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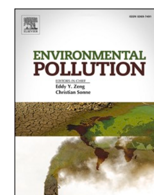

Environmental exposure to cadmium reduces the primary antibody-mediated response of wood mice (*Apodemus sylvaticus*) from differentially polluted locations in the Netherlands☆

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ABSTRACT

The Wood mouse (*Apodemus sylvaticus*) is a widespread mammalian species that acts as a reservoir host for multiple infections, including zoonotic diseases. Exposure to immunotoxins, like for instance trace metals, may reduce the ability of the host to mount proper responses to pathogens, potentially increasing the transmission and prevalence of infections. Antibody-mediated responses are crucial in preventing and limiting infections, and the quantification of the primary antibody response is considered a sensitive predictor of immunosuppression. The current study aims to investigate effects of cadmium exposure on the antibody-mediated responses of wood mice inhabiting polluted and non-polluted areas in the Netherlands. Wood mice were captured alive at different locations and immunized to sheep red blood cells (SRBC) to induce a primary antibody response. SRBC-specific antibody-producing cells, or plaque forming cells (PFC), were quantified and related to kidney cadmium levels. Differential circulating main leukocyte populations were also characterised. Cadmium concentrations in mice kidneys differed between mice captured at different locations, and increased with individual body mass, likely associated with age-related time of exposure. Effect of cadmium was apparent on the percentages of B cell counts in blood. Because of potential natural immune heterogeneity between wild rodent populations, mice immune responses were analysed and compared grouped by captured locations. Capture location had significant effect on the total counts of white blood cells. Increasing cadmium exposure in wood mice captured from polluted sites was associated with a decrease of splenic PFC counts. This field research shows that wood mice antibody responses can be impaired by cadmium exposure, even at low environmental levels, by affecting B cell functioning mainly. Impaired B cell function can make exposed mice more susceptible to infections, potentially increasing the reservoir function of their populations. It also shows that immunomodulatory effects in the field should be assessed site specifically.

1. Introduction

Pollution with trace metals is common in populated areas, and is mainly related to human-related activities (Tchounwou et al., 2012). Metals may accumulate in the environment over time and wildlife species inhabiting such areas can be exposed to elevated levels (Wijnhoven et al., 2007). Terrestrial animals living nearby the sources, particularly small mammals, can be chronically exposed through their life cycles, often without noticeable effects when exposure is relatively low (Burger, 2008). The immune system however, can still be modulated by such low and chronic environmental exposures, potentially hampering protective host responses upon immune activation, for example during infections (Lehmann et al., 2011). Trace metals have been shown to modulate the immune system, lowering the resistance to infections in humans and animals (Heilmann, 2012; Krueger and Wade, 2016; Simonyte and Planciuniene, 2010). Many wild rodent species are known natural hosts of infectious agents, including relevant zoonotic infectious agents (Morand et al., 2015). The modulated immune system of wild small mammals environmentally exposed to immunotoxic trace metals might not be able to limit infectious agents, thereby facilitating their transmission, increasing the prevalence within the species and also to other species, including humans (Bean et al., 2013; Mandl et al., 2015).

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The mammalian immune system is a complex network that includes different specialized mobile and tissue-resident cell types, immune organs, and many signalling pathways that work together to provide protection against infectious agents (Chaplin, 2010). A modulation of the immune system, meaning a change in any of these constituents, may be sufficient to decrease the efficiency of some protective functions, such as the humoral or cellular responses, in limiting infections and their transmission (Selgrade, 2007). The humoral immune responses, mediated by B cells and the production of antibodies (or antibody-mediated responses), are extremely relevant in the immunity against particular types of infections, including extracellular parasites and bacteria (Sebina and Pepper, 2018). The measurement of the primary humoral response, defined as a naive immune response to an antigen that is in contact with the immune system for the first time, is conveyed as the best predictor of immunosuppression caused by the exposure to immunotoxicants (Herzyk and Holsapple, 2007). At the same time, this method can be employed in wildlife small mammal species to assess and compare the effectivity of the primary humoral response between differentially exposed populations, for instance in animals living in diverse contaminated areas (Biser et al., 2004; Boughton et al., 2011).

