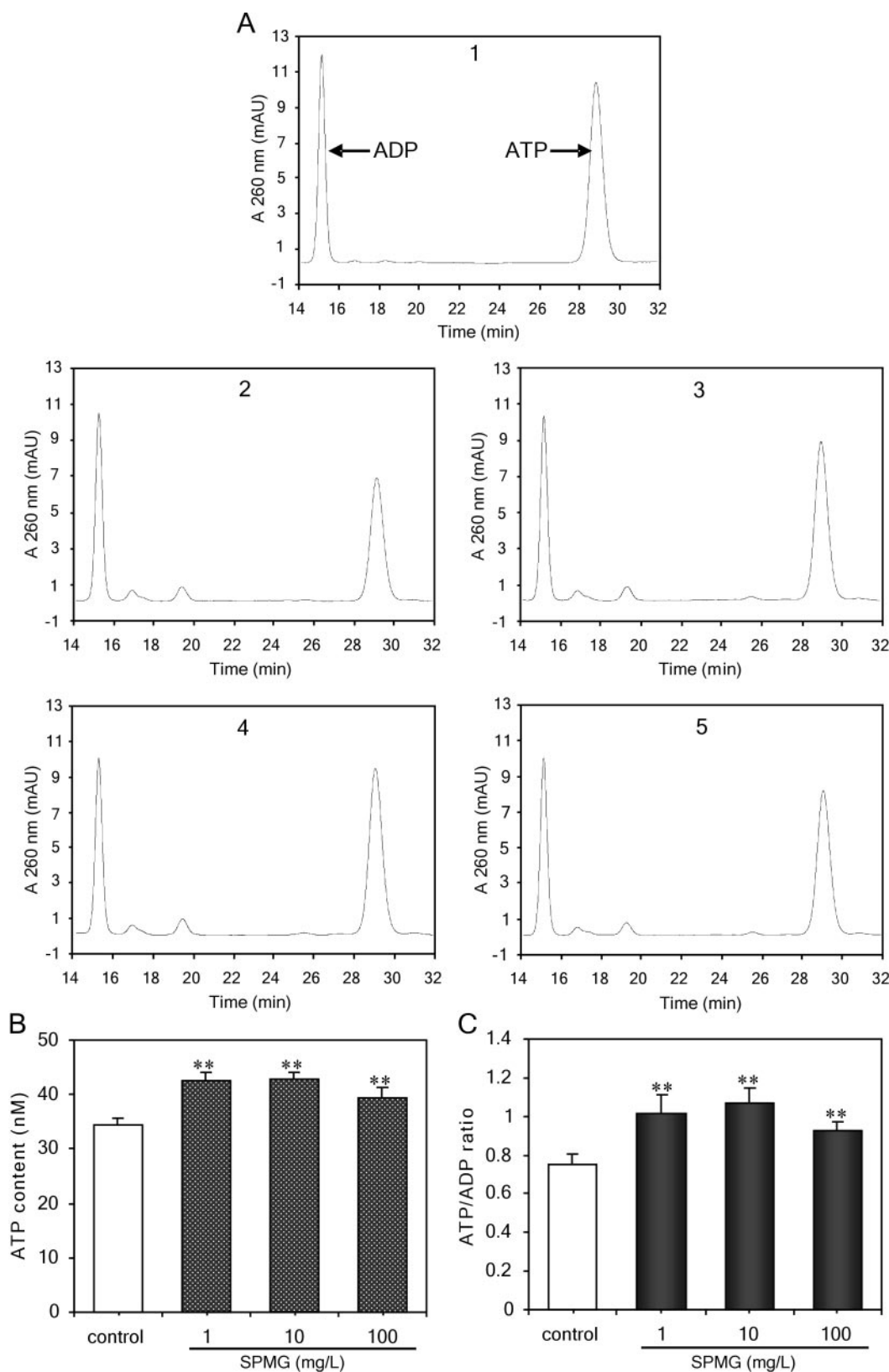
**Sulfated Polymannuroguluronate Protects Mitochondria against Cyto c Release and Inhibits T-Cell Apoptosis.** Numerous polysaccharides have been shown to exert antiapoptotic activities by protecting cells from oxidative damage (Liu et al., 1997; Sun et al., 2004). More importantly, SPMG has manifested potential anti-AIDS efficacy in persons infected with HIV where apoptotic T-cell death seems frequent. We therefore want to elucidate whether SPMG is able to protect T cells against apoptosis and thus explain its anti-AIDS activities in detail. Because aging T lymphocytes represent a good surrogate model for evaluation of oxidative stress- and apoptosis-involved events in mitochondria in many diseases, including HIV infection (Schindowski et al., 2001; Sastre et al., 2003), we selected this type of T cell in the subsequent experiments.

The percentage of hypodiploidy reflecting the degree of apoptosis was measured by flow cytometric analysis after PI staining. Results indicated that the percentage of hypodiploid cells in the control group was  $40.47 \pm 5.27$ , whereas those of hypodiploid cells in SPMG-treated groups (1, 10, and 100 mg/l) were significantly lower than that of the control (*P* < 0.01), yielding  $9.59 \pm 1.51$ ,  $10.63 \pm 1.72$ , and  $18.95 \pm 2.16\%$ , respectively (Fig. 1B). Electrophoresis of endonuclease-mediated DNA fragmentation was also performed to assess the effect of SPMG on T-cell apoptosis. Results showed that 1, 10, and 100 mg/l SPMG markedly suppressed DNA fragmentation and the formation of DNA ladders (Fig. 1C). All these results indicated that SPMG at optimum concentrations exerted strong antiapoptotic activities.

