Title: Strain differences of cadmium-induced toxicity in rats: Insight from spleen and lung immune responses

Author: Jelena Demenesku Aleksandra Popov Aleksandrov Ivana Mirkov Marina Ninkov Lidija Zolotarevski Dragan Kataranovski Ilija Brceski Milena Kataranovski

PII: S0378-4274(16)30128-X
DOI: http://dx.doi.org/doi:10.1016/j.toxlet.2016.05.022
Reference: TOXLET 9386

To appear in: *Toxicology Letters*

Received date: 19-2-2016
Revised date: 16-5-2016
Accepted date: 20-5-2016

Please cite this article as: Demenesku, Jelena, Aleksandrov, Aleksandra Popov, Mirkov, Ivana, Ninkov, Marina, Zolotarevski, Lidija, Kataranovski, Dragan, Brceski, Ilija, Kataranovski, Milena, Strain differences of cadmium-induced toxicity in rats: Insight from spleen and lung immune responses.Toxicology Letters http://dx.doi.org/10.1016/j.toxlet.2016.05.022

STRAIN DIFFERENCES OF CADMIUM-INDUCED TOXICITY IN RATS: INSIGHT FROM SPLEEN AND LUNG IMMUNE RESPONSES

Jelena Demenesku [a], Aleksandra Popov Aleksandrov [a], Ivana Mirkov [a], Marina Ninkov [a], Lidija Zolotarevski [b], Dragan Kataranovski [a, c], Ilija Brceski [d], Milena Kataranovski [a, e, *]

[a] Immunotoxicology group, Department of Ecology, Institute for Biological Research "Sinisa Stankovic", University of Belgrade, 142 Bulevar despota Stefana, 11000 Belgrade, Serbia

[b] Institute of Pathology, Military Medical Academy, 17 Crnotravska, 11000 Belgrade, Serbia

[c] Institute of Zoology, Faculty of Biology, University of Belgrade, 16 Studentski trg, 11000 Belgrade, Serbia

[d] Faculty of Chemistry, University of Belgrade, 3 Studentski trg, 11000 Belgrade, Serbia

[e] Institute of Physiology and Biochemistry, Faculty of Biology, University of Belgrade, 16 Studentski trg, 11000 Belgrade, Serbia

*****Corresponding author:

Milena Kataranovski, PhD

Immunotoxicology group, Department of Ecology, Institute for Biological Research "Sinisa Stankovic", University of Belgrade, 142 Bulevar despota Stefana, 11000 Belgrade, Serbia.

Tel.: +381 11 2078375;

Fax: +381 11 2761433;

E-mail address: milena@ibiss.bg.ac.rs

Highlights

- Strain differences in acute cadmium immunotoxicity are explored in DA and AO rats
- Cadmium affected spleen and lungs cells immune response in DA and AO rats
- The degree of some changes differ between strains in both compartments
- Data depict complex influence of genetic background on the cadmium immunotoxicity

Abstract

The impact of genetic background on effects of acute i.p. cadmium administration (0.5 mg/kg and 1 mg/kg) on basic immune activity of spleen and lungs was examined in two rat strains, Albino Oxford (AO) and Dark Agouti (DA), known to react differently to chemicals. More pronounced inhibition of Concanavalin A (ConA)-induced and Interleukin (IL)-2 stimulated spleen cell proliferation as well as higher levels of nitric oxide (known to decrease cell`s proliferative ability) in DA rats at 1 mg/kg, along with greater inhibition of ConA-induced Interferon (IFN-γ)-production by total and mononuclear (MNC) spleen cells and IL-17 production by spleen MNC in DA *vs.* AO rats at this dose show greater susceptibility of this strain to Cd effects on spleen cells response. More pronounced infiltration of neutrophils/$CD11b^+$ cells to lungs of DA rats treated with 1 mg/kg of Cd and decreased IL-17 lung cell responses noted solely in DA rats speaks in favor of their higher susceptibility to this metal. However, lack of strain disparity in lung cells IFN-γ responses show that there are regional differences as well. Novel data from this study depict complexity of the influence of genetic background on the effects of cadmium on host immune reactivity.

Keywords: Cadmium; Rats; Albino Oxford (AO) and Dark Agouti (DA) strains; Spleen; Lungs

1. Introduction

Cadmium (Cd), an industrial pollutant and component of cigarette smoke, is one of the most toxic metals in the environment (Morselt, 1991). It adversely affects a number of organs and tissues such as liver and kidneys (which are cadmium prime targets), as well as lungs, testes and brain (U.S. Department of Health and Human Services, 1997; WHO, 1992). Effects of cadmium on the components of immune system were less investigated and showed both suppression (Marth et al., 2001; Pathak and Khandelwal, 2007) and stimulation (Olszowski et al., 2012).

The susceptibility of tissues to cadmium toxicity is associated with several host physiological parameters including age and sex (Gochfeld, 1997). Differences in cadmium hepatotoxicity were observed between young and adult mice (Shaikh et al., 1993) and rats (Yamano et al., 1998) and in hepatotoxicity and nephrotoxicity between male and female rats (Gubrelay et al., 2004). Host genetic variability was considered as a factor that could identify the degree of susceptibility to metals in general (Gochfeld, 1997;), but the factors that render some animals more prone to toxicity than the others are not well known. Strain differences noted in hepatotoxicity and testicular toxicity of cadmium in rats were shown as related to the levels of its accumulation in liver (Shaikh et al., 1993; Shimada et al., 2004) and testes (Shimada et al., 2009, 2011). However, several studies showed that cadmium burden is less important than host genetic background, as strain differences in hepatotoxicity (Kuester et al., 2002;) and testicular toxicity (King et al., 1998; Liu et al., 2001;) were noted in the presence of similar tissue concentrations of this metal. Differential susceptibility of some tissues to cadmium has been ascribed to differences in the activity of these tissues cells . Kupffer cells (liver macrophages) were shown to be the major determinant of differential susceptibility of Fischer 344 and Sprague-Dawley rats (which accumulated similar levels of cadmium in liver) to hepatotoxicity of cadmium (Kuester et al., 2002). Differential intensity of leukocyte infiltration into lungs accounted for strain differences in the susceptibility of C57BL/6 and DBA mice (which accumulated similar levels of metal) to cadmium-induced pulmonary injury (McKenna et al., 1997). These two studies showed not only the contribution of cells of immune system to strain differences in cadmium tissue toxicity in experimental rodents, but they also suggested the influence of genetic variations to cadmium

toxicity to immune system. Indeed, one early study showed differential effects of cadmium administration on thymic lymphocytes of Brown-Norway and Lewis rats, although the same amount of this metal was observed in thymuses of both strains (Morselt et al., 1988). Examination of *in vitro* cadmium exposure on cytokine responses by human peripheral blood mononuclear cells showed high level of inter-individual variability of cytokine mRNA levels (coefficient of variation between distinct blood donors of up to 68%) (Marth et al., 2001). Despite these early data, the impact of genetic background on cadmium toxicity to cells of immune system is largely unknown.

Differential effects of cadmium on components of immune system (suppression, stimulation or lack of the impact) were reported in the literature, most probably due to differences in sources of immune cells (blood, spleen, thymus, lungs) used in the studies. Differential effects of cadmium in the same tissue (spleen) might be ascribed to differences in the activities examined(Demenesku et al., 2014) In view of the above cited findings the aim of the present paper was to examine the impact of genetic background on immunomodulatory potential of cadmium by analyzing its effects on spleen and lung immune responses comparatively in Albino Oxford (AO) and Dark Agouti (DA) rats (strains which are known to differ in immune-mediated tissue responses). To this aim, basic aspects of immune activity of spleen and lung leukocytes were analyzed in animals of these strains following acute intraperitoneal (*i.p.*) cadmium administration. The data obtained showed complexity of the influence of genetic background on the effects of cadmium on host immune reactivity, which is a novel finding.

2. Materials and methods

2.1 Chemicals

Cadmium chloride ($CdCl_2$) was purchased from Serva, Feinbiochemica (Heidelberg, Germany) and was prepared in sterile pyrogen-free saline (Hemofarm AD, Vršac, Serbia). Concanavalin A (ConA), deoxyribonuclease I (DNase I), *o*-dianisidine dihydrochloride, three-(4,5-dimethyl-thiazol-2-yl)-2,5 diphenyl-tetrazolium bromide (MTT), myeloperoxidase (MPO), N-(1-naphtyl) ethylenediamine

dihydrochloride, sulfanilamide (*p*-aminobenzenesulfonamide) were all purchased from Sigma (Sigma Chemical Co., St. Louis, MO, USA). Collagenase (type IV) purchased from Worthington Biochemical Corporation (Lakewood, USA), hydrogen peroxide (H_2O_2) from Zorka Farma Sabac (Serbia) and sodium nitrite from Fluka Chemika (Buchs, Switzerland) were used in experiments. Culture medium RPMI-1640 (Biowest, Nuaillé, France) supplemented with 2 mM glutamine, 20 μg/ml gentamycine (Galenika a.d., Zemun, Serbia), 5% (v/v) heat inactivated fetal calf serum (Biowest, Nuaillé, France) was used in cell culture experiments. For use in experiments ConA was dissolved in RPMI-1640 medium. All solutions for cell culture experiments were prepared under sterile conditions and sterile filtered (Minisart, pore size 0.20 μm, Sartorius Stedim Biotech, Goettingen, Germany) before use. ^{3}H-thymidine (GE Healthcare, Little Chalfont, UK) was prepared in culture medium. Monoclonal antibody OX-42 (mouse anti-rat CD11b/CD11c) was purchased from AbD Serotec (Serotec Ltd., Oxford, UK). Lysis buffer (used for red blood cell lysis), phycoerythrin (PE) labeled $F_{(ab')2}$ goat anti-mouse IgG and mouse anti-rat CD25 were from eBioscience (eBioscience Inc., San Diego, CA, USA). Recombinant human interleukin-2 (IL-2; *E. coli*-derived, Ala21-Thr153) was obtained from R&D Systems (Minneapolis, USA).