In order to gain a deeper understanding in how pollution with trace metals can affect the immune system of environmentally exposed wild small mammals, the present study investigated immunomodulatory effects of cadmium in wild ranging wood mice (*Apodemus sylvaticus*), environmentally exposed to known levels of cadmium (van den Brink et al., 2010). For this, internal cadmium levels in kidneys of wood mice trapped alive from different locations with known levels of cadmium contamination were determined. Cadmium-induced immunomodulation was evaluated *ex vivo* with the quantification of the primary antibody-mediated response against a model antigen. Other immunological assays, such as ELISA and PCR for the determination of key molecules, were not suitable because of the limited availability of species-specific reagents for wild small mammals (Garnier et al., 2017). Results obtained will elucidate whether the humoral immune system in wild caught wood mice can be modulated by the environmental exposure to cadmium and will be related to the possible mode of toxicity of cadmium in wild caught small mammals.

2. Materials and methods

2.1. Experimental design

The present study was conducted with environmentally exposed wood mice (*A. sylvaticus*) captured at two different locations with known differences in cadmium levels (van den Brink et al., 2010). The first selected location was Plateaux, south of Valkenswaard, province of North Brabant (51°57′49″N, 5°44′28″E), which is known for being polluted with cadmium due to the proximity to a zinc smelter plant (van den Brink et al., 2010). The second location was the experimental station of Wageningen University at Sinderhoeve in Renkum, province of Gelderland (51°95′58.92″N, 5°45′16.62″E). The location does not have a history of contamination and was employed as a control non-polluted site (van den Brink et al., 2011). A total of 28 mice were captured; 15 from Plateaux and 13 from Sinderhoeve, all captured during August-October, 2019. Mice were captured with Longworth small mammal live-traps activated during the night. Traps were supplied with hay bedding, peanut butter bait and apple pieces to provide food and moisture. Captured mice were transported within the traps to Sinderhoeve where they were kept individually in outdoor, one square meter housing units, resembling outside conditions (including soil and sand bedding, weeds and tree branches). The traps were left open inside the housing units to be used as nest and to ease the recapturing of each mouse during consecutive experimental procedures. Mice were provided with non-contaminated water and food (rodent seed mix and fruit pieces) *ad libitum*, renewed twice per week. To reduce the stress after transport the mice had an acclimatization period of three to five days before the first procedures. Since cadmium generally has a very low excretion rate (~0.001 % of body Cd per day) the internal cadmium concentrations are not expected to decrease during the acclimatization period (Chandler et al., 2016). Cadmium internal levels were determined in mice kidneys to provide actual, individual exposure concentrations.

After the acclimatization period, mice were sensitized to sheep red blood cells (SRBC) with an intraperitoneal (i.p.) injection. For this purpose each mouse was recaptured and anaesthetised with inhaled isoflurane, followed by an i.p. injection with 200 μL of SRBC (10^9 cells/mL), freshly prepared the same day. SRBC solution was prepared with 5 mL of fresh sheep blood in Alsever's (Biotrading, the Netherlands), washed with 45 mL of cold sterile HBSS in a 50 mL tube and centrifuged at 1000×*g* for 10 min at 4 °C. Supernatants were discarded and the washing step was repeated for three times. Washed SRBCs were resuspended in 5 mL HBSS yielding a cell concentration of approximately 10^9 cells/mL. Each mouse was injected with 200 μL SRBC suspension i.p.

After being sensitized each mouse was placed back into the housing unit and observed until recovered from anaesthesia. Seven days after immunisation, the mice were recaptured and anaesthetised again using the same procedure described above. Under anaesthesia each mouse was weighed, sexed and blood was drawn from the orbital venous sinus with a capillary tube into an EDTA treated blood collecting tube. After bleeding, mice were sacrificed by cervical dislocation. Spleen and kidneys were dissected and weighed; spleens were kept in 3 mL HBSS on ice and kidneys were frozen at −80 °C until assayed.

Experiments were conducted according to Dutch legislation on the use of test animals, approved by the Animal Experimental Committee (DEC) (Project 2017.W-0065.002).

2.2. Cadmium determination in kidneys

Chemical analysis was performed for cadmium determination in kidneys. Kidneys are target organs for cadmium and were selected to allow comparison to other studies in wood mouse (Rogival et al., 2007; van den Brink et al., 2011). Briefly, kidneys were freeze-dried and digested in aqua-regia in microwave in Teflon vessels. Digested samples were analysed with inductively coupled plasma atomic emission spectrometer (ICP-AES), and when concentrations were below the detection limits the analysis was carried with inductively coupled plasma mass spectrometry (ICP-MS) (van den Brink et al., 2010). Cadmium concentrations are expressed as dry weight concentrations in μg/g d.w.

