

# The effect of magnetic nanoparticles of $\text{Fe}_3\text{O}_4$ on immune function in normal ICR mice

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**Abstract:** We investigated the effect of magnetic nanoparticles of  $\text{Fe}_3\text{O}_4$  ( $\text{Fe}_3\text{O}_4$ -MNPs) on the mice immune system. Imprinting control region (ICR) mice were assigned randomly into four groups and treated with normal saline or low, medium, or high doses of  $\text{Fe}_3\text{O}_4$ -MNPs, respectively. After intravenous administration of  $\text{Fe}_3\text{O}_4$ -MNPs for 72 hours, the peripheral T cells and the induction of primary immune responses in mice were investigated by flow cytometry and determined using enzyme-linked immunosorbent assay, respectively. The results showed that the ratio of spleen to body weight was not different between the experimental groups and control group ( $P > 0.05$ ). The lymphocyte transformation rates in the suspension of spleen were higher in low-dose group than those in the control group ( $P < 0.05$ ), while the proliferation of splenocytes was low in the medium and high groups when compared to the control group ( $P < 0.05$ ). In peripheral blood, both the proportions of subset  $\text{CD4}^+$  and  $\text{CD8}^+$  T lymphocytes in the low-dose group were higher than those in the control group, whereas there was no difference in the number of  $\text{CD4}^+$  T cells between the medium- and low-dose groups. Interestingly, the  $\text{Fe}_3\text{O}_4$ -MNPs enhanced the production of interleukin-2 (IL-2), interferon- $\gamma$ , and IL-10 but did not affect the production of IL-4 in peripheral blood. It is concluded that  $\text{Fe}_3\text{O}_4$ -MNPs could influence immune functions of normal ICR mice in a dose-dependent manner.

**Keywords:** magnetic nanoparticle of  $\text{Fe}_3\text{O}_4$ , immune function, splenocyte proliferation, cytokine

Nanotechnology offers an efficient alternative for cancer diagnostics and tumor target treatment due to the unique properties of nanostructures, such as large surface-to-volume ratio, porous structure, embedded effect, and size effect, which have been recognized as offering potential promising applications in biomedical engineering. Much effort has been extended to the development of novel nanocomposites and biomaterials for DNA detection,<sup>1</sup> intracellular labeling,<sup>2</sup> drug carrier,<sup>3</sup> cancer targeting,<sup>4</sup> imaging,<sup>5</sup> and so on. Therefore, magnetic nanoparticles of  $\text{Fe}_3\text{O}_4$  ( $\text{Fe}_3\text{O}_4$ -MNPs) as a kind of biocompatible nanomaterial which is feasible to characterize and easily functionalize, may offer an exciting development toward developing an effective drug delivery system while biocompatible superparamagnetic particles like magnetite could be utilized in tissue-specific release of therapeutic agents and magnetic field assisted radionuclide therapy.<sup>6-9</sup>

As a novel material, though we have already proved that  $\text{Fe}_3\text{O}_4$ -MNPs have no cytotoxicity, the exact function of  $\text{Fe}_3\text{O}_4$ -MNPs on immune function has not yet been adequately clarified. In the present paper, we investigated the effects of  $\text{Fe}_3\text{O}_4$ -MNPs on the immune system in imprinting control region (ICR) mice to elucidate the interactions between  $\text{Fe}_3\text{O}_4$ -MNPs and immune system and to provide theoretic evidence for the clinical applications.

## Materials and methods

### Experimental agents

Experimental agents were sourced from the following locations: RPMI1640 (Gibco Chemical Co., Carlsbad, CA, USA); Anti-CD3 (PE-Cy5), Anti-CD4, Anti-CD8 (Pharmingen, San Diego, CA); Calf serum (Gibco Chemical Co); Enzyme-linked immunosorbent assay kit (Gibco, CA, USA); Con A (Sigma Chemical Co., St Louis, MO, USA).

### Preparation of Fe<sub>3</sub>O<sub>4</sub>-MNPs

Based on our previous studies,<sup>10-11</sup> the synthesis of Fe<sub>3</sub>O<sub>4</sub>-MNPs was prepared by the electrochemical deposition under oxidizing conditions. Before being applied in the present experiment, the magnetite nanoparticles were well-distributed in RPMI-1640 medium freshly added with 10% heated-inactivated fetal bovine serum (FBS) using ultrasound treatment in order to obtain Fe<sub>3</sub>O<sub>4</sub>-MNPs colloidal suspension.