2.2 Animals and cadmium treatment

All animal procedures were complied with the Directive 2010/63/EU on the protection of animals used for experimental and other scientific purposes and was approved by the Ethical Committee of the Institute for Biological Research "Sinisa Stankovic" (IBISS), University of Belgrade, Serbia. Twelve to fourteen weeks-old male Dark Agouti (DA) and Albino Oxford (AO) rats, conventionally housed at IBISS, in controlled environment (21/24 °C temperature, a 60 % relative humidity and 12-hour (h) light:dark cycle) were used. All rats had *ad libitum* access to standard rodent chow and filtered water throughout the study.

Four to six animals were assigned per group per experiment in two independent experiments. Sterile filtered $CdCl_2$ was administered by *i.p.* injection at a concentration at which animals received 0.5 or 1 mg of cadmium/kg body weight (b.w.) in a 0.5 ml dosing

volume. Control group was administered with pyrogen-free saline solely. All measurements were carried out 48 h post-cadmium exposure in animals anesthetized by *i.p.* injection of 40 mg/kg b.w. of thiopental sodium (Rotexmedica, Tritau, Germany).

2.3. Cadmium determination

Atomic absorption spectrometry graphite tube technique (AAS Varian 1275; graphite tube, GTA-95, Palo Alto, CA, USA) was used for cadmium content determination in liver, spleen, kidneys and lungs. Lyophilized tissue samples were homogenized and digested in a microwave digestion system (MBS-9, CEM Innovators, Buckingham, UK) in a mix of concentrated HCl and HNO_3 (metal-free), filtered and diluted using metal-free ultrapure water. Reference materials were used as control samples: SeronormTM Trace Elements Serum L-1 and ClinChek Plasma Control. Method limit of detection (LOD) was 0.1 mg/kg (0.00089 mmol/kg) and limit of quantification (LOQ) 0.3 mg/kg (0.00267 mmol/kg). The concentrations were expressed as μmol of Cd per kg of wet tissue.

2.4. Clinical biochemistry

Heparinized plasma obtained from whole blood samples collected at necropsy, was assessed for levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activity using an autoanalyzer (Ciba Corning Express, Oberlin, OH) and commercially available reagents. Specific gravity, pH, white blood cell number, protein, nitrite, blood/hemoglobin, urobilinogen, bilirubin, glucose, and ketone content in urine was measured in samples freshly collected (by micturition over Petri dish) 48 h post-cadmium exposure using semi quantitative Multistix® 10SG urine strips (Bayer Diagnostics, Tarrytown, NY, USA).

2.5. Peripheral blood leukocyte counts

Total peripheral blood leukocyte cell counts were determined using an improved Neubauer hemocytometer (Neubauer, Wertheim, Germany). The differential cell counts were determined in air-dried whole-blood smears stained according to a standard May

Grünwald-Giemsa/MGG (Alkaloid AD, Skopje, The Former Yugoslav Republic of Macedonia) protocol by differentiating at least 300 cells.

2.6. Histology

Samples of livers, kidneys, spleens and lungs were cut out and immediately fixed in 4% formaldehyde (pH 6.9), processed for embedding in paraffin wax and subsequently sectioned at 5 μm. Hematoxylin and eosin (H&E)-stained histology slides were analyzed (in a blinded manner) by a certified histopathologist using a Coolscope digital light microscope (Nikon Co, Tokyo, Japan).

2.7. Spleen cell isolation and culture

Spleens were removed, blotted dry and weighed. The relative spleen weight was calculated according to the following formula: organ weight (in mg)/b.w. (in g). Spleen cell differential counts were determined by differentiating at least 1000 cells from air-dried spleen blood smears (prepared from a fresh section of spleen) stained according to MGG protocol. Spleen cells were obtained by mechanical disintegration of spleen tissue through a cell strainer (BD Falcon, BD Bioscience, Bedford, USA), and suspended in complete RPMI-1640 culture medium. Cell viability was determined by Trypan blue dye exclusion and was more than 95%.

For proliferation measurements, total spleen cells (3×10^5 cells/well) were cultured in medium solely (spontaneous proliferation), medium supplemented with 2.5 μg/ml ConA (ConA-stimulated), or in medium with 0.25 μg/ml ConA and 20 ng/ml of IL-2 (IL-2-stimulated proliferation). After 48 h of culture 0.5 μCi of ^{3}H-thymidine per well was added and incubation was continued for additional 16 h. Incorporation of ^{3}H-thymidine into cellular DNA was measured by liquid scintillation counting (LKB, Wallac, Turku, Finland) and proliferation was expressed as counts per minute (c.p.m.).

For examination of nitric oxide (NO) production, spleen cells (3×10^5 cells/well) were cultured in 96-well microplates (Sarstedt Inc., Newton, NC) for 48 h in medium supplemented with 2.5 μg/ml ConA.

Mononuclear spleen cells were obtained by centrifugation (700 × g, 20 min, 20 °C) of spleen cell suspension over Percoll density separation medium (Sigma Chemical Co., St. Louis, MO, USA).

Total and mononuclear spleen cells (3×10^5 cells/well) were cultured for 48 h in medium solely (non-stimulated cytokine production) or in the presence of 1 μg/ml ConA (ConA-stimulated cytokine production).

2.8. Lung leukocyte isolation and culture

Lungs were removed, cleared of blood and weighed. The relative lungs' weight was calculated according to the following formula: organ weight (in mg)/b.w. (in g). Tissue (left lung) was homogenized on ice using an IKA T18 basic homogenizer (IKA Works INC., Wilmington NC) in sterile saline. Homogenates were then subjected to sonication (3 × 15 s on ice at 30% of maximum intensity amplitude) by a laboratory sonicator (Bandelin electronic, UW 2070, Berlin, Germany).

Right lung was finely minced and incubated with gentle mixing (magnetic stirrer) for half an hour at 37 °C in culture medium supplemented with 1.0 mg/ml collagenase and 30 μg/ml DNase. Cells were resuspended in complete RPMI-1640 culture medium and counted by improved Neubauer hemocytometer. Cell viability was determined by Trypan blue dye exclusion and was always >90%. Differential cell counts were determined by examining at least 300 cells from lung cells cytospin preparations stained with MGG protocol.

Mononuclear cells were obtained by centrifugation of lung cell suspension over Percoll density separation medium (700 × g, 20 min, 20 °C).

Total and mononuclear lung cells were resuspended in complete RPMI-1640 culture medium and cultured for 48 h in medium solely (non-stimulated cytokine production) or in the presence of 1 μg/ml ConA (ConA-stimulated cytokine production).

2.9. MTT assay for cell viability

A quantitative colorimetric assay described for lymphocyte cell lines (Denizot and Lang, 1986) in which tetrazolim salt MTT is metabolically reduced by cell's mitochondrial dehydrogenases to colored end product formazan, was used as a measure of isolated leukocytes' metabolical viability. Spleen (3×10^5 cells/well) or lung (4×10^5 cells/well) cells were incubated with 500 μg/ml of MTT for 3 h in 96-well plate. Formazan produced by cells was dissolved by overnight incubation in 10% SDS-0.01N HCl. Absorbance was measured spectrophotometrically at 540/650 nm, by an ELISA 96-well plate reader (GRD, Rome, Italy).

2.10. Myeloperoxidase (MPO) activity

MPO activity was assessed on the basis of the oxidation of *o*-dianisidine dihydrochloride by cells (Bozeman et al., 1990). MPO was evaluated by addition of 33 μl of spleen or lung cell lysate (obtained by repeated freezing and thawing) or the lung tissue homogenate to 966 μl of substrate solution (0.167 mg/ml *o*-dianisidine dihydrochloride and 0.0005% H_2O_2 in 50 mM potassium phosphate buffer, pH 6.0). Absorbance was read at 450 nm by an ELISA 96-well plate reader during ten min at three-min intervals against the MPO standard. Values were expressed as MPO units (U)/10^6 cells or MPO U/g of tissue.

2.11. Nitric oxide (NO) production

As an indicator of NO formation, the concentration of the stable NO oxidation product, nitrite, was measured using Griess assay (Hibbs et al., 1988) in medium conditioned for 48 h by total spleen cells. Aliquots (50 μl) of conditioned medium were mixed with an equal volume of Griess reagent (a mixture of 0.1% N-(1-naphtyl) ethylenediamine dihydrochloride in water and 1% sulfanilamide in 5% phosphoric acid) and incubated for 10 min at room temperature. Following incubation, the absorbance was measured at 540 nm/670 nm by an ELISA 96-well plate reader. The amount of present nitrite was obtained by extrapolation from a standard curve constructed in parallel with known concentration of sodium nitrite solutions.

