ORIGINAL ARTICLE

The effects of carboxymethyl chitosan on the regulation of the proliferation, differentiation and cytokine expression of peripheral blood mononuclear cells

Wenhua Xu^{1,2}, Baoqin Han², Yee Liang³, Xiaoying Kong², Mi Rong² and Wanshun Liu²

To elucidate the effects of carboxymethyl chitosan (CMCTS) on the proliferation, differentiation and cytokine secretion of peripheral blood mononuclear cells (PBMCs), leukomonocytes were separated and cultured. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays, acridine orange/ethidium bromide staining, flow cytometry and enzyme-linked immunosorbent assay was used to evaluate different concentrations of CMCTS A (molecular weight (MW) = 50 000) and CMCTS B (MW = 500 000) in the proliferation, apoptosis, differentiation and cytokine secretion of PBMCs. The results demonstrated that CMCTS A and CMCTS B at concentrations of 500 and 100 $\mu\text{g ml}^{-1}$, respectively, significantly enhanced the proliferation of PBMCs. Both CMCTS A and CMCTS B inhibited the apoptosis of PBMCs, and the optimal inhibitory concentration was 500 $\mu\text{g ml}^{-1}$. Furthermore, the inhibitory efficacy of CMCTS A was higher than that of CMCTS B. CMCTS A induced the differentiation of PBMCs with CD4 antibody at concentrations of 500 and 100 $\mu\text{g ml}^{-1}$. Furthermore, the differentiation of PBMCs with CD3–CD56 antibodies was induced by both CMCTS A and CMCTS B. Both CMCTS A and CMCTS B at concentrations of 500 $\mu\text{g ml}^{-1}$ induced interleukin (IL)-2 secretion, with CMCTS A having a more marked effect than that of CMCTS B. CMCTS A at a concentration of 500 $\mu\text{g ml}^{-1}$ induced interferon (IFN)- γ secretion. In conclusion, CMCTS with a low MW (50 000) and at a concentration of 500 $\mu\text{g ml}^{-1}$ positively regulated the proliferation, differentiation and IFN- γ and IL-2 secretion of the PBMCs *in vitro*.

Polymer Journal (2013) **45**, 226–232; doi:10.1038/pj.2012.118; published online 11 July 2012

Keywords: carboxymethyl chitosan; IFN- γ ; IL-2; immunity; PBMCs

INTRODUCTION

The magnitude of the immunity and the integrity of the immune system have a close connection with the health of human life. The inhibition of immune function is the primary cause of the decrease in immune function as well as cancer and other diseases.

Chitosan and its derivatives have a comprehensive effect on the regulation of immune functions. These compounds promote the proliferation of macrophagocytes and fibroblasts, induce cellular and antitrope immunity and the complement system and exterminate pathogenic bacteria. Consequently, chitosan and its derivatives function to induce the immune system and promote the repair process of the immune system after being affected by diseases. In addition, chitosan with 70% deacetylation can promote the production of interferons (IFNs) and cytokines.

A previous study has reported that traditional carbohydrates, such as starch and cellulose, could not activate T cells and could not stimulate the body to generate immunological memory. Therefore,

traditional carbohydrates are thymus-independent antigens. However, zwitterionic polysaccharides could activate CD4⁺ T cells and produce relevant inflammatory factors or anti-inflammatory factors to mediate the occurrence or prevention of abscessus.¹

There have been some reports describing the solubility, chelation and antiplogistic ability of CM-chitosan, but there have been few studies on its biological properties as a type of immunomodulator. CM-chitosan and CM-chitin exhibit the polymeric properties of zwitterions; therefore, they can have structures with different dimensions and configurations, which endow them with several biological activities.²

The purpose of this study was to investigate the immunological enhancement effects of carboxymethyl chitosan (CMCTS) *in vitro*. In this study, CMCTS A (molecular weight (MW) = 50 000) and CMCTS B (MW = 500 000) were prepared at different concentrations and used to analyze their effects on the proliferation and secretion of cytokines. The results of this study have provided important evidence

¹Department of Public Health, Medical College, Qingdao University, Qingdao, China; ²College of Marine Life Sciences, Ocean University of China, Qingdao, China and ³Affiliated Hospital of Medical College of Qingdao University, Qingdao, China

Correspondence: Professor W Liu, College of Marine Life Sciences, Ocean University of China, Qingdao 266003, China.