It has become clear that mitochondria play a central role in cell apoptosis by releasing mitochondrial apoptogenic pro-

teins. Cyto c is the most important and essential component of the apoptosome (Liu et al., 1996). Therefore, we next examined the influence of SPMG on the mitochondrial release of cyto c using Western blot analysis. As shown in Fig. 1D (top), the cytosolic cyto c levels of the SPMG-treated

groups (1, 10, and 100 mg/l) were significantly lower than that of the control group, manifesting that SPMG was able to protect mitochondria and inhibit mitochondrial release of cyto c in T cells, which accounts for the antiapoptotic activities of SPMG.



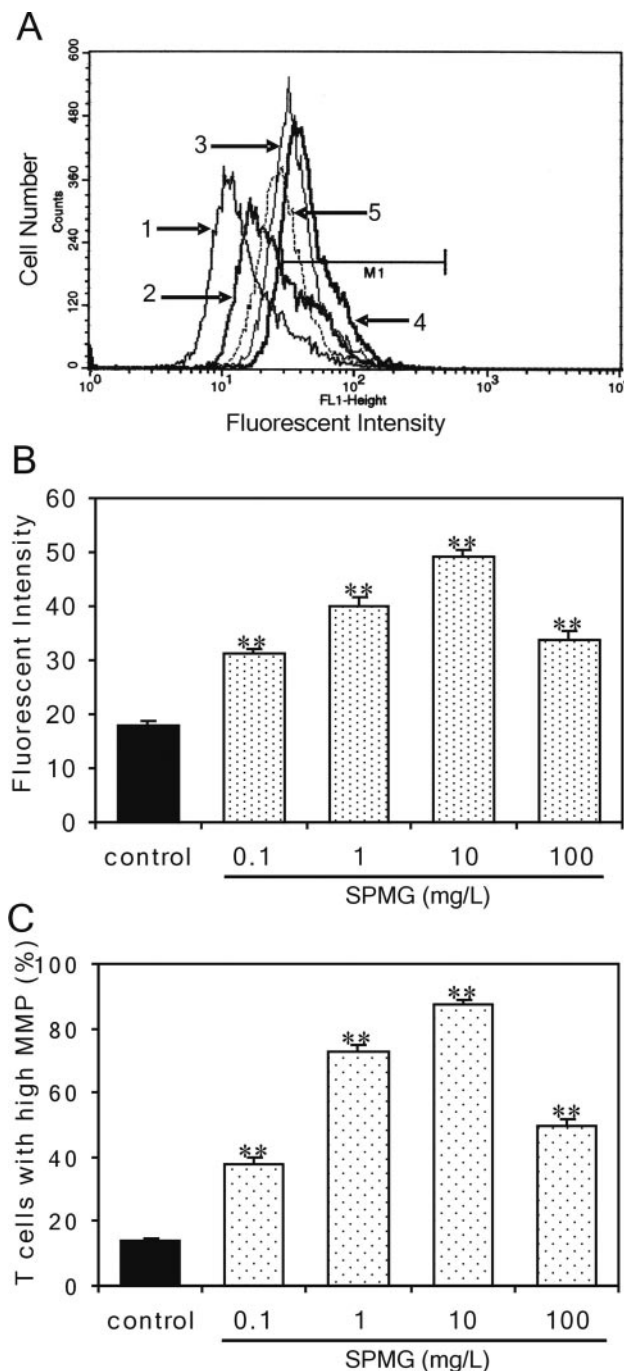
**Fig. 2.** Effects of SPMG on the ATP level and ATP/ADP ratio in T lymphocytes. A, T lymphocytes treated with 1, 10, and 100 mg/l SPMG were harvested, and adenine nucleotides were extracted in 0.5 M perchloric acid on ice for 10 min. After being neutralized to ~pH 6.5–6.8, the supernatant was examined by HPLC on a CAPCELL PAK C18 SG column. The absorbance of the eluents was monitored at 260 nm. Four parallel samples were prepared in each group, and the data shown are representative of three independent experiments with similar results. A1, standard sample; A2 control; A3, 1 mg/l SPMG; A4, 10 mg/l SPMG; and A5, 100 mg/l SPMG. B, comparison of ATP content. C, comparison of ATP/ADP ratio. \*\*,  $P < 0.01$  compared with control.

**Sulfated Polymannuroguluronate Enhances ATP Level and ATP/ADP Ratio in T Lymphocytes.** ATP level and ATP/ADP ratio are generally accepted as a very sensitive measure for the cellular energy state (Frenzel et al., 2002). Studies indicated that the cellular ATP level is an important determination for cell death and a marker of mitochondrial activity as well. The impairment of ATP level and loss of energy charge are considered as early events in apoptosis and sufficient ATP depletion will lead to apoptotic cell death (Comelli et al., 2003; Don et al., 2003; Zhelev et al., 2004). In fact, the agents facilitating the increase in ATP and keeping it at a high level will protect cells against apoptosis (Gabryel et al., 2002). Therefore, we wanted to examine whether antiapoptosis of SPMG might be attributed to the involvement of ATP. For this, we investigated the effects of SPMG on the intracellular ATP level and ATP/ADP ratio. Figure 2A shows the chromatograms obtained by HPLC with isocratic elution. The incubation of T lymphocytes with SPMG (1, 10, and 100 mg/l) resulted in a significant increase in ATP content after 12-h treatment ( $P < 0.01$ ), manifesting a rise in the efficiency of oxidative phosphorylation. The highest elevating rate was  $24.19 \pm 1.25\%$  compared with control (Fig. 2B). The ADP level exhibited only a slight decrease ( $P > 0.05$ ), but it was accompanied by a significant rise in the ATP/ADP ratio ( $P < 0.01$ ) after SPMG treatment (Fig. 2C). These data suggested that the protective effect of SPMG is related to the preservation and restoration of high ATP levels, favoring its ability to combat T-cell apoptosis.