### Animals and animal care

Female and male ICR mice, which were age-matched (eight weeks of age) and weight-matched (18–22 g), were purchased from Shanghai National Center for Laboratory Animals. Animals were kept with a 12-hour light/dark cycle and received water and food ad libitum in a semi-barrier system. The experiments were performed in adherence to the guidelines for the Care and Use of Laboratory Animals of the National Institute of Health.

### Experimental groups and preparation for blood samples

Mice were randomly assigned to one of four groups (n = 10 per group). The doses of 5.14 mg/kg (low dose group), 20.7 mg/kg (medium dose group), and 51.4 mg/kg (high dose group) Fe<sub>3</sub>O<sub>4</sub>-MNPs, were dissolved in normal saline and intravenously (iv) injected into mice once. Meanwhile, those injected with 0.2 mL normal saline alone formed the control group. After being monitored for 72 hours, the eyeballs of mice were extirpated for blood collecting at the end of this period and spleens were aseptically removed immediately, blotted, and weighed and then used for various analyses. Blood samples obtained from the mice were centrifuged (1500 rpm) for 5 minutes at 4°C to separate plasma and blood cells. The blood cells were used for analyzing surface markers of lymphocytes and the plasma was stored at –80°C for determination of cytokines.

### Lymphocyte proliferation assay

Single-cell suspensions were prepared from the spleens in RPMI-1640 medium. Briefly, a cell suspension was produced

by puncturing the spleen with a 20-gauge needle gently flushing the organ with ice-cooled (4°C) culture medium solution. The suspension was freed from debris by centrifugation at 1000 rpm for 20 minutes at 4°C. And the remaining splenocyte suspensions were washed twice and adjusted to  $2 \times 10^6$  cells/mL with RPMI-1640. The splenocyte cell suspension was placed in a 96-well microtiter plate in 200 µL aliquots, and cultured in the presence or absence of T-cell mitogen (concanavalin A [ConA], Sigma, USA) (5 µg/mL). Meanwhile the wells receiving complete RPMI-1640 were regarded as control. Cells were cultured for 68 hours at 37°C in a 5% humidified CO<sub>2</sub> atmosphere, following which 10 µL MTT (0.5 mg/mL) were added to each well at 37°C in the dark for at least 4 hours, the formazan crystals were dissolved in 150 µL dimethyl sulfoxide (Sigma Aldrich) and the reduction of MTT was quantified by absorbance at a wavelength of 570 nm using a microplate reader (Model-550; Bio-Rad Laboratories, Hercules, CA, USA). The results were expressed as a mean differential optical density ( $OD_{\text{mitogen}} - OD_{\text{control}}$ ).

### The proportions of lymphocyte subset

Phenotypic analyses of blood lymphocytes were performed using FCM. Briefly, the cells were incubated with PE or FITC-conjugated monoclonal antibodies [Anti-CD3 (PE-Cy5), Anti-CD4 (FITC), or Anti-CD8 (PE)] for 10 minutes, washed three times, and then resuspended in FACS permeabilizing solution before determination. At least 10,000 cells were analyzed for each MoAb staining using a FACScan flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA). Results were expressed as mean fluorescence intensity for a given molecule per cell.

### Assessment of cytokines

The levels of interleukin-2 (IL-2), interleukin-4 (IL-4), interleukin-10 (IL-10), and interferon-γ (INF-γ) in serum were measured in duplicate using enzyme-linked immunosorbent assay kit according to the manufacturer's instruction. Briefly, 50 µL samples or standard control were added to 50 µL assay diluents for each well, incubated at room temperature for 2 hours; after thorough washing, 100 µL conjugate was added to each well for incubation of 2 hours. Then 100 µL substrate solution was added to each well and incubated for 30 minutes. Finally, a 100 µL stop solution was added to each well and the optical density was measured using ELISA reader (Bio-Rad Laboratories, Hercules, CA, USA) with dual wavelength of 450 nm.

### Statistical analysis

Data were analyzed using the Statistical Package for Social Science (version 13.0; SPSS Inc., Chicago, IL, USA). The

significant difference between groups was analyzed using one-way ANOVA;  $P$  values  $<0.05$  were considered statistically significant.