E-mail: wanshunliu@hotmail.com

Received 21 December 2011; revised 19 April 2012; accepted 26 April 2012; published online 11 July 2012

for the use of these compounds in clinical practice. Further investigation of the effects of CMCTS on lymphocytes is still required to better understand its targets at the cellular and molecular levels.³

MATERIALS AND METHODS

Materials

O,N-CMCTS A (MW = 50 KD, degree of carboxymethylation = 105.65%, degree of deacetylation = 97.74%), O,N-CMCTS B (MW = 500 KD, degree of carboxymethylation = 106.79%, degree of deacetylation = 98.48%), chitosan A (MW = 50 KD, degree of deacetylation = 97.89%) and chitosan B (MW = 500 KD, degree of deacetylation = 98.93%) were supplied by the Qingdao Biotemed Biomaterial Co., Ltd (Qingdao, China). A mouse interleukin-2 (IL-2) enzyme-linked immuno sorbent assay (ELISA) kit and a mouse IFN- γ ELISA kit were procured from R&D (Lorton, VA, USA). All other chemicals were of reagent grade.

Preparation of peripheral blood mononuclear cells (PBMCs)

A volume of 50 ml of human venous blood was collected and anticoagulated using sodium citrate. The blood was then centrifuged at 700 g for 20 min at room temperature. Three layers formed: the superstratum plasma layer, the middle buffy coat layer and the RBCs pellet. The superstratum was inactivated at 56 °C for 30 min and then maintained at room temperature for 15 min followed by coagulation at 800 g for 25 min at 4 °C. The superstratum was obtained as autologous plasma. Phosphate-buffered saline (PBS) was added to the middle layer to a volume of 25 ml and then mixed with 20 ml of PBMC separation medium. The mixture was then coagulated at 800 g for 20 min. The middle layer PBMC was transferred to another centrifuge tube, washed with 20 ml of PBS and then coagulated with 500 g for 8 min; finally, the bottom layer was obtained. The cells were washed three times and then re-suspended in 12 ml of GT-T551 medium (GIBCO, Pascagoula, MD, USA). The cells were transferred into three culture flasks, and each of the flasks was supplemented with 1600 μ l of autologous plasma.⁴ All cells were grown in a humidified atmosphere at 37 °C (5% CO₂) under pyrogen-free conditions. The cells were routinely checked for and were free of mycoplasma.⁵

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) analysis

The PBMCs were cultured in six 96-pore TPCS at a concentration of 10⁶ cells ml⁻¹. Among the six plates, two plates were controls containing PBMCs that were cultured using normal methods. In the test group, the PBMCs in two plates were cultured using CMCTS A, which has a MW of 50 kDa, and the other two plates were cultured using CMCTS B, which has a MW of 500 kDa. Both the CMCTS A group and the CMCTS B group were divided into four concentrations: 1000, 500, 100, 50 and 10 μ g ml⁻¹. The control group cells were treated with normal culture media that did not contain CMCTS. The cells were observed, and images were collected under a microscope daily. To assess the effects of the different concentrations of CMCTS A and CMCTS B on the proliferation and viability of the PBMCs, the samples were analyzed using the MTT mitochondrial activity assay method at days 2 and 4 after cell seeding.

Acridine orange (AO)/ethidium bromide (EB) staining

The PBMCs were cultured in three 96-pore TPCS at a concentration of 10⁶ per ml. The three plates were the control, which contained the PBMCs that were cultured using normal methods, the CMCTS A group (the PBMCs were cultured with CMCTS A), and the CMCTS B group (the PBMCs were cultured with CMCTS B). Both the CMCTS A group and the CMCTS B group were divided into five concentrations: 1000, 500, 100, 50 and 10 μ g ml⁻¹.⁶ The control group cells were treated using normal culture media that did not contain CMCTS. AO was dissolved in a PBS solution at a concentration of 100 mg l⁻¹, and EB was dissolved in a PBS solution at a concentration of 100 mg l⁻¹; the two solutions were then mixed at the same concentration and the AO/EB dye mixtures were obtained. A volume of 4 μ l of the AO/EB dye mixture was mixed with 100 μ l of the cell suspensions at the eight day after cell seeding. One drop of the stained cells was placed on a glass slide and covered

with a cover slip. Images were then collected under a fluorescence microscope. A total of 200 cells were counted to identify the cell morphology: VN, a living cell that had green, normally structured chromatin; VA, an apoptotic cell at the prophase that had green but pyknotic chromatin; and NVN, a dead, but not apoptotic, cell that had orange, normally structured chromatin. NVA, an apoptotic cell in the late phase that had orange but pyknotic chromatin. The apoptosis rate was calculated as follows: $P = (NVN + NVA) / (VN + VA + NVN + NVA) \times 100\%$.