2.3. Haematocrit and WBC

The haematocrit, representing the percentage of erythrocytes per blood volume, was measured in blood capillaries centrifuged at 13,000 rpm for 3 min. The percentage of erythrocytes in the capillary was determined comparing with the total volume filled in the capillary set as 100 %. Nucleated, white blood cells were counted manually per sample (>100 per sample). For this, whole blood samples were treated with 10–20 % of Türk's solution (Sigma Aldrich, the Netherlands), and counted with a haemocytometer under microscope and expressed as white blood cells per micro litre (WBC/μL).

2.4. Haemolysis/haemagglutination

For the haemolysis haemagglutination titers determination, plasma samples were prepared from whole blood samples. For this 0.5 mL of blood was centrifuged at 2,000×*g* for 15 min in 1.5 mL Eppendorf tubes. Supernatant plasma was aliquoted in tubes and stored at −80 °C until assayed. In the assay, 2-fold plasma dilutions were prepared with HBSS buffer. Briefly, 25 μL of each plasma dilution was mixed with 25 μL 1 % sheep blood in a U-shape 96 well-plate. The samples were incubated at 37° with 100 % humidity for 1 h. Haemolysis and haemagglutination titers were visually determined for each sample (Vos, 2007).

2.5. Plaque forming assay

The primary humoral response to sheep red blood cells (SRBC) was measured *ex vivo* according to Ladics (2007). Briefly, each spleen kept in HBSS on ice was homogenized to generate a spleen cell suspension. For this, each spleen was cut into pieces over the frosted side of a glass slide and with the help of another glass slide the spleen pieces were smashed to remove the spleen capsule. The 3 mL of HBSS in which each spleen was contained was used to suspend the spleen cells and to transfer the cell suspension back to the tube. The spleen suspensions were washed with 10 mL of cold HBSS and centrifuged at 500×*g* for 10 min at 4 °C. After centrifugation the supernatants were discarded and the pelleted cells were resuspended in 10 mL HBSS and passed through a 50 μm cell strainer into another tube. The cell suspensions were centrifuged again and supernatants discarded. The washed pelleted cells were resuspended in 2.5 mL of cold HBSS and kept on ice until assayed.

Sheep red blood cells were prepared in a similar way as for the immunisation described above. Sheep blood in Alsevers' from the same batch for immunisation of each mouse was used in the assay. SRBCs at a cell concentration of approximately 10^9 cells/mL were prepared in HBSS and kept on ice.

The semisolid media in which the PFA was performed was prepared with 100 mL of HBSS HEPES buffer (487.6 mL HBSS + 2.4 mL $NaHCO_3$ 7.5 % + 10 mL HEPES 1 M) pH 7.4, 500 mg bacto agar and 1.6 mL dextran solution pH 6.9 (900 mg dextran in 30 mL NaCl 0.85 %). The media was mixed in a glass beaker using a magnetic-stirrer hot plate set at 200 °C until agar was dissolved, and lowered to 70 °C afterwards.

Glass test tubes (75 × 12 mm) were placed in a block heater at 37 °C. 350 μL of warm agar media was poured in each tube, in combination with 25 μL of SRBC solution, 25 μL of reconstituted guinea pig complement sera (Sigma Aldrich, the Netherlands), briefly mixed and finally 100 μL of the individual spleen cell suspension was added in different dilutions (undiluted, 1:1, 1:10 and 1:20). The solutions were briefly mixed and poured over a 10 cm diameter plastic Petri dish and immediately covered with a 25 × 60mm glass coverslip, avoiding bubble formation. Three replicates per dilution per sample were prepared. The dishes were incubated overnight at 37 °C and 100 % humidity. Plaques of haemolysis were counted the next day. Plaques of haemolysis or plaque forming cells (PFC), accounting for SRBC-specific IgM antibody forming B cells were counted. Splenocytes from each cell suspensions were manually counted with Türk's solution staining in a haemocytometer, as performed with WBC. PFC data was expressed as PFC per million splenocytes.