**Sulfated Polymannuroguluronate Promotes the Mitochondrial Membrane Potential of T Cells.** The MMP is the driving force for mitochondrial ATP synthesis and plays a decisive role in cell survival (Perl et al., 2002). The disruption of MMP can induce apoptosis by influencing oxidative phosphorylation and subsequent ATP synthesis (Nagy et al., 2003). Therefore, we then investigated the effect of SPMG on the MMP in T lymphocytes using rhodamine 123 staining assay. FCM analysis indicated that SPMG at concentrations of 0.1, 1, 10, and 100 mg/l remarkably elevated the MMP of T lymphocytes (Fig. 3A). As shown in Fig. 3B, the fluorescent intensity of all T cells in the SPMG-treated groups (0.1, 1, 10, and 100 mg/l) was  $31.07 \pm 1.19$ ,  $39.96 \pm 1.51$ ,  $48.99 \pm 1.37$ , and  $33.90 \pm 1.42$  au, respectively, which was much stronger than that of the control group ( $17.96 \pm 0.97$  au;  $P < 0.01$ ). The cell percentage with high MMP was increased from  $13.68 \pm 1.27$  to  $38.04 \pm 1.88$ ,  $72.42 \pm 2.23$ ,  $87.34 \pm 1.81$ , and  $49.88 \pm 1.92$  after SPMG (0.1, 1, 10, and 100 mg/l) treatment, respectively (Fig. 3C). The enhancement of the MMP in T cells, therefore, accounts for the antiapoptotic action of SPMG.

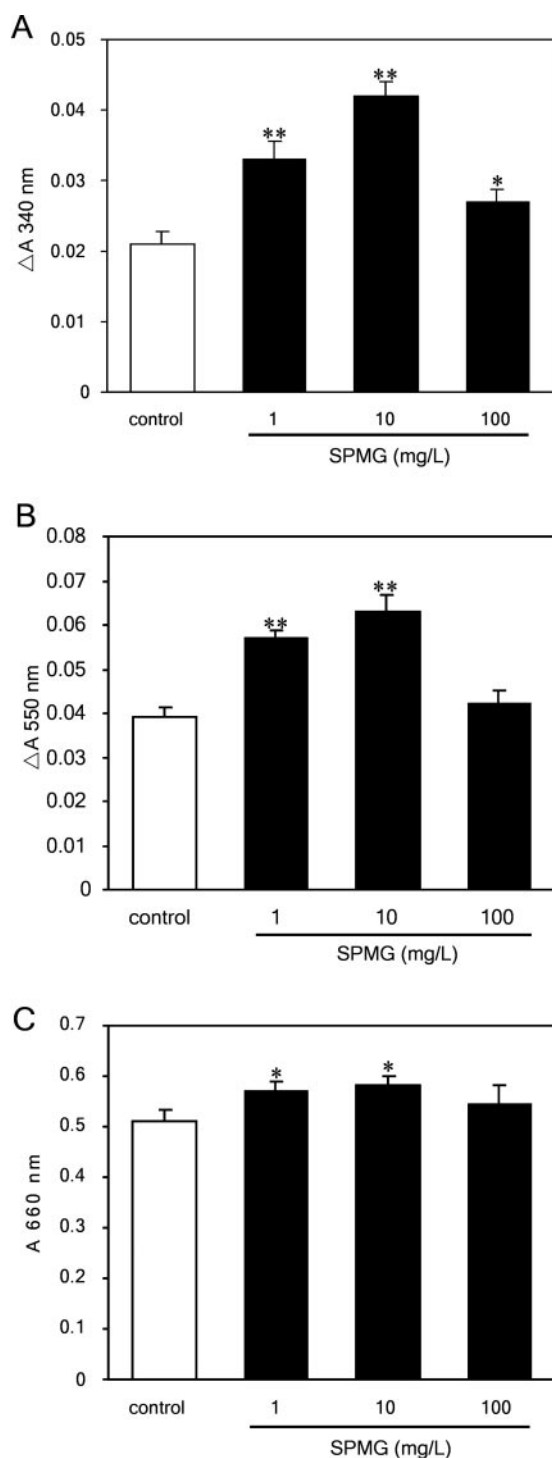
**Sulfated Polymannuroguluronate Enhances the Activities of Mitochondrial Complex I, III, and V.** Mitochondrial respiratory enzymes have been verified to play crucial roles during ATP synthesis. The enhancement in their enzymic activities favors an increase in ATP level (Du et al., 1999). SPMG was proven to elevate ATP levels in T cells and to exert protective activities against apoptosis, which prompted us to hypothesize that SPMG might affect mitochondrial respiratory enzymes. Here, we investigated the influence of SPMG on the activities of complexes I (NADH dehydrogenase), III (cytochrome *c* reductase), and V (mitochondrial ATP-synthase). As shown in Fig. 4, A and B, SPMG significantly protected and increased the enzymatic activities of complex I and III. The absorbance reduction in the SPMG-treated groups (1 and 10 mg/l) was much greater

than that of the control group ( $P < 0.01$ ). In addition, SPMG at 1 and 10 mg/l obviously enhanced the enzymatic activities of complex V. The absorbance in the SPMG-treated groups (1 and 10 mg/l) ( $0.57 \pm 0.02$  and  $0.58 \pm 0.02$ ) was higher than that of the control group ( $0.51 \pm 0.02$ ;  $P < 0.05$ ) (Fig. 4C). All these data indicated that SPMG facilitated the activities of



**Fig. 3.** Effect of SPMG on the MMP of T cells. A, T lymphocytes were incubated with 1, 10, and 100 mg/l SPMG for 12 h, and rhodamine 123 was added at a final concentration of  $1 \mu\text{M}$  and incubated for 30 min. Next, T lymphocytes were harvested, washed, and evaluated by FCM. The fluorescence intensity of all T cells (B) and the percentage of the T cells with high MMP (C) are shown. Four parallel samples were prepared in each group, and the data shown are a representative of three separate experiments with similar results. A1, control; A2, 0.1 mg/l SPMG; A3, 1 mg/l SPMG; A4, 10 mg/l SPMG; and A5, 100 mg/l SPMG. \*\*,  $P < 0.01$  compared with control.