## Results

### The characteristic of $\text{Fe}_3\text{O}_4$ -MNPs

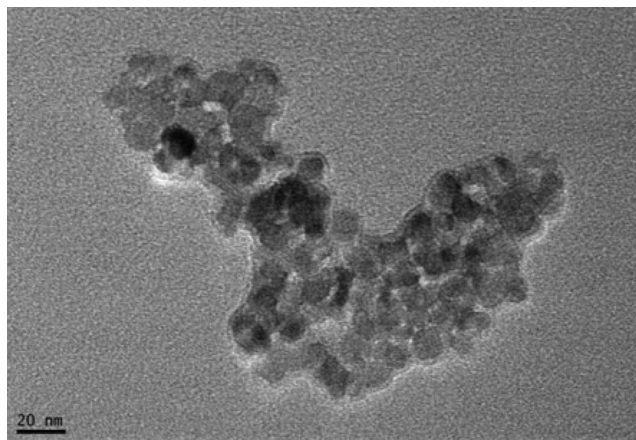
A colloidal suspension of  $\text{Fe}_3\text{O}_4$ -MNPs was achieved by using ultrasound treatment and the magnetization and the size of  $\text{Fe}_3\text{O}_4$ -MNPs were found to be  $25.6 \times 10^{-3}$  emu/mg and 20 nm, respectively (Figure 1).

### The changes of spleen weight in $\text{Fe}_3\text{O}_4$ -MNPs-treated ICR mice

Mice treated with  $\text{Fe}_3\text{O}_4$ -MNPs appeared healthy and their body weight gain patterns were similar to controls (data not included). The spleens of  $\text{Fe}_3\text{O}_4$ -MNPs-treated mice showed same appearance as the controls. Both the spleen weight and the ratio of spleen weight to body weight showed no significant difference between the experimental groups and the controls (Table 1). It suggested that  $\text{Fe}_3\text{O}_4$ -MNPs did not cause splenomegaly, which was due to the deposition of damaged erythrocytes or to recruitment and/or proliferation of splenic cells.

### Splenocyte proliferation

A significant increase of splenocyte proliferative capacity was noticed after administration of  $\text{Fe}_3\text{O}_4$ -MNPs in low dose ( $P < 0.05$ ; low dose versus control). Both administration of medium-dose and high-dose  $\text{Fe}_3\text{O}_4$ -MNPs affect splenocyte proliferation and reduced the splenocyte proliferative capacity compared with the control group ( $P < 0.05$ ; control versus medium-dose/high-dose  $\text{Fe}_3\text{O}_4$ -MNPs) (Table 2).<sup>12</sup>



**Figure 1** TEM Image of magnetic nanoparticles of  $\text{Fe}_3\text{O}_4$ . Bar = 20 nm (X40000 times).

### The proportions of lymphocyte subsets in peripheral blood

There were no differences between the low-dose group and the medium-dose group in the proportions of  $\text{CD4}^+$  T-cell subset in peripheral blood of ICR mice, and both had more  $\text{CD4}^+$  T lymphocytes than the control group. But the high-dose group showed no difference compared with control ( $P > 0.05$ ) (Figures 2 and 3). Furthermore the proportions of  $\text{CD8}^+$  T lymphocytes subset were slightly lower after the commencement of high dose. Though both low- and medium-dose groups indicated that they have more  $\text{CD8}^+$  T lymphocytes than those of control group, and the low-dose group showed significantly more when compared to the medium-dose group ( $P < 0.05$ ) (Figures 4 and 5).

### Cytokine release

To detect whether  $\text{Fe}_3\text{O}_4$ -MNPs can alter cytokine production patterns in peripheral blood, enzyme-linked immunosorbent assay was conducted.  $\text{Fe}_3\text{O}_4$ -MNPs altered the production of IL-2, INF- $\gamma$ , and IL-10. Interestingly, low dose of  $\text{Fe}_3\text{O}_4$ -MNPs significantly increased the levels of INF- $\gamma$ , IL-2, IL-10 and IL-4 ( $P < 0.05$ ) when compared to medium or high doses of  $\text{Fe}_3\text{O}_4$ -MNPs ( $P < 0.05$ ), and there were no significant differences between medium and high doses of  $\text{Fe}_3\text{O}_4$ -MNPs ( $P > 0.05$ ), suggesting that low dose of  $\text{Fe}_3\text{O}_4$ -MNPs significantly increased the ability of splenocytes to release cytokines. But no significant changes of IL-4 were observed between the experiment groups and the control group (Figure 6).