2.6. Flow cytometry

Flow cytometry analysis was performed to immunophenotype the main immune cell subpopulations and their glutathione (GSH) content from wood mice blood samples. Cellular GSH content was chosen as a relevant endpoint because (1) of the relationship between its high affinity to cadmium and the related redox mechanisms of toxicity (Nemmiche, 2017), (2) its relevance in regulating immune responses at cellular level (Mak et al., 2017), and (3) the potential variations of GSH levels among murine immune cells (García-Mendoza et al., 2019). Two panels of fluorescent-conjugated antibodies and intracellular GSH stain were employed per blood sample. The first panel was used to determine cell subpopulations and the second panel to quantify GSH content per subpopulation. Briefly, for the first panel 50 μL of whole blood was transferred to a 1.5 mL Eppendorf tube, adding 1 μL TruStain FcX (anti-mouse CD16/32) antibody (Biolegend, U.S.A.) used for Fc blocker, mixed and left for incubation for 10 min on ice. After incubation a cocktail of primary fluorescent-labelled antibodies was added to each sample. The cocktail included 2 μL of each antibody: Alexa Fluor 488 anti-mouse CD45 (panleukocyte marker) (FL-1 detector), PE anti-mouse CD3 (T cells marker) (FL-2 detector), PerCP/Cy5.5 anti-mouse CD45 R/B220 (B cells marker) (FL-3 detector) and Alexa Fluor 647 anti-mouse CD11b (myelocytes marker) (FL-4 detector) (all four antibodies from Biolegend, U.S.A.). Tubes were briefly mixed and incubated for 30 min in the dark on ice. After incubation, 1 mL of RBC Lysis/Fixation working solution 1x (prepared according to manufacturer protocol) (Biolegend, U.S.A.) was added and the tubes were incubated 15 min in the dark at room temperature. Tubes were then centrifuged at 400×*g* for 5 min, supernatants were aspirated and discarded and the pelleted cells were washed with 1 mL of sterile cold DPBS calcium-magnesium-free buffer, supplemented with EDTA in a final concentration of 1 mM, pH 7.2 (here after just called DPBS-buffer). After the washing step, the cells were resuspended in 500 μL buffer solution and kept on ice until acquiring data by flow cytometry (Pockley et al., 2015).

For the second panel 50 μL blood was added to a 1.5 mL Eppendorf tube together with 1 mL of cold DPBS-buffer. Tubes were centrifuged at 400×*g* for 5 min and supernatants aspirated and discarded. Pelleted cells were resuspended in 500 μL DPBS-buffer containing 1 μM Cell Tracker Green CMFDA dye, briefly mixed and incubated at 37 °C in the dark for 30 min (Invitrogen, the Netherlands). Cell Tracker Green CMFDA fluorescent dye reacts with thiol groups of which GSH is the most abundant (Aoyama and Nakaki, 2015; Sebastià et al., 2003). After incubation the tubes were centrifuged at 400×*g* for 5 min, supernatants aspirated and the cell pellets were resuspended in 100 μL of DPBS-buffer. Later 1 μL TruStain FcX (anti-mouse CD16/32) antibody was added to each tube, briefly mixed and incubated for 10 min in the dark on ice. After the incubation, a cocktail of primary fluorescent-labelled antibodies was added to each sample. The antibody cocktail added to each sample included 2 μL of the same antibodies as in the first panel, except Alexa Fluor 488 anti-mouse CD45 which was replaced by Cell Tracker Green CMFDA dye, which is measured by the same detector (FL-1). After adding the antibody cocktails, the tubes were briefly mixed and incubated on ice for 30 min in the dark. Following the incubation, 1 mL of RBC Lysis/Fixation Solution 1x and from this part the protocol continues similarly to the first panel.

Flow cytometry data was acquired with BD Accuri C6 cytometer and analysed with BD Accuri C6 software (BD Biosciences, U.S.A.). In the first panel employed to obtain cell subpopulation proportions were included T cells ($CD45^+$ $CD3^+$ (double positive)); B cells ($CD45^+$ CD45 R/B220+ (pan-B cell marker (Rodig et al., 2005)); myeloid cells ($CD45^+$ CD11b+). Myeloid cells were gated in FSC/SSC into monocytes and neutrophils. For the second panel, the mean content of GSH was calculated for lymphocytes, combining T cells ($CD3^+$) and B cells (CD45R+), monocytes and neutrophils (CD11b+). For this panel the cells subpopulations were also gated according their distribution in FSC/SSC.

