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Effects of subchronic exposure of nonylphenol on the expression of immune-related factors and estrogen receptors in the spleen of rats

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Abstract

Background: Previous studies have shown that EDCs may activate nuclear transcription factor, such as activator protein-1 (AP-1), nuclear factor of activated Tcells (NF-AT) and nuclear factor kappa B (NF-κB) in the process of immune damage. At the same time, some experts believed that estrogen may play an important role in this process. As a typical representative of EDCs, nonylphenol (NP) has not been reported. The aim of this work was to explore the relationship between the immune inflammatory damage and the changes in estrogen expression in male rats during the chronic exposure to NP at environmental concentrations. Sixty SPF Sprague–Dawley rats were divided into five groups ($n = 12$ per group): blank control group (corn oil), low-dose NP exposure group (0.4 mg/kg/d), medium-dose NP exposure group (4 mg/kg/d), high-dose NP exposure group (40 mg/kg/d), and estradiol control group (E_2: 30 μg/kg/d).

Results: Compared with the control group, rat spleen organ coefficient, number of spleen nodules, relative area of lymph nodes and white pulp were relatively reduced in the L (NP, 0.4 mg/kg) and H (NP, 40 mg/kg) exposure dose groups ($P < 0.001$). Lymphocytes were rich in cytoplasm, mitochondria were swollen, part of the cristae was reduced, and rough endoplasmic reticulum was expanded. The serum levels of IgG ($P < 0.001$) and IgM ($P = 0.002$) showed a downward trend. The percentage of Th cells ($CD3^+CD4^+$) was significantly decreased ($P < 0.001$), and the percentage of B lymphocytes shows an opposite trend ($P < 0.001$). Giemsa staining showed that the number of neutrophils ($P < 0.001$) was increased. The expressions of estrogen receptor ER-α and ER-β protein in the spleen increased significantly ($P < 0.001$). The expressions of AP-1 protein and NF-AT protein in the spleen were increased, and the expression of NF-KB protein was decreased ($P < 0.001$). The expressions of IL-4, ER-α and ER-β ($P < 0.001$) levels in serum increased. The mRNA-seq bioinformatics detection showed the final differentially expressed immune-inflammatory-related genes between the control and H-NP groups as follow: down-regulated: TLR4, Gata3, IL12, up-regulated: TNF-a, IL10, INOS. The mRNA expressions of ER-α, ER-β, NF-KB, IL4, AP-1, TLR4, Gata3, and NF-AT were consistent with the results of mRNA-seq analysis. NP content was correlated with the expressions of ER-α, ER-β, IL4, AP-1, NF-AT, TLR4, NF-KB, as well as IL-12 proteins in the spleen tissue ($[r] < 1$, $P < 0.05$).

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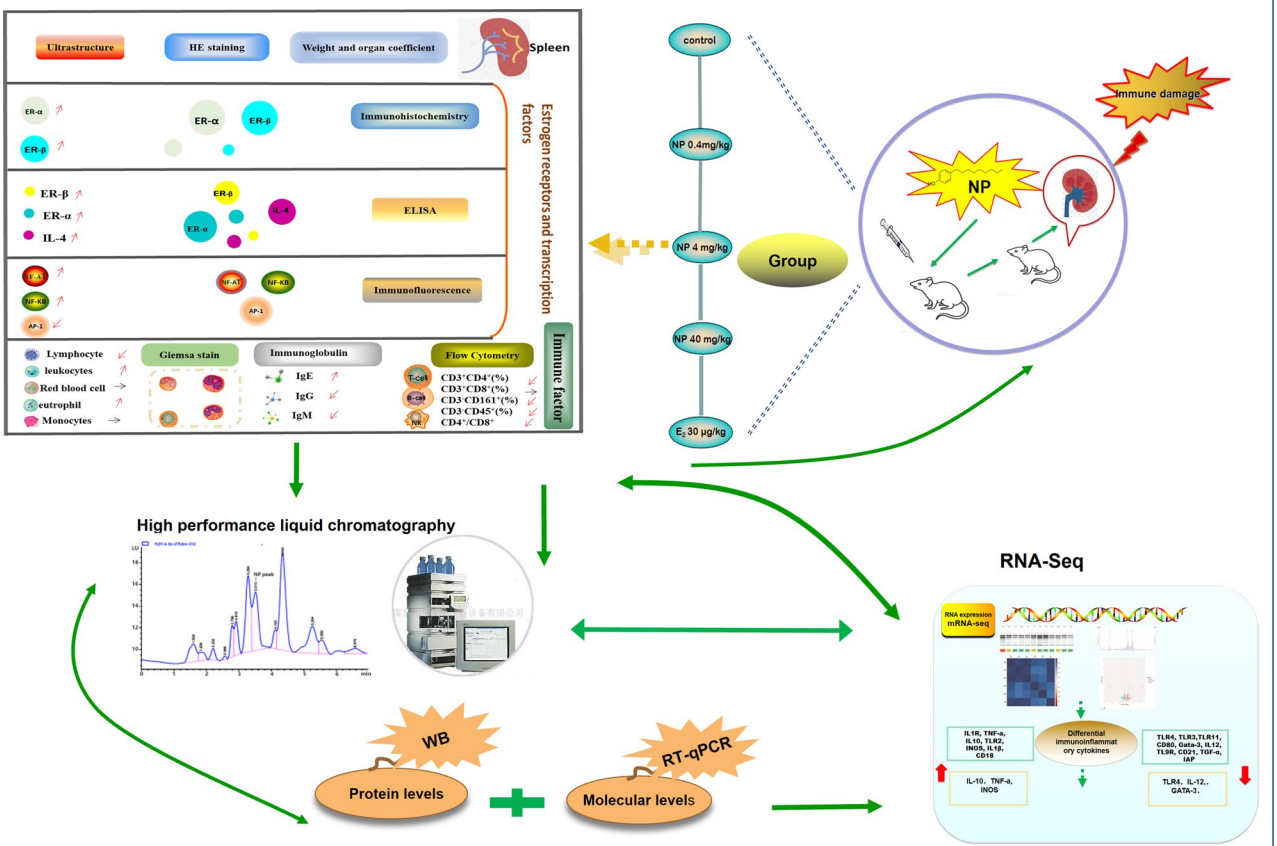
Conclusions: Chronic exposure to NP at environmental concentration could cause immune dysfunction, resulting in immunotoxicity and inflammatory effects, and lead to changes in the activity of transcription factors and differential immune inflammatory factors in rats.

Keywords: Nonylphenol, Immune-related factors, Estrogen receptor, Spleen

Graphical Abstract



Introduction

As a typical representative of EDCs, nonylphenol (NP) is one of the main metabolites of nonylphenol polyoxyethylene ethers (NPEs), and it is extremely difficult to degrade in the environment. As technical syntheses are based on nonene obtained from trimerization of propene, NP consists of a very complex mixture of isomers with differently branched nonyl groups, and the isomer composition varies depending on the manufacturer. Furthermore, in the environment, isomer specific degradation alters the isomer mixture again. This results in a very complex contamination problem concerning the endocrine-disrupting potential of NP. Theoretically, there are 211 possible constitutional isomers and approximately 100 have been observed in environmentally relevant matrices [1–10]. A large number of studies have confirmed that EDCs have estrogen effects, can competitively bind to estrogen receptors, and affect the metabolism of estrogen in the body. One of its main mechanisms of function is the interaction with steroid hormone receptors (such as androgens, estrogen, and adrenal hormones), and environmental estrogens are currently the most concerned sex hormones [11–20]. It can regulate the activity of various immune cells such as T cells, B cells, and macrophages by reducing the synthesis and secretion of various cytokines and immunoglobulins [21, 22].

The structural expression, functional regulation and variation of ER-type receptors in different target tissues and their relationship with the occurrence and development of diseases have been research hotspots in recent years [23–26]. Humans show strong gender differences in infection and autoimmunity [27]. Experts speculated that

sex hormones may regulate immune responses [17, 28]. Molero et al. proved that the expression of ERs in neutrophils depended on the woman's menstrual cycle [29]. A recent study showed that the sex-specificity of BPA exposure changes the microanatomical structure of spleen cells in CD1 mice [30]. ERα was found to be sensitive to 17β-estradiol in the ER of male neutrophils, confirming that xenoestrogens, as endogenous estrogen, exhibited different effects that may depend on gender [31]. The phenotypic differences in EDCs exposure between men and women may be related to the impact of gender on immune function [32]. Bisphenol A (BPA) has been shown to change the expression of estrogen receptors in terms of sex and dosage. Recently, it has been discovered that BPA may change the function of T cells by regulating the expression of ERs [33]. The inhibition of Th1 cells and the enhancement of Th2 cells caused by NP exposure may be due to the effect of ERs [34, 35]. These results indicated that the estrogen receptor may be a potential target of EDCs immunomodulation [11, 14, 36–41].