Flow cytometry

PBMCs (at least 1×10^6 cells) cultured with 1000, 500, 100, 50 or 10 μ g ml⁻¹ of CMCTS A or CMCTS B were collected. PBMCs cultured with 1000, 500, 100, 50 or 10 μ g ml⁻¹ CTS A or CTS B were also collected. The control group cells were treated using normal culture media that did not contain CMCTS. The cells were then washed using a PBS buffer, which was subsequently incubated with anti-human-CD3-fluorescein isothiocyanate, CD4-phycoerythrin and CD8-phycoerythrin or CD56-phycoerythrin monoclonal antibody in the same buffer. Finally, the cells were analyzed using a flow cytometer to detect the subgroup of helper T lymphocytes (CD3⁺CD4⁺), inhibitory T lymphocytes (CD3⁺CD8⁺) and the natural killer (NK) cell subgroup (CD3⁺CD56⁺).⁷

ELISA detection

The PBMCs (1×10^6 per well) were cultured in the presence of CMCTS A or CMCTS B at a concentration of 1000, 500, 100, 50 or 10 μ g ml⁻¹. The control group cells were treated using normal culture media that did not contain CMCTS. After 48 h of incubation at 37 °C, the supernatants were collected and then measured for IFN- γ and IL-2 using a sandwich ELISA kit following the manufacturer's instructions. The concentrations were calculated by interpolating from the standard curves based on the recombinant cytokine dilutions run in parallel on the same plate.^{4,8}

RESULTS

Cell compatibility and MTT analysis

The results revealed that the growth of the cells in the CMCTS A and CMCTS B experimental groups at the four concentrations was better than that of the control group at both the second and the fourth day. All of the cells were uniformly round and arranged as a tight monolayer (Figures 1 and 2). The results from the MTT analysis and the SPSS software analysis (SPSS Inc., Chicago, IL, USA) (Figures 3 and 4) indicated that both CMCTS A and CMCTS B at concentrations of 500 and 100 μ g ml⁻¹ clearly ($P < 0.05$) induced the proliferation of the PBMCs compared with the control group. Furthermore, the cell growth was better in the medium containing CMCTS A than in the medium containing CMCTS B.

AO/EB staining

The apoptosis rates of the PBMCs cultured with CMCTS A at concentrations of 1000, 500, 100, 50 and 10 μ g ml⁻¹ were 15.7%, 1%, 2.5%, 10.5% and 13.9%, respectively. The apoptosis rates of the PBMCs cultured with CMCTS B at concentrations of 1000, 500, 100, 50 and 10 μ g ml⁻¹ were 17.8%, 2%, 6%, 16% and 17.6%, respectively. Furthermore, the apoptosis rates of the two groups were both lower than that of the control, which was 38% (Figures 5 and 6 and Table 1). The results revealed that the survival rate of the PBMCs was the highest at the concentration of 500 μ g ml⁻¹ of CMCTS, and that CMCTS A had a stronger effect than that of CMCTS B.

Flow cytometry

The flow cytometry results are presented in Table 2. Among our tested media, the medium containing CMCTS A at a concentration of 500 μ g ml⁻¹ resulted in the best promotion effects for the differentiation of CD4⁺ PBMCs ($P < 0.05$; Figure 7). However, the expression

Figure 1 The cell compatibility of the CMCTS A-treated PBMCs ($\times 10$). (a), control; (b–f), CMCTS A at concentrations of 1000, 500, 100, 50 and $10 \mu\text{gml}^{-1}$ on the second day, respectively. (g), control; (h–l), CMCTS A at concentrations of 1000, 500, 100, 50 and $10 \mu\text{gml}^{-1}$ at the fourth day, respectively. A full color version of this figure is available at *Polymer Journal* online.