2.12. Flow Cytometry

Adhesion molecule CD11b expression measurement was achieved by flow cytometry analysis of peripheral blood leukocytes following lysis of red blood cells. Obtained cells (1×10^6) were incubated with mouse anti–rat CD11b on ice for 30 min, washed and stained with PE labeled $F_{(ab')2}$ goat anti-mouse IgG for further 30 min. To determine numbers of $CD25^+$ cells, 1×10^6 spleen cells were incubated on ice for 30 min with PE labeled mouse anti-rat CD25 antibody. Following staining, cells were washed, fixed with 1 % paraformaldehyde and kept in the dark at 4 °C until analysis. Fluorescence intensity was assayed in the CyFLOW SPACE system (Partec, Munich, Germany). Cells in histogram with position characteristic for granular cells or lymphocytes and monocytes (mononuclear cells) were analyzed for percentages of $CD11b^+$ cells. A minimum of 10,000 events/sample was acquired each time and analyzed using FlowMax software (Partec).

2.13. Enzyme–linked immunosorbent assay (ELISA)

Cytokine concentrations were determined in medium conditioned by spleen or lung cells using commercially available ELISA sets for rat IFN-γ (R&D Systems, Minneapolis, USA) and mouse IL–17 cross-reactive with rat IL-17 (eBioscience Inc., San Diego, CA, USA) according to the manufacturer's instructions. Cytokine titer was calculated by a reference to a standard curve constructed using known amounts of recombinant IFN-γ and IL-17.

2.14. Reverse transcription and real time polymerase chain reaction (RT-PCR)

Total RNA (1 μg) isolated from (5×10^6) total and mononuclear lung and spleen cells with mi-Total RNA Isolation Kit (Metabion, Martinsried, Germany) following manufacturer’s instructions, was reverse transcribed using random hexamer primers and MMLV (Moloney Murine Leukemia Virus) reverse transcriptase according to the manufacturer's instructions (Fermentas, Vilnius, Lithuania). Prepared cDNAs were amplified using Power SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, California, USA)

based on the recommendations of the manufacturer in a total volume of 20 μl in an ABI PRISM 7000 Sequence Detection System (Applied Biosystems). Thermocycler conditions comprised an initial step at 50 °C for 5 min, followed by a step at 95°C for 10 min and subsequent 2-step PCR program at 95 °C for 15 s and 60 °C for 60 s for 40 cycles. PCR primers (forward/reverse) used were: IFN-γ: 5'-AACAGTAAAGCAAAAAAGGATGCA-3'/5'-TGTGCTGGATCTGTGGGTTGT-3'; IL-17: 5'-CTACCTCAACCGTTCCACTTCAC-3'/5'-CCTCCCAGATCACAGAAGGATATC-3'; MT – 1: 5'-GAACTGCAAATGCACCTCCTGC-3'/5'-CAAGACTCTGAGTTGGTCCG-3'; MT – 2: 5'-TGCAAGAAAAGCTGCTGTTCC-3'/5'-TTACACCATTGTGAGGACGCC-3'; inducible nitric oxide synthase (iNOS): 5'-TTCCCATCGCTCCGCTG-3'/5'-CCGGAGCTGTAGCACTGCA-3'; β-actin (housekeeping): 5'-CCCTGGCTCCTAGCACCAT-3'/5'-GAGCCACCAATCCACACAGA-3'. Accumulation of mRNA was detected in real time and results were analyzed with 7500 System Software (Applied Biosystems) and calculated as 2^{-dCt}, where dCt was difference between threshold cycle (Ct) values of specific gene and endogenous control (β-actin). Data for mRNA from cells of cadmium administered animals are expressed as relative value to mRNA from control animals (considered as 1).

2.15. Data display and statistical analysis

Results were expressed as mean values (pooled from two independent experiments) ± SD. The correlations were analyzed using Pearson correlation test. Statistical analysis was performed using STATISTICA 7.0 (StatSoft Inc., Tulsa, OK) and statistical significance was defined by a Mann-Whitney U test. A *P*-value < 0.05 was considered significant.

3. Results

3.1. General considerations

Administration of cadmium resulted in accumulation of this metal in the liver, kidneys, spleen and lungs of both AO and DA rats with higher (compared to 0.5 mg of cadmium/ kg) concentrations in rats administered with the dose of 1 mg/kg (Fig. 1). Similar levels of this metal were noted in tissues of both strains, except in spleens of AO rats administered with 1 mg of cadmium/kg, where slightly, though significantly ($P < 0.05$), lower concentrations were noted in cadmium-treated AO (16.9 ± 3.3 μmol/kg) compared to DA rats (23.8 ± 3.9 μmol/kg). Somewhat higher ($P < 0.05$) concentrations of cadmium were measured in lungs of AO rats administered with 1 mg of cadmium/kg (6.1 ± 1.0 μmol/kg) compared to DA rats (4.5 ± 0.8 μmol/kg) (Fig. 1).

No changes were noted in plasma levels of aspartate aminotransferase (AST) or alanine aminotransferase (ALT) at lower cadmium dose. At 1 mg/kg, increased plasma levels of both hepatic enzymes were noted in animals of both strains (275.1 ± 110.7 U/l compared to controls 157.1 ± 63.3 U/l, $P < 0.05$ in AO and 376.7 ± 110.8 U/l compared to controls 204.4 ± 45.5 U/l, $P < 0.001$ in DA rats for AST, and 100.8 ± 36.6 U/l *vs.* 50.7 ± 11.1 U/l in controls, $P < 0.05$ in AO and 151.8 ± 37.9 U/l *vs.* 76.1 ± 22.6 U/l in controls, $P < 0.001$ in DA rats for ALT), but values were lower ($P < 0.05$) in AO compared to DA rats. No changes in urinary biochemical parameters in either of strains were observed at either of cadmium doses (data not shown).

3.2. Histology

Histological examination of livers of rats treated with 1 mg of cadmium/kg, showed presence of neutrophil infiltration in DA rats, while leukocyte presence was less obvious in AO rats (Fig. 2A, B). Histological examination of spleens (Fig. 2C, D) revealed neutrophil accumulation in red pulp of spleens of both strains. Analysis of spleen smears of cadmium-treated animals showed increases (compared to respective controls) not only in granulocytes (16.7 ± 2.5 % compared to 12.5 ± 0.7 % in AO rats, $P < 0.05$ and 18.2 ± 4.4 % compared to 10.9 ± 4.1 % in DA rats, $P < 0.01$), but in numbers of metamyelocytes as well (4.7 ± 0.8 % *vs.* 2.4 ± 0.3 %

in AO rats, $P < 0.05$ and 5.5 ± 1.9 % *vs.* 3.7 ± 1.5 % in DA rats, $P < 0.05$) with similar values in both strains. Histological examination of lungs (Fig. 2E, F) showed interstitial widening with leukocyte, predominantly neutrophil, infiltration. Neutrophils appear in focuses mainly perivascularly in AO rats and in the interstitium of DA rats. Histological evaluation of kidneys showed no differences between treated and control animals of both strains (data not shown).

3.3. Spleen cell responses of AO and DA rats following cadmium administration

Administration of 1 mg of cadmium/kg resulted in increase in relative spleen weight in cadmium-treated compared to control rats of both strains and in cellularity in AO rats (Table 1). No changes were observed in spleen cell viability of either of strains.

Measurements of metallothionein (MT) spleen cell response revealed significantly higher levels of mRNA for both MT isoforms in spleen cells of cadmium-treated rats of both strains (however lower levels of mRNA for MT-2 in AO compared to DA rats at higher cadmium dose were noted).

Examination of spleen proliferative characteristics (Fig. 3) revealed no changes in spontaneous activity in either of strains. Stimulation with ConA resulted in increase in response both in controls and in cadmium-treated rats of both rat strains, but proliferation was lower (compared to controls) in groups of rats of both strains which received 1 mg of cadmium/kg (Fig. 3A). The degree of reduction of proliferative response, , however, was lower ($P < 0.01$) in AO rats (by 55.4 ± 2.7%) compared to 78.1 ± 5.4% in DA rats. When expression of CD25 (activation-inducible chain of IL-2 receptor), as well as proliferation in response to IL-2 in spleen cells of rats treated with higher cadmium dose were measured (Fig. 3B), a decrease in percentages of splenocytes that express these molecules was noted in cadmium-treated rats of both strains, but IL-2 stimulated proliferation was not affected in AO in contrast to DA rats (Fig. 3B).

Measurements of spleen cell production of NO, which might inhibit cell proliferation, in medium conditioned by ConA-stimulated spleen cells (Fig. 3C) revealed increase in cultures of cells from cadmium-treated DA rats solely. Examination of iNOS mRNA showed lack of changes in AO rats in contrast to significantly higher levels in cadmium-treated *vs.* control DA rats. Examination of

another aspect of oxidative activity of spleen cells, MPO activity, showed increase in cells of cadmium-administered animals of both strains (1.2 ± 0.4 compared to cells of the controls 0.6 ± 0.2, $P < 0.05$ in AO and 1.6 ± 0.6 compared to controls 0.4 ± 0.1, $P < 0.01$ in DA rats) with no strain differences.

Examination of IFN-γ spleen cell responses (Fig. 4A) revealed no changes in spontaneous activity in either of strains at either of cadmium doses. Similar levels (compared to those in controls) of ConA-stimulated IFN-γ production were noted at cadmium dose of 0.5 mg/kg compared to controls. Lower (compared to controls) levels of IFN-γ production were noted at higher cadmium dose in both strains, but decrease was less pronounced in AO compared to DA rats (Fig. 4A). Decrease of IFN-γ mRNA was noted at this cadmium dose in cells of both strains (Fig. 4B). No effects of cadmium administration were seen on IL-17 responses (either at the level of protein product or at mRNA level), though a tendency ($P = 0.06$) of an increase of IL-17 mRNA was seen in spleen cells of DA rats at higher cadmium dose (Fig. 4C, D).