Nuclear factor of activated Tcells (NF-AT), Activator protein-1 (AP-1), and Nuclear Factor Kappa B (NF-κB) in T cells are multi-functional and important nuclear transcription factors involved in inflammation, immune response and stress-related multiple gene expression. These nuclear transcription factors' abnormal activation or complete inhibition is related to the occurrence of many diseases [42–44]. The regulation of IL-4 by Th cells of bis (2-ethylhexyl) phthalate (DEHP)—exposed mice depended on the activation of the NF-AT of activated T cells [45–47]. EDCs blocked the MAPK and NF-κB pathways while activated the NF-AT [48]. Lee et al. found that 4-Octylphenol (4-OP) promoted the production of T cells by activating NF-AT, thereby enhancing the amount of IL-4 [49]. Prenatal exposure of mice to EDCs interfered with the regulation of estrogen receptors, NF-AT, AP-1, and NF-κB nuclear factors, affected Fas/Fas gene ligands, and the differentiation of T cells, and has a significant effect on immune function in the long term [50].

Exposure to EDCs may cause damage to the body's immune function [51–54]. Related domestic and foreign studies have shown that EDCs such as BPA and PAE may cause changes of the AP-1, NF-AT, and NF-κB in the process of immune damage. A large amount of data in the literature confirmed the effect of EDCs on the estrogen nuclear receptors in immune cells [55–57]. At the same time, some experts believed that estrogen may play an important role in this process [58]. As a typical representative of EDCs, NP has estrogen-like activity [59, 60]. There are few reports on the changes of transcription factors and estrogen during the process of NP-induced immune function damage [42, 47, 58, 61–64]. In this study, the main route of exposure to NP was simulated by gavage administration through animal experiments, and sub-chronic long-term low-concentration NP exposure was carried out. To investigate whether long-term exposure to low concentrations of NP affects immune function, and the changes and associations between immune factors and estrogen receptors. At the same time, combined with high-throughput screening technology, the overall effect was evaluated for their toxicological effects and relationships, and the possible mechanism model of estrogen in the process of NP acting on immune factors was conceived. To explore the association effects of NP subchronic exposure on estrogen expression, transcription factor changes, immune injury and inflammation in rats.

Materials and methods

Reagents

Nonylphenol (99% purity, Product Code: BT5655) was purchased from the Shandong Xiya Chemical Industry Co., Ltd. (Shandong, China). Estradiol (E_2) was purchased from Zhejiang Lianshuo Biotechnology Co., Ltd. (96.3% purity, Product Code: 2RR9-37EB), the antibody of lymphocyte population detected by flow cytometry was purchased from BD Company (Becton Dickinson, US): CD3 antibody (Product Code: 554833), CD161a antibody (Product Code: 565413), CD45R antibody (Product Code: 561876), CD4 antibody (Product Code: 561833), CD8a antibody (Product Code: 561611). Hematoxylin and Eosin Staining Kit (Product Code: G1018) Purchased from Wuhan Guge Biological Co., Ltd. (Hubei, China). BCA protein quantification kit was purchased from Beijing Soleibo Technology Co., Ltd. CD8a antibody (Product Code: PC0020). Estrogen receptor alpha (ERα) and estrogen receptor beta (ERβ) antibodies (Product Code: ab13504 and ab53358), Toll-like receptor-4 antibody (Product Code: ab8376), AP-1 antibody (Product Code: ab21981) and NF-κB antibody (Product Code: ab16502), NF-AT antibody (Product Code: ab59204) IL-4 antibody (Product Code: ab9622) Purchased from Abcam (Cambridge, MA, USA). HP-1100 High Performance Liquid Chromatography (HPLC) was purchased from Agilent Technologies (Palo Alto, CA, USA). Rat estrogen receptor beta (ERβ) (Product Code: JL45045) and Rat estrogen receptor alpha (ERα) enzyme-linked immunosorbent assay kit was purchased from China Jianglai Biotechnology Co., Ltd (Shanghai, China). (Product Code: JL26212). Reverse transcriptase kit and amplification reagent kit were purchased from Takara (Bio, Inc., Japan, Product Code: 9109 and RR820A). Trizol reagent was purchased from Thermo-Invitrogen, USA (Product Code: 15596018). All other chemicals were commercially available.

Experimental design

Experimental animals

This experiment was proved the Animal Experiment Ethics Committee of Zunyi Medical University [No. Lun Shen (2018) No. 2-166]. All experiments were conducted under the guidelines and regulations of Zunyi Medical University. To avoid the interference of internal estrogen, 60 male Sprague–Dawley rats of 4 weeks were provided by Hunan Changsha Tianqin Biological Technology Co., Ltd. [License No. SCXK (Xiang) 2014-0011].

Animal grouping

After 1 week of adaptive feeding, they were randomly divided into 5 groups (control, 3 NP dose and E_2 groups) with 12 animals in each group. The control group (C) was given corn oil 5 ml/kg daily by gavage, and the NP low (L), medium (M) and high (H) dose groups were given NP 0.4, 4, 40 mg/kg/day. The gavage volume was 5 ml/kg/bw/day and gavage was conducted at 8 o'clock every morning, and continued for 180 days. Animal room conditions were: room temperature: 25 ± 3 °C, 55 ± 5% humidity, 12 h dark/light cycle, free feeding and drinking. Since the plastic contains NP, the rats were fed in polyacrylamide cages to reduce interference.

Subchronic exposure dose setting

In the Pearl River of China, maximum NP content and sediment in water were 3 μg/l and 12 mg/kg, while sediment in fish was 9 mg/kg [65]. The previous research of our lab showed that the 200 mg/kg of NP exposure affected the normal development of the immune organs of the rats' offspring [66]. In addition, The Danish Institute of Toxicology, Safety and Toxicology proposed the allowable daily intake of NP for humans was 5 μg/kg bw/day, and the tolerable daily intake of rats was calculated as 0.5 mg/kg bw according to the safety factor (human:rat = 1:100)/day. Since the tolerable daily NP intake in humans is 5 μg/kg bw/day, human exposure to NP amounts to 40 mg/kg/d were based on the maximum daily NP tolerance (5 μg bw/day) for an adult weighing 60 kg (this dose considers NP accumulation and the body's long-term multi-pathway exposure to NP) [60, 67–70]. In this study, the human NP exposure dose was much lower than the 40 mg/kg/d NP exposure dose, and the tolerable daily intake of mice was calculated to be 0.5 mg/kg bw/day according to the safety factor (human:mice = 1:100). The other dose groups were 10 and 100 times higher than this dose group; hence, the NP doses were 0.4, 4, and 40 mg/kg bw/day, respectively. As reported, the 17α-ethinylestradiol concentrations in wastewater ranged from 0.00 to 0.52 ng/l, and the NP concentrations ranged from 287 to 2058 ng/l. The NP/17α-ethinylestradiol levels in wastewater were approximately 0–3958 times higher [71, 72]. The E_2 treatment group dosage was set at 30 μg/kg regarding the maximum exposure concentration of NP and 17α-ethinylestradiol.

Coefficient of the rats body weight and the spleen

The weight of the rats was monitored before each gavage. After 180 days of overnight fasting, the rats were anesthetized with 20% urethane and their whole blood was collected from the abdominal aorta. After standing for 2 h, the blood serum was collected by centrifugation. The spleen tissues were quickly taken on ice, dried on filter papers and weighed. The pieces of 0.5 × 0.5 cm spleen was fixed in 10% formaldehyde. The remaining spleen tissues were frozen at − 80 °C for later use. The organ coefficient was calculated according to the formula $C = (m_1/m_2) \times 100\%$, where C represented the organ coefficient, m_1 represented the wet weight of the spleen, and m_2 represented the weight of the rat.

Pathological alterations of spleen tissue

Histopathologic observation

After soaked in 10% formaldehyde for 24 h, the rat spleen tissues were dehydrated, and embedded in paraffin. The sections were stained with hematoxylin and eosin and observed with an optical microscope (HT7700, Hitachi, Tokyo, Japan). There were 3 replicates of each sample. Image-pro plus 6.0 software (Media Cybernetics, Rockville, MD) was used for quantitative analysis [71].

Ultrastructural observation

Approximately 1 cubic millimeter of rat spleen tissues were fixed in 2.5% neutral glutaraldehyde. The tissues were rinsed with 0.1 M phosphate buffered solution (PBS), dehydrated with an ascending graded alcohol series (30%, 50%, 70%, 90%, 100%), and then followed by permeation, embedding, curing, sectioning, electron staining. After drying, the sections were observed by a transmission electron microscope (Hitachi Co., Ltd., Tokyo, Japan) [71].