Figure 2 The cell compatibility of the CMCTS B experimental groups and the controls. (a), control; (b–f), CMCTS B at concentrations of 1000, 500, 100, 50 and $10 \mu\text{gml}^{-1}$ on the second day, respectively. (g), control; (h–l), CMCTS B at concentrations of 1000, 500, 100, 50 and $10 \mu\text{gml}^{-1}$ on the fourth day, respectively. A full color version of this figure is available at *Polymer Journal* online.

Figure 3 The cell compatibility of the experimental groups with CMCTS A after a 2- and 4-day culture period in comparison with the control group.

Figure 4 The cell compatibility of the experimental groups with CMCTS B after a 2- and 4-day culture period in comparison with the control group.

Figure 5 AO/EB staining of the experimental groups of CMCTS A and the controls. (a), control; (b–f), CMCTS A at concentrations of 1000, 500, 100, 50 and $10\mu\text{g ml}^{-1}$ on the second day, respectively.

Figure 6 AO/EB staining of the experimental groups of CMCTS B and the controls. (a), control; (b–f), CMCTS B at concentrations of 1000, 500, 100, 50 and $10\mu\text{g ml}^{-1}$ on the second day, respectively.

Table 1 Cell Counting of the leukomonocyte cells after AO/EB staining

	VN	VA	NVN	NVA	P (%)
MW500 000, $500\mu\text{g ml}^{-1}$	197	1	1	1	1
MW500 000, $500\mu\text{g ml}^{-1}$	195	1	2	2	2
MW500 000, $100\mu\text{g ml}^{-1}$	192	3	2	3	2.5
MW500 000, $100\mu\text{g ml}^{-1}$	186	2	4	8	6
MW500 000, $50\mu\text{g ml}^{-1}$	172	7	9	12	10.5
MW500 000, $50\mu\text{g ml}^{-1}$	156	12	16	16	16
Control	98	5	36	40	38

Abbreviations: AO, acridine orange; EB, ethidium bromide; MW, molecular weight.

Table 2 Flow cytometric methods for CD4, CD8 and CD3–CD56 of the leukomonocyte cells

	CD4 ⁺ (%)	CD8 ⁺ (%)	CD3–CD56 ⁺ (%)
Control	14.33 ± 0.95	80.23 ± 0.92	0.43 ± 0.071
CMCTS 500 (MW = 50 000)	$17.82 \pm 0.89^*$	83.07 ± 0.86	$1.12 \pm 0.097^*$
CMCTS 100 (MW = 50 000)	14.73 ± 0.93	82.52 ± 0.89	$0.97 \pm 0.082^*$
CMCTS 50 (MW = 50 000)	14.64 ± 0.83	80.83 ± 0.91	0.44 ± 0.074
CMCTS 10 (MW = 50 000)	14.22 ± 0.79	80.67 ± 0.79	0.42 ± 0.063
CMCTS 500 (MW = 50 0000)	14.85 ± 0.87	81.26 ± 0.74	$0.95 \pm 0.089^*$
CMCTS 100 (MW = 500 000)	14.47 ± 0.95	82.48 ± 0.95	$0.78 \pm 0.082^*$
CMCTS 50 (MW = 500 000)	14.55 ± 0.74	81.47 ± 0.83	0.47 ± 0.068
CMCTS 10 (MW = 500 000)	14.39 ± 0.75	82.35 ± 0.86	0.46 ± 0.073

Abbreviations: CMCTS, carboxymethyl chitosan; MW, molecular weight.
 $P < 0.05$ vs control.

Figure 7 Flow cytometry for CD4⁺ PBMCs of the CMCTS A, CMCTS B and CTS experimental groups and the controls.

Figure 9 Flow cytometry for CD3-CD56⁺ PBMCs of the CMCTS A, CMCTS B and CTS experimental groups and the controls.

Figure 8 Flow cytometry for CD8⁺ PBMCs of the CMCTS A, CMCTS B and CTS experimental groups and the controls.

