Immunological changes of chronic oral exposure to depleted uranium in mice

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1. Introduction

Depleted uranium (DU) is the residue that remains after the refining and enriching of 235U from natural uranium; the content of 235U is usually 0.2–0.3%. Due to its high penetrability and low price as a raw material, DU has been widely used in counterweights, radiation-protective clothing, and military activities (serving as an armour material and an ammunition component) (Bleise et al., 2003). However, during its production and use, uranium may be released into the environment due to failure to follow standard procedures, thus causing environmental pollution. The physical half-life of DU is up to 4.49 × 10^6 years, and the element remains in the environment for a long time, contaminating soil, groundwater, flora, and fauna, which eventually enter the human body through the food chain, leading to chronic contamination of local residents (Di Lella et al., 2005).

The radioactivity of DU is approximately 60% that of natural uranium, but DU has the same heavy-metal toxicity as natural uranium (Priest, 2001; Squibb et al., 2012). During acute high-dose exposures, the kidney is the main target organ of the chemical toxicity of DU, which may cause severe tubular necrosis (Hao et al., 2012a) and mitochondrial damage (Shaki et al., 2012). Low-dose chronic exposure may cause a series of harmful effects, such as neurobehavioural abnormalities, genetic toxicity, reproductive toxicity, and cancer (Houpert et al., 2005; Lestaet al., 2005; Hao et al., 2009, 2012b; Mould, 2001; Gagnaire et al., 2013) reported that low-dose DU exposure had an impact on oxidative stress, detoxification, and the defence system of zebrafish; moreover, the researchers stressed that further research on immunotoxicity (or immune markers) would elucidate these effects of uranium. Our previous research

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(Hao et al., 2012a) has confirmed that in addition to primary accumulation in the kidney, DU also accumulates in the liver and spleen, suggesting that DU might have certain effects on the immune system.

Several studies have confirmed that DU has a toxic effect on immune cells in vitro. Kalinich et al. (2002) found that macrophages can uptake uranium and subsequently undergo apoptosis. Gazzin et al. (2004) determined that DU causes abnormal expression and release of tumour necrosis factor (TNF-α) and interleukin (IL)-6 from macrophages. Wan et al. (2006) demonstrated that exposure to low-dose DU affects the immune function through regulation of the expression of cytokines (e.g., involved in signal transduction, interleukin expression, chemokines, chemokine receptors, and neurotrophic factors).

However, few published studies exist on the impact of DU on immune function and inflammation in live animals. Monleau et al. (2006) found that inhalation of insoluble DU causes a time-dependent increase in a variety of inflammatory cytokines in rat lung tissue. A rat model of chronic exposure was established by long-term intake of uranium-containing water (40 mg/l); at 3, 6, and 9 months, the effect of uranium exposure on various inflammatory pathways [prostaglandins, histamine, cytokines and nitric oxide (NO)] was evaluated. The results revealed that chronic ingestion of DU causes time-dependent changes in a variety of inflammatory pathways (Dublineau et al., 2007). DU enters the body through the oral route. Direct ingestion of contaminated food and soil should also be considered in addition to drinking contaminated water. However, there is still no report on the effects of ingesting DU-contaminated food on the immune system.

In addition, it is a speculated that the “Gulf War Syndrome” might be caused by the systematic shift of T helper (Th) 2 cytokines by Th1 cytokines because the clinical symptoms are markedly similar to those of autoimmune diseases (Rook and Zumla, 1997). In vitro, after cluster of differentiation (CD) 4+ T cells and macrophages are exposed to DU, there is increased expression of IL-5 and IL-10, which strongly suggest a shift to Th2 cells during the initial stages of T cell differentiation (Wan et al., 2006). For other heavy metals, such as lead, studies on mouse bone marrow-derived dendritic cells also revealed a shift to Th2 cells during the immune response (Gao et al., 2007). In this study, we hypothesised that DU may modulate immune cell cytokine expression, especially Th1 and Th2 cytokines, to influence the immune system function. However, Dublineau et al. (2006) reported that, there was no biological consequences in the cytokine expression [IL-10, transforming growth factor (TGF)-β, interferon (IFN)-γ, TNF-α] in Peyer’s patches and in mesenteric lymph nodes of rats after chronic ingestion of DU by drinking water (40 mg/l).

Therefore, the objective of this study was to establish a mouse model in which mice were exposed to long-term ingestion of DU-containing feed, to evaluate the overall impact of DU exposure on the entire immune system of the mice after 4 months, and to verify whether the DU exposure caused an imbalance between Th1 and Th2 cytokines. We set up 4 different dose groups based on the DU concentration. The control group consumed normal feed with a uranium concentration of approximately 0 mg/kg. The uranium concentration that was used in the DU group (3 mg/kg) was mainly based on the average concentration of uranium in the natural soil (3 mg/kg; Bleise et al., 2003). The uranium concentration that was used in the DU300 groups (30 mg/kg) was mainly based on the concentration range of uranium in the topsoil of the western Kosovo region (0.69–31.47 mg/kg; Di Lella et al., 2005) and on the uranium concentration (40 mg/l) that is the uranium concentration commonly used in drinking water in studies (Wade-Gueye et al., 2012; Barillet et al., 2011) of chronic exposure [which was twice the highest environmental concentration in Finland (Juntunen, 1991)]. Finally, in accordance with the 10-fold uranium concentration gradient for each dose group, the DU300 groups were exposed to 300 mg/kg; this 300 mg/kg concentration was still far lower than that of the highest uranium concentration in the topsoil of the Kosovo region (assessed in November 2000), which was approximately 18,000 mg/kg (Sansone et al., 2001). Four months after the animals were fed the DU-containing feed, immunotoxicological experiments were conducted to comprehensively evaluate the animals’ innate immunity and cellular and humoral immune function to analyse the subtypes of the immune cells and the expression of the cytokines and thus further explore the molecular mechanisms of the immunotoxicity of DU. The results of this study suggest that DU plays a role in increasing the incidence of autoimmune diseases, infectious diseases, and tumours, which lays the foundation for future studies of the biological effects of chronic DU exposure.