## Discussion

Close attention has been paid to current nanoparticle techniques. It is well known that nanoparticles could present a versatile nanoscale surface for biomolecular recognition because of the numerous potential benefits in merging biomacromolecules and nanoparticles. Meanwhile, much effort has been explored to the development of new nanocomposites and their application in many research fields such as DNA detection, intracellular labeling, drug carrier, and so on.

The magnetic nanoparticle of  $\text{Fe}_3\text{O}_4$  we tested has good biocompatibility and no cytotoxicity.<sup>13,14</sup> Our group have proved that  $\text{Fe}_3\text{O}_4$ -MNPs could increase the intracellular effective concentration of chemotherapeutic drugs *in vitro* to reverse MDR. The focus of this study was to investigate whether  $\text{Fe}_3\text{O}_4$ -MNPs have effects on mice immune system, especially the T cell functions after given different dose.

**Table 1** Effects of Fe<sub>3</sub>O<sub>4</sub>-MNPs on the spleen weight and the ratio of spleen weight to body weight (n = 10, mean ± SD)

Groups	Spleen weight (g)	The ratio of spleen weight to body weight (×10 <sup>-3</sup> )
Control (saline)	0.0940 ± 0.0152	3.3102 ± 0.4478
Low dose of Fe <sub>3</sub> O <sub>4</sub> -MNPs (5.14 mg/kg)	0.0720 ± 0.0286	2.3793 ± 0.8027*
Medium dose of Fe <sub>3</sub> O <sub>4</sub> -MNPs (20.7 mg/kg)	0.0900 ± 0.0187	3.1488 ± 0.5225*
High dose of Fe <sub>3</sub> O <sub>4</sub> -MNPs (51.4 mg/kg)	0.1120 ± 0.0084	4.0481 ± 0.8744*

**Note:** \**P* > 0.05, when compared to the control.

The total body weights of mice were not changed significantly, and the spleen/body weight ratio was unchanged. Actually, quantification of body weight and organ weight forms an integral part of any toxicological study providing an initial assessment of overall animal health status, as well as potential pathology. Descriptions of the tier approach to immunotoxicity evaluation should incorporate measurements of body weight, weights of spleen, thymus, kidney, and liver, as well as organ/body weight ratios in an initial screen.<sup>15</sup>

Proliferation of lymphocytes following exposure to mitogenic stimuli is an important methodology for the assessment of cell-mediated immunity. This assay has enjoyed frequent use in immunotoxicology studies for its ease of performance and relative reproducibility.<sup>16</sup> Therefore, we have investigated the effects of Fe<sub>3</sub>O<sub>4</sub>-MNPs on lymphoproliferation in spleen following exposure to Con-A. The results showed that the proliferation of lymphocytes was significantly increased when mice were injected with 5.14 mg/kg Fe<sub>3</sub>O<sub>4</sub>-MNPs compared with the controls. However, the other two groups did not act in a similar way.

To detect the levels of proportion of CD4<sup>+</sup> or CD8<sup>+</sup> T cells, which are markers for T cell lymphocytes. Fe<sub>3</sub>O<sub>4</sub>-MNPs influenced the function of helper T cells or suppresser T cells, when the less the Fe<sub>3</sub>O<sub>4</sub>-MNPs were given, the more of an

increase in T cell numbers was seen, suggesting that low concentration of Fe<sub>3</sub>O<sub>4</sub>-MNPs can regulate T cell functions in ICR mice.

The Th1 cytokines promote cellular immunity by activating macrophages, cytotoxic CD8<sup>+</sup> T lymphocytes, and so on, while the Th2 cytokines enhance humoral immunity, including activation and class switching of antibody producing B cells.<sup>17</sup> Th1 cells are defined by their ability to secrete the inflammatory cytokines IL-2 and INF-γ and are involved in cellular immunity, some autoimmune disease, and in chronic inflammatory disorders. The Th2-biased cells preferentially produce IL-4, IL-5, IL-10, and IL-13 and participate in humoral response and antibody production.<sup>18</sup> In the present study, low dose of Fe<sub>3</sub>O<sub>4</sub>-MNPs was found to strongly affect the production of Th1 cytokine, and also affect some Th2 cytokine release, suggesting that Fe<sub>3</sub>O<sub>4</sub>-MNPs might be involved in inflammations associated with infections.

