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# A Short-Term Extremely Low Frequency Electromagnetic Field Exposure Increases Circulating Leukocyte Numbers and Affects HPA-Axis Signaling in Mice

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There is still uncertainty whether extremely low frequency electromagnetic fields (ELF-EMF) can induce health effects like immunomodulation. Despite evidence obtained in vitro, an unambiguous association has not yet been established in vivo. Here, mice were exposed to ELF-EMF for 1, 4, and 24 h/day in a short-term (1 week) and long-term (15 weeks) set-up to investigate whole body effects on the level of stress regulation and immune response. ELF-EMF signal contained multiple frequencies (20–5000 Hz) and a magnetic flux density of 10 μT. After exposure, blood was analyzed for leukocyte numbers (short-term and long-term) and adrenocorticotropic hormone concentration (short-term only). Furthermore, in the short-term experiment, stress-related parameters, corticotropin-releasing hormone, proopiomelanocortin (POMC) and CYP11A1 gene-expression, respectively, were determined in the hypothalamic paraventricular nucleus, pituitary, and adrenal glands. In the short-term but not long-term experiment, leukocyte counts were significantly higher in the 24 h-exposed group compared with controls, mainly represented by increased neutrophils and CD4 ± lymphocytes. POMC expression and plasma adrenocorticotropic hormone were significantly lower compared with unexposed control mice. In conclusion, short-term ELF-EMF exposure may affect hypothalamic-pituitary-adrenal axis activation in mice. Changes in stress hormone release may explain changes in circulating leukocyte numbers and composition. Bioelectromagnetics. 37:433–443, 2016. © 2016 The Authors. *Bioelectromagnetics* Published by Wiley Periodicals, Inc.

**Keywords: ELF-EMF; in vivo exposure; leukocytes; immune response; stress**

Conflicts of interest: Dr. Cuppen owns stock in, and is employed by, Immunent and Neiding BV. Neiding would have an interest in demonstrating effects of EMF on the immune system. However, Dr. Cuppen has only been involved in the preparation and instrumentation of the experiments, not in the execution, analysis, or discussion of the results.



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## INTRODUCTION

To date, extremely low frequency electromagnetic fields (ELF-EMF), produced by high voltage power lines and electronic devices, are a subject of discussion on the possible consequences they might have on human health. Exposure to EMF is low but inevitable. Though some epidemiological studies suggest an association between ELF-EMF exposure and childhood leukemia [Ahlbom et al., 2000; Greenland et al., 2000; Ahlbom et al., 2008; Hardell and Sage, 2008; Li et al., 2009], conclusive biological evidence is lacking [Vijayalaxmi and Scarfi, 2014]. In fact, no mechanism of interaction has been found.

Within the western population, about 1.5–5% of individuals are self-proclaimed electrohypersensitive (EHS) or, as defined by the World Health Organization, suffering from idiopathic environmental intolerance with attribution to EMF [Hillert et al., 2002; Levallois et al., 2002; Schreier et al., 2006]. They experience complaints that are attributed to the presence of various EMFs in their direct surroundings. These complaints include, but are not limited to, headache, fatigue, stress, sleep disturbances, and skin symptoms like burning sensations and rash. Conversely, complaints of EHS patients are not specific and a direct relationship with exposure to EMF has not been demonstrated. Various provocation studies were not able to show a direct association between EMF exposure and symptoms or complaints [Genuis and Lipp, 2012].

Previous studies suggest that functioning of the immune system is modulated by ELF-EMF exposure (200–5000 Hz, 5 μT) characterized by reduced mortality in fish and improved feed conversion rate in chickens with coccidiosis infection; however, the mechanism behind these observations remains unclear [Cuppen et al., 2007; Elmusharaf et al., 2007].

### Potential Effects of ELF-EMF on Immune Cells Have Already Been Investigated In Vitro

Neutrophil extracellular trap formation has been shown to be enhanced by ELF-EMF exposure [Vijayalaxmi and Scarfi, 2014; Golbach et al., 2015]. Furthermore, there are indications that production of reactive oxygen species and inflammatory cytokines under exposure to 50 Hz ELF-EMF is increased [Petrini et al., 1997; Frahm et al., 2009; Patruno et al., 2009]. However, these results are in contrast with studies that could not find any effects [Ikeda et al., 2003; de Kleijn et al., 2011; Bouwens et al., 2012]. It is therefore still being called into question whether ELF-EMF exposure can induce changes at cellular level and more importantly, that these changes have direct consequences for human health. Interestingly, ELF-EMF at flux densities 100–1000 μT have been suggested to affect brain functioning in anterior cingulate and ipsilateral insula, and it has been demonstrated that exposure can alter neuroprocessing in humans [Robertson et al., 2010a,b]. Coping with physical, chemical, or biological disturbances involves an extensive repertoire of physiological, endocrinological, and immunological responses. Bi-directional neuroendocrine-immune interaction is vital to regulatory networks, ensuring homeostasis both during stressful and non-stressful conditions. Under normal homeostatic conditions, there is a balance in the functioning of immune response between the induction of normal pro-inflammatory processes and compensatory anti-inflammatory processes. Under stress conditions, activation of the hypothalamic-pituitary-adrenal (HPA) axis leads to production of increased amounts of stress hormones, mainly cortisol. As a result, increased levels of glucocorticoids in circulation can influence the amount and composition of circulating leukocytes and inhibit pro-inflammatory responses [Griffiths et al., 1997; Webster et al., 2002]. The HPA axis is an important route to transduce neural input to specific signals that can influence peripheral systems like immune response. In the HPA axis, corticotropin-releasing hormone (CRH) is released from the paraventricular nucleus of the hypothalamus, resulting in the induction and secretion of adrenocorticotropic hormone (ACTH) from the anterior pituitary gland. ACTH on its turn stimulates production and release of glucocorticoids from the adrenal cortex [Kageyama and Suda, 2009].