2. Materials and methods

2.1. Animals

Male Kunming mice weaned at 3 weeks of age were obtained from the Institute of Zoology [The Third Military Medical University, SCCX (Chongqing) 2007-0003, China]. The mice were acclimated to the laboratory for 7 days prior to the start of the experiment and found to be in good health were selected for use. The mice’s weights were in the range 18–21 g at the beginning of the experiments. The mice were housed in plastic cages (ten mice per cage) under controlled conditions with a 12:12-h (light:dark) cycle, an ambient temperature of 20–25 °C, and a relative humidity of 55%. The mice had free access to water and food throughout the experimental period. Food intake, water intake, body weight, and health status were recorded daily. Over the four months after ingestion of DU, the mice were euthanised by rapid decapitation or anaesthetised with ether for blood collection. The animal experiments were conducted in conformity with the National Institutes of Health guidelines (NIH Pub. No. 85-23, revised 1996) and with the agreement by the Animal Care and Use Committee of the Third Military Medical University.

2.2. Contamination

DU233U: 99.75%; 234U: 0.20%, and trace 232U, specific activity of 1.24 × 108 Bq/g was purchased from the China National Munitions Corporation, Beijing. The preparation of DU-spiked food followed as previous study (Hao et al., 2009). In brief, DU was dissolved in nitric acid as uranyl nitrate and then spiked in food evenly. The resulting chemical speciation of uranyl nitrate mixed with food was uranyl nitrate hexahydrated [UO2(NO3)2 · 6H2O]. For animal exposure, four different solutions were prepared to obtain four concentrations of uranium in food: 0 mg/kg (control group), 3 mg/kg (DU1 group), 30 mg/kg (DU2 group) and 300 mg/kg (DU3 group). After food consumption and weight were considered, the mice were exposed to DU in their food at approximate doses of 0, 0.4, 4, and 40 mg/kg body weight/day for four months, respectively.

2.3. Relative weight of spleen and thymus

Over the four months after ingestion of DU, the mice of each group (n = 10) were anaesthetised with ether and blood samples were collected from femoral vein. Serum was prepared for biochemical analyses below. Then spleen, thymus and sternum from mice were lightly dissected and spleens and thymus were weighed and normalised to the body weight. Spleen, thymus and sternum were used for uranium analyses below.

2.4. Biochemical assays

Serum concentrations of urea nitrogen (BUN), creatinine (CR), alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were measured by an automated spectrometric system (KoneLab 20, Thermo Electron Corporation, Cergy-Pontoise, France) with the manufacturer’s biological chemistry reagents.

2.5. Uranium analyses

The uranium content was measured in the kidney, sternum, thymus and spleen. Samples (25–400 mg) were digested by the addition of 3 ml of concentrated nitric acid in a CEM MARS Xpress Microwave Accelerated Reaction System (CEM Corporation, Matthews, NC, USA) using following procedure: (1) microwave power at 1600 W, ramp 5 min to reach 120 °C and remained at 120 °C for 2 min; (2) microwave power at 1600 W, ramp 2 min to reach 150 °C and remained at 150–C for 2 min. Uranium content in samples was determined using an inductively coupled plasma mass spectrometer (ICP-MS, Thermo Finnigan MAT, Bremen, Germany). The limit for the instrument was 0.002 ppb. Values are expressed as ng g−1 of fresh sample material.
In addition, to verify the source of uranium, the $^{239/240}$U/$^{235}$U isotopic ratio was also measured by ICP-MS.

2.6. Cytotoxicity assay of splenic natural killer (NK) cells

Spleens were harvested aseptically from euthanized mice of each group (n = 10) and single cell suspensions were prepared as previously described (Hao et al., 2012a). The cell preparations from each mouse were analysed individually. NK cell-mediated cytotoxicity was determined in a colorimetric assay based on the measurement of lactate dehydrogenase (LDH) released from the cytosol of lyzed YAC-1 target cells (Chinese Academy of Sciences, Shanghai, China) into the supernatant according to the method of previous study (Konjevic et al., 1997; Lv et al., 2012). Briefly, splenic and YAC-1 cells were coincubated at ratios of 40:1 in complete RPMI 1640. After a 4-hour incubation period in a humidified chamber (37 °C, 5% CO2), cell suspension was used to account for spontaneous LDH release activity. The spontaneous LDH release activity correlates with cytotoxicity of NK cell (Konjevic et al., 2012). The LDH release activity was determined using an LDH cytotoxicity assay kit (Beyotime, Haimen, Jiangsu, China) according to the manufacturer’s instruction. The absorbance was measured at 490 nm by a microplate reader (Bio-rad 550, Bio-Rad Laboratories, California, USA) within 1 h. The percentage of specific lysis was expressed using the formula: Cytotoxicity (%) = LDH activity in supernatant/LDH activity in supernatant + LDH activity in cell lysate) × 100.