2.7. Statistics

Linear regressions were calculated using SPSS ((IBM SPSS Statistics, version 25). Differences between locations were assessed by ANOVA (SPSS). Significance level was set at $\alpha = 0.05$. Prior to all analyses, data were checked for normality. In case this assumption was not met, the data was log-transformed prior to statistical analyses.

3. Results

3.1. Biomass and kidney cadmium levels

The biomass of the wood mice was approximately 17.7 ± 0.64 and did not differ significantly between Sinderhoeve and Plateaux locations (18.03 ± 0.96 versus 17.5 ± 0.84; ANOVA: $F_{prob} = 0.359$, d.f. = 32, $p = 0.553$). The internal levels of cadmium (10-logarithmic transferred) were on average 0.71 ± 0.11 and were significantly higher in animals from Plateaux (1.20 ± 0.44 (Plateaux) versus 0.30 ± 0.46 (Sinderhoeve); ANOVA: $F_{prob} = 32.36$, d.f. = 32, $p < 0.0001$). Per location, individual cadmium concentrations in kidneys were significantly related to the mouse body mass (Fig. 1). The slopes were significantly positive

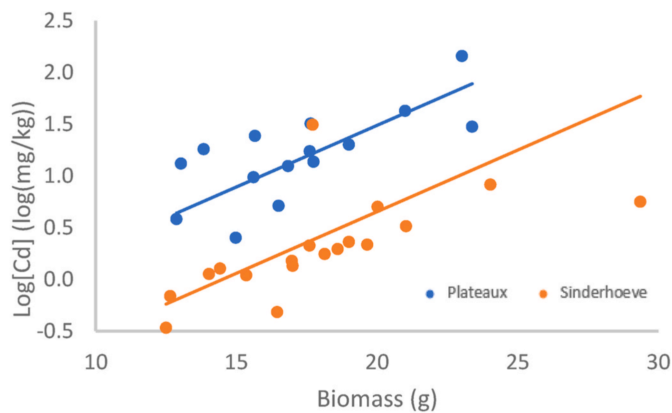

Fig. 1. Relationship between kidney concentrations (log (mg/kg)) (dry weight) and biomass (g) in wood mice from the two locations. For statistics of the regressions see text.

(0.08 ± 0.016; Regression: $t_{value} = 4.89$; d.f. = 32; $p < 0.001$)) and did not differ between locations. The intercepts of the linear regressions were significantly different (−0.188 versus −1.144 ± 0.120: $t_{value} = 7.98$, $p < 0.0001$; overall regression: d.f. = 32; F-value: 40.126, $F_{prob} < 0.0001$) illustrating the differences in exposures between locations.

3.2. *Haematological and flow cytometric observations*

There was not a clear significant relationship between cadmium exposure and WBC in either gender of mice captured from Plateaux or Sinderhoeve (overall regression: total d.f. = 32, F-value = 1.267, F_{prob} = 0,304). Similarly, haematocrit and haemolysis/haemagglutination did not show any significant relationship with renal cadmium concentrations (Haematocrit overall regression: total d.f. = 32, F-value = 1.85, $F_{prob} = 0.160$; haemagglutination overall regression: total d.f. = 29, F-value = 0.874, $F_{prob} = 0.467$).

The flow cytometry analysis allowed the immunophenotyping of different subpopulations cells in peripheral blood of each sample by immunostaining with fluorescent labelled antibodies. i. This included T cells, B cells and myeloid cells, in order to determine changes in cell type specific populations in blood. WBC-numbers appeared higher in animals from Sinderhoeve when compared to Plateaux ($p = 0.061$, Table 1, and Annex 1), while kidney cadmium concentrations were negatively related to the percentage of B cells in blood ($p = 0.076$, Table 1).

Pooling T cells and B cells into lymphocytes, and dividing myeloid cells into neutrophils and monocytes showed that GSH content was significantly lower in neutrophils when compared to lymphocytes and monocytes (ANOVA: $F_{prob} = 56.79$, total d.f. = 110, $p < 0.001$, posthoc: LSD). No effect of gender nor Cd-concentrations on GSH levels could be established. GSH-levels were significantly correlated between cell types (Table 2) indicating that individuals with higher levels of GSH in one cell type likely also contained more GSH in others.