Detection of the immune inflammatory cells by Giemsa staining

One drop of fresh blood was added into the clean glass slide near the ground glass end, mixed with Jimsa A and Jimsa B, respectively, stained for about 3–5 min, rinsed the solution, and dried by air, and then about 200 cells were counted under the microscope [73].

Detection of serum ER-α, ER-β and IL-4 by enzyme-linked immunosorbent assay (ELISA)

Serum samples were prepared by centrifuging the whole blood samples. The serum was centrifuged after being

stored in a refrigerator at 4 °C overnight. Serum IL-4, ER-α, ER-β levels were measured by ELISA kits under the manufacture's protocols. Data were collected and calculated [74].

Detection of the serum levels of immunoglobulins (IgE, IgG and IgM)

One ml of the standard protein solution at the concentration 0, 0.4, 0.8, 1.2, 1.6 and 2.0 mg/ml were added to 6 colorimetric tubes, respectively, with 3 parallel tubes for each concentration. 4 ml of Biuret reagent were added after making up to 3 ml of each tube. The measurement was carried out at the wavelength of 546 nm in the UV spectrometer (Unico UV-2100, Shanghai).

Detection of the typing of lymphocyte subsets by flow cytometric

The peripheral blood lymphocytes were suspended in 1 ml pre-cooled Stain Buffer (The composition of aqueous buffered solution was phosphate buffered solution (PBS) fetal bovine serum and $\leq 0.09\%$ sodium azide) centrifuged at 300 g/min for 10 min at 4 °C. This step was repeated twice. Finally, the final cell concentration was adjusted to 2×10^7 cells/ml with pre-cooled Stain Buffer. 100 µl of the cell suspension was added to a flow tube, blotted with specific surface antibody, and incubated for 20 min in the dark (on ice). The cells were washed twice with Stain Buffer, 1 ml/tube/time, centrifuged at $300 \times g$ for 5 min. Finally the cells were resuspended with 0.5 ml Stain Buffer and tested on a flow cytometer (BD FACS Calibur, USA) [75, 76].

Detection of NP concentration in spleen by high-performance liquid chromatography (HPLC)

According to Yu et al. methods [71], 0.05 g spleen tissues were homogenized with 2 ml of *n*-hexane-diethyl ether (volume ratio 7:3), and centrifuged at 4000 rpm for 10 min. The supernatant was transferred to another clean glass tube, dried in a 40 °C water bath, and dissolved in 0.5 ml acetonitrile. The specimen, filtered with a 0.22 µm membrane (pre-rinsed with acetonitrile) into the sample bottle, was tested by a HP-1200 system (Agilent, Santa-Clara, CA, USA).

Detection of the expression levels of ER-α and ER-β proteins in spleen by immunohistochemical analysis

Paraffin sections were blocked with serum, blotted with primary antibody and secondary antibody. The nucleus were stained with DAPI. Finally, specimens were observed under a microscope and calculated Integral-Optical Density (IOD) [59, 77, 78].

Detection of the expression levels of AP-1, NF-AT and NF-KB proteins in spleen by immunofluorescence analysis

Paraffin sections were blocked with serum first, then marked by a histochemical pen and autofluorescence quencher was added to the sections followed by primary antibody and secondary antibody. The nuclei were stained with DAPI. Finally, specimens were observed under a fluorescence microscope and calculated Integral-Optical Density (IOD) [66, 77].

Detection of the expression levels of inflammatory-related genes (ER-α, ER-β, AP-1, NF-AT, NF-κB, IL-10 and IL-12) in spleen

50–100 mg of spleen tissues were homogenized with 1 ml of TRIzol (Invitrogen, USA) on ice and kept at room temperature for 10 min; 200 µl chloroform was added and centrifuged. RNA integrity was measured by mixing 1–20 µl of Qubit RNA IQ Assay kit samples RNA IQ working solution and then measuring on a Qubit 4 fluorometer. The results were expressed as RNA IQ values ranging from 1 to 10, similar to other RNA quality scores, with values closer to 10 indicating higher sample integrity. GAPDH was as the housekeeping gene, and the primer sequences are shown in Additional file 4: Table S1. The relative quantitative formula: $Q = 2^{-\Delta\Delta Ct}$, $-\Delta\Delta Ct =$ (average Ct value of target gene in experimental group-average Ct value of reference gene in experimental group) − (average Ct value of target gene in control group-average Ct value of reference gene in control group) was used to calculate the expression of each gene [71].

Detection of the expression levels of inflammatory-related proteins (ER-α, ER-β, AP-1, NF-AT, NF-κB, IL-10 and IL-12) in spleen

The expressions of synapse-associated proteins (ER-α, ER-β, AP-1, NF-AT, NF-κB, IL-10 and IL-12) in spleen tissues were detected by Western Blot. Total protein was extracted from 50 to 100 mg of spleen with 600 µl lysis buffer. Protein was analyzed by Image-lab (BIO-RAD, Version: 5.1.0) and Image software (JNIH, Bethesda, USA). GAPDH was as an internal reference to calculate the expression [71, 79].

Analysis of spleen tissue by RNA-Seq

The total RNA was extracted and RNA library was constructed using KAPA Stranded RNA-Seq Library Prep Kit (Illumina). mRNA-seq was conducted under the Illumina HiSeq X Ten sequencing platform, and the sequencing volume was set to 6G reads [80–82], and

the results of RNA-Seq are shown in Additional file 5: Table S2.

Statistical analysis

All experimental data were shown as mean ± SEM. Statistical analysis was performed using SPSS 22.0 software (SPSS Inc., Chicago, IL, USA). Variance test and one-way analysis of variance (One-Way ANOVA) were used in two-group comparisons. For further pairwise comparisons between groups, if the variances were equal, the Tukey test was used for pairwise comparisons, and if the variances were not uniform, Dunnett's T3 was used for pairwise comparisons. Pearson correlation analysis was used for the correlation of normal measurement data, and Spearman correlation analysis was used for the correlation of non-normal measurement data. $P<0.05$ was considered statistically significant.

Results

Effects of NP on body weight and organ coefficient in rats

There was an increased tendency of their body weight when rats exposed to NP for 30–120 days, but there was no significant difference in groups ($P>0.05$). When rats were exposed to NP for 160 days, there was a significant difference in body weights in groups ($F=8.487$, $P<0.001$). However, compared with the control group, the weight of the spleen after 180 days of subchronic exposure to NP showed a tendency to decrease, and there were significant differences in groups ($F=12.166$, $P<0.001$), as shown in Fig. 1A, B.

Histopathologic alterations of spleen

The HE staining results of spleen tissues were analyzed. The number of lymphocytes and macrophages in the NP-exposed group was significantly reduced, the marginal zone of the follicle was obviously widened, plasma cell infiltration can be seen, and the central artery wall had a tendency to gradually thicken. The splenic sinus was congested and dilated, and a few neutrophil infiltrations were seen in the red pulp. Image-pro plus 6.0 software was used to quantitatively analyze the relative area of splenic white pulp, the relative area of splenic lymph nodes, and the number of spleen nodules in the spleen tissues of each group (Additional file 1: Fig. S1; Additional file 2: Fig. S2). The results showed the relative area of the spleen tissue of the rats in each NP group decreased compared with that of the control group ($F=26.03$, $P<0.001$). The relative area of lymph nodes in the M (NP, 4 mg/kg) and L (NP, 0.4 mg/kg) groups gradually decreased ($F=25.76$, $P<0.001$), and the number of spleen nodules decreased to a certain extent compared with the control group ($F=16.065$, $P<0.001$), as shown in Fig. 1C.

Effects of NP on the ultrastructure of spleen

In the control group lymphocyte nucleoplasm ratio was high, cytoplasm had a few organelles, mitochondria and rough endoplasmic reticulum were more and no obvious abnormalities. The lymphocytes in the Low NP group were slightly rich in cytoplasm, mitochondria were slightly swollen, part of the cristae was reduced and vacuolated, and the rough endoplasmic reticulum had no obvious abnormalities. In the Middle NP group, the lymphocyte nuclear chromatin agglomerated with agglomerated edges, cytoplasmic mitochondria were swollen, cristae were reduced, vacuolization, mitochondrial membrane was incomplete, and the rough endoplasmic reticulum was slightly expanded. In the High NP group, lymphocytes were slightly rich in cytoplasm, mitochondria were slightly swollen, some cristae were slightly reduced, focal vacuolation, and rough endoplasmic reticulum were slightly expanded. The nuclear chromatin in the estradiol group appeared clumping and pyknosis, as shown in Fig. 1D.

Effects of NP on the serum immune inflammatory cells

The number of neutrophils in the M and L NP groups increased compared to the control, and there were significant differences in the groups group ($F=21.35$, $P<0.001$). There were no significant difference of white blood cell count ($F=2.477$, $P=0.077$), lymphocyte count ($F=1.953$, $P=0.141$), monocyte count ($F=1.106$, $P=0.369$), and red blood cell count ($F=0.141$, $P=0.965$) among the groups. The results of cell counting are shown in Fig. 2A.