**Fig. 4.** Effects of SPMG on complex I, III, and V activities. T-cell mitochondria were isolated after treated with SPMG (1, 10, and 100 mg/l) as mentioned under *Materials and Methods*. A, mitochondrial complex I activity was measured by the addition of 50  $\mu\text{M}$  CoQ1 to the reaction mixture, and the changes in absorbance at 340 nm were examined. B, after the reaction was initiated by addition of 15  $\mu\text{M}$  ubiquinol to the reaction mixture, the activity of mitochondrial complex III was measured by examining the changes of absorbance at 550 nm. C, to examine the activity of complex V, the reaction was initiated by the addition of 10 mM ATP and 10 mM  $\text{MgCl}_2$  and stopped by the addition of ice-cold 5% perchloric acid. The supernatant was obtained, and the absorbance was measured at 660 nm after the addition of molybdate reagent. Four parallel samples were prepared in each group, and the data shown are representative of three independent experiments with similar results. \*,  $P < 0.05$  and \*\*,  $P < 0.01$  compared with control.

these mitochondrial respiratory enzymes, allowing the preservation and restoration of high ATP levels of T cells by SPMG.

**Sulfated Polymannuroguluronate Exerts Antioxidative Activities.** Many studies have shown that mitochondria are one of the major sources of damaging free radicals and ROS in cells, and they are also a major target of these species (Cardoso et al., 1999; Sastre et al., 2003). In fact, mitochondria suffer oxidative damage more easily because of their continual exposure to the accumulated ROS, which contributes to apoptosis of cells (Murphy and Smith, 2000). All these notions, together with the fact that SPMG simultaneously enhanced MMP and mitochondrial respiratory enzymes activities, led us to presume that SPMG might exert antioxidative actions. To confirm this, we next investigated the antioxidative activities of SPMG. Flow cytometric analysis showed that SPMG treatment significantly decreased the fluorescent intensity coming from DHR conversion. The fluorescent intensity in SPMG (1, 10, and 100 mg/l) groups ( $46.57 \pm 2.42$ ,  $41.83 \pm 2.34$ , and  $45.92 \pm 1.96$  au) was much fainter than that of the control group ( $58.61 \pm 3.15$  au;  $P < 0.01$ ), indicating the capability of SPMG to reduce aging-induced augment in mitochondrial ROS levels (Fig. 5, A and B). It is noteworthy that such antioxidative activities of SPMG provided another mechanistic explanation of its anti-apoptosis activities.

Next, we further studied the free radical scavenging activity of SPMG in cell-free system, with reductive glutathione as control. SPMG exerted a significant inhibition on  $\text{O}_2^-$  chemiluminescence in a concentration-dependent manner with an  $\text{IC}_{50}$  of 650 mg/l (Fig. 5C). And as shown in Fig. 5D, SPMG exhibited a strong scavenging action on  $\cdot\text{OH}$  with an  $\text{IC}_{50}$  of 450 mg/l. The inhibitory effect of SPMG on  $\cdot\text{OH}$  chemiluminescence augmented with increasing amounts of SPMG. Both of the scavenging activities of SPMG on  $\text{O}_2^-$  and  $\cdot\text{OH}$  were a little lower than those of reductive glutathione. It was also shown that SPMG had a stronger scavenging activity on  $\cdot\text{OH}$  than that on  $\text{O}_2^-$ . The free radical-scavenging abilities of SPMG might block ROS generation from the start and thus reduce the accumulation of ROS in mitochondria, accounting for the antioxidative activities of SPMG.