Because cadmium administration results in accumulation of granulocytes (which might modulate or contribute to cytokine responses), mononuclear cell cytokine responses were measured next. Similar pattern of mononuclear cell IFN-γ (Fig. 4E, F) and IL-17 (Fig. 4G, H) response was observed, compared to total spleen cells, except in DA rats, where lower levels of ConA-stimulated production of IL-17 were observed.

3.4. Lung leukocyte responses of AO and DA rats following cadmium administration

No changes in relative weight of lungs of both cadmium-treated AO and DA rats were noted (Table 2). While no differences were noted in numbers of leukocytes recovered from lungs of AO rats at either of cadmium doses, increased numbers of leukocytes were recovered from lungs of DA rats administered with 1 mg of cadmium/kg. No changes were observed in lung leukocyte viability. Measurements of MT response at lower cadmium dose revealed similar values of MT-1 mRNA, and a tendency of lower values of MT-2 mRNA ($P = 0.06$ in AO and $P = 0.052$ in DA rats) compared to controls. Significant increase of mRNA of both MT isoforms in

lung cells of rats of both strains administered with 1 mg of cadmium/kg was detected, but higher values were noted in AO rats. Analysis of cytospin preparations of lung leukocytes from cadmium-treated rats revealed increased numbers of neutrophils only from lungs of rats treated with higher cadmium dose, but values were much lower in AO compared to DA rats. Drop in percentages of lymphocytes was seen at this dose in DA rats solely, while monocyte/macrophage numbers were lower in both stains. Measurements of MPO activity in homogenates of lung tissue from rats treated with 1mg of cadmium/kg confirmed neutrophil infiltration judging by increased ($P < 0.01$ *vs.* controls) values in cadmium-treated rats of both strains but values in AO (3.3 ± 0.3 U/g of tissue) were lower ($P < 0.01$) compared to 7.6 ± 0.9 U/g in DA rats. In addition, higher MPO activity was noted in leukocytes recovered from lungs of cadmium-treated rats of AO (1.6 ± 0.5 compared to cells of the controls 0.9 ± 0.4, $P < 0.05$) and DA strain (4.4 ± 0.9 compared to controls 0.7 ± 0.2, $P < 0.05$), but the levels were lower ($P < 0.05$) in the former strain.

Because of the constant exchange of peripheral blood and lung leukocytes (mainly neutrophils), changes in differential peripheral blood leukocyte numbers were examined in rats treated with higher cadmium dose (Fig. 5). Increased numbers of total and neutrophilic leukocytes were noted in blood of cadmium-treated animals of both AO (Fig. 5A, B) and DA (Fig. 5D, E) strains, but the increase was less pronounced in AO rats. High ($r = 0.94$) and significant ($P < 0.001$) correlation was observed between peripheral blood and lung neutrophils of DA rats ($Y = 5.35 + 0.68 \times X$) treated with cadmium. Lower level of correlation ($r = 0.78$) between neutrophillic leukocytes in these two compartments was seen in AO rats, but it was still significant ($P < 0.01$, $Y = 18.96 + 1.11 \times X$). Measurements of CD11b showed increased numbers of $CD11b^+$ cells within granular cell population from blood, but the numbers were higher in DA (Fig. 5F) rats compared to AO (Fig. 5C).

Examination of IFN-γ responses by lung cells showed similar levels of spontaneous IFN-γ production at cadmium dose of 0.5 mg/kg compared to controls, while lower levels were noted at higher cadmium dose in both strains (Fig. 6A). Similar pattern of changes of this cytokine was noted following stimulation with ConA and the effect was seen at lower Cd dose in DA rats, solely. To see if decreased production at 1 mg of cadmium/kg was noted because of lower percentages of lymphocytes, IFN-γ production by

mononuclear cells was measured next (Fig. 6B), and results showed decreased spontaneous and ConA-stimulated production in treated animals of both strains. Decreases of IFN-γ mRNA were noted in both total (Fig. 6C) and mononuclear (Fig. 6D) lung cells in both strains.

No effect was seen on IL-17 production by total lung leukocytes of AO rats at either of cadmium doses, while a decrease was seen at 1 mg of cadmium/kg in DA rats (Fig. 6E). The effect on lung mononuclear leukocytes at higher metal dose was seen as well (Fig. 6F). In addition, level of IL-17 mRNA was decreased in total and mononuclear lung cells of DA rats (Fig. 6G, H).

4. Discussion

In the present study, toxicity of acute cadmium administration was examined by analyzing changes in spleen (most often explored in the context of cadmium immunotoxicity) and lungs (known target for cadmium) in two rat strains with disparate susceptibility to environmental stimuli. Slight differences of cadmium concentrations in the tissue might have accounted for higher levels of MT response in spleen of DA rats and in lungs of AO rats, but genetic factors seemed most important contributors to differences between these strains in the toxicity of this metal to these immune compartments The presence or absence of some of the effects or their intensity , however, depended on the tissue and the specific cell activity examined.

Increases in spleen cellularity in AO rats might have resulted from cell migration to spleens and/or less pronounced suppression of cell proliferation. Lack of increase in spleen cellularity in DA rats might be ascribed to lower responsiveness to IL-2 (major lymphocyte growth factor) and to increased production of nitric oxide (known to inhibit lymphocyte proliferation). The effects of somewhat higher cadmium levels in spleen of DA rats might have contributed to more pronounced decrease in spleen cell proliferation of this strain as well, although greater differences (near or more than 50%) in cadmium tissue deposition were reported as responsible for strain differences in cadmium hepatotoxicity and testicular toxicity (Shimada et al., 2004, 2009). Lower percentages of CD25 (inducible chain of IL-2 receptor) positive spleen cells noted in both strains exposed to cadmium along with a decrease in IL-2-driven

proliferation in DA rats solely, implies strain differences in pathways involved in IL-2/IL-2R signaling. Indeed, differences between AO and DA rats in IL-2 responses were noted previously (Vukmanović et al., 1989). Production of NO by spleen cells from cadmium-treated DA rats might have deepened lower responsiveness of splenocytes from this strain to IL-2, as interference of NO with IL-2/IL-2R signaling pathways was reported (Bingisser et al., 1998; Mazzoni et al., 2002). Differential levels in produced NO between strains rely on iNOS transcription, in line with data that showed differences between murine strains in NO production and iNOS gene transcription in other experimental systems (Evans et al., 1993; Zidek et al., 2000).

Lower level of inhibition of IFN-γ production by both total and mononuclear spleen cells of AO rats in response to stimulation with ConA (though similar level of decrease in the cytokine mRNA were observed in both strains) show that differential responsiveness to stimuli is responsible for disparate cytokine production between strains. Lack of the general effect of cadmium administration on spleen cell production and expression of IL-17, show, that at least for leukocytes of this compartment, IL-17 is not directly affected. A decrease of responsiveness of IL-17 production by spleen mononuclear cells of DA rats show, however, that cadmium administration might affect this cytokine in spleen.

Neutrophil infiltration into the lungs of both strains depicts proinflammatory activity of cadmium in this tissue, but with marked strain differences. Lower numbers of neutrophil leukocytes recovered from lungs of AO rats might have resulted from lower degree of increases in peripheral blood neutrophils in these rats. Lungs harbor intravascular reservoirs of leukocytes, predominantly neutrophils (termed ‘marginated pool’) that constantly exchange with circulating neutrophils (Kuebler, 2005). High and significant correlation between numbers of neutrophils in the peripheral blood and those recovered from lungs, corroborate such considerations. Lower number of neutrophils in lungs of AO rats might have resulted from lower percentages of $CD11b^{+}$ cells in the peripheral blood of these rats, as these molecules are involved in neutrophil extravasation and migration to peripheral tissue (Arnaout, 1990). Inhibition of IFN-γ, at both transcription as well as protein product levels in mononuclear cells of both spleen and lungs, implies that this cytokine

is an absolute target for cadmium, in line with numerous data from human peripheral blood mononuclear cells (Boscolo et al., 2005; Hemdan et al., 2006; Marth et al., 2001; Pathak and Khandelwal, 2008; Shen et al., 2001). Lack of differences between AO and DA rats in the degree of decrease of ConA-stimulated production of IFN-γ by lung leukocytes (in contrast to spleen cells where they were observed), showed that strain-related differences in the particular activity depend on the immune compartment in which it is examined. Differences in IL-17 gene transcription and protein product expression by lung leukocytes (together with effects on ConA-stimulated production of cytokine by spleen cells) , for the first time, showed strain differences in the effect of cadmium on IL-17 response.

5. Conclusion

Strain differences in the effects of acute cadmium administration were shown in two compartments of immune system: spleen and lungs. Although contribution of slight differences of cadmium concentrations in the tissue could not be ruled out, genetically based differences seemed more important contributing factors. The impact of strain, however, differs between these compartments and in relation to the activity examined. On the basis of less intense suppression or lack of the effect on some aspects of spleen and lung cell activity, AO rats seemed less susceptible to cadmium. This suggests that attention should be paid on selection of animal strain in studies of cadmium immunotoxicity.

Conflict of interest

The authors declare that they have no conflict of interest.

Acknowledgements

The authors would like to thank Ms. Jasmina Ninkov for her assistance in language editing and proofreading this paper. This study was supported by the Ministry of Education, Science and Technological Development of the Republic of Serbia, Grant #173039.