Figure 10 ELISA for IFN- γ of the PBMCs of the CMCTS A and CMCTS B experimental groups and the controls.

of CD8⁺ in the cultured PBMCs was not affected by either CMCTS A or CMCTS B at any of the tested concentrations (Figure 8). Furthermore, the differentiation of the CD3⁺CD56⁺ PBMCs (NK cells) was promoted by the medium containing either CMCTS A or CMCTS B at concentrations of 500 or 100 $\mu\text{g ml}^{-1}$ ($P < 0.05$) (Figure 9). In conclusion, CMCTS A induced the differentiation of both CD4⁺ PBMCs and NK cells, whereas CMCTS B only induced the differentiation of NK cells.

ELISA

The values of IFN- γ and IL-2 from the ELISA were compared with the standard curve, and then the secretion concentrations of IFN- γ and

Figure 11 ELISA for IL-2 of the PBMCs of the CMCTS A and CMCTS B experimental groups and the controls.

IL-2 were obtained. The secretion concentration of the IFN- γ of the PBMCs cultured in the media with CMCTS A at a concentration of $500 \mu\text{g ml}^{-1}$ was significantly higher ($P < 0.05$) than that of the control. However, the secretion level of the IFN- γ of the PBMCs cultured in the media with CMCTS B at all of the tested concentrations was not significantly different from that of the control (Figure 10). Furthermore, the secretion concentration of the IL-2 of the PBMCs cultured in the media with both CMCTS A and CMCTS B at a concentration of $500 \mu\text{g ml}^{-1}$ was significantly higher than that of the control ($P < 0.05$) (Figure 11). Therefore, CMCTS A could induce the PBMCs to produce both IFN- γ and IL-2 at a concentration of $500 \mu\text{g ml}^{-1}$, whereas CMCTS B at a concentration of $500 \mu\text{g ml}^{-1}$ could only induce the PBMCs to produce IL-2.

DISCUSSION

The MTT experimental results revealed that both CMCTS A and CMCTS B significantly induced the proliferation of the PBMCs. The most appropriate concentration was $500 \mu\text{g ml}^{-1}$. The CMCTS with a low MW (50000) had a more obvious effect than that of the CMCTS with a high MW (500000). The AO/EB staining results suggested that CMCTS A at a concentration of $500 \mu\text{g ml}^{-1}$ performs the best for the inhibition of cell aging among the tested conditions. The flow cytometry technique has been aptly described as 'microscopy of fast moving objects'. Flow cytometry can provide a considerable amount of information about specific cell types and can measure up to seven parameters for each cell type.⁹ The flow cytometry results revealed that CMCTS A at a concentration of $500 \mu\text{g ml}^{-1}$ induced the differentiation of CD4⁺ PBMCs. However, the differentiation of CD3⁺ CD56⁺ PBMCs was induced by both CMCTS A and CMCTS B at concentrations of 500 and $100 \mu\text{g ml}^{-1}$. The ELISA analysis results indicated that CMCTS A at a concentration of $500 \mu\text{g ml}^{-1}$ stimulated the secretion of both IFN- γ and IL-2. In contrast, CMCTS B only stimulated IL-2 secretion. The results suggested that the effects of CMCTS on the proliferation, differentiation and cytokine secretion of the PBMCs are closely related to its MW and concentration. Among the conditions tested in this study, CMCTS with a low MW had a more obvious effect than that of CMCTS with a high MW, and $500 \mu\text{g ml}^{-1}$ was the most appropriate concentration.

The experimental results suggested that CMCTS acted directly on the PBMCs. That is, CMCTS may be recognized by cell surface receptors such as toll-like receptors, which can induce cytokine secretion. The flow cytometry results demonstrated that CMCTS induced the differentiation of CD4⁺ and CD3-CD56⁺ PBMCs and secretion of IFN- γ . This result coincided with the ELISA result. In addition, published papers have reported that CMCTS acts on antigen-presenting cells to indirectly induce the proliferation of PBMCs.