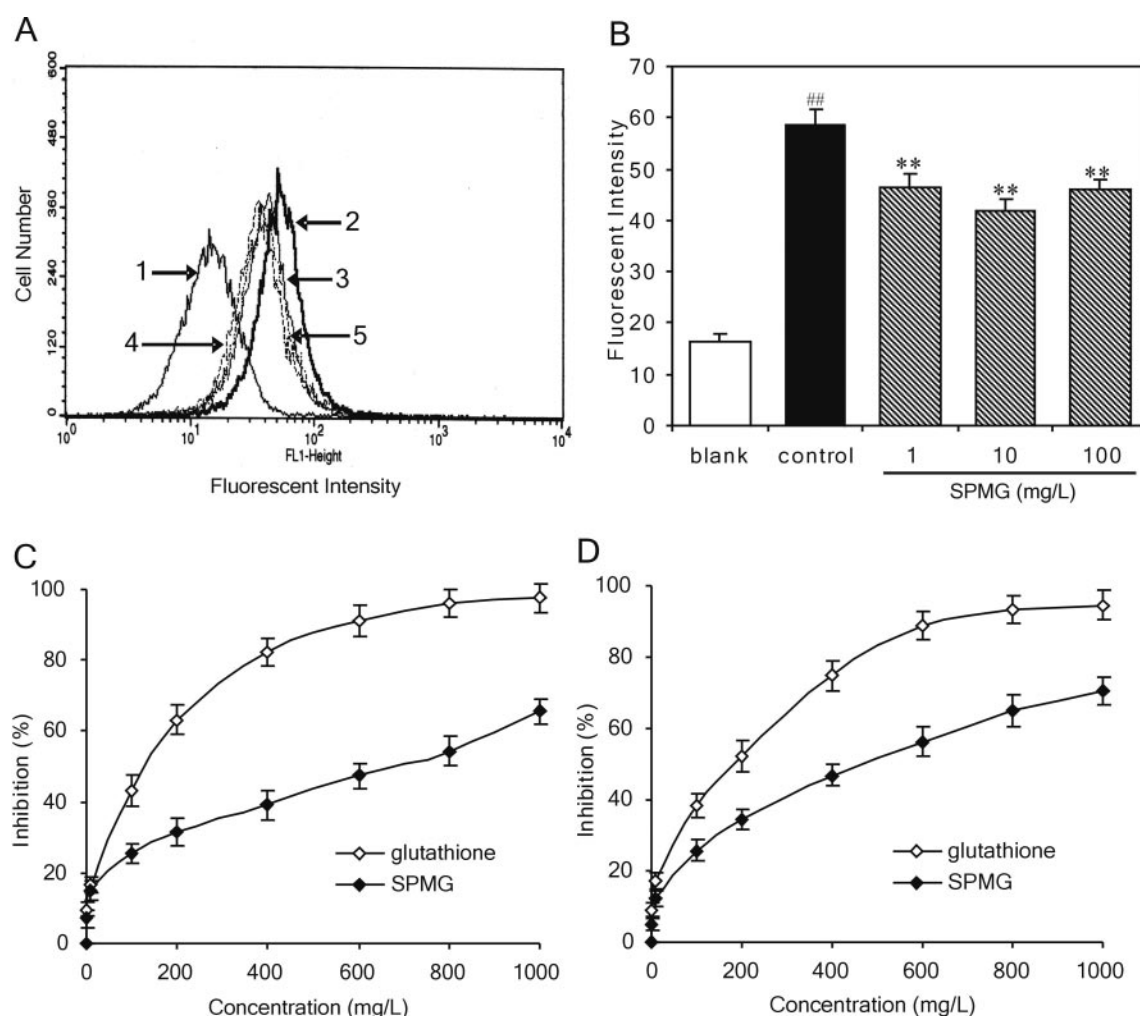
**Sulfated Polymannuroguluronate Enters T-Cell Mitochondria.** It is known that polysaccharides can exert their bioactivities via directly binding to receptors or partners in immunocytes (Honda et al., 1994; Willment et al., 2001). All these led us to postulate that there may be SPMG binding sites (receptors) in T lymphocytes. We first examined the possible binding of SPMG to T cells. FCM analysis showed that the fluorescent intensity of T lymphocytes in the SPMG-FITC group ( $22.46 \pm 1.41$  au) was much stronger than that of the control group ( $7.72 \pm 0.23$  au;  $P < 0.01$ ) (Fig. 6A). We then confirmed this finding using confocal microscopy analysis. As shown in Fig. 6B, T lymphocytes of the SPMG-FITC group showed bright greenish yellow fluorescence, whereas control group exhibited no fluorescence, indicating a significant amount of SPMG binding in T lymphocytes. It is noteworthy that the analysis of the confocal microscope slices further verified that SPMG entered T cells and was engaged with binding partners in cell cytoplasm (optical sections of 5  $\mu\text{m}$  in the  $z$ -axis through the center of T cells are shown). All these observations then raised the possibility that SPMG might enter the mitochondria.

We then confirmed the possibility of SPMG entering the mitochondria with FITC labeled probe of SPMG. Data from spectrofluorometer examination showed that after incubation with SPMG-FITC, the fluorescence intensity of mitochondrial homogenate was enhanced gradually compared with control (Fig. 6, C and D). The increase in incubation time (from 3 to 12 h) and SPMG concentration (from 1 to 100 mg/l) resulted in incremental increase in the fluorescence intensity of the mitochondrial homogenate of T cells (from  $0.39 \pm 0.07$  to  $5.47 \pm 0.22$  and from  $1.39 \pm 0.09$  to  $5.47 \pm 0.22$ , respectively). These data confirmed the entrance of SPMG into T-cell mitochondria in a time- and concentration-dependent manner. The capability of SPMG to target mitochondria, together with its antioxidative actions, can effectively protect mitochondria from oxidative damage and subsequently protect T cells against apoptosis.

**Sulfated Polymannuroguluronate Binds to the Mitochondrial Import Receptor and ADP/ATP Carrier in T-Cell Mitochondrial Membrane.** The above-mentioned studies substantially supported that SPMG entered the mi-

tochondria. We next want to identify and characterize the possible engagement of SPMG with mitochondria. For this, membrane protein preparation was first obtained from T lymphocytes. After solubilization, the preparation was applied to the SPMG-Sepharose affinity column and eluted with linear gradient of NaCl (0.15–2 M) in Tris buffer. As a result, numerous proteins were eluted at NaCl concentrations between 0.15 and 1 M. At the range from 1 to 2 M NaCl, a single symmetric peak with relatively less proteins was obtained, which was subsequently applied to LC-MS/MS analysis (Fig. 7A).

A full-scan spectrum from LC-MS/MS analysis is shown in Fig. 7B. It was confirmed by MS/MS analysis that SPMG can engage with both the mitochondrial import receptor and AAC, which lie in the outer and inner mitochondrial membrane, respectively. Table 1 lists all the identified peptides by MS/MS matching of these two proteins. The MS/MS spectrum of the mass peak at  $m/z$  2372.61 of mitochondrial import receptor and that at  $m/z$  2798.10, which is involved in AAC, are shown in Fig. 7, C and D, respectively, as examples.