References

Arnaout, M.A., 1990. Structure and function of the leukocyte adhesion molecules CD11/CD18. Blood 75, 1037–1050.

Bingisser, R.M., Tilbrook, P.A., Holt, P.G., Kees, U.R., 1998. Macrophage-derived nitric oxide regulates T cell activation *via* reversible disruption of the Jak3/STAT5 signaling pathway. J. Immunol. 160, 5729–5734.

Boscolo, P., Di Giampaolo, L., Qiao, N., Reale, M., Castellani, M.L., Lucci, I., Travaqlini, P., Kouri, M., Verna, N., Volpe, A.R., Carmignani, M., Paganelli, R., Di Gioacchino, M., 2005. Inhibitory effects of cadmium on peripheral blood mononuclear cell proliferation and cytokine release are reversed by zinc and selenium salts. Ann. Clin. Lab. Sci. 35, 115–120.

Bozeman, P.M., Learn, D.B., Thomas, E.L., 1990. Assay of the human leukocyte enzymes myeloperoxidase and eosinophil peroxidase. J. Immunol. Methods 126, 125–133.

Demenesku, J., Mirkov, I., Ninkov, M., Popov Aleksandrov, A., Zolotarevski, L., Kataranovski, D., Kataranovski, M., 2014. Acute cadmium administration to rats exerts both immunosuppressive and proinflammatory effects in spleen. Toxicology 326, 96–108.

Denizot, F., Lang, R., 1986. Rapid colorimetric assay for cell growth and survival: modifications to the tetrazolium dye procedure giving improved sensitivity and reliability. J. Immunol. Methods 89, 271-277.

Djokic, J., Ninkov, M., Mirkov, I., Popov Aleksandrov, A., Zolotarevski, L., Kataranovski, D., Kataranovski, M., 2014. Differential effects of cadmium administration on peripheral blood granulocytes in rats. Environ. Toxicol. Pharmacol. 37, 210–219.

Djokic, J., Popov Aleksandrov, A., Ninkov, M., Mirkov, I., Zolotarevski, L., Kataranovski, D., Kataranovski, M., 2015. Cadmium administration affects circulatory mononuclear cells in rats. J. Immunotoxicol. 12, 115–123.

Evans, T.J., Strivens, E., Carpenter, A., Cohen, J., 1993. Differences in cytokine response and induction of nitric oxide synthase in endotoxin-resistant and endotoxin-sensitive mice after intravenous gram-negative infection. J. Immunol. 150, 5033–5040.

Gochfeld, M., 1997. Factors influencing susceptibility to metals. Environ. Health Perspect. 105, 817–822.

Gubrelay, U., Mehta, A., Singh, M., Flora, S.J., 2004. Comparative hepatic and renal toxicity of cadmium in male and female rats. J. Environ. Biol. 25, 65–73.

Hemdan, N.Y., Emmrich, F., Sack, U., Wichmann, G., Lehmann, J., Adham, K., Lehmann, I., 2006. The *in vitro* immune modulation by cadmium depends on the way of cell activation. Toxicology 222, 37–45.

Hibbs, J.B.M. Jr., Taintor, R.R., Vavrin, Z., Rachlin, E.M., 1988. Nitric oxide: a cytotoxic activated macrophage effector molecule. Biochem. Biophys. Res. Commun. 157, 87–94.

King, L.M., Anderson, M.B., Sikka, S.C., George, W.J., 1998. Murine strain differences and the effects of zinc on cadmium concentrations in tissues after acute cadmium exposure. Arch. Toxicol. 72, 650–655.

Kuebler, W.M., 2005. Inflammatory pathways and microvascular responses in the lung. Pharmacol. Rep. 57, 196–205.

Kuester, R.K., Waalkes, M.P., Goering, P.L., Fisher, B.L., McCuskey, R.S., Sipes, I.G., 2002. Differential hepatotoxicity induced by cadmium in Fischer 344 and Sprague-Dawley rats. Toxicol. Sci. 65, 151–159.

Lafuente, A., González-Carracedo, A., Romero, A., Esquifino, A.I., 2003. Effect of cadmium on lymphocyte subsets distribution in thymus and spleen. J. Physiol. Biochem. 59, 43–48.

Liu, J., Corton, C., Dix, D.J., Liu, Y., Waalkes, M.P., Klaassen, C.D., 2001. Genetic background but not metallothionein phenotype dictates sensitivity to cadmium-induced testicular injury in mice. Toxicol. Appl. Pharmacol. 176, 1–9.

Marth, E., Jelovcan, S., Kleinhappl, B., Gutschi, A., Barth, S., 2001. The effect of heavy metals on the immune system at low concentrations. Int. J. Occup. Med. Environ. Health 14, 375–386.

Mazzoni, A., Bronte, V., Visintin, A., Spitzer, J.H., Apolloni, E., Serafini, P., Zanovello, P., Segal, D.M., 2002. Myeloid suppressor lines inhibit T cell responses by an NO-dependent mechanism. J. Immunol. 168, 689–695.

McKenna, I.M., Waalkes, M.P., Chen, L.C., Gordon, T., 1997. Comparison of inflammatory lung responses in Wistar rats and C57 and DBA mice following acute exposure to cadmium oxide fumes. Toxicol. Appl. Pharmacol. 146, 196–206.

Morselt, A.F.W., Leene, W., De Groot, C., Kipp, J.B.A., Evers, M., Roelofsen, A.M., Bosch, K.S., 1988. Differences in immunological susceptibility to cadmium toxicity between two rat strains as demonstrated with cell biological methods. Effect of cadmium on DNA synthesis of thymus lymphocytes. Toxicology 48, 127–139.

Morselt, A.F., 1991. Environmental pollutants and diseases. A cell biological approach using chronic cadmium exposure in the animal model as a paradigm case. Toxicology 70, 1–132.

Olszowski, T., Baranowska-Bosiacka, I., Gutowska, I., Chlubek, D., 2012. Pro-inflammatory properties of cadmium. Acta Biochem. Pol. 59, 475–482.

Pathak, N., Khandelwal, S., 2007. Role of oxidative stress and apoptosis in cadmium induced thymic atrophy and splenomegaly in mice. Toxicol. Lett. 169, 95–108.

Pathak, N., Khandelwal, S., 2008. Impact of cadmium in T lymphocyte subsets and cytokine expression: differential regulation by oxidative stress and apoptosis. Biometals 21, 179–187.

Shaikh, Z.A., Jordan, S.A., Tewari, P.C., 1993. Cadmium disposition and metallothionein induction in mice: strain-, sex-, age-, and dose-dependent differences. Toxicology 80, 51–70.

Shen, X., Lee, K., König, R., 2001. Effects of heavy metal ions on resting and antigen-activated $CD4^+$ T cells. Toxicology 169, 67–80.

Shimada, H., Takamure, Y., Shimada, A., Yasutake, A., Waalkes, M.P., Imamura, Y., 2004. Strain differences of cadmium-induced hepatotoxicity in Wistar-Imamichi and Fischer 344 rats: involvement of cadmium accumulation. Toxicology 203, 189–197.

Shimada, H., Narumi, R., Nagano, M., Yasutake, A., Waalkes, M.P., Imamura, Y., 2009. Strain difference of cadmium-induced testicular toxicity in inbred Wistar-Imamichi and Fischer 344 rats. Arch. Toxicol. 83, 647–652.

Shimada, H., Hata, I., Hashiguchi, T., Imamura, Y., 2011. Genetic background of resistance to cadmium-induced testicular toxicity in inbred Wistar-Imamichi rats. Arch. Toxicol. 85, 1195–1199.

U.S. Department of Health and Human Services, 1997. Toxicological Profile for Cadmium. Draft for Public Comment. Agency for Toxic Substances and Disease registry, Atlanta, USA.

Vukmanović, S., Mostarica Stojković, M., Lukić, M.L., 1989. Experimental autoimmune encephalomyelitis in “low” and “high” interleukin 2 producer rats. I. Cellular basis of induction. Cell. Immunol. 121, 237–246.

WHO, 1992. Environmental Health Criteria 134, Cadmium, first ed. World Health Organization, Geneva, Switzerland.

Yamano, T., Shimizu, M., Noda, T., 1998. Comparative effects of repeated administration of cadmium on kidney, spleen, thymus and bone marrow in 2-, 4- and 8-month-old male Wistar rats. Toxicol. Sci. 46, 393–402.

Zidek, Z., Franoková, D., Boubelik, M., 2000. Genetic variations in *in-vitro* cytokine-induced production of nitric oxide by murine peritoneal macrophages. Pharmacogenetics 10, 493–501.

Figure captions:

Fig. 1. Cadmium tissue content. Results are expressed as mean ± S.D. from two experiments with four to six animals per group per experiment. Significance at: ** $P < 0.01$ *vs.* control (cadmium 0 mg/kg), ^^ $P < 0.01$ for higher *vs.* lower dose, and # $P < 0.05$, ## $P < 0.01$ for AO *vs.* DA rats.

Fig. 2. Histology of liver, spleen and lungs of AO and DA rats administred with cadmium. Leukocyte infiltration in liver of (A) AO rats and (B) DA rats (× 200). Neutrophillic leukocytes in the red pulp of spleens of (C) AO and (D) DA rats (× 400). (E) Focuses of neutrophils perivascularly in the lungs of AO rats (× 200). (F) Neutrophils in the interstitium of the lungs of DA rats (× 200).