IFN- γ is a pleiotropic cytokine that is endowed with potent immunomodulatory effects on a variety of immune cells *in vitro* and *in vivo*.^{10,11} This cytokine exerts its multiple biological activities by either positively or negatively controlling the expression of many genes and proteins.¹²

Generally, IFN- γ is secreted by T cells and NK cells. The optimally induced IFN- γ production in T cells occurs when these cells receive a combination of different types of signals, such as a specific or nonspecific ligand for the receptor, a balanced combination of cytokines and/or contact with accessory cells through adhesion molecules.¹⁰ In contrast, a single signal, either IL-2, PHA, PMA or calcium ionophores, can function to induce NK cells to produce this protein.^{13,14}

IL-2 is the principal growth factor for T and NK lymphocytes and is responsible for regulating the magnitude and duration of the immune response.¹⁵ The importance of IL-2 as a key cytokine for T-cell activation and immune function has extensive experimental support.¹⁶ IL-2 has a role as an auto- and paracrine growth factor during the first 48–72 h of T-cell activation.¹⁷ IL-2 stimulation via the IL-2 receptor drives the clonal expansion of the activated T cells and prompts cell differentiation to acquire effector functions.¹⁸ The appropriate production of IL-2 is an important determinant of the magnitude of T-cell-dependent immune responses.¹⁹

The results in this study suggested that CMCTS can be recognized by the polysaccharide-ligand receptors on the surface of the cell membrane to launch the signal expression pathway of IFN- γ . IL-2 secreted by T cells induces the proliferation of T cells and NK cells and subsequently induces these cells to secrete IFN- γ . Both IFN- γ and IL-2 function on T cells and NK cells to induce proliferation and inhibit apoptosis.

By inducing cell cycle progression and protecting the cells from apoptosis, IL-2 is involved in the successful execution of an immune response. Upon binding to its receptor, IL-2 activates a variety of signal transduction pathways, including the Ras/mitogen-activated protein kinase/extracellular signal-regulated kinase and Janus kinase/signal transducer and activator of transcription cascades.²⁰ The mechanism for IFN- γ to kill cancer cells and induce the apoptosis of cancer cells consists of IFN- γ reducing the level of osteoclastogenesis in response to TNF- α treatment *in vivo*. IFN- γ inhibits TNF- α -induced osteoclastogenesis in mice with T cells that had been exposed to anti-CD4 and anti-CD8 antibodies. These results provide evidence that IFN- γ directly inhibits osteoclastogenesis and induces cell apoptosis by Fas/FasL signals, leading to the indirect regulation of bone resorption.²¹ Therefore, IL-2-dependent proliferation of PBMCs and IFN- γ -induced apoptosis of cancer cells act together to maintain the immune function.

Taking the above descriptions into account, CMCTS could be used as an immunomodulator. Compared with traditional glycoconjugates, such as starch and cellulose, this type of zwitterionic polysaccharide has special advantages for the preparation of vaccines. As CMCTS is a T-cell-dependent antigen, it does not need to be combined with proteins to form glycoconjugates. Therefore, we can use the capsular polysaccharide of the infected bacteria to regulate immunity.³

CONCLUSIONS

In conclusion, CMCTS can promote the proliferation, differentiation and cytokine secretion of PBMCs and inhibit the apoptosis of PBMCs. These effects are influenced by the MW and concentration of the CMCTS. In medical surgery, CMCTS may be prepared as a type of immunomodulator to promote the application of polysaccharides for immunoregulation.

ACKNOWLEDGEMENTS

This work was supported by the Ocean University of China. We thank Professor Wanshun Liu and Professor Baoqin Han, Ocean University of China, for technical assistance on confocal microscopy and the staff at the Affiliated Hospital of Qingdao University for the preparation of leukomonocytes.