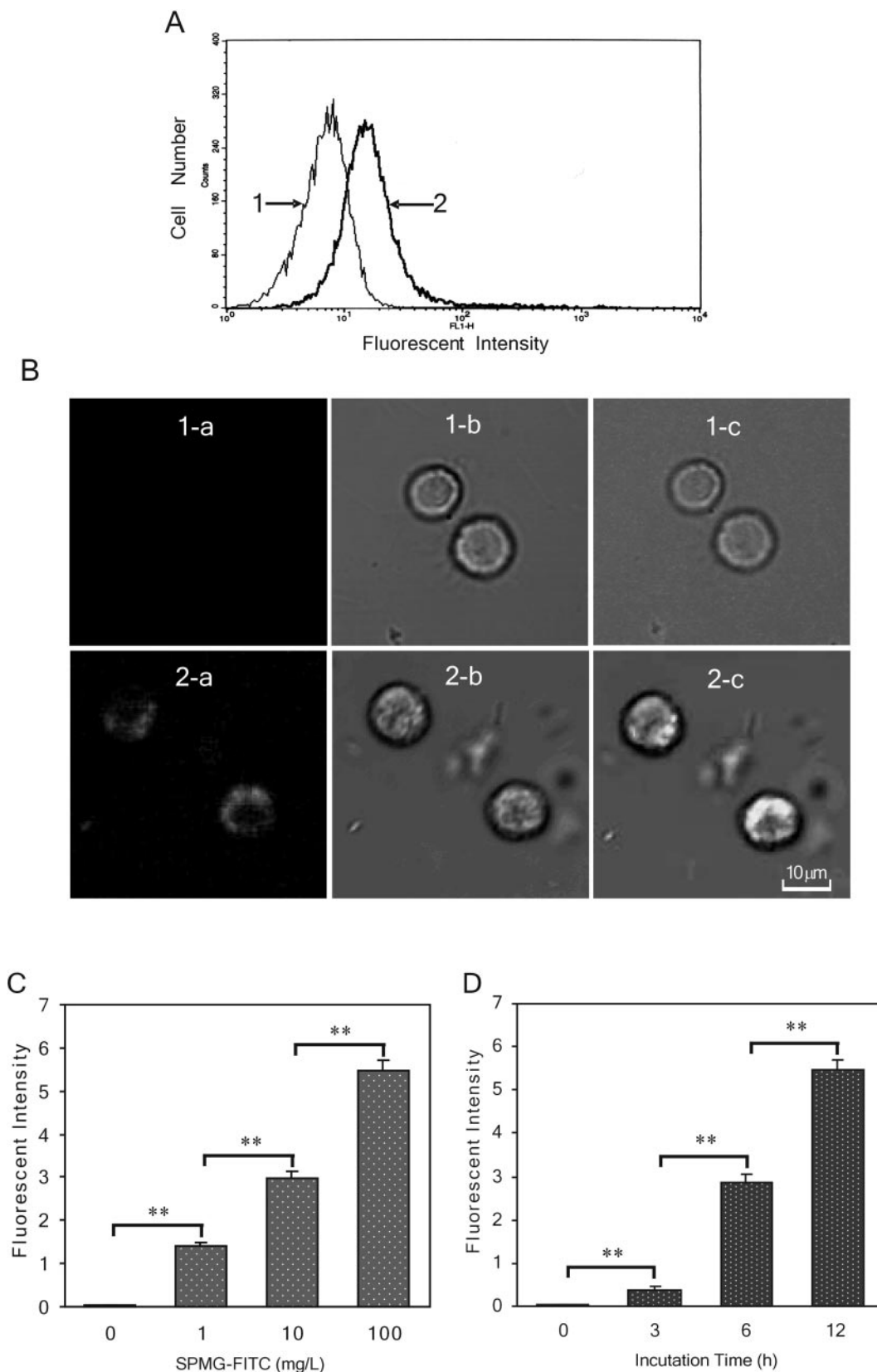
**Fig. 5.** Analysis of antioxidative activities of SPMG. A and B, SPMG at 1, 10, and 100 mg/l were added to T cells and incubated for 12 h at 37°C. Then, T lymphocytes were harvested and loaded with 10  $\mu$ M DHR 123 for 30 min at 37°C. Next, T cells were washed with PBS and cellular fluorescence was acquired using FCM. The scavenging ability of SPMG on superoxide radical ( $O_2^-$ ) was evaluated in pyrogallol-luminol system (C), and the scavenging activity of SPMG on hydroxyl radical ( $\cdot OH$ ) was evaluated in  $V_C$ - $Cu^{2+}$ -yeast suspension-luminol- $H_2O_2$  system (D). Reductive glutathione at the same concentrations was used as control. Four parallel samples were prepared in each group, and the data shown are representative of three independent experiments with similar results. A1, blank; A2, control; A3, 1 mg/l SPMG; A4, 10 mg/l SPMG; and A5, 100 mg/l SPMG. <sup>##</sup>,  $P < 0.01$  compared with blank and <sup>\*\*</sup>,  $P < 0.01$  compared with control in B.