Comparisons of serum levels of immunoglobulins (IgE, IgG and IgM)

With the increase of the NP exposure dose, the serum levels of immunoglobulin IgG ($F=48.582$, $P<0.001$) and IgM ($F=5.709$, $P=0.002$) decreased relative to the control group, and the difference in the groups was statistically significant. There was an increasing trend of the

(See figure on next page.)

Fig. 1 Effects of subchronic NP exposure for 180 days on the spleen tissues of SD male rats. **A** Alteration in body weight of rats exposed to NP for 180 days ($n=8$). **B** Comparisons of organ weights and organ coefficients ($n=8$). **C** HE staining of spleen tissues and quantitative analysis of HE staining ($n=9$) [HE × 100, relative area of spleen white pulp, relative area of splenic lymph node, number of spleen nodules, relative area of marginal zone (mm^2) = relative area of white pulp (mm^2)-area of lymph node (mm^2)]. **D** Transmission electron microscope images of rat spleen tissues after subchronic exposure to NP for 180 days. Data were presented as the mean ± SEM, one-way ANOVA, [a]vs. control group, $P<0.05$; [b]vs. low NP group, $P<0.05$; [c]vs. middle NP group, $P<0.05$; [d]vs. high NP group, $P<0.05$

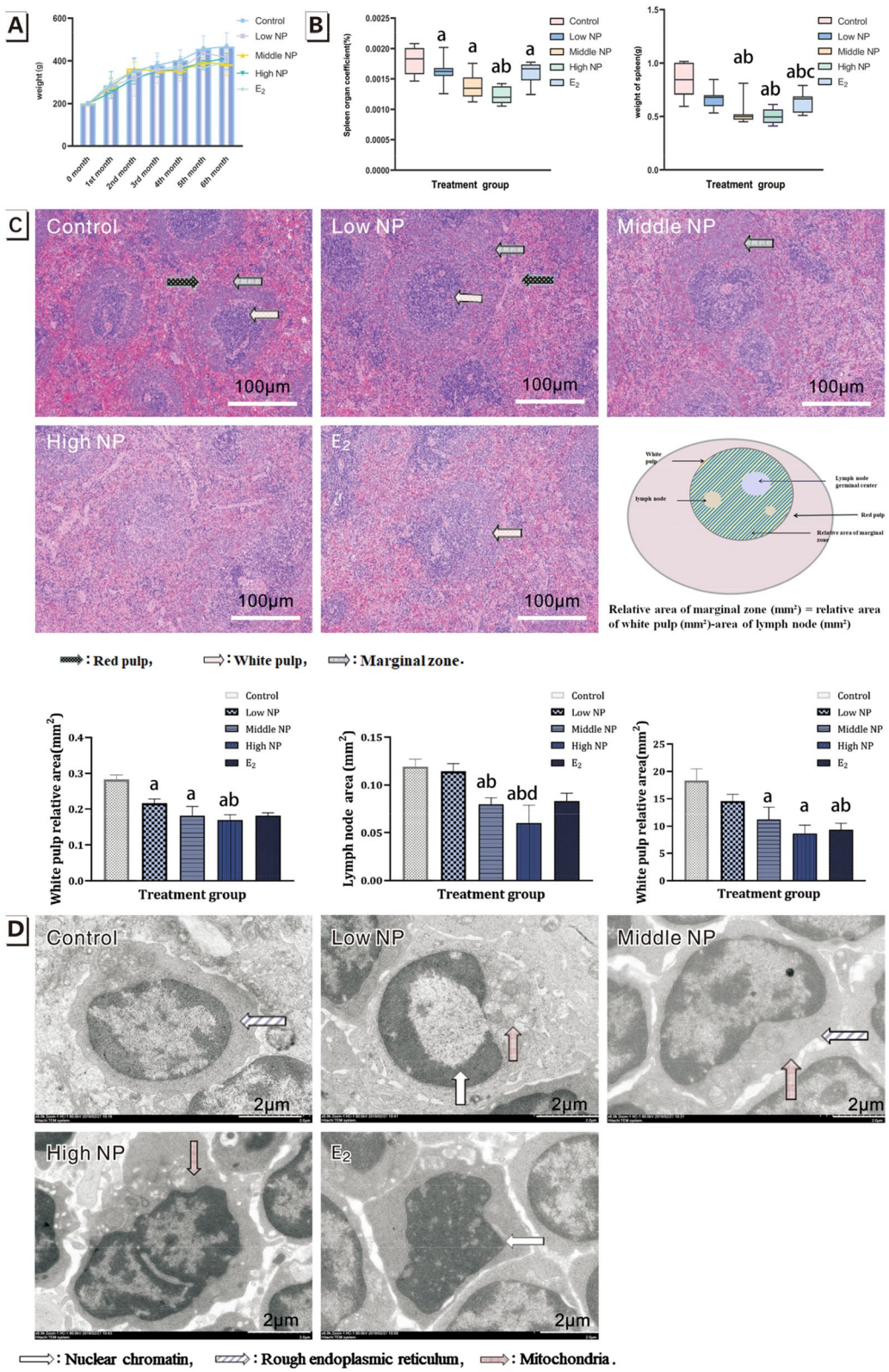


Fig. 1 (See legend on previous page.)

serum immunoglobulin IgE content compared with the control group, and the differences among the groups were statistically significant ($F=93.559$, $P<0.001$), as shown in Fig. 2B.

Effects of NP on the typing of lymphocyte subsets

The percentage of Th cells ($CD3^+CD4^+$) in each NP-exposed group was significantly lower than that of the control group, and the difference in the groups was statistically significant ($F=27.137$, $P<0.001$). The percentage of Ts cells ($CD3^+CD8^+$) in each NP-exposed dose group was lower than the control group C group, and the difference among the groups was not statistically significant ($F=0.878$, $P=0.491$). Compared with the control group, the ratio of $CD4^+$ to $CD8^+$ in H NP group had a downward trend, and the difference was significant ($F=3.941$, $P=0.013$). The percentage of B lymphocytes ($CD3^-CD161^+$) in each NP-exposed group was significantly higher than that of the control group, and the difference between the groups was statistically significant ($F=39.814$, $P<0.001$). The percentage of NK cells ($CD3^-CD45^+$) in each NP-exposed group was lower than that in the control group, and the difference was not statistically significant ($F=83.746$, $P<0.001$), as shown in Fig. 2C, D.

Effects of NP on the serum levels of ER-α, ER-β and IL-4

The levels of IL-4 ($F=568.4$, $P<0.001$), ER-α ($F=38.9$, $P<0.001$), and ER-β ($F=31.51$, $P<0.001$) in serum were higher than those in the control group, and the differences among the groups were significant, as shown in Fig. 3A.

Effects of NP on the expression levels of ER-α and ER-β proteins in spleen

The ERα was mainly expressed in the nucleus of granulocytes (shown by the arrow), which was diffusely distributed and strongly positive. Compared with the control group, the ER-α staining in each NP group gradually increased with the increase of the NP dose ($F=189.3$, $P<0.001$). The ER-β was mainly expressed in the cytoplasm of splenic lymphocytes (shown by the arrow), which was widely distributed and strongly positive. As the NP dose increased, the intensity of staining of the ER-β gradually increased ($F=414.9$, $P<0.001$), as shown in Fig. 3B, C.

Effects of NP on the expression levels of AP-1, NF-AT and NF-KB proteins in spleen

The expressions of the three transcription factors positively expressed in the cytoplasm of splenic lymphocytes. Compared with the control group, the fluorescence staining intensity of NF-AT ($F=189.3$, $P<0.001$) and AP-1 ($F=354.9$, $P<0.001$) in the spleen of rats gradually increased in each NP group. The fluorescence intensity of NF-κB staining gradually weakened. The difference among the groups was significant ($F=413.8$, $P<0.001$), as shown in Fig. 3D, E.

Effects of NP on the expression levels of inflammatory-related genes (ER-α, ER-β, AP-1, NF-AT, NF-Kb, IL-10 and IL-12) in spleen

The mRNA expressions levels of ER-α ($F=10.95$, $P<0.001$), ER-β ($F=4.891$, $P=0.005$), IL-10 ($F=4.223$, $P=0.01$), AP-1 ($F=3.543$, $P=0.02$), NF-AT ($F=2.839$, $P<0.05$), IL-4 ($F=4.212$, $P=0.01$), TLR4 ($F=5.884$, $P=0.002$), NF-κB ($F=6.653$, $P<0.001$), IL-12 ($F=3.137$, $P=0.02$) and GATA-3 ($F=4.223$, $P=0.01$) in each NP group were more higher than in the blank control group ($P<0.05$), as shown in Fig. 4A.