Fig. 3. The effect of cadmium on proliferative characteristics of spleen cells in AO and DA rats. (A) Spontaneous and ConA-stimulated proliferation. (B) Number of $CD25^+$ cells and IL-2- stimulated proliferation. (C) Nitric oxide (NO) level in spleen cells conditioned medium, and iNOS mRNA levels. Results are expressed as mean ± S.D. Significance at: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ *vs.* control (cadmium 0 mg/kg), and # $P < 0.05$ for AO *vs.* DA.

Fig. 4. The effect of cadmium on spleen cell IFN-γ and IL-17 protein product and mRNA levels in AO and DA rats. Total spleen cells IFN-γ (A) and IL-17 (C) production, and mRNA expression (B and D, respectively). Mononuclear spleen cells IFN-γ (E) and IL-17 (G) production, and mRNA expression (F and H, respectively). Results are expressed as mean ± S.D. Significance at: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ *vs.* control (cadmium 0 mg/kg), and # $P < 0.05$, ### $P < 0.001$ for AO *vs.* DA rats.

Fig. 5. The effect of cadmium on peripheral blood leukocyte number and expression of CD11b molecule in AO and DA rats. Total leukocyte number, differential cell count and number of $CD11b^+$ cells in peripheral blood of AO (A, B, C respectively) and DA

rats (D, E, F respectively). Results are expressed as mean ± S.D. Significance at: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ *vs.* control (cadmium 0 mg/kg), and # $P < 0.05$, ## $P < 0.01$ for AO *vs.* DA rats.

Fig. 6. The effect of cadmium on lungs cell IFN-γ and IL-17 protein product and mRNA levels in AO and DA rats. Total and mononuclear lungs cells IFN-γ production (A and B, respectively) and mRNA expression (C and D, respectively). Total and mononuclear lungs cell IL-17 production (E and F, respectively) and mRNA expression (G and H, respectively). Results are expressed as mean ± S.D. Significance at: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ *vs*. control (cadmium 0 mg/kg).

Table 1.

The effect of cadmium administartion on spleen weight, cellularity, cell viability and metallothionein (MT) response

	AO			DA		
	Cadmium dose (mg/kg)			Cadmium dose (mg/kg)		
Parameters	0	0.5	1	0	0.5	1
Relative weight (mg/g body weight)	1.70 ± 0.32	2.23 ± 0.55	2.46 ± 0.99***	1.68 ± 0.16	1.74 ± 0.13	2.02 ± 0.25***
Cellularity (× 10^6/per spleen)	299.3 ± 99.0	290.2 ± 83.5	440.1 ± 130.3***	271.5 ± 49.7	205.9 ± 98.8	288.8 ± 73.5
Cell viability (A 540nm)	0.29 ± 0.05	0.26 ± 0.08	0.28 ± 0.00	0.23 ± 0.02	0.27 ± 0.06	0.24 ± 0.09
MT – 1 mRNA expression (relative values)	1.00 ± 0.49	2.48 ± 1.48**	2.26 ± 1.77*	1.00 ± 0.20	2.46 ± 0.75*	2.36 ± 1.00**
MT – 2 mRNA expression	1.01 ± 0.51	2.92 ± 1.33**	3.93 ± 2.30$^{**\#\#}$	1.03 ± 0.19	3.72 ± 2.97**	12.00 ± 2.19$^{**\wedge\wedge}$

(relative values)

Results are expressed as mean ± S.D. from two experiments with four to six animals per group per experiment. Significance at: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ *vs.* control (cadmium 0 mg/kg), ^^ $P < 0.01$ for higher (1mg/kg) *vs.* lower dose (0.5 mg/kg), and ## $P < 0.01$ for AO *vs.* DA.

Table 2.

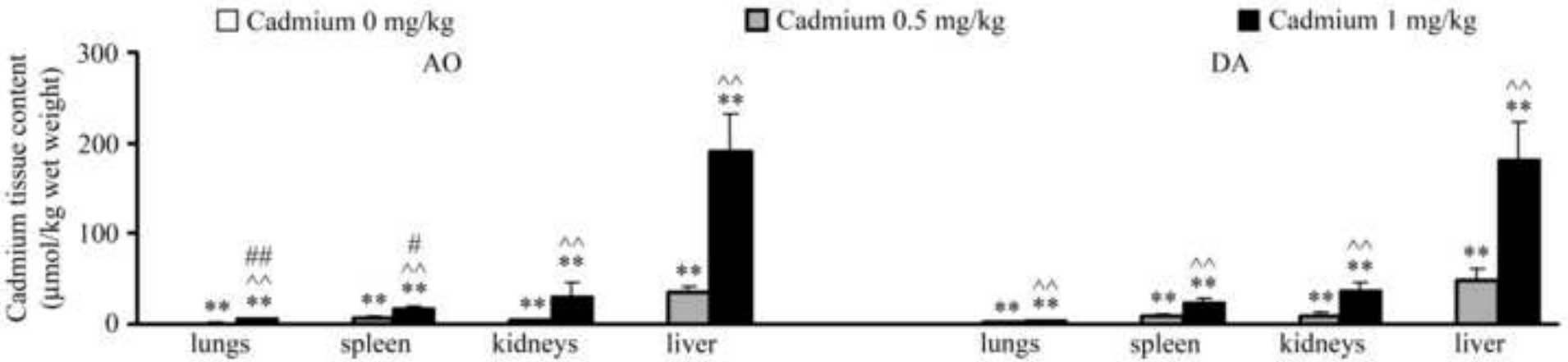
The effect of cadmium administration on lung weight, leukocyte recovery, cell viability, metallothionein (MT) response and differential leukocyte count.

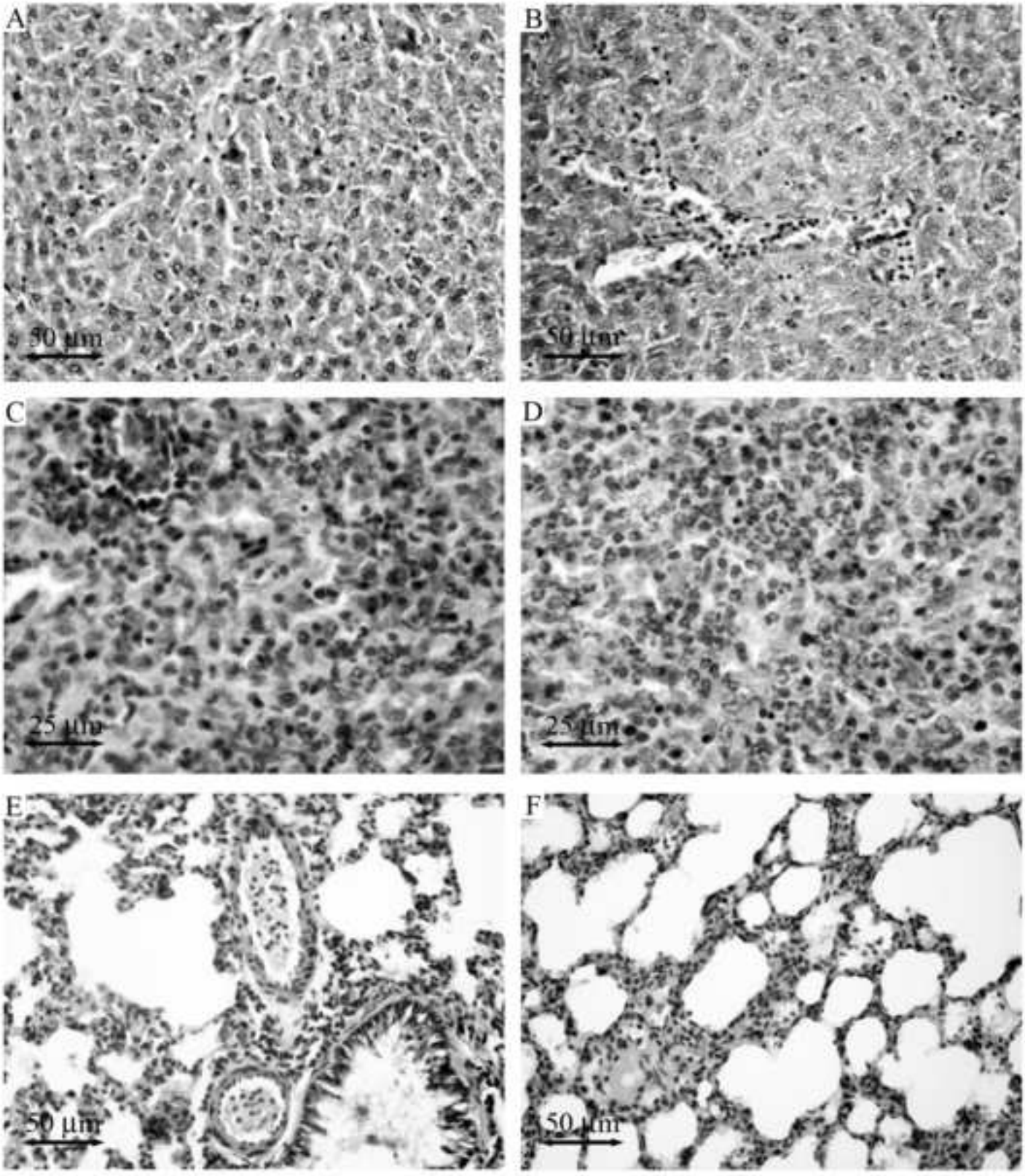
	AO			DA		
	Cadmium dose (mg/kg)			Cadmium dose (mg/kg)		
Parameters	0	0.5	1	0	0.5	1
Relative weight (mg/g body weight)	6.10 ± 1.87	6.62 ± 1.07	5.90 ± 1.12	6.30 ± 1.60	5.80 ± 0.45	6.63 ± 1.71
Leukocyte recovery ($\times 10^6$/ per lung)	16.2 ± 8.9	18.4 ± 5.3	20.0 ± 9.2	16.7 ± 5.0	15.9 ± 2.7	29.1 ± 5.2***
Cell viability (A 540nm)	0.62 ± 0.09	0.49 ± 0.16	0.55 ± 0.10	0.55 ± 0.13	0.43 ± 0.15	0.43 ± 0.14
MT – 1 mRNA expression (relative values)	1.00 ± 0.62	0.96 ± 0.06	4.58 ± 2.41**##	1.00 ± 0.26	1.34 ± 1.25	1.69 ± 0.25**