2.7. Assessment of phagocytic activity

Mice of each group (n = 10) were sacrificed by rapid decapitation, followed by a peritoneal wash after inoculation with sterile phosphate buffer saline (PBS). To obtain the macrophages, Cells were then washed three times in PBS by centrifugation (1000 rpm for 5 min) and counted. The uptake of the neutral red dye, which accumulates in cell lysosomes, was used to evaluate the phagocytic activity of the macrophages by colorimetry according to the method of previous study (Bussolari et al., 2008). Briefly, macrophages (2 × 10^5 cells/well) were cultured on a 96-well flat bottomed microplate and incubated for 30 min with 10 μl of neutral red staining solution (Beyotime, Haimen, Jiangsu, China). Then cells were fixed with Baker’s formal-calcium solution for 30 min and washed twice. The neutral red solution was extracted from cells by 100 μl acidified alcohol to each well. The optical density (OD) at 550 nm of samples was determined by a microplate reader.

2.8. Determination of total serum immunoglobulin (Ig) M, IgG, IgE

Mice of each group (n = 10) were anaesthetised with ether and blood samples were collected from femoral vein. Serum was prepared and stored at −80 °C until measurement. Total serum IgM, IgG and IgE levels were respectively measured using the enzyme linked immunosorbent assay (ELISA) kits (Innovative Research, Inc., Michigan, USA), according to the manufacturer’s instruction as previously described (Ma et al., 2012). The conversion from optical density to concentration was calculated from a linear regression formula using purified mouse IgM, IgG or IgE standards.

2.9. Lymphocyte proliferation test

MITT [3-(4,5-dimethyl-2-thiazoly)-2,5-diphenyl-2H-tetrazolium] assay was used to determine the lymphocyte proliferation as previously described (Hao et al., 2012a). Briefly, one hundred microlitres of splenic cells (2 × 10^5 cells/ml) was harvested from euthanized mice of each group (n = 10) and cultured in triplicate in 96-well culture plates in complete RPMI-1640 supplemented with lipopolysaccharide (LPS; Sigma–Aldrich, St. Louis, MO, USA) or concanavalin A (ConA; Sigma–Aldrich, St. Louis, MO, USA) at 5 μg/ml final concentration. Con A stimulates the proliferation of T lymphocytes, while LPS stimulates B lymphocytes. The proliferation was determined using an MTT Cell Proliferation and Cytotoxicity Assay Kit (Beyotime, Haimen, Jiangsu, China) according to the manufacturer’s instructions. The absorbance was measured at 570 nm by a microplate reader. Stimulation Index was calculated for each sample as: S.I = As/Av, where As is the absorbance of stimulated cells by ConA or LPS, and Av is the absorbance of unstimulated cells.

2.10. Delayed type hypersensitivity (DTH)

The mice of each group (n = 10) were sensitised by intraperitoneal injection of 2% (volume ratio) sheep red blood cells (SRBCs; Lanzhou National Hycclone Bio-engineering Co., LTD, Lanzhou, Gansu, China) suspended in 200 μl of saline (about 1 × 10^9 SRBCs). After four days, 20% SRBCs suspended in 20 μl of saline were injected into the left hind paw, and the resulting oedema was measured using a pressure sensitive micrometre (The Dyer Company, Lancaster, PA, USA) after 24 h. The procedure used was slightly improved as previously described (Lagrange et al., 1974).

2.11. Splenic cells phenotyping analyses

Single cell suspensions of splenic cells in each group (n = 10) were prepared as described above. The relative distributions of lymphocytes in mice spleens were determined by FACSscan analyses as previously described (Tejón et al., 2003). Splenic cells were stained using combinations of the following monoclonal conjugated antibodies (BD Biosciences Pharmingen, San Jose, CA, USA): anti-CD-3-APC-Cy7, anti-CD4-FITC, anti-CD8-PerCP-Cy5.5, anti-igM-APC and anti-IgD-PE. Briefly, splenocyte suspensions of 3 × 10^6 cells/ml in PBS were prepared, and spleen cellularity was determined using trypsin blue dye exclusion method. Then an aliquot of 100 μl of splenocyte suspensions (3 × 10^6 cells) were labelled with the monoclonal antibodies (either with anti-CD-3-APC-Cy7, anti-IgM-APC and anti-IgD-PE, or with anti-CD3-APC-Cy7, anti-CD4-FITC and anti-CD8-PE-PerCP-Cy5.5) for 30 min at 4 °C, followed by PBS wash (three times). Data were acquired using a FACScalibur (BD Biosciences Pharmingen, San Jose, CA, USA) and analysed employing FlowJo 7.6.4 software (Tree Star Inc., Ashland, OR, USA).

2.12. Determination of NO and cytokine

Single cell suspensions of peritoneal macrophages in each group (n = 10) were prepared as described above. Macrophages (2 × 10^6 cells/ml) were incubated with LPS (5 μg/ml) for 24 h. Then the culture supernatant was collected for determination of cytokine. NO was determined by the Griess method as previously described (Luna et al., 2012). Nitrite was used to assess NO and absorbance was measured at 550 nm by a microplate reader. The cytokines IL-1β, IL-6, IL-18 and TNF-α in culture supernatants were determined using ELISA kits (R&D Systems, Abingdon, UK) according to the manufacturer’s instructions. The absorbance was measured at 450 nm by a microplate reader. The limit of detection for the cytokines shown was IL-1β (1.25 pg/ml), IL-6 (7.8 pg/ml), IL-18 (15.6 pg/ml) and TNF-α (10.9 pg/ml).