## Conclusion

In conclusion, our results indicate Fe<sub>3</sub>O<sub>4</sub>-MNPs can influence immune functions of mice in a dose-dependent manner. Further study indicated that a high dose of Fe<sub>3</sub>O<sub>4</sub>-MNPs has no significant influence on the immune systems of the mice. These data could be useful for improving biomedical applications of Fe<sub>3</sub>O<sub>4</sub>-MNPs, but these immunological effects of Fe<sub>3</sub>O<sub>4</sub>-MNPs should be further conducted both *in vivo* and *in vitro*.

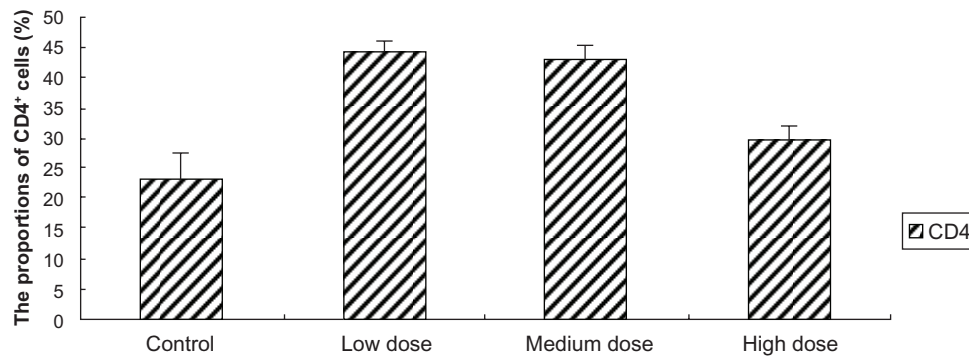
## Acknowledgments

This work was supported by 973 National Key Fundamental Research Project of China (No. 2006CB933205), 863 Project of People's Republic of China (No. 2007AA022007), National Nature Science Foundation of People's Republic of China (No. 30740062, 30872970), and Special-Purpose Science Research Foundation for High School (No. 20070286042).

**Table 2** Influence of Fe<sub>3</sub>O<sub>4</sub>-MNPs on the rate of lymphocyte transformation in spleen suspension (n = 10, mean ± SD)

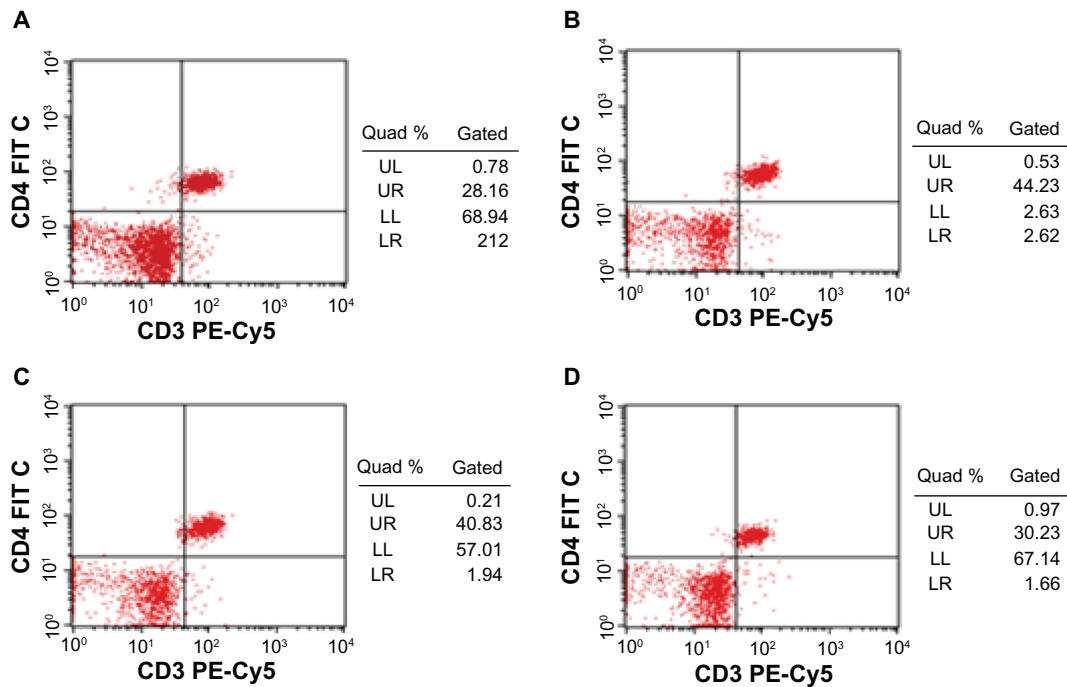
Groups	The rate of lymphocyte transformation
Control (saline)	0.0193 ± 0.001027
Low dose of Fe <sub>3</sub> O <sub>4</sub> -MNPs (5.14 mg/kg)	0.0398 ± 0.005155*
Medium dose of Fe <sub>3</sub> O <sub>4</sub> -MNPs (20.7 mg/kg)	0.0111 ± 0.003029*
High dose of Fe <sub>3</sub> O <sub>4</sub> -MNPs (51.4 mg/kg)	0.0046 ± 0.001517*