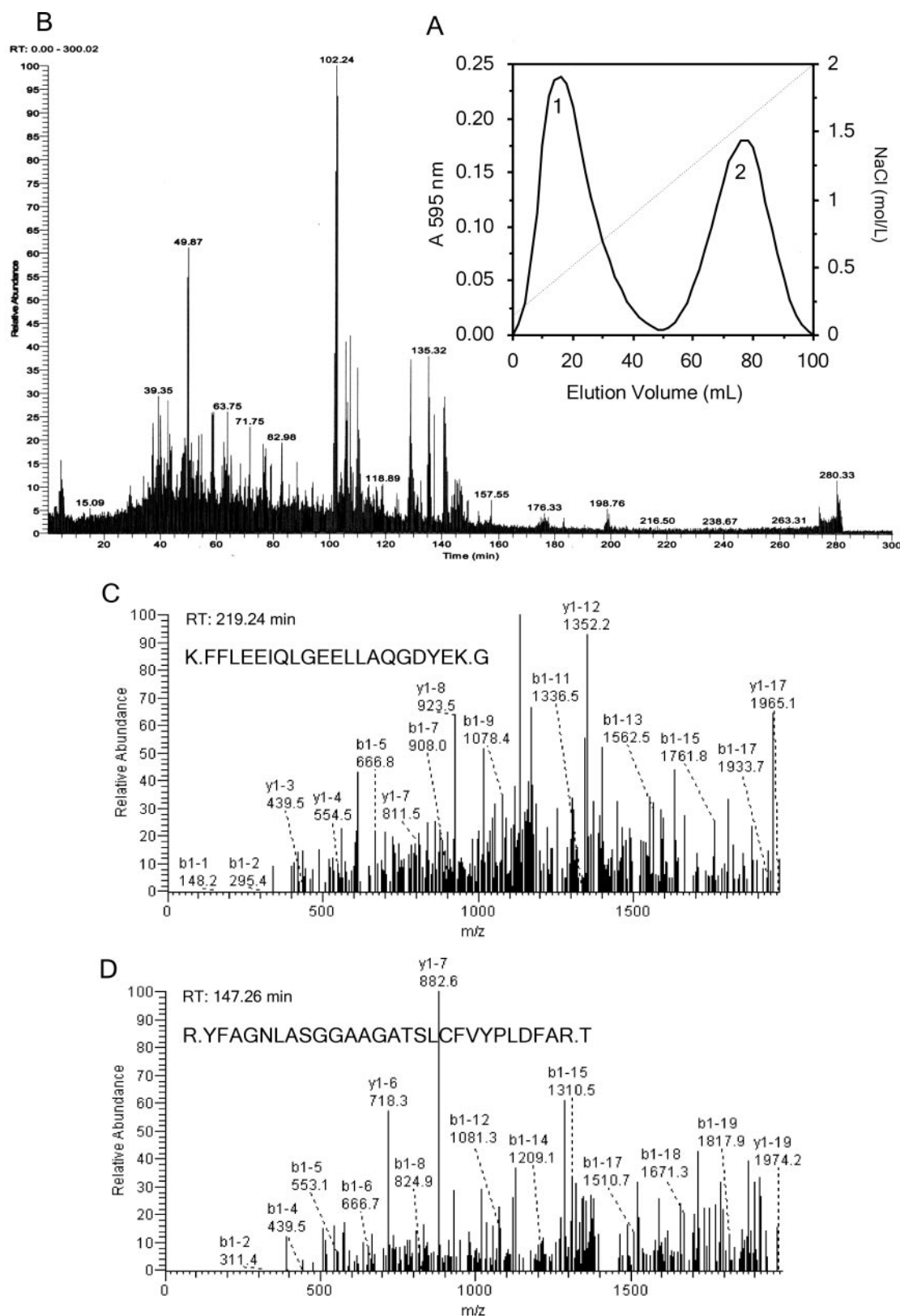
The binding of SPMG to these two transporting receptors in both outer and inner membrane of mitochondria might give us a good explanation of the transport mechanism of SPMG into the mitochondria.

## Discussion

The key function of mitochondria in cells is to provide ATP by oxidative phosphorylation (Saraste, 1999; Perl et al.,



**Fig. 6.** Binding and entrance of SPMG to T cells and to T-cell mitochondria. A, thymocytes were seeded, and SPMG-FITC was added at 100 mg/l. After 6-h incubation at 37°C, T cells were harvested, washed, and analyzed by FCM. An aliquot of each sample was spotted on a slide, analyzed, and photographed under a confocal laser scanning microscope. B, optical sections of 5  $\mu$ m in the z-axis through the center of T cells were shown. To elucidate the entrance of SPMG into T-cell mitochondria, T lymphocytes were incubated with 1, 10, and 100 mg/l SPMG-FITC for 12 h at 37°C (C) or with 100 mg/l SPMG-FITC at 37°C for 3, 6, or 12 h, respectively (D). Next, T cells were harvested, washed, and homogenized, and mitochondria were isolated and lysed by homogenizing on ice. The supernatant was obtained and measured by spectrofluorometer. Four parallel samples were prepared in each group, and the result shown is representative of three separate experiments with similar results. 1, control; 2, SPMG-FITC. \*\*,  $P < 0.01$  compared with the front group in C and D.



**Fig. 7.** Purification and LC-MS/MS analysis of SPMG binding mitochondrial import receptor and ADP/ATP carrier protein. The solubilized thymocyte membrane preparations were applied to a SPMG-Sepharose affinity column, and the SPMG-bound proteins were eluted with 0.15 to 2 M linear gradient of NaCl in Tris buffer, pH 7.4, containing 0.1% Triton X-100 (v/v). The eluted proteins were estimated with 0.01% (w/v) Coomassie Brilliant Blue G-250 in ethanol, phosphoric acid, and water [1:2:20 (v/v)], and the absorbance was measured at 595 nm (A). The fraction 2 with higher affinity was applied to LC-MS/MS analysis. The proteins were reduced, alkylated, and digested. After separation by a reverse-phase capillary column, the sample was analyzed by electrospray ionization-MS/MS. The data shown are representative of three independent experiments with similar results. A, elution curve of the membrane proteins binding to SPMG. B, full-scan spectrum by LC-MS/MS analysis. C, MS/MS spectrum of the mass peak at  $m/z$  2372.61 of mitochondrial import receptor. D, MS/MS spectrum of the mass peak at  $m/z$  2798.10 of AAC.

2002). Mitochondria are also a major source of free radicals and ROS and a target for their damaging effects (Frenzel et al., 2002; Fang and Beattie, 2003). A large body of evidence indicates that during aging, free radical and ROS production by mitochondria sharply increases. These findings are accompanied by the impairment in mitochondrial function and morphology, including a decline in MMP, an increase in mitochondrial release of apoptogenic molecules, a decrease in activities of electron transport chain complexes, and a subsequent depletion of mitochondrial energy production (Schindowski et al., 2000; Sastre et al., 2003). All these as a consequence trigger cell apoptosis to a great extent both in vivo and in vitro (Schindowski et al., 2001). Therefore, cells from aging rats have been accepted as a good model for evaluating the mitochondrial oxidative stress-associated apoptosis.