Table 1
Statistical output of linear regression models (Dependent = intercept+a* locations + b*sex + c*kidney + residual). Listed are the F-statistic of overall regression and t-values of individual parameters.

Dependent	Location (*t*-value)	Sex (*t*-value)	Kidney Cd (*t*-value)	Total d.f.	Regression (F-statistic)
WBC (#/μl)	<u>1.946</u>	−0.016	1.307	32	0.304
T cells (%)	−0.275	0.068	−0.430	32	0.063
B cells (%)	−0.522	1.257	<u>−1.838</u>	32	1.882
Myeloid cells (%)	0.706	−0.144	0.855	32	0.253

Underlined t-values are significant at $0.05 < p < 0.10$.

Table 2
Pearson' correlation factors between GSH-levels in different cell types.

Lymphocytes	1		
Monocytes	0.615**	1	
Neutrophils	0.359*	0.774**	1
	Lymphocytes	Monocytes	Neutrophils

**: $p < 0.01$, * $0.01 < p < 0.05$.

3.3. *Spleen plaque forming assay (PFA)*

The PFA analysis was performed grouping the animals according the origin capture location (e.g. Sinderhoeve versus Plateaux). Site specific analyses were performed because immune state of the animals may differ between location, related to local immune-stressors and pathogens. The separate analyses show clear significant decreases in the number of plaque forming cells (PFC) with higher concentrations of cadmium, both in the Sinderhoeve group and Plateaux animals (Fig. 2, statistical output Table 3).

4. Discussion

This study was initiated to gain insight whether cadmium can modulate the immune system and the antibody-mediated immune responses in environmentally exposed wood mice (*A. sylvaticus*), captured at different locations with known differences in the level of cadmium pollution. The wood mouse was selected as species of interest because it is a widespread small mammalian species, allowing to collect them at different locations, but even more so because of its relevance in hosting infectious agents (González-Barrio et al., 2021; Maaz et al., 2016; Schmidt et al., 2014). Cadmium concentrations in wood mice kidneys were significantly different between capture locations, with the higher concentrations at Plateaux, similar to an earlier study (van den Brink et al., 2010). The internal cadmium concentrations were related to the body mass of the mice (Fig. 1), which is assumed to be related with mice age. Older mice generally weigh more and have been exposed for longer time, increasing time dependent cadmium accumulation (Blagojević et al., 2012). Although cadmium concentrations in kidneys were significantly different between capture locations, the effect of the time of exposure (as indicated by body mass) on the uptake of cadmium was similar among capture locations, as shown by the comparable slopes of the regressions for both locations (Fig. 1). This would indicate that the time dependent accumulation of Cd is similar at different locations.

Regarding immunomodulating effects of cadmium exposure, no apparent changes in white blood cell counts (WBC) with increasing cadmium concentrations were observed. Increasing WBC counts

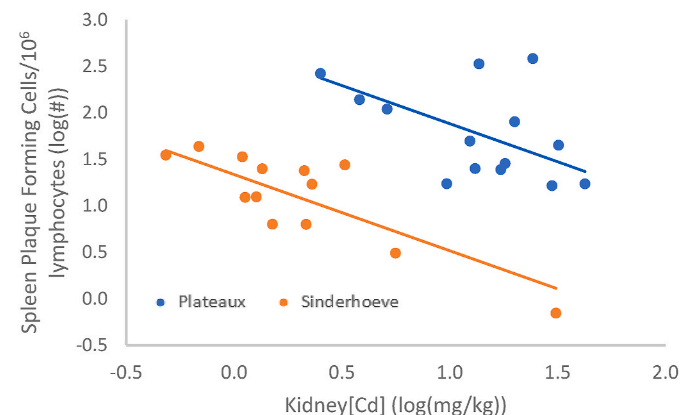

Fig. 2. Linear regressions of plaque forming assay (PFA) related to cadmium kidney concentrations (mg/kg d.w.), expressed as plaque forming cells (PFC) per million spleen lymphocytes, for animals captured in Sinderhoeve (n = 13) and both sites at Plateaux combined (n = 14).