2.13. Data analyses

All data were analysed with SPSS 12.0 (SPSS Inc., Chicago, IL, USA). Results are expressed as means ± standard deviation (SD). Statistical analysis for homogenous variance data was performed by one-way ANOVA and Tukey’s HSD test for multiple comparisons. Results were considered to be statistically significant at p < 0.05 (two-sided).

3. Results

3.1. DU chronic exposure did not change the body weight, organ weight, and blood-biochemistry parameters

During the entire exposure period in each group of animals, no behavioural or mental disorders were observed, the food and water consumption was normal, and the body hair was soft and smooth—all with no obvious clinical signs and symptoms. After 4 months of exposure, the body, thymus, and spleen weights of the mice in each group exhibited no significant differences (Table 1). The renal-function test results (including BUN and CR levels) for the mice in each group were within the normal range, with no significant difference being observed between the groups (Table 1). Similar results were obtained for the liver-function (including ALT and AST levels) tests, suggesting that under the conditions of this study, chronic exposure to DU had no apparent effect on the liver and kidney function.

3.2. DU chronic exposure increased in the uranium content of tissues

After 4 months of consumption of DU-containing feed, there was a certain degree of uranium accumulation in the kidney, spleen, thymus, and sernum in each group of animals (Fig. 1). DU, once absorbed, was distributed throughout the entire body, particularly the kidney and bone (Vicente-Vicente et al., 2010). This study also revealed that the concentration of uranium was the highest in the
kidney, closely followed by sternum, and the uranium concentration in the DU300 group was significantly higher than that in the other groups \((p < 0.05)\). The uranium concentration of the control group (in the kidney, spleen, thymus, and sternum) was notably low (at the normal background level) with significant differences compared with the other groups \((p < 0.05)\). Uranium also significantly accumulated in the spleen and thymus in the DU30 group and the DU3 group, and the uranium accumulation in each tissue tended to increase with increasing doses of exposure. Combined with our previous studies (Hao et al., 2009), these results provided firm evidence of a positive correlation between the dose of DU exposure and the levels of DU accumulation in the various tissues in vivo.

Besides the uranium accumulation in tissues, the \(^{235}\text{U}/^{238}\text{U}\) isotopic ratio changed evidently after 4 months of DU exposure (Table 2). The \(^{235}\text{U}/^{238}\text{U}\) isotopic ratio of the control group in tissues was relatively constant, and decreased significantly after DU exposure. With increasing DU accumulation, the \(^{235}\text{U}/^{238}\text{U}\) isotopic ratio in tissues tended to decrease, especially in the spleen and thymus. Due to the higher DU accumulation, the \(^{235}\text{U}/^{238}\text{U}\) isotopic ratio in the kidney and sternum after DU exposure was nearly 0.002 (\(^{235}\text{U}/^{238}\text{U}\) in the DU material).

### 3.3. DU chronic exposure influenced the cytotoxicity of NK cells

The cytotoxicity of splenic NK cells was assessed by measuring their killing capacity using YAC-1 target cells. The results revealed a downward trend of the cytotoxicity of NK cells with increasing doses of DU consumption. The cytotoxicity of NK cells in the DU300 group decreased to approximately one-half that in the control group, with significant differences compared with the other groups \((p < 0.05)\), whereas there was no significant difference between the DU3 or DU30 groups and the control group (Fig. 2).

#### 3.4. DU chronic exposure altered the macrophage activity

It is established that macrophages are important targets of uranium poisoning (Kalinich et al., 2002). Long-term exposure to DU has a significant impact on the function of peritoneal macrophages (Table 3). We mainly detected the secretion of NO, and the change in the secretion of TNF-\(\alpha\), IL-1\(\beta\), IL-6, and IL-18 in peritoneal macrophages after LPS stimulation in each group. The results revealed that after a long-term exposure to DU, the secretion levels of NO in all the groups were significantly lower than that in the control group \((p < 0.05)\), and the secretion level of NO was particularly low in the DU300 group, which was approximately one-third of that in the control group. In addition, the abilities of mouse peritoneal macrophages to secrete TNF-\(\alpha\), IL-18, and IL-18 were significantly reduced in the DU300 group \((p < 0.05)\), and the ability to secrete TNF-\(\alpha\) in the DU30 group was significantly lower than that in the control group \((p < 0.05)\). However, there was no significant difference in the level of IL-6 secreted by macrophages or in the phagocytic activity of neutral red particles (measured by OD at 550 nm) among the groups.

#### 3.5. DU chronic exposure changed the total serum IgM, IgG, and IgE levels

After 4 months of exposure to DU, the serum immunoglobulin levels were significantly affected (Fig. 3). With the increasing DU exposure dose, there was a trend towards an increase in the total serum IgG level in the mice, which was increased approximately 25% in the DU300 group. The total serum IgG level in the DU30 group was also significantly higher than that in the control group \((p < 0.05)\), whereas there was no significant difference between the DU3 and DU30 groups. The most striking change after chronic DU exposure was the total serum IgE level. Compared with

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**Table 1**

Effect of DU chronic exposure on the body weight, organ weight, and blood-biochemistry parameters in mice.\(^a\)