- 1 Liu, X. Y., Sun, C. X., Li, J., Ni, W. H., Zhu, J. J. & Zhou, Y. F. Preparation of zwitterionic polysaccharides and their effects on lymphocyte proliferation *in vitro* and *in vivo*. *J. Northeast Normal Univ.* **42**, 110–114 (2010).
- 2 Chang, J., Liu, W. S., Han, B. Q. & Liu, B. The evaluation on biological properties of carboxymethyl-chitosan and carboxymethyl-chitosan. *J. Ocean Univ. Chin* **7**, 404–410 (2008).
- 3 Xu, W. B., Xu, L. H., Lu, H. S., Ou-Yang, D. Y., Shi, H. J., Di, J. F. & He, X. H. The immunosuppressive effect of gossypol in mice is mediated by inhibition of lymphocyte proliferation and by induction of cell apoptosis. *Acta Pharmacol. Sin* **30**, 597–604 (2009).
- 4 Xiao, B. G., Ma, C. G., Xu, L. Y., Hans, L. & Lu, C. Z. IL-12/IFN- γ /NO axis plays critical role in development of Th1-mediated experimental autoimmune encephalomyelitis. *Mol. Immunol.* **45**, 1191–1196 (2008).
- 5 Derek, B., Fran, D. & Frances, B. Involvement of both intrinsic and extrinsic pathways in IFN- γ -induced apoptosis that are enhanced with cisplatin. *Eur. J. Cancer* **41**, 1474–1486 (2005).
- 6 Zhang, J. H., Yu, J., Li, W. X. & Cheng, C. P. Evaluation of Mn²⁺ stimulated and Zn²⁺ inhibited apoptosis in rat corpus luteal cells by flow cytometry and fluorochromes staining. *Chin. J. Physio.* **141**, 121–126 (1998).
- 7 Tsiavou, A., Hatziagelaki, E., Chaidaroglou, A., Koniavitou, K., Degiannis, D. & Raptis, S. A. Correlation between intracellular interferon- γ (IFN- γ) production by CD4⁺ and CD8⁺ lymphocytes and IFN- γ gene polymorphism in patients with type 2 diabetes mellitus and latent autoimmune diabetes of adults (LADA). *Cytokine* **31**, 135–141 (2005).
- 8 Gary, T. B., Norma, L. G., Stephen, L. H. & Denise, L. D. Expression of the chemokine MIG is a sensitive and predictive marker for antigen-specific, genetically restricted IFN- γ production and IFN- γ -secreting cell. *J. Immunol. Methods* **257**, 55–69 (2001).
- 9 Terry, H., Susan, M. & Amanda, J. R. The application of flow cytometry to histocompatibility testing. *Transpl. Immunol.* **8**, 3–15 (2000).
- 10 Yong, H. A. & Hardy, K. J. Role of interferon-gramma in immune cell regulation. *J. Leukoc. Biol.* **58**, 373–378 (1995).
- 11 Billiau, A. Interferon- γ : biology and role in pathogenesis. *Adv. Immunol.* **62**, 61–130 (1996).
- 12 Sandra, G. & Filippo, B. IFN γ Expression in Macrophages and Its Possible Biological Significance. *Cytokine and Growth Factor Rev.* **9**, 117–123 (1998).
- 13 Trinchieri, G., Matsumoto-Kobayashi, M., Clark, S. C. & Seehra, J. Response of resting human peripheral blood natural killer cells to interleukin 2. *J. Exp. Med.* **160**, 1147–1169 (1984).
- 14 Young, H. A. & Ortaldo, J. R. One-single requirement for interferon-gramma production by human large granular lymphocytes. *J. Immunol.* **139**, 724–727 (1987).
- 15 Lindholm, C. K. IL-2 receptor signaling through the Shb adaptor protein in T and NK cells. *Bilchem. Biophys. Res. Commun.* **296**, 929–936 (2002).
- 16 Ma, A., Koka, R. & Burkett, P. Diverse functions of IL-2, IL-15, and IL-7 in lymphoid homeostasis. *Annu. Rev. Immunol.* **24**, 657–679 (2006).
- 17 Jose, C. C. & George, C. T. Transcriptional regulation of IL-2 in health and autoimmunity. *Autoimmun. Rev.* **8**, 190–195 (2009).
- 18 Smith, K. A. Interleukin-2. *Curr. Opin. Immunol.* **4**, 271–276 (1992).
- 19 Choi, J. M., Kim, H. J., Lee, K. Y., Choi, H. J., Lee, I. S. & Kang, B. Y. Increased IL-2 production in T cells by xanthohumol through enhanced NF-AT and AP-1 activity. *Int. Immunopharmacol.* **9**, 103–107 (2009).
- 20 Michelle, M. F., Forest, R. & Kathleen, L. M. IL-2 activation of a P13K-dependent STAT3 serine phosphorylation pathway in primary human T cells. *Cellular* **15**, 625–636 (2003).
- 21 Kohara, H., Kitaura, H., Fujimura, Y., Yoshimatsu, M., Morita, Y., Eguchi, T., Masuyama, R. & Yoshida, N. IFN- γ directly inhibits TFN- α -induced osteoclastogenesis *in vitro* and *in vivo* and incuces apoptosis mediated by Fas/Fas ligand interactions. *Immunol. Lett.* **137**, 53–61 (2011).