Effects of NP on the expression levels of inflammatory-related proteins (ER-α, ER-β, AP-1, NF-AT, NF-κB, IL-10 and IL-12) in spleen

Compared with the control group, the expression of ER-α ($F=193.8$, $P<0.001$), ER-β ($F=158.6$, $P<0.001$), NF-AT ($F=7.149$, $P<0.001$), IL-4 ($F=11.43$, $P<0.001$) and AP-1 ($F=27.94$, $P<0.001$) protein in the spleen gradually increased in each NP-exposed group, and there were statistical differences among the groups. The protein expressions of TLR4 ($F=90.81$, $P<0.001$), NF-κB ($F=162.5$, $P<0.001$) and IL-12 ($F=17.55$, $P<0.001$) gradually decreased, and the differences among the groups were statistically significant, as shown in Fig. 4B, C.

Analysis of spleen tissue by RNA-Seq

A total of 6 samples (3 biological replicates) of the control group and the NP exposure group were analyzed. The differentially expressed genes were selected by DESeq2. In this

(See figure on next page.)

Fig. 2 Effect of subchronic NP exposure for 180 days on the immune function of SD male rats. **A** Immune inflammatory cells counting in Giemsa staining ($n=8$). **B** Comparisons of immunoglobulin IgE, IgG, IgM contents in rat serum ($n=6$). **C** Type of rat lymphocyte in each dose group and each lymphocyte changes. **D** Types of rat lymphocyte populations in each dose group and quantitative analysis of changes in indicators of lymphocyte populations. The first line was T lymphocyte typing, the second line was B lymphocyte typing, and the third line was NK cell typing. Among them, CD3 was the surface marker of splenic T lymphocytes, and CD45R was the surface marker of splenic B lymphocytes; CD161a was the surface marker of NK cells. APC was allophycocyanin; FITC was fluorescein isothiocyanate; PE was phycoerythrin. Data were presented as the mean ± SEM, one-way ANOVA, [a]vs. control group, $P<0.05$; [b]vs. low NP group, $P<0.05$; [c]vs. middle NP group, $P<0.05$

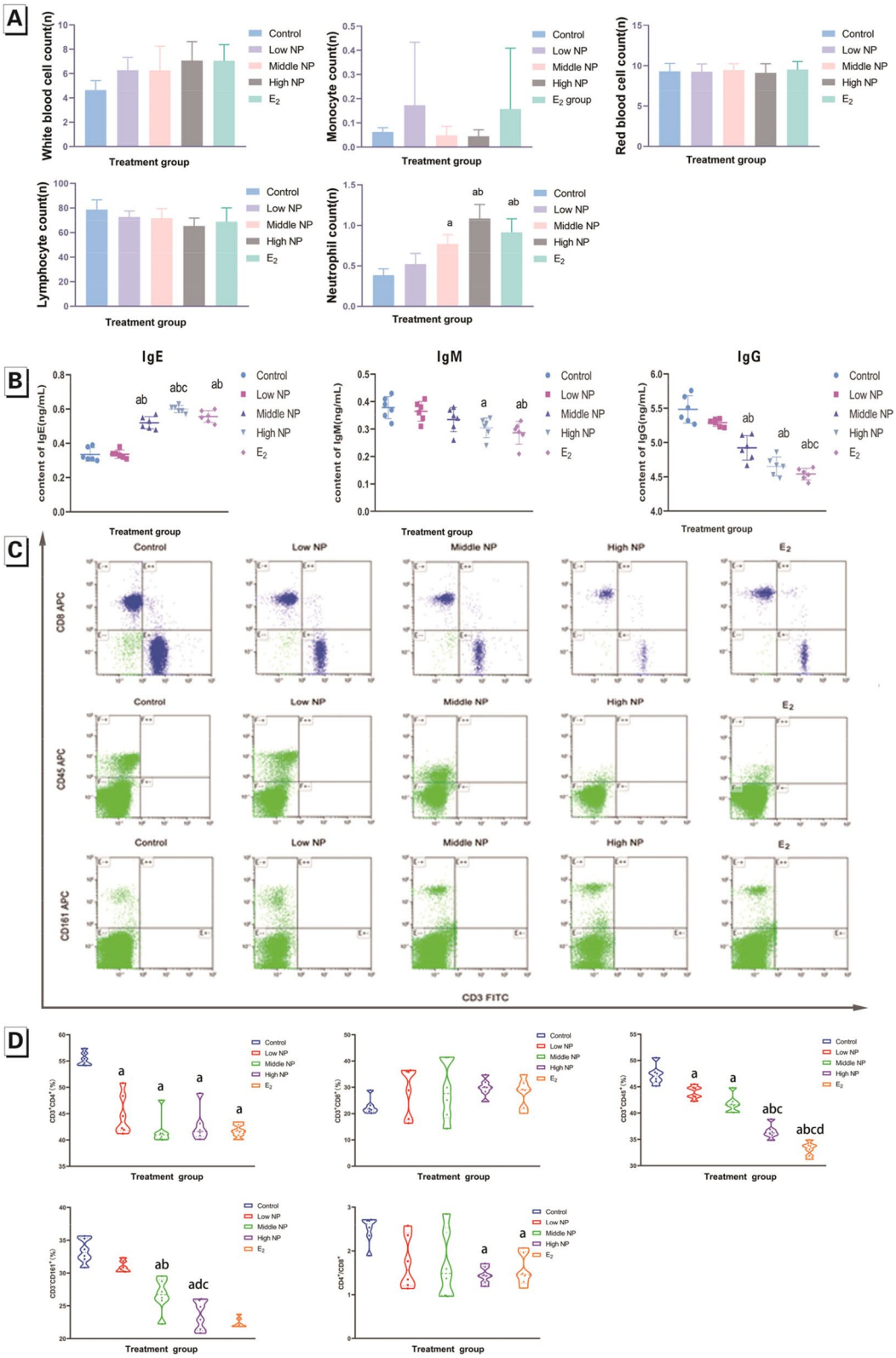


Fig. 2 (See legend on previous page.)

analysis, the screening conditions were P value < 0.05 and fold-change greater than 2 times and less than 0.5 times. The pathway analysis of the differential genes was based on the KEGG database (http://www.genome.jp/kegg/), and the pathways that were significantly related to the differential genes were screened from the database. In the final comparison, 810 differential transcripts were obtained, of which 363 genes were up-regulated and 447 genes were down-regulated. As shown in the volcano map (Fig. 5D), a correlation analysis heat map (Fig. 5A), and a cluster map of differential conversion rates (Fig. 5C) based on the differentially expressed genes, there was a significant difference between the control group and the NP group.

As shown in Fig. 5, the biological processes, cell composition, and molecular functions analyzed from GO were integrated through multiple functional levels of cells, tissues, organs, and species, combined with the significant difference P value, and pathway analysis (as shown in Fig. 5B, E) differential gene signal pathway analysis bubble diagram and gene signal pathway diagram (Fig. 5G, H), select significant cytokines, as shown in Fig. 5F, which were up-regulated: IL1R, TNF-a, IL10, TLR2, INOS, IL1β, CD18, CXCL4, down-regulated: TLR4, TLR3, TLR11, CD80, Gata-3, IL12, TL9R, CD21, TGF-α, IAP, XIAP. According to the specific $\log_2$FoldChange, P value, P_{adj} value (as shown in Fig. 5F and Additional file 6: Table S3), the 3 up-regulated TNF-a, IL10, INOS, and the 3 down-regulated: TLR4, Gata-3, IL12 were finally screened.

NP concentration in the spleen of rat

When rats were subchronically exposed for 180 days, the content of NP of rats in the M and H groups has increased compared to the control group, and there were significant differences in the groups (F = 997.4, P < 0.001), as shown in Additional file 3: Fig. S3A, B.

Correlation analysis of NP concentration in rat spleen and relative expression of estrogen receptor, IL-4, NF-κB, AP-1 and other immune factors

The relative protein expressions of AP-1 (r = 0.74, P < 0.001), IL-4 (r = 0.508, P = 0.011), ER-β (r = 0.861, P < 0.001) and ER-α (r = 0.848, P < 0.001) were positively correlated with the concentration of NP in the spleen of rats exposed to NP for 180 days. The relative protein expressions of NF-κB (r = −0.929, P = 0.015), IL-12 (r = −0.804, P < 0.001) and TLR-4 (r = −0.925, P < 0.001) were negatively correlated with the concentration of NP in the spleen of rats exposed to NP for 180 days, as shown in scatter plot (Fig. 4D).