<table>
<thead>
<tr>
<th>Group</th>
<th>BW (g)</th>
<th>RSW (mg/g)</th>
<th>RTW (mg/g)</th>
<th>ALT (IU/l)</th>
<th>AST (IU/l)</th>
<th>BUN (mmol/l)</th>
<th>CR ((\mu)mol/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>46.0 ± 6.6</td>
<td>3.29 ± 0.94</td>
<td>2.57 ± 0.65</td>
<td>33.0 ± 2.5</td>
<td>124.5 ± 18.3</td>
<td>8.8 ± 1.2</td>
<td>10.3 ± 3.1</td>
</tr>
<tr>
<td>DU3</td>
<td>46.4 ± 4.4</td>
<td>3.10 ± 0.41</td>
<td>2.32 ± 0.48</td>
<td>35.8 ± 2.6</td>
<td>128.8 ± 39.3</td>
<td>8.7 ± 0.9</td>
<td>12.0 ± 1.0</td>
</tr>
<tr>
<td>DU30</td>
<td>44.5 ± 5.8</td>
<td>2.98 ± 0.53</td>
<td>2.69 ± 0.50</td>
<td>33.8 ± 4.7</td>
<td>135.0 ± 21.3</td>
<td>9.3 ± 1.0</td>
<td>9.8 ± 1.5</td>
</tr>
<tr>
<td>DU300</td>
<td>41.7 ± 3.5</td>
<td>2.96 ± 0.84</td>
<td>2.46 ± 0.49</td>
<td>37.3 ± 6.0</td>
<td>131.5 ± 24.4</td>
<td>8.9 ± 1.0</td>
<td>10.7 ± 1.6</td>
</tr>
</tbody>
</table>

\(^a\) Values are expressed as the mean ± SD (\(n = 10\)). Statistical analysis was performed by one-way ANOVA. There was no difference between the groups. BW, body weight; RSW, relative spleen weight; RTW, relative thymus weight; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BUN, blood urea nitrogen; CR, creatinine.

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**Fig. 1.** After 4 months of depleted uranium (DU) exposure, the uranium contents in the spleen, thymus, sternum and kidney rose greatly. Spleen, thymus, sternum and kidney were harvested from mice and the uranium contents were analysed by inductively coupled plasma mass spectrometry (ICP-MS). The data are expressed as the mean ± SD (\(n = 10\)). The error bars represent the SD. *\(p < 0.05\), compared with the control group; *\(p < 0.05\), compared with the control group.

**Fig. 2.** Depleted uranium (DU) chronic exposure altered the cytotoxicity of natural killer (NK) cells. Splenic cells were isolated from mice after 4 months of DU exposure and the cytotoxicity of splenic NK cells was evaluated by measurement of lactate dehydrogenase (LDH) released from the cytosol of lysed YAC-1 target cells. The data are expressed as the mean ± SD (\(n = 10\)). The error bars represent the SD. *\(p < 0.05\), compared with the control group; *\(p < 0.05\), with one-way ANOVA and Tukey’s HSD test for multiple comparisons.
the control group, the serum IgE level was significantly increased in the DU$_3$, DU$_{30}$, and DU$_{300}$ groups \((p < 0.05)\), and its level in the DU$_{300}$ group was increased by approximately 200%. However, there was no significant difference between the levels of total serum IgM among the groups.

### 3.6. DU chronic exposure altered the proliferation of splenic T and B cells

Interestingly, after the long-term consumption of DU-containing feed, the proliferative ability of the mouse splenic cells stimulated with ConA and LPS decreased with the increase of the consumption dose (Fig. 4). ConA and LPS respectively stimulated the proliferation of splenic T and B cells. Furthermore, the results revealed that in the DU$_{30}$ and DU$_{300}$ groups, the stimulation indexes of T cells were significantly lower, while the stimulation index of B cells were significantly higher, than in the control groups; these differences were statistically significant \((p < 0.05)\). However, there was no significant change in the stimulation index of T cells in the DU$_3$ group, and the stimulation index of B cell was still higher than that in the control group \((p < 0.05)\).

### 3.7. DU chronic exposure influenced the delayed-type hypersensitivity

SRBCs were used to induce DTH in the mice, and at 24 h after the second injection of SRBCs, the plantar thickening ratio in the DU$_{300}$ group was significantly less than that in the control group, as well as those in the DU$_{30}$ and DU$_3$ groups \((p < 0.05)\). By contrast, there was no significant difference between the DU$_{30}$ or DU$_3$ group and the control group (Fig. 5).

### 3.8. DU chronic exposure altered the percentages of the subtypes of splenic T and B cells

Flow cytometry revealed that after long-term exposure to DU, the mouse splenic B cell surface receptor (BCR) changed. With

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**Fig. 3.** After 4 months of depleted uranium (DU) exposure, the total serum immunoglobin (Ig) M, IgG, and IgE levels changed in different degrees. Serum was collected from femoral veins of mice and IgM, IgG, and IgE levels were respectively measured using the enzyme linked immunosorbent assay (ELISA). The data are expressed as the mean ± SD \((n = 10)\), and the error bars represent the SD. *\(p < 0.05\), compared with the control group; \#* \(p < 0.05\), with one-way ANOVA and Tukey’s HSD test for multiple comparisons.

**Fig. 4.** Depleted uranium (DU) chronic exposure induced opposite changes to the proliferation of splenic T and B cells. Splenic cells were isolated from mice after 4 months of DU exposure and the stimulation index of T cells were measured by MTT assay as stimulated by concanavalin A (ConA) and lipopolysaccharide (LPS), respectively. The data are expressed as the mean ± SD \((n = 10)\), and the error bars represent the SD. *\(p < 0.05\), compared with the control group; \#* \(p < 0.05\), with one-way ANOVA and Tukey’s HSD test for multiple comparisons.
with analyses on sensitivity error as described in Section 2. The data are expressed as the mean ± SD (n = 10), and the error bars represent the SD. *p < 0.05, compared with the control group; #p < 0.05, with one-way ANOVA and Tukey's HSD test for multiple comparisons.