Table 3
Statistical output of linear regression model log (AFC) = Constant + A*log [Cd]+B*Location + residual.

Coefficient	Estimate	Std. error	t-value	P-value
Constant	4.070	0.433	9.390	<0.0001
Log ([Cd])	−0.820	0.187	−4.383	<0.0001
Location	−1.367	0.215	−6.731	<0.0001

Total df. 26; F-value overall regression: 20.370 ($F_{prob} < 0.0001$).

however, have been observed in rats experimentally exposed to trace metals mixtures, including lead and cadmium, under lab conditions (Kim et al., 2009; Yuan et al., 2014). Humans, chronically exposed to cadmium also showed increased counts of WBC, mainly due to higher counts of monocytes (Parks et al., 2006). Such increase of WBC and monocytes has been associated with an increase of myelopoiesis, at the expenses of lymphopoietic precursors, resulting in an increase of granulocytes and monocytes and a decrease in B cells and T cells, also shown in mice chronically exposed to cadmium (Zhang et al., 2016). Lower numbers of B cells in blood have also been reported in rats as well as mice exposed to cadmium (Lafuente et al., 2004), however this could not be confirmed in the current study. It appears that in the current study, environmental exposure to cadmium did not affect WBC or the composition of the different sub-populations of cells. This may be due to fact that free ranging animals may be exposed to higher levels of different pathogens and antigens when compared to animals exposed in the lab or humans. Impacts of cadmium on the structural composition of the cellular populations of the immune system may therefore be somewhat limited.

In the analysis of cell subpopulations also a difference in cellular glutathione (GSH) content between cell types was observed, with neutrophils containing significantly lower GSH levels than monocytes and lymphocytes. In an earlier *in vitro* study, monocyte/macrophages contained the highest levels of GSH when compared to other cell types (García-Mendoza et al., 2019). Besides, increasing cadmium concentrations depleted the content of GSH of innate immune cells, in which GSH acts as a protective system for the bactericidal mechanisms of monocyte/macrophages based on reactive oxygen and nitrous species (Ferret et al., 2002; Zhu et al., 2008). Increasing kidney cadmium concentrations in wood mice in the current study however, were not correlated with a significant decrease of GSH content in cells of the innate immune system, which may indicate the ability of the animals to supplement the cellular GSH. Based on this, the chronic impact of Cd on the redox status of cells of the innate immune system may be limited.

Although the structural aspects of the immune system of the wood mice, e.g. WBC and composition of the populations of the different cell types, did not significantly differed between the sites, the analysis of specific primary humoral responses was performed site-specific analysis due to the marked differences in the response in the PFC-assay between wood mice from the different capture locations. Mice exposed to the lower cadmium concentrations had higher numbers of plaque forming cells per million splenocytes (PFC/10^6 splenocytes), decreasing with the increment of cadmium internal levels in kidneys of wood mice from both sites (Fig. 2). The absolute levels of PFC differed significantly between sites, but the effect of cadmium exposure was similar for each site. In previous studies, considerably inter- and intra-species variation in different wild mice species has been observed in the primary humoral response to SRBC in the plaque forming assay (Lochmiller et al., 1991). Furthermore, differences in humoral immunity have also been observed between neighbouring populations wild populations of house mice (*Mus musculus domesticus*) (Abolins et al., 2018). Such variation in the immune responses between wildlife populations has been attributed to the antigenic environments where the animals live in and to the availability of resources for the animals (Viney and Riley, 2017), likely explaining the absolute differences between the Sinderhoeve and Plateaux.