Discussion

As far as we know, this was the first time to explore the effects of low-dose NP exposure, combined with the regulation of estrogen receptors, NF-AT, AP-1, and NF-κB transcription factors, on the immune and inflammatory damage of the body. In this study, for the first time, transcriptome sequencing technology, was used to screen differential gene expression between the control group and the 40 mg/kg NP exposure group—in the spleen, using advanced methods, such as KEGG database and pathway analysis. The difference in RNA expression further revealed the specific effect of estrogen receptor and NP on the regulation of immune inflammation.

The Endocrine Society and the US Environmental Protection Agency (USEPA) proposed that EDCs have complicated influences on the immune system. Indeed, it is easy to overlook the no observed adverse effect level (NOAEL) [60, 81, 83, 84]. It is currently challenging to determine dangerous levels of NP exposure that have daily and long-term effects on humans due to influences by both exposure dose and exposure period (early exposure and longer term exposure) [85–88]. Immunotoxicology is a marginal discipline based on toxicology and immunology [84, 89]. Female mice are more susceptible to autoimmune diseases than male mice despite the pathological differences [28, 32]. In previous studies on the effects of NP on immune function, exposure levels far exceeded the actual environmental exposure levels, exposure periods were too short, and the selected species were primarily females. To accurately approximate the environmental exposure dose of NP in the general population, a concentration slightly higher than the ambient concentration (but in the same order of magnitude) was adopted [90–93]. Specifically, the doses were set at 0, 4, and 40 μg/kg day, and the duration of exposure was set at 180 d. SD male mice of stable species were selected to eliminate gender interference and explore lower doses and longer exposure. In addition, combining immune function alterations with gene expression changes. To explore the

(See figure on next page.)

Fig. 3 Changes of transcription factors, estrogen receptor ER-α, ER-β and IL-4 in rats after subchronic exposure to NP for 180 days (×200). **A** Changes of ER-α, IL-4, and ER-β in rat serum (n = 6). **B** Immunohistochemistry of ER-α and ER-β in the spleen (×200). **C** Immunohistochemical quantitative analysis of ER-α and ER-β protein expression in the spleen. **D** Immunofluorescence of NF-κB, NF-AT, and AP-1 in the spleen (×200). **E** Immunohistochemical quantitative analysis of NF-κB, NF-AT, AP-1 protein expression in the spleen (n = 9). Data were presented as the mean ± SEM, one-way ANOVA, [a]vs. control group, P < 0.05; [b]vs. low NP group, P < 0.05; [c]vs. middle NP group, P < 0.05

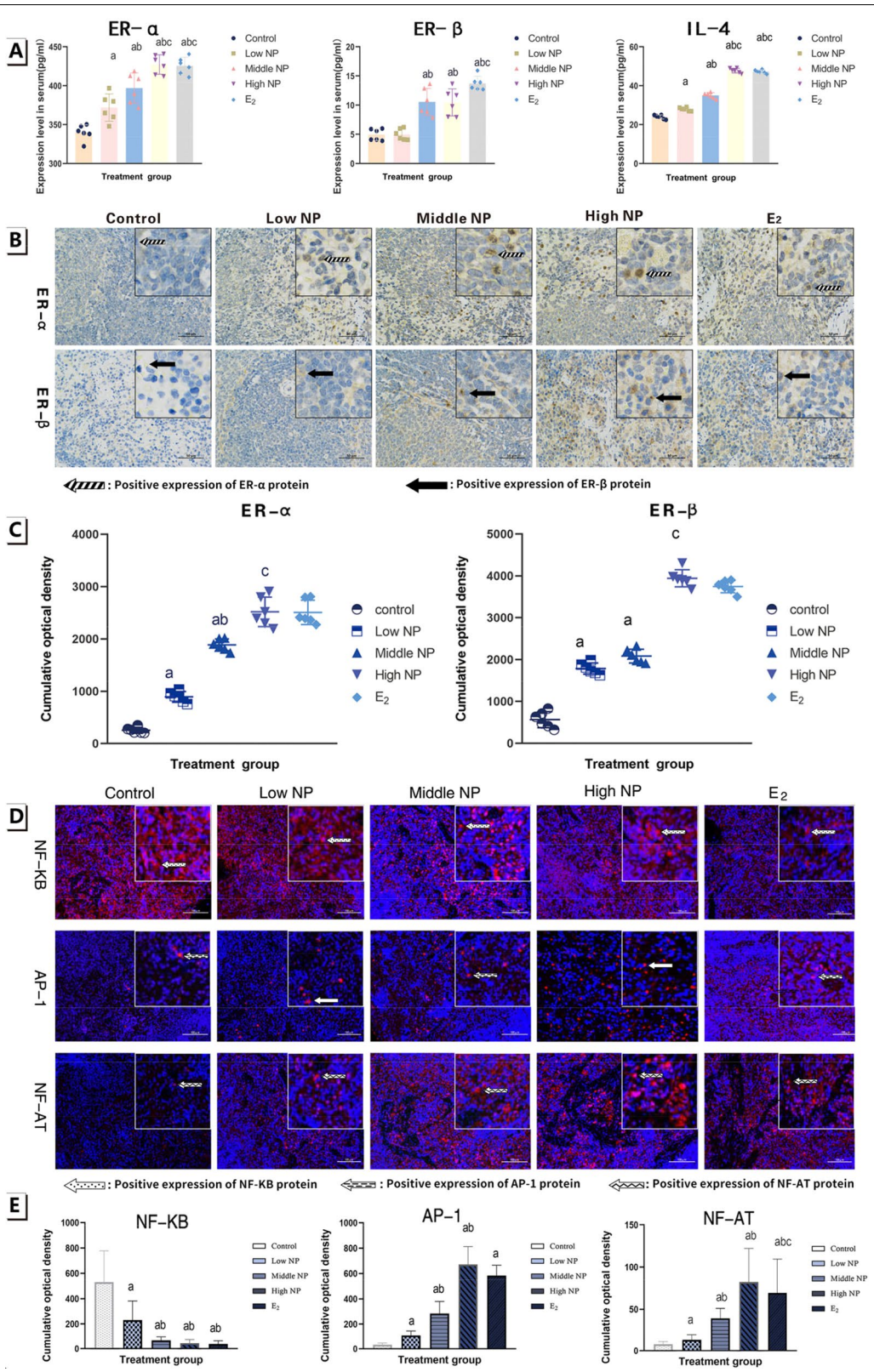


Fig. 3 (See legend on previous page.)