T lymphocytes infected with HIV can enhance production of ROS, which may result in severe oxidative stress in HIV-infected patients. Such increase in oxidative damage can cause the depletion of ATP level and loss of energy charge, threatening T-cell homeostasis and integrity and leading to apoptotic cell death of patients. All these favor further viral replication and accelerate the progression of AIDS (Pace and Leaf, 1995; Olinski et al., 2002). Strategies to prevent mitochondria from oxidative damage and to restore mitochondrial functions may provide new therapies for HIV infection (Murphy and Smith, 2000). In fact, a very wide range of antioxidants have been claimed to inhibit HIV infection in T cells and to offer protection against the development of AIDS (Schreck et al., 1992; Jaruga et al., 2002). It is conceivable that compounds increasing ATP supply will exert antiapoptotic functions against severe T-cell depletion caused by HIV infection (Gabryel et al., 2002). In our studies, the SPMG supplementation significantly enhanced ATP/ADP ratio and kept ATP at a high level. This finding supports a theory that the maintenance of ATP energy supply accounts for the antiapoptotic activities of SPMG.

The MMP is increasingly recognized to be the driving force for mitochondrial ATP synthesis and to play a decisive role in cell survival (Perl et al., 2002). Disruption of MMP is thought to be a significant factor in the induction of apoptosis (Nagy et al., 2003). SPMG dramatically increased the MMP of T cells, accompanied by the reduction of the cyto c release from mitochondria. We suspect that this mechanism underlies the abilities of SPMG to promote ATP synthesis and to protect T cells against apoptosis. In addition, mitochondrial respiratory enzymes have been verified to play crucial roles in ATP synthesis. The enhanced enzymic activities will facilitate the efficiency of oxidative phosphorylation and consequently ATP level and energy charge (Du et al., 1999; Monteiro et al., 2004). In the present study, SPMG potentially increased the

activities of respiratory enzymes, including complex I, III, and V, which may give us a good explanation for the elevated actions of SPMG on ATP levels in T cells.

The mitochondrial electron transport chain is an important source of ROS, which in turn drives the mitochondria to be continually exposed to the accumulated ROS. Therefore, the mitochondria are more vulnerable to oxidative damage than the rest of the cell (Cardoso et al., 1999; Murphy and Smith, 2000; Fang and Beattie, 2003). In fact, ROS-induced collapse of MMP; mitochondrial release of apoptotic factors, including cyto c; and intracellular ATP depletion are commonly accepted as the causes of apoptotic cell death (Frenzel et al., 2002; Comelli et al., 2003; Nagy et al., 2003). Numerous polysaccharides have been identified to scavenge free radicals and protect cells from death, due to their ability to degrade the excessive free radicals and ROS (Liu et al., 1997). SPMG protected mitochondria from oxidative damage by targeting mitochondria and scavenging free radicals effectively, including  $O_2^-$  and  $\cdot OH$ . These abilities of SPMG might block ROS generation from the start and thus reverse the accumulation of ROS in mitochondria, accounting for the antioxidative and antiapoptotic activities of SPMG.

Increasing evidence supports the concept that polysaccharides can exert their bioactivities via directly binding to receptors or partners in immunocytes (Honda et al., 1994; Willment et al., 2001). In the present study, we found that SPMG not only binds to and enters T cells but also targets the mitochondria. Further investigation using SPMG affinity chromatography and subsequent LC-MS/MS analysis supported a theory that SPMG binds to both mitochondrial import receptor and ADP/ATP carrier protein, which lie in the outer and inner membrane of mitochondria, respectively. These notions indicate that targeting of SPMG on T-cell mitochondria is a receptor-mediated event, which in turn underlies the antiapoptotic activities of SPMG on T cells.

In summary, we have demonstrated for the first time that SPMG exhibited antiapoptosis of T cells via targeting mitochondria by scavenging free radicals and decreasing ROS accumulation, thereby protecting mitochondria against cyto c release and improving the ATP energetic status. The restoration of SPMG on ATP depletion is probably caused by its enhancement in MMP and activation of the mitochondrial respiratory enzymes via binding to the mitochondrial importer receptor and AAC receptor in mitochondria. All these effects might shed new light on understanding the anti-AIDS activities of SPMG, particularly its correction of the immune deficiency and reversal of the excessive T-cell depletion upon HIV infection. The explicit mechanisms of SPMG underlying apoptosis-involved physiological depletion of T lymphocytes in the course of viral infection need to be further elucidated,

TABLE 1

LC-MS/MS characterization of SPMG binding mitochondrial import receptor and ADP/ATP carrier protein

Peptide Sequence	MH <sup>+</sup>	Charge	Xcorr	Delta Cn	Ions
Mitochondrial import receptor					
KFFLEEIQLGELLAQGDDYK	2372.61	2	2.4203	0.4860	13/38
RIVSAQSLAEDDVE	1376.45	1	2.2394	0.2902	11/24
ADP/ATP carrier protein					
KDFLAGGVAAAISKT	1220.40	1	2.4739	0.2121	13/24
KLLQVQHASKQ	1137.36	2	2.3371	0.1657	13/18
RKGTDIMYTGTLDLCWRK	1818.04	2	4.2899	0.4993	21/28
RYFAGNLASGGAAGATSLCFVYPLDFART	2798.10	2	3.5019	0.4348	18/52
RYFPTQALNFAFKD	1447.66	1	2.4522	0.3115	13/22



which will provide proof of principle for SPMG in immune silencing-associated anti-AIDS function.

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