3.9. DU chronic exposure caused mark changes in the cytokines secreted by the splenic cells

The levels of IFN-γ, TNF-α, IL-4, and IL-10 released by the stimulated-splenic cells were detected by ELISA (Fig. 7), and the results revealed that the level of IFN-γ in the DU300 group significantly decreased to approximately one-third of that in the control group with significant differences when compared with the other groups (p < 0.05). The level in the DU30 group was also significantly lower than that in the control group (p < 0.05), whereas there was...
Fig. 7. Depleted uranium (DU) promotes Th (T helper) 2 skewing in the spleen after 4 months of exposure. Splenic cells were isolated from mice and the levels of IFN-γ, TNF-α, IL-4, and IL-10 released by the stimulated-splenic cells were detected by the enzyme linked immunosorbent assay (ELISA) as described in Section 2. The data are expressed as the mean ± SD (n = 10), and the error bars represent the SD. *p < 0.05, compared with the control group; # p < 0.05, with one-way ANOVA and Tukey’s HSD test for multiple comparisons.

no significant difference between the DU3 group and the control group. The change in TNF-α level was similar to that of IFN-γ, and the TNF-α level decreased by approximately 50% and 20% in the DU300 group and the DU30 group, respectively, whereas the TNF-α level in the DU3 group did not change significantly. By contrast, the IL-4 level gradually increased with the increase in the exposure dose, with the increase reaching 1.5, 2, and 3 times that of the control group in the DU3, DU30, and DU300 groups, respectively; these differences were significant (p < 0.05). The IL-10 level also showed an increasing trend with the increasing dose of exposure, particularly in the DU300 group, in which the IL-10 level was increased to approximately 2.5 times that of the control group with a significant difference compared with the other groups (p < 0.05). There was no significant difference between the DU30 or the DU3 group and the control group.

4. Discussion

To the best of our knowledge, this study is the first to evaluate the impact of chronic DU exposure on the immune system in mice through exposure to DU in the diet. The results revealed that after 4 months of consuming the DU-containing feed, the immune function of the mice was changed in a concentration-dependent manner. When the DU dose in the feed reached 300 mg/kg, the immune function of the mice was significantly inhibited, compromising the innate immune function of the mice, thereby leading to the abnormalities in the acquired immune function and increasing the number of splenic mlgM+ cells and the proportion of mlgM/ mlgD+ double-positive cells; however, the number of splenic CD3+ cells and the ratio of splenic CD4+/CD8+ T cells were decreased. In addition, the release of cytokines from the spleen was abnormal, inhibiting the levels of Th1-derived cytokines while increasing the levels of Th2-derived cytokines, thereby promoting the shift to Th2 cells. However, the dose of less than 30 mg/kg in the DU-containing feed exhibited little or no impact on the immune function. This study verified the hypothesis that with sufficient doses and durations of exposure, DU may cause a systemic shift of Th1 cytokines to Th2 cytokines.

Exposure to DU by consumption is an important mode of internal DU contamination. Though less likely, children may ingest contaminated soil directly through their hands, and the potentially harmful effects cannot be ignored (Bleise et al., 2003). However, we found that after 4 months of exposure through consumption, the animals in all the groups exhibited no obvious clinical signs and symptoms; furthermore, the serum biochemical examination demonstrated that chronic exposure to DU had no significant impact on the liver and kidney function. Long-term follow-up on the health status of Gulf War veterans revealed that their urinary uranium concentrations were high, but their renal function was normal with no clinical health effects associated with uranium (McDiarmid et al., 2011), which is consistent with the results of the present study.

The measurement of uranium concentration in the tissues with ICP-MS showed that after 4 months of consumption of DU-containing feed, a significant accumulation of uranium occurred in the kidney, spleen, thymus, and sternum in the mice; moreover, with the consumption of increased doses of DU, the uranium concentration tended to increase while the 235U/238U isotopic ratio tended to decrease. The uranium concentration and 235U/238U were sensitive indicators to assess the pollution of uranium. The results of the present study suggest a potential risk from chronic DU exposure. Zhu et al. (2009) measured the uranium concentration at various time points after the implantation of DU chips into mice, and found uranium accumulation in the bone and the spleen, which gradually increased with time.

In addition, the present study conducted a more comprehensive evaluation of the immune function of mice after chronic exposure to DU. First, this study evaluated the innate immune function of the mice, particularly the function of NK cells and macrophages. The results revealed that the innate immune function of the DU300 group (300 mg/kg) was significantly inhibited. NK cells have immune surveillance and killing effects on tumour cells and virus-infected cells without the antigen sensitisation or the presence of antibodies. We demonstrated that the cytotoxicity of the NK cells gradually decreased with the increase in the exposure dose, suggesting that a consumption dose of 300 mg/kg or higher may lead to tumour susceptibility; however, this hypothesis requires further validation in future studies. The results of this study were consistent with the immunotoxicity of heavy metal cadmium. After newborn Sprague-Dawley rats were exposed to a low concentration of cadmium (10 ppb) for 24 days through breastfeeding, the results revealed a gender-related impact on the cytotoxic effect of NK cells on both day 28 and day 63 (Pillet et al., 2005). Holásková et al. (2012) reported that chronic exposure to low-dose cadmium in the parental generation causes a reduced proportion of splenic NK cells in the offspring mice, most likely leading to reduced tumour resistance. However, earlier studies revealed that NK cells are apparently not sensitive to the immunotoxicity that is caused by chronic exposure to lead or to lead combined with cadmium (Yücesoy et al., 1997; Neilan et al., 1983). We reason that in addition to gender, these differences may be mainly due to the channel of exposure, the dose of exposure, the duration of exposure, and the age at which exposure to the heavy metal occurs in the animal model. Additionally, DU is radioactive, which may also be one of the reasons for its unique effect compared with other heavy metals.