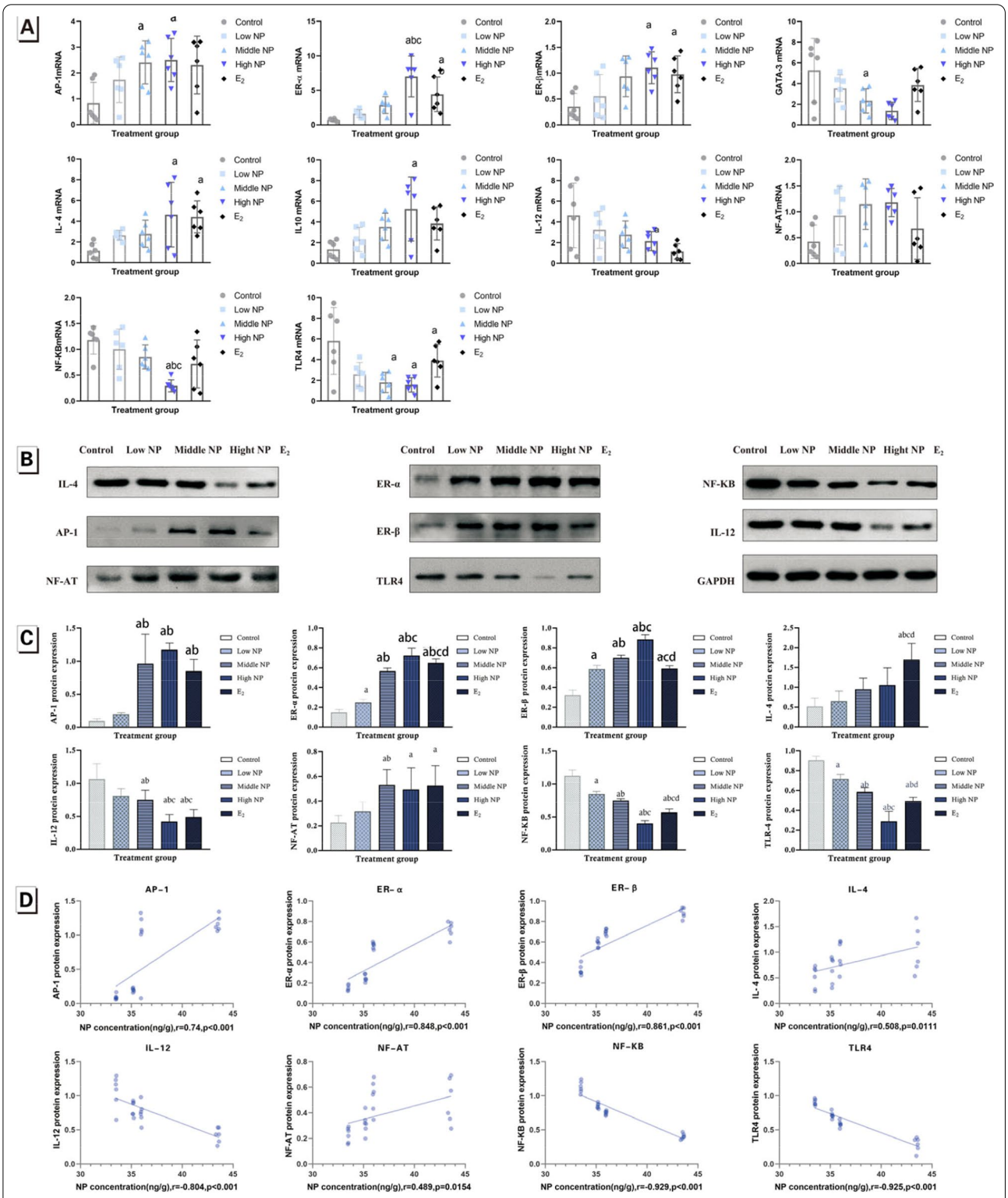


Fig. 4 Differential expressions of immune factors, transcription factors, and estrogen receptors in the spleen and their correlation with NP concentration. **A** RT-qPCR of ER-α and ER-β, NF-κB, NF-AT, AP-1, and differential immune cytokines ($n=6$). **B** Immunoadsorption of ER-α, ER-β, NF-κB, NF-AT, AP-1 and IL-12 etc. **C** Protein quantitative analysis of ER-α, ER-β, NF-κB, NF-AT, AP-1 and IL-12 etc. ($n=6$). **D** Correlation analysis of NP concentration in rat spleen tissues with protein expressions of ER-α, ER-β, NF-κB, NF-AT, AP-1 and IL-12 etc. Data were presented as the mean ± SEM, one-way ANOVA, [a]vs. control group, $P<0.05$; [b]vs. low NP group, $P<0.05$; [c]vs. middle NP group, $P<0.05$; [d]vs. high NP group, $P<0.05$

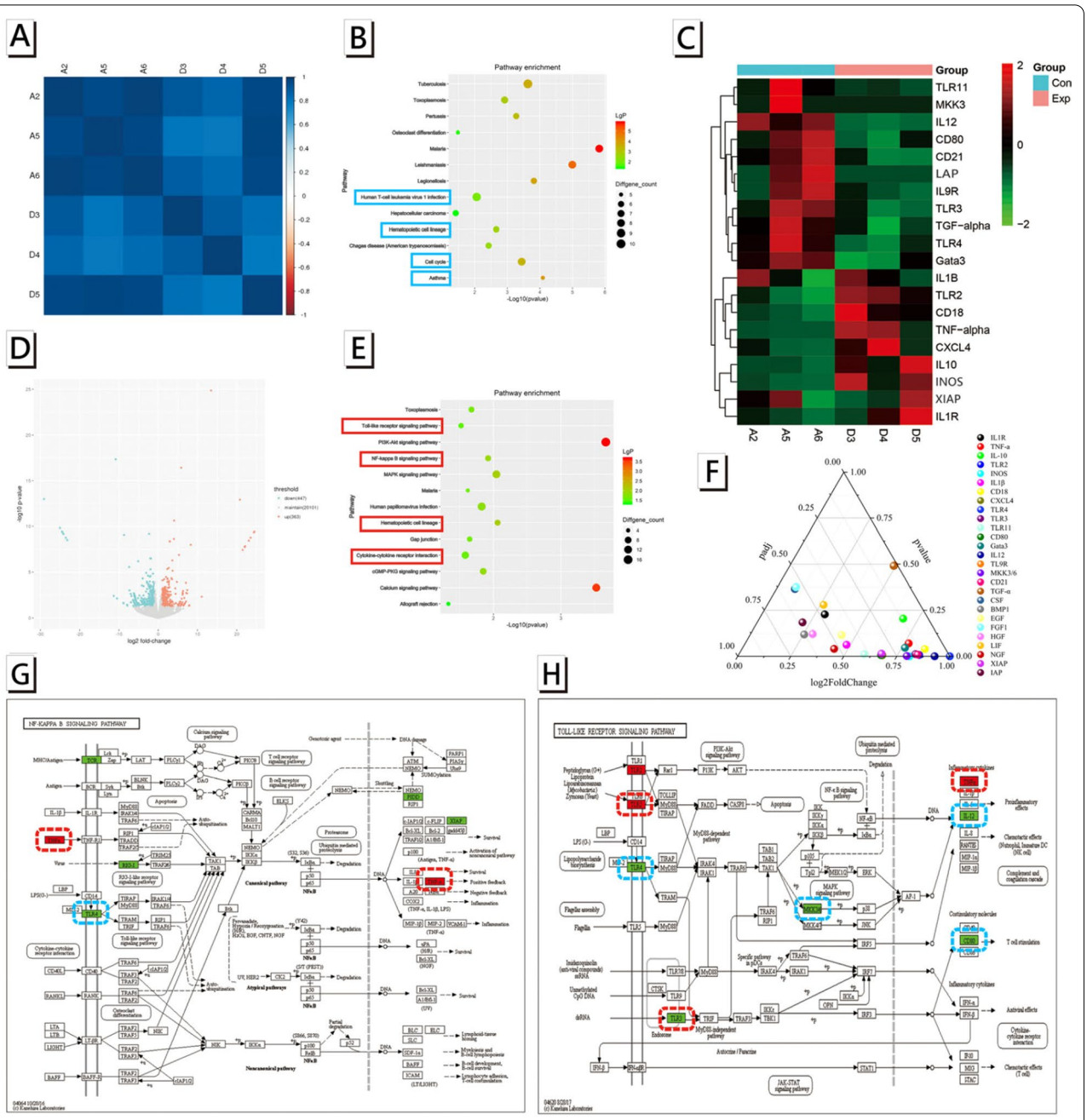


Fig. 5 RNA-Seq to analyze the changes of related immune cytokines in the spleen. **A** Correlation analysis chart of differential gene expression. **B** Bubble chart of pathway analysis of differential gene signal of down-regulation. **C** Heat map of immune-related differential cytokines. **D** Volcano map of differential gene expression. **E** Bubble chart of pathway analysis of up-regulated differential gene signal. **F** Three-dimensional image of screening of related immune cytokines. **G** NF-κB signal pathway diagram. **H** Toll-like receptor signal pathway diagram (**A** represented the blank control, **D** represented the 40 mg/kg NP group). In the figure, the blue dashed box was the down-regulated significance mark, and the red dashed box was the up-regulated significance mark. $\log_2$FoldChange: the multiple of the difference of $\log_2$, the value of this study was greater than 2 times or less than 0.5 times, *P* value: indicating the level of significance. P_{adj}: *P* value correction value; Gene symbol: gene name; IL1R: IL1 receptor; TLR2: toll-like receptor; INOS: inducible nitric oxide synthase; IL1β: interleukin-1 beta; EGF: fibroblast growth factor; TNF-a: TNF-alpha; CXCL4: Chemokine (C–X–C motif) ligand 4; TLR4: toll-like receptor-4; MKK3/6: mitogen-activated protein Kinase Kinase 3/6; TGF-α: transforming growth factor alpha; CSF: colony-stimulating factor; BMP: bone morphogenetic protein; HGF: hepatocyte growth factor; LIF: leukemia inhibitory factor; NGF: nerve growth factor; IAP: inhibitor of apoptosis proteins; XIAP: X-linked inhibitor of apoptosis proteins

value of data support for gene expression profiles to predict changes in immune factors.

As the body's largest immune organ, the spleen accounts for the largest amount of lymphatic tissue in the body, and is considered the body's immune center [94–96]. All immunocytes accumulate, mainly producing immune responses to various antigenic substances and secreting many immune effectors. Indeed, organ weight positively correlated with cellular immune function [64, 97–99]. The pathological changes suggested that the subchronic exposure of NP caused atrophy and pathological irreversible damage to the spleen of rats, which could affect immune damage and abnormalities in regulatory functions. Although changes in body weight and organ coefficients were non-functional tests, their changes may be the earliest and most sensitive way to reflect the toxic effects of exogenous compounds [96, 100–102]. In this study, the changes in the organ coefficients of the NP groups were different from those of the control group. However, there were no significant differences in body weight during the first 160 days of intragastric administration, and the differences were significant after 160 days. The reason may be the lower exposure dose and the exposure time, indicating that the low toxic dose needed to be used for a longer time to better show the toxic effect.