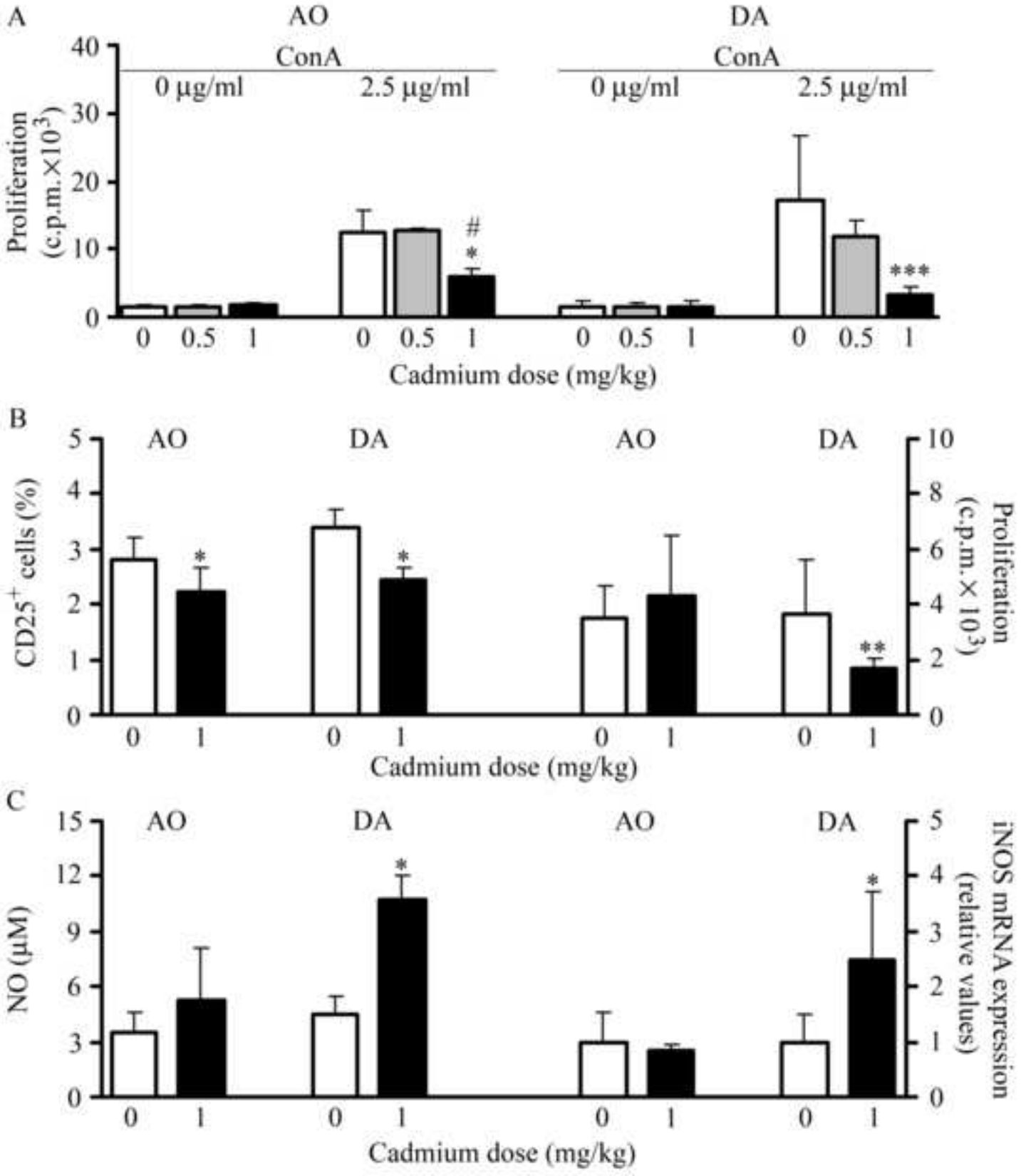
MT – 2 mRNA expression (relative values)	1.00 ± 0.51	0.33 ± 0.43	4.89 ± 1.66$^{**\#}$	1.00 ± 1.00	0.32 ± 0.48	2.29 ± 0.62*
Differential leukocyte count (%)						
Neutrophils	6.9 ± 3.5	8.8 ± 2.4	32.4 ± 7.9$^{**\#\#}$	11.1 ± 7.6	10.8 ± 6.1	60.1 ± 8.4***
Lymphocytes	55.9 ± 12.3	51.1 ± 18.5	48.9 ± 10.6	63.1 ± 8.7	61.0 ± 18.5	25.9 ± 8.1**
Monocytes/Macrophages	37.1 ± 13.7	40.1 ± 7.3	18.6 ± 7.4*	25.8 ± 10.6	28.0 ± 3.7	14.0 ± 5.7*

Results are expressed as mean ± S.D. from two experiments with four to six animals per group per experiment. Significance at: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ *vs.* control (cadmium 0 mg/kg), and # $P < 0.05$, ## $P < 0.01$ for AO *vs.* DA.

Figure 1


Cadmium 0 mg/kg
Cadmium 0.5 mg/kg
Cadmium 1 mg/kg
Cadmium tissue content (µmol/kg wet weight)
300
200
100
0
AO
DA
lungs
spleen
kidneys
liver
lungs
spleen
kidneys
liver