The mechanisms behind the reduction of PFC counts could be attributed to reported cadmium-induced inhibition of the DNA synthesis in B cells (Banfalvi et al., 2007; Daum et al., 1993), supressing proliferation and reducing overall B cell numbers and antigen-specific antibody-producing B cells. This result is in line with the observed negative association between cadmium levels in mice kidneys and the percentage of B cells in blood (Table 1). On the other hand, the counts of total white blood cells (WBC/μL) appeared to be higher in animals from the Sinderhoeve location (Table 1), supporting the concept of immune heterogeneity between different mice populations, described before. Although the location shows an association with numbers of WBCs in blood, it did not affect their apparent functioning as shown in the PFA (Fig. 2). A study on wild white-footed mice (*Peromyscus leucopus*) environmentally exposed to trace metals in contaminated areas, found no effects on the primary humoral response, measured with the spleen plaque forming assay (Biser et al., 2004). However, the levels of cadmium detected in that study were comparable to the lowest levels found in wood mice captured in Sinderhoeve and in wood mice captured from other non-contaminated referential areas (Rogival et al., 2007). As mentioned earlier, immune responses can vary between individuals and populations, driven by various factors. Body condition and age are some of the most important factors, in which better body condition and increasing age are associated with improved immune status (Abolins et al., 2018). Other factors are resources availability, antigenic environment, seasonality and as shown in the current study, immunotoxic pollutants. In this case, the primary humoral responses of wood mice were shown to be affected by the environmental exposure to cadmium. The positive effect that better body condition (higher weight) and increasing age could have on the overall humoral responses was shown to be impaired by increasing cadmium concentrations. Impaired or less effective humoral responses may be associated with increased susceptibility or lowered resistance to extracellular bacteria and parasitic infections that normally are controlled by specific antibody responses. For instance, a lower host resistance to the parasite *Heligmosomoides polygyrus* was reported in wood mice environmentally exposed to trace metals, attributed to a decreased humoral immunity (Tersago et al., 2004). The immunity to some important zoonotic infections in natural rodent reservoir hosts have also been reported to be mediated by humoral/antibody responses (Andrianaivoarimanana et al., 2012; Brunet et al., 1995; Clerc et al., 2018; Mandl et al., 2015; Richer et al., 2014). Therefore, the decrease of the humoral response of environmentally exposed wood mice to trace metals could lead to a decreased host resistance to certain types of infections, facilitating the transmission and increasing the prevalence of infections in polluted areas.

In conclusion, the environmental exposure to the trace metal cadmium at environmentally relevant concentrations appeared to impact the proportions of the sub-populations of B cells of wood mice. In addition, the primary antibody-mediated response induced by SRBC showed location specific decreases upon cadmium exposure, indicating impacted functioning of B cells, likely related to a decrease in the antibody response through IgM/IgG immunoglobulins. Site-specific effects should be considered in future studies, due to the antigenic variability found in the field, making different wild populations also variable in how their immune systems respond. Affected and decreased humoral responses in wild small mammal species, due to environmental exposure to immunotoxic compounds, can decrease the host resistance to infections, potentially favouring the transmission between animals of the same species and to other species including the human (Bean et al., 2013; Feingold et al., 2010; Mandl et al., 2015). Particular scientific attention should be pointed to study the immunity of wild small mammal reservoir hosts populations in areas with increased prevalence of relevant infections combined with high levels of cadmium or other immunotoxic pollutants.

Author contribution

DGM: conceptualization, methodology, investigation, formal

analysis, writing; HvdB: investigation, resources; NvdB: conceptualization, methodology, writing, supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A

Location		N	LogCd (mg.kg)		Ht (%)		Heamagglut (titer)		WBC/ul		T-cell (%)		B-cell (%)		Monocytes (%)		Neutrophils (%)		Lymph_GSH (abs)		Mono_GSH (abs)		Neutro_GSH (abs)	
	Gender		Mean	Stderror	Mean	Stderror	Mean	Stderror	Mean	Stderror	Mean	Stderror	Mean	Stderror	Mean	Stderror	Mean	Stderror	Mean	Stderror	Mean	Stderror	Mean	Stderror
Plateaux	F	9	1.12	0.18	46.89	1.55	6.00	0.31	5436	625	45.2	5.3	23.1	2.1	3.2	1.1	5.4	1.5	5.69	0.03	5.66	0.09	5.07	0.10
	M	6	1.32	0.09	45.08	0.89	6.42	0.45	4838	980	29.0	5.2	21.7	3.5	5.9	4.1	12.7	5.3	5.62	0.07	5.55	0.12	5.01	0.15
Sinderhoeve	F	5	0.53	0.25	42.20	1.729162	6.40	0.48	5555	656	39.7	3.3	27.3	2.4	5.4	1.6	9.2	2.1	5.66	0.04	5.66	0.09	5.24	0.08
	M	8	0.14	0.12	42.88	1.063812	7.00	0.57	6456	679	48.8	4.5	24.1	4.5	5.3	1.7	4.4	0.6	5.60	0.06	5.62	0.09	5.14	0.08

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