The results of the lymphocyte typing in this study indicated that the subchronic exposure of NP has broken the immune balance of T, B, and NK cells in SD male rats. The ratio of $CD4^+/CD8^+$ reflected the state of immune balance, and its value decreased, which suggested that the initiation of immune response and the induction effect were also reduced [83, 86]. Some scholars have shown that NP can inhibit the mitosis of lymphocytes by acting on ER-α on mouse T cells and B cells, reducing the number of effector cells produced by T cells and B cells, and ultimately affecting the immune function [94, 103]. In addition, studies have found that estrogen stimulated expressions of IFN-γ and IL-12 in $CD8^+$ T cells, thereby enhancing the activity of B lymphocytes [104, 105]. IL-12 is an important initiator of cellular immune responses, as demonstrated by high-throughput sequencing results detecting upregulation of the immune factors TNF-a and IL10 in IL12 downregulation experiments [106, 107]. Sundstedt has demonstrated that AP-1 and NF-κB transcription factors disturbed the expression of activated CD4+ T cells in vivo [108]. The expression of AP-1, NF-κB, NF-AT, and octamer-binding transcription factors, is involved in the regulation of IL-2 gene promoter activity, resulting in the reduction of IL-2 expression at the mRNA and protein levels [44]. Dokter et al. found that IL-10 and IL-4 inhibited the effect of LPS-induced IL-10 and IL-4 on activator protein-1 (AP-I), nuclear factor IL-6 (NF-IL6), and nuclear factor NF-κB expression by suppressing the transcriptional rate of the IL-6 gene [109]. Alteration of these transcription factors might inhibit IL-2 expression in T cells. In addition, CD4+ T is the precursor of IL-2 and IL-4, and IL-2 could produce IL-4 [107, 110, 111].

According to the data of estrogen receptors ER-α and ER-β in immunohistochemical experiments, it could be inferred that subchronic low-dose exposure of NP has caused alterations in the expression of estrogen receptors, the balance and homeostasis of each lymphocyte population, and Th1/Th2 cell balance and $CD4^+/CD8^+$ ratio. Therefore, estrogen receptors may be involved in this process of disturbing the balance.

Peripheral blood is the main source of lymphocytes and can provide interspecies comparisons [55, 112]. The results of peripheral blood lymphocyte typing and immunoglobulin detection showed that white cells and monocytes in the peripheral blood of NP-exposed rats increased significantly, and the number of lymphocyte decreased. Serum IgM and IgG levels declined with the increase of NP exposure dose and serum IgE levels increased. All the above indicated that NP exposure had an immunosuppressive effect similar to E_2, by reducing the body's ability to recognize, killing and promptly removing mutant cells from the body to prevent tumors and other diseases [113, 114]. Studies have shown that NP can inhibit mouse type 1 helper T cells (Thl) from secreting INF-γ and T cells (Th2) to produce IL-4, showing an immunosuppressive effect [103, 112]. The increase of IL-4 was related to the increase of IgE concentration in animal plasma [113–116]. This was consistent with the results of the Elisa experiment, immunoglobulin detection and peripheral blood test in this study.

When naive CD4+ T cells differentiate into effector Th2 cells, they are prone to allergic sensitization. IL-4 was secreted during the degranulation of eosinophils. In the Th2 differentiation stage, IL-4 played an important role [117, 118], which was the same as the results of increase of IL-4 in the spleen in immunofluorescence, RT-qPCR and western blot in this study. The normal expression of inflammatory factors is essential for body homeostasis as they may cause cellular or tissue damage and may induce major diseases (e.g., cancer and metabolic diseases) [54, 119–121]. As a key gene of the TLR family, TLR4 activates MAPK, PI3K, and NF-κB pathways, thus affecting cell activity and apoptosis [61, 122–125]. Relevant studies have shown that vitamin D reduced the inflammatory response and lung cell apoptosis in asthmatic mice through the high mobility group protein B1 (HMGB1)/TLR4/NF-kB) signaling pathway [124, 126]. Toll-like receptors (TLR2, 4) could interact with HMGB1 and the receptors of advanced glycation end products (RAGE) to activate the nuclear factor NF-κB signaling pathway and

induce the release of downstream inflammatory mediators [59, 61, 126]. This also proved once again the pathway in this study was consistent to the asthma pathway and the TLR4-NF-κB pathway.

Immune factors can regulate many biological processes of hematopoietic cells and mediate cellular activation, differentiation and survival [34, 97, 98]. More and more studies show that, Involved in the induction of nitric oxide, the production of reactive oxygen species, etc. It is regulated by the mobilization of reactive calcium through the opening of Ca^{2+} channels on the intracellular calcium storage membrane by immune factor signals [29, 127]. GRP75-induced ER-mitochondrial Ca^{2+} transfer may be an important factor in Th1/Th2 imbalance in asthma patients. In addition, HMGB1 specifically promotes Th2 cytokines release through GRP75-induced enhancement of ER-mitochondrial Ca^{2+} transfer and increase in ROS [128]. Oxidative stress, inflammation, and hypoxia can lead to proteins unfolding or misfolding that interfere with endoplasmic reticulum (ER) homeostasis, thereby triggering ER stress. ER stress and autophagy are mechanistically interrelated, and exposure to low doses of NP may cause stress and autophagy. The role of NP exposure in the tumor microenvironment regulation during immune damage has not been elucidated [129–140]. It has been demonstrated that the activation of C kinase 1 scaffold protein Receptor (RACK1), an EDC target in the immune milieu, is an important molecular player in cancer progression [141–144]. Urriola et al. reported that BPA and NP induce apoptosis in prostate and ovarian cancer cell lines, a process dependent on ADAM17 activation [145, 146]. Sun et al. found that epidermal growth factor receptor (EGFR) and ERK up-regulation is associated with estrogenic responses during 17α-ethinylestradiol- and 4-NP-stimulated mock lung adenocarcinoma cell production [147]. Since microenvironmental dynamics highly influence tumor development, estrogen-sensitive peripheral cells, such as breast cancer cells and vascular endothelial cells, are also involved in angiogenesis and tumorigenesis [145, 146, 148–151]. Moreover, the tumor microenvironment itself contains stromal fibroblasts, endothelial cells, immunocytes, and acellular components of the extracellular matrix [152–154]. Hence, the specific mechanisms by which subchronic NP exposure causes chronic immune inflammation in mice, induces estrogen receptor and transcription factor changes in vivo, and disrupts the Th1/Th2 balance, CD4+/CD8+ ratio, and tumor microenvironmental homeostasis are complex and need to be further explored [145, 146].

The immune system is closely related to the nerve and endocrine systems, and the three systems influence each other to form a neuroendocrine immune network [51, 54, 64, 75, 99, 100, 155–157]. As an important sex steroid hormone, estrogen plays an important role in the occurrence and development of autoimmune diseases by directly regulating the development of immune organs, immune cells and the secretion of immune-related factors [11, 41, 56, 119]. However, the mechanism of estrogen receptor in NP on immune cytokines needs further research. In addition, technical NP contains over 20 para-substituted isomers. The estrogenic activity of the isomers is heavily dependent on facets of the structure of the nonyl side chain, such as the degree of branching and bulkiness. Only total level of 4-NP was detected rather than all the isomers in animal experiment, which is a limitation of our study [4–7].

Conclusions

Subchronic exposure of 40 mg/kg/day NP for 180 days can cause immune dysfunction in rats, resulting in immunotoxicity and inflammatory effects, and lead to changes in the activity of transcription factors, such as AP-1, NF-AT, NF-κB and differential immune inflammatory factors in rats. In this process, estrogen receptors may play an important role and participate in immune inflammatory by affecting the expressions of transcription factors and immune factors.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12302-022-00610-6.

Additional file 1: Figure S1. 4200 instrument result graph.

Additional file 2: Figure S2. Peak diagram of sample quality test results.

Additional file 3: Figure S3. Detecting the accumulation of NP in the spleen by HPLC ($n=6$).

Additional file 4: Table S1. Primer sequences.

Additional file 5: Table S2. Quality analysis of spleen tissue samples by RNA-Seq.

Additional file 6: Table S3. Reference value of immune cytokines.

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Not applicable.

Authors' contributions
JX and JY designed the study. XF, CN, DY, HW, PW, ML, JY and JX analyzed and interpreted the data. XF and CN conducted the laboratory work. JY wrote the manuscript, JX revised the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials
The data sets used and/or analyzed during the current study available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate
The Ethics Committee of the Zunyi Medical University approved the study (2015-1-017). All methods were performed in accordance with guidelines and regulations of the Zunyi Medical University.

Consent for publication
All the authors read and approved this paper.

Competing interests
The authors declare that they have no competing interests.

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