Previous studies on the effect of DU on macrophages mainly revealed the impact of soluble uranium on megakaryocytic cells (N8R383 or J774) or peritoneal macrophages via in vitro experiments (Kalinich et al., 2002; Gazin et al., 2004; Wan et al., 2006). The present study evaluated the immune function of mouse peritoneal macrophages after long-term exposure to DU and demonstrated that as the exposure dose increased, the ability of macrophage to secrete NO, TNF-α, IL-1β, and IL-18 decreased. All of these factors are involved in the antipathogenic effector functions of macrophages (Kawai and Akira, 2010). Therefore, inhibition of the secretory function of macrophages suggests that uranium
exposure weakens the capability of animals to fight against infection. In agreement with the results of this study, Dublineau et al. (2007) reported that, after rats were exposed to DU for 6 months through drinking water (40 mg/l), the secretion of NO was decreased in ileal tissue, which may be observed because uranium caused a reduction in NO-secreting cells (macrophages) in the ileal tissue, as well as a reduction in inducible NO synthase (iNOS) activators [C–C motif ligand 2 (CCL-2)] and an increase in NO inhibitors (IL-10). In the experiments of lead-induced immunotoxotoxicity, the inhibition of the NO secretion from macrophages was considered to be a sensitive indicator (Dietert and Pienpenbrink, 2006). The exposure of peritoneal macrophages to lead (20 μM) for 72 h led to decreased NO secretion, a decreased phagocytic index, and significantly increased catalase levels, which may increase the incidence of infectious diseases (Bussolaro et al., 2008). However, the phagocytosis experiments of neutral red particles in this study did not demonstrate decreased phagocytic activity in the DU300 group, suggesting that the phagocytic activity was not as sensitive to the DU-induced immunotoxicity as was the NO secretion and TNF-α secretion, which was similar to the lead-induced immunotoxicity of the macrophages (Dietert and Pienpenbrink, 2006). In addition, studies of the immune function of children suffering from irritable bowel syndrome have shown that a low dose of γ internal irradiation significantly changes their innate immune function and also leads to significant decreases in the macrophage activity and phagocytic index (Sheikh Sajadieh et al., 2010). However, Gazin et al. (2004) found that exposure of the NR8383 macrophages to uranium (50 μM) for 24 hours causes increased secretion of TNF–α, whereas the secretion of IL-1β and IL-10 is not affected by uranium exposure. We believe that these inconsistent results stem from the fact that the present study employed in vivo experiments to investigate the effect of long-term exposure to relatively low doses of DU.

Second, long-term exposure to DU caused changes in the humoral immune function of the mice. In particular, when the dose of uranium in the feed exceeded 30 mg/kg, the total serum IgG and IgE levels increased, the proliferative capacity of splenic B cells was enhanced, and the proportion of mlgM+mlgD+ double-positive B cells increased; the serum IgG level did not change significantly in the DU3 group (3 mg/kg), but the serum IgE level was significantly increased. IgG is the product of the secondary immune response, and IgE mainly mediates allergic reactions. The increase in the IgG and IgE levels strongly suggested that chronic exposure to DU might increase the susceptibility to allergic disease. At present, the researchers of lead exposure–induced immunotoxicity have not reached a consensus regarding the change in the total serum IgM and IgG levels. Generally, a sufficiently high dose and long exposure time leads to a decrease in the total serum IgM and IgG levels, while a short-term exposure at a low dose increases the total serum IgM and IgG levels. However, an increased serum IgG level has been recognised as the one of the significant markers of lead–induced immunotoxicity (Dietert and Pienpenbrink, 2006). This study also found that the chronic exposure to DU led to greater proliferative ability of splenic B cells stimulated by LPS, further suggesting that the DU exposure may promote the B cell–mediated humoral immune function. This result is different from those from acute exposure to large doses of DU. The results of our previous study (Hao et al., 2012a) showed that in four days after intraperitoneal injection of DU (10 mg/kg body weight), the proliferative ability of the splenic B cells was decreased. Further research on the characteristic B cell surface marker, BCR, showed that chronic exposure to DU increases the total number of spleen B lymphocytes and the ratio of mature B cells (mlgM+mlgD+) to the total number of B cells. This finding was the first discovery of the impact of chronic DU exposure on B-cell maturation, and the function of the mature B-cells in recognising antigens and mediating specific immune responses was thereby affected. The impact of DU on humoral immunity was apparently similar to that of radiation. Exposure to low doses of gamma external irradiation (10 CGy, 1 cGy/min) activated the thymus–dependent humoral immune and enhanced polyclonal B-cells in mice (Sharetski et al., 2000). It should be clarified that both immunosuppression and immune stimulation are immunotoxic reactions (Gleichmann et al., 1989).