**Note:** \**P* < 0.05, when compared to the control.



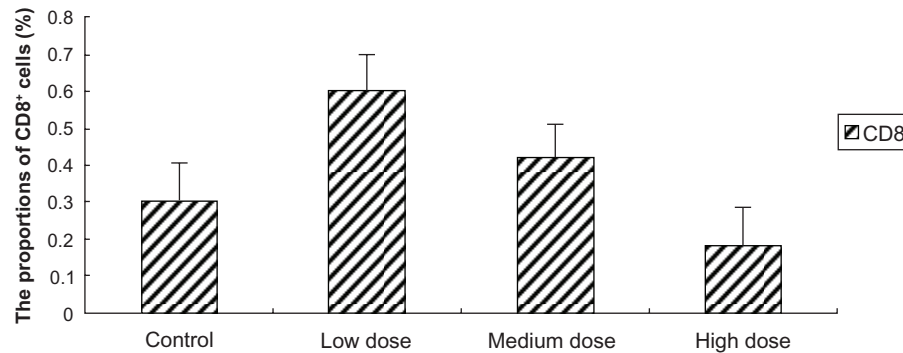
**Figure 2** The effects of Fe<sub>3</sub>O<sub>4</sub>-MNPs on the proportion of CD4<sup>+</sup> T lymphocyte subset in peripheral blood.

**Notes:** Control: 0.2 mL saline; Low dose: low dose of Fe<sub>3</sub>O<sub>4</sub>-MNPs (5.14 mg/kg); Medium dose: medium dose of Fe<sub>3</sub>O<sub>4</sub>-MNPs (20.7 mg/kg); High dose: high dose of Fe<sub>3</sub>O<sub>4</sub>-MNPs (51.4 mg/kg). CD4<sup>+</sup>, CD8<sup>+</sup> represent CD4, CD8-positive T cells.



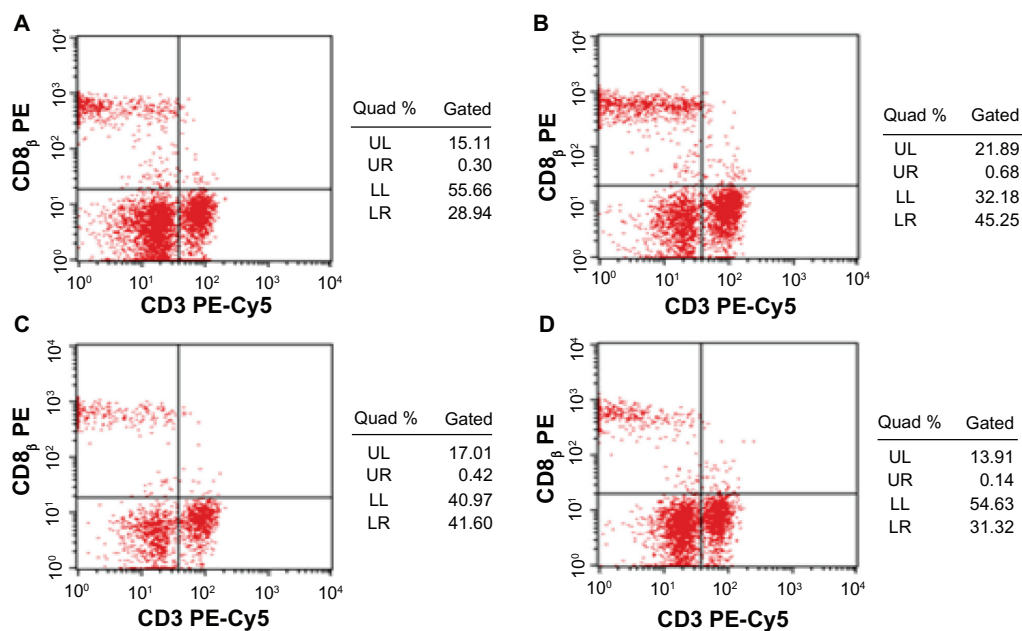
**Figure 3** Effect of Fe<sub>3</sub>O<sub>4</sub>-MNPs on the proportion of CD4<sup>+</sup> T lymphocyte subset in peripheral blood by FCM.