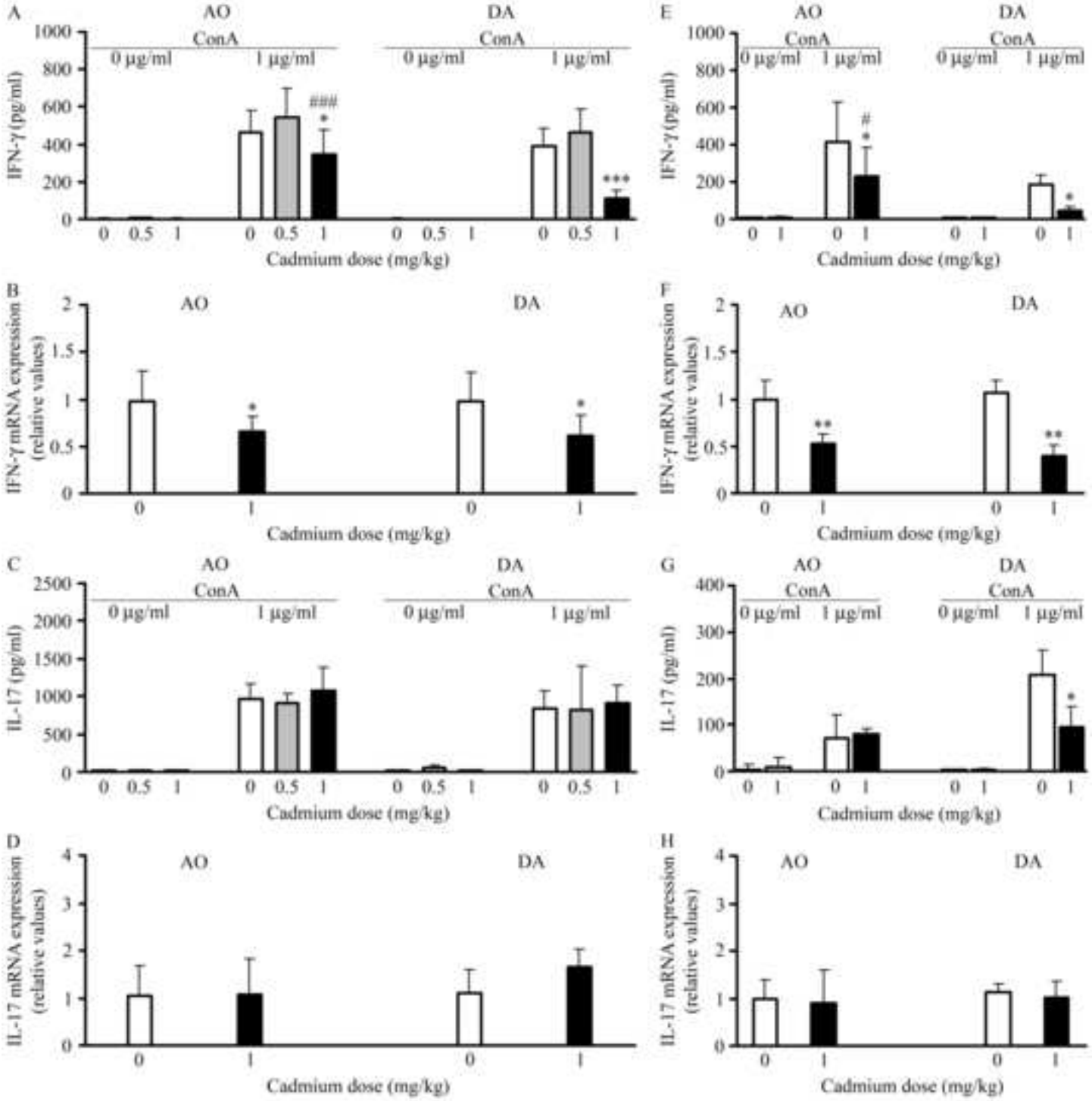


Figure 5

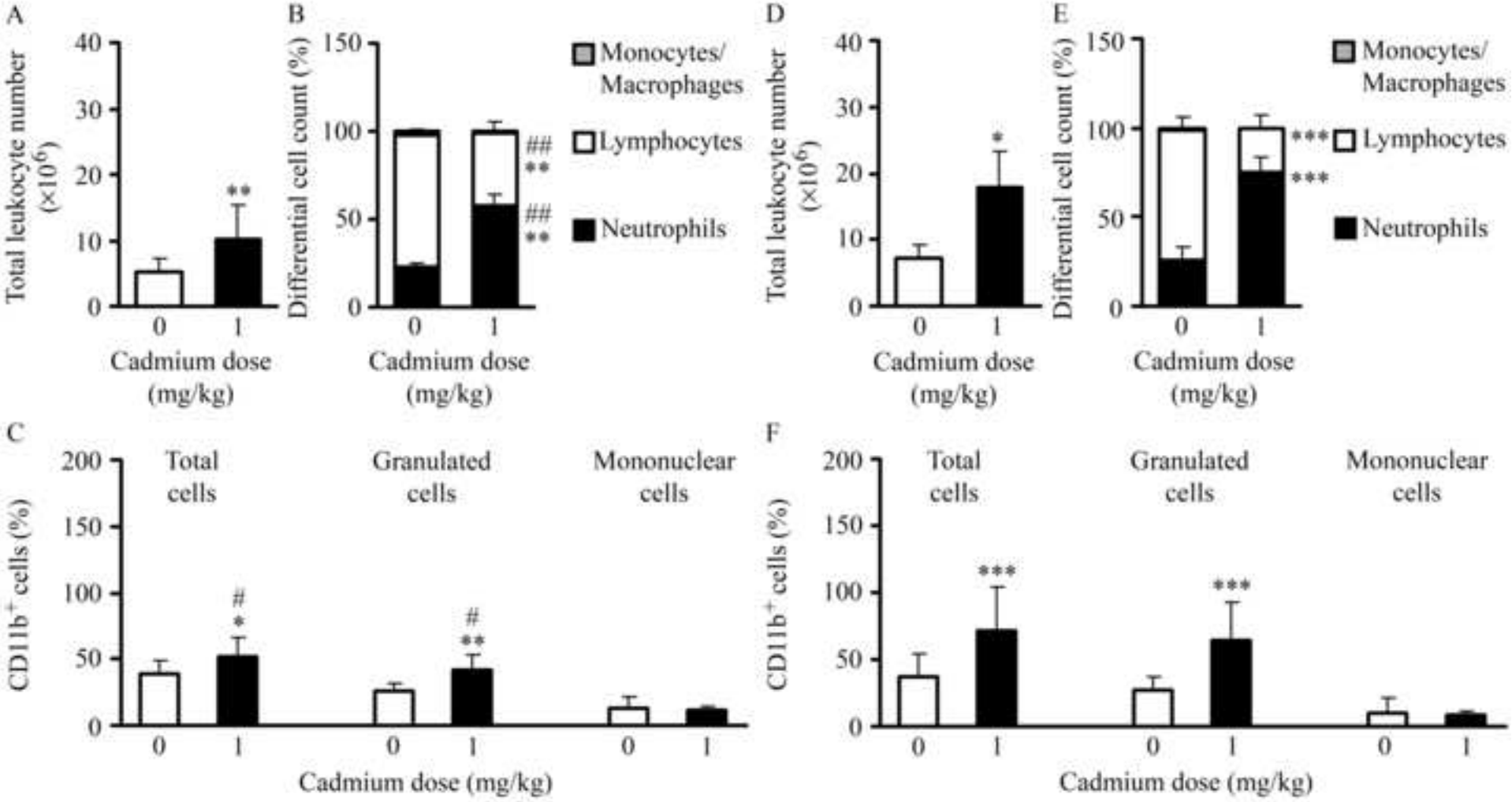
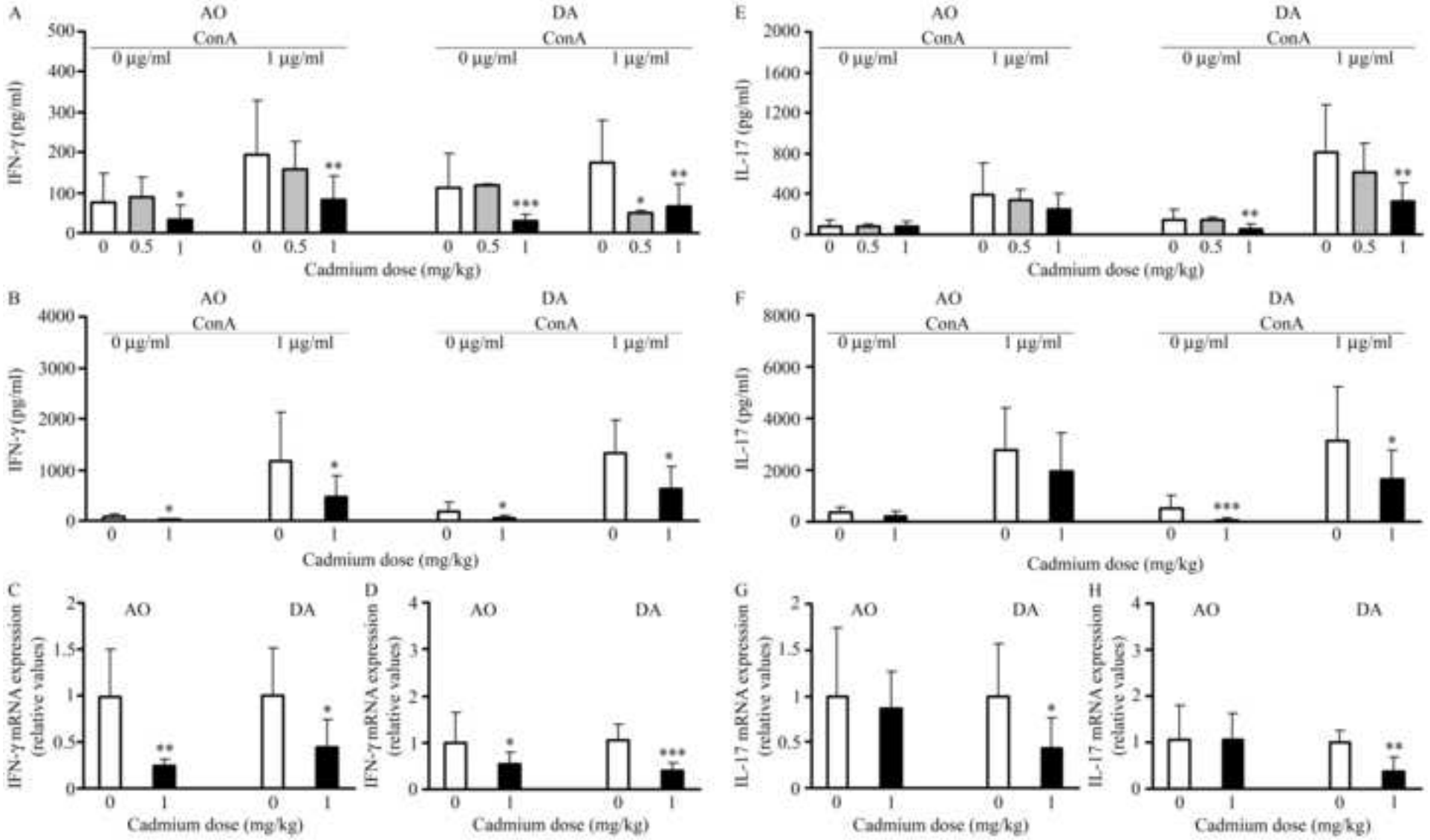

A
Total leukocyte number (×10^6)
40
30
20
10
0
**
0
1
Cadmium dose (mg/kg)
B
Differential cell count (%)
150
100
50
0
##
**
##
**
0
1
Cadmium dose (mg/kg)
Monocytes/ Macrophages
Lymphocytes
Neutrophils
D
Total leukocyte number (×10^6)
40
30
20
10
0
*
0
1
Cadmium dose (mg/kg)
E
Differential cell count (%)
150
100
50
0
***
***
0
1
Cadmium dose (mg/kg)
Monocytes/ Macrophages
Lymphocytes
Neutrophils
C
CD11b+ cells (%)
200
150
100
50
0
Total cells
Granulated cells
Mononuclear cells
#
*
#
**
0
1
0
1
0
1
Cadmium dose (mg/kg)
F
CD11b+ cells (%)
200
150
100
50
0
Total cells
Granulated cells
Mononuclear cells
***
***
0
1
0
1
0
1
Cadmium dose (mg/kg)

Figure 6


A
AO
DA
ConA
0 μg/ml
1 μg/ml
IFN-γ (pg/ml)
Cadmium dose (mg/kg)
B
C
IFN-γ mRNA expression (relative values)
D
E
IL-17 (pg/ml)
F
G
IL-17 mRNA expression (relative values)
H