Third, long-term exposure to DU led to changes in the cellular immune function in the DU300 group (300 mg/kg), including decreased proliferative ability of ConA-stimulated splenic T cells, suppression of delayed–type hypersensitivity, decrease in the number of CD3+ cells, and decrease in the ratio of CD4+/CD8+ splenic T cells. In the DU300 group (30 mg/kg), the proliferative ability of splenic T cells was also significantly decreased, suggesting reduced responsiveness of the T cells to mitogens. No significant change in the DU3 group (3 mg/kg) was observed. In the DU300 group, the inhibition of DTH that was primarily mediated by T cells suggested dysfunctional T-cell sensitisation, proliferation, and release of lymphokines or aggregation of lymphocytes through chemotactic effects, and this process mainly depended on the involvement of Th1 cells (Dietert and Pienpenbrink, 2006). Similar to the results of this study, pregnant female rats that are exposed to lead acetate (250 ppm) via drinking water from inception of the pregnancy to birth produced offspring in which the Th1 cells were suppressed at week 13 (Chen et al., 2004). Furthermore, many studies (Chen et al., 1999; Lee et al., 2001) have demonstrated that chronic lead exposure decreases the responsiveness of delayed–type hypersensitivity, which is believed to occur through the inhibition of Th1 cytokine IFN-γ. This study also revealed that 4 months of exposure to more than 300 mg/kg uranium in the diet decreases the proportion of the total splenic T lymphocytes (CD3+ cells). Moreover, the proportion of CD4+CD8– T lymphocytes was decreased, the proportion of CD4–CD8+ T lymphocytes was increased, and the ratio of CD4+/CD8– splenic T cells was decreased, suggesting an imbalance of the subsets of CD4+ and CD8+ T cells, which would cause a decrease in the cellular immune function mediated by the CD4+ T cells and a significantly weakened anti-viral infection capacity of the CD4+ T cells. Consistent with the results of this study, Wan et al. (2006) conducted in vitro experiments on CD4+ splenic T cells and reported that exposure to DU (500 μM) for 24 hours led to apoptosis and necrosis of the CD4+ T cells. Moreover, a survey of 148 males with occupational exposures to lead has demonstrated significant reductions in the ratio of serum CD4+/CD8+ T cells and in the levels of IFN-γ and TNF-α, while the proportion of CD8+ T cells and the IL-10 levels are significantly increased (García-Lestón et al., 2011). However, to the best of our knowledge, no immunological analyses of the uranium-exposed population have been conducted.

Finally, long-term exposure to DU led to significant changes in the level of cytokines released by stimulated splenic cells in the mice. In general, when the DU dose in feed was higher than 30 mg/kg, the chronic exposure decreased the expression of Th1 cytokines (IFN–γ, TNF-α) and increased the expression of Th2 cytokines (IL-4, IL-10) with a shift of Th1 cytokines to Th2 cytokines. To the best of our knowledge (Mosmann and Coffman, 1989; Abbas et al., 1996), Th1 cells mediate the immune response related to cytotoxicity and local inflammation and are involved in the formation of cellular immunity and delayed–type hypersensitivity. Th1 cells also activate iNOS in macrophages to promote their secretion of NO, thereby yielding the above-described results, including decreased proliferative ability of T cells, decreased responsiveness of DTH, and macrophage dysfunction—which are adequately explained by the inhibition of Th1 cytokines. The main function of Th2 cells is to stimulate B cells to proliferate and, subsequently, to generate antibodies, the production of which is associated with humoral immunity. Th2 cells may assist the mouse B cells to synthesise IgA, IgG, and IgE.
IgG, and IgE and may negatively regulate cytotoxic T cells (CTL) and NK cells. Therefore, the increased levels of Th2 cytokines offers a good explanation for the increase in the total serum IgG and IgE levels, as well as the weakened cytotoxic effect of the NK cells. Similar to the results of this study, numerous studies (Heo et al., 1997; Dietert and Piepenbrink, 2006; Gao et al., 2007) have demonstrated that exposure to low doses of lead causes a significant shift of Th1 cytokines to Th2 cytokines. However, chronic ingestion of DU by drinking water (40 mg/l) did not lead to modifications in the cytokine gene expression in Peyer’s patches (Dublineau et al., 2006). The differences may be due to the different exposure routes and evaluation tissue. In addition, before determination of cytokine, splenic cells were stimulated with ConA or PMA and ionomycin, which would increase the differences between groups. The limitation of the present study is that only one time point was evaluated; thus, the results do not reflect the dynamic changes in immune function based on the age of the animal and the exposure time to DU.

In summary, after 4 months of exposure to low doses of DU (lower than 30 mg/kg) through the diet in young mice, the impact of DU exposure on the immune function of the body was relatively small. However, when a higher dose (300 mg/kg) of DU was applied, significant changes in immune function ensued, mainly manifested as decreased innate immune function, inhibition of cellular immune function, and abnormal humoral immune function, which mainly occurred because of the imbalance of Th1/Th2 cells in vivo, leading to the deregulation of various immune functions in the body. The DU-induced shift of Th cells (towards Th2) may lead to increased susceptibility to autoimmune disease, cancer, and infectious diseases. The mechanism for Th1/Th2 imbalance was complex, and the transcription factors (GATA3, STAT1, STAT4, STAT6, T-bet, c-Maf, and so on) might play a relevant role in this process. Further study is necessary to elucidate the mechanism of DU exposure on Th1/Th2 balance.

In addition, our results suggest that the immune system is one of the systems most sensitive to damage induced by chronic uranium poisoning. Therefore, the present study indicates that in-depth investigations examining the immune function of the population that is chronically exposed to uranium should be performed, which may lead to the discovery of valuable biomarkers.

Conflict of interest

The authors declare no conflict of interest.

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