**Notes:** **A)** 0.2 mL saline; **B)** low dose of Fe<sub>3</sub>O<sub>4</sub>-MNPs (5.14 mg/kg); **C)** medium dose of Fe<sub>3</sub>O<sub>4</sub>-MNPs (20.7 mg/kg); **D)** High dose of Fe<sub>3</sub>O<sub>4</sub>-MNPs (51.4 mg/kg).



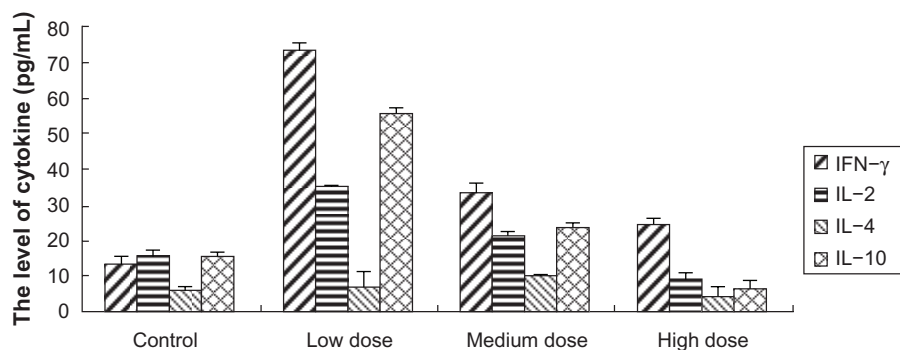
**Figure 4** Effect of Fe<sub>3</sub>O<sub>4</sub>-MNPs on the proportion of CD8<sup>+</sup> T lymphocyte subset in peripheral blood.

**Notes:** Control: 0.2 mL saline; Low dose: low dose of Fe<sub>3</sub>O<sub>4</sub>-MNPs (5.14 mg/kg); Medium dose: medium dose of Fe<sub>3</sub>O<sub>4</sub>-MNPs (20.7 mg/kg); High dose: high dose of Fe<sub>3</sub>O<sub>4</sub>-MNPs (51.4 mg/kg). CD4<sup>+</sup>, CD8<sup>+</sup> represent CD4, CD8-positive T cells.



**Figure 5** Effect of  $\text{Fe}_3\text{O}_4$ -MNPs on the proportions of  $\text{CD8}^+$  T lymphocyte subset in peripheral blood by FCM.

**Notes:** **A)** 0.2 mL saline; **B)** low dose of  $\text{Fe}_3\text{O}_4$ -MNPs (5.14 mg/kg); **C)** medium dose of  $\text{Fe}_3\text{O}_4$ -MNPs (20.7 mg/kg); **D)** High dose of  $\text{Fe}_3\text{O}_4$ -MNPs (51.4 mg/kg).



**Figure 6** Effect of  $\text{Fe}_3\text{O}_4$ -MNPs on the production of cytokine in peripheral blood.

**Notes:** Control: 0.2 mL saline; Low dose: represents low dose of  $\text{Fe}_3\text{O}_4$ -MNPs (5.14 mg/kg); Medium dose: represents medium dose of  $\text{Fe}_3\text{O}_4$ -MNPs (20.7 mg/kg); High dose: represents high dose of  $\text{Fe}_3\text{O}_4$ -MNPs (51.4 mg/kg).

## Disclosure

The authors confirm no conflicts of interest in this work.

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