Induction of the HPA axis results in increased glucocorticoid hormone levels, and predominantly leads to immunosuppression [Viveros-Paredes et al., 2006]. In rodents, corticosterone is the most important steroid stress hormone. For example, in mice, corticosterone has been demonstrated to decrease the number of circulating leukocytes [Van Dijk et al., 1979], and in rats, forced swim test induced stress, causing changes in the proportion of circulating leukocytes, with the most dominant increase in neutrophils [Connor et al., 1998] and monocytes [Van Dijk et al., 1979]. There is indication that 30 days of ELF-EMF and static EMF exposure in mice decrease numbers of total circulating leukocytes but increase the amount of neutrophils [Hashish et al., 2007]. Moreover, there is evidence of increased plasma levels of corticosterone after long term ELF-EMF exposure [de Bruyn and de Jager, 1994; Mostafa et al., 2002]. ELF-EMF exposure, via interaction with the HPA axis, may indirectly influence immune functioning.

We hereby hypothesize that a low intensity ELF-EMF exposure may affect stress regulation and, as a result, will affect number and activation of leukocytes. Similar effects may also play a role in the development of complaints related to EMF exposure in humans, especially in EHS patients.

In the current study, we present an experimental exposure model in mice in which we specifically investigate HPA axis parameters and leukocyte counts in vivo after both short- and long-term exposure with ELF-EMF signals that contain steep ramps and sharp peaks. These simulate spikes that can occur in fields from so-called "dirty" power nets in the human environment.

## METHODS

### EMF Exposure System

The system (system included generator and coils from Immunent, Veldhoven, the Netherlands), was targeted to provide sufficient homogeneity in the exposure area and low fringe fields at locations of controls and different experimental groups.

Mice were placed in plastic cages where they could move in a rectangular area ($150 \times 250$ mm). These cages were placed within PVC cylinders (diameter 320 mm outside and 300 mm inside) on the central plane and on planes 200 mm above and below.

Around the cylinder a solenoid coil was wound, 30 windings on 20 mm pitch, 0.5 mm diameter copper wire. This coil was driven with 0.2 A rms to achieve rms flux density 10 μT in the center.

We set the homogeneity requirement to have $10 \pm 2$ μT. To support ventilation, 200 holes of 15 mm diameter were drilled in each tube between windings.

Since frequencies used were low and coils and cages contained no magnetic materials, homogeneity could be very well-determined by Biot Savart calculations (Biot Savart software from www.ripplon.com, New Westminster, Canada). In Figure 1, it is shown that homogeneity requirement was met.

In our system, the current required to generate 10 μT was very low (0.2 A). This generated 0.1 W heat in the coil, which was separated from the cages by a 10 mm-thick PVC cylinder. Consequently, heat and vibrations were deemed negligible. At a distance of at least 4 m from active coils, flux density was below detection limit.

Experimental setup contained 12 coils connected to a signal generator and four control coils with the same characteristics but not connected to the signal generator. The system could deliver various exposure regimes inside the coils (automated periods of exposure).

The signal $V(t)$ applied to the coil was described previously [Bouwens et al., 2012] and is the sum of 4 simultaneously generated square wave signals with equal amplitudes and periodicities $f_i = 401$, 432, 700, and 750 Hz, respectively. As mentioned in the introduction, steep ramps and sharp corner peaks in square wave signals could be considered to mimic effects of "dirty electricity" power nets. Values $f_i$ were selected because pilot experiments had been performed with 700 and 750 Hz in the Netherlands, and 401 and 432 Hz are their counterparts, scaled with relative strength difference of the static earth magnetic field between Bloemfontein and the Netherlands, because we suspected some form of (cyclotron) resonance phenomenon to be in effect. Plateau reproduction of square waves was accurate up to 1%, and rise time was 10 μ s.

At the low frequencies we used, magnetic flux density signal $B(x,t)$ could be assumed to be the product of the coil current $I(t)$ and a location-specific constant $k(x)$, including coil homogeneity. Relation between coil current $I(t)$ and voltage signal $V(t)$ was determined by the differential equation

$$d\,I(t)/dt = V(t) * R/L.$$

This is a linear differential equation so that each square wave component in $V(t)$ contributes an independent additive current component $b_i(t)$ to the coil current $I(t)$. Functions $b_i(t)$ are given by the following formulas, where the period $T_i = 1/f_i$ and $a_i$ are a constant which is dependent on $T_i$ and proportional to the amplitude of $U_i(t)$:

$$b_i(t) = a_i^*(2\exp(-\omega_o t) - (1 + \exp(-\omega_o T_i/2)))$$

$$\text{for } 0 \le t \le T_i/2, \text{with } \omega_o = R/L,$$

$$b_i(t) = -b_i(t - T_i/2)$$

$$\text{for } T_i/2 \le t \le T_i.$$

In Figure 1B, a graphical representation is given for a single component $b_i(t)$.

Coils used in this work have $R = 2.8\,\Omega$ and $L = 0.119$ mH, so $R/L = 8403$.

In order to obtain the same signal waveform $B(t)$ as in earlier experiments where $R/L = 1900$, a straightforward analogue circuit was used between digital signal generation and final amplifiers. This circuit feeds the input signal into an RC circuit with the same characteristic time $\omega_o$ and takes a linear combination of the result and the original signal to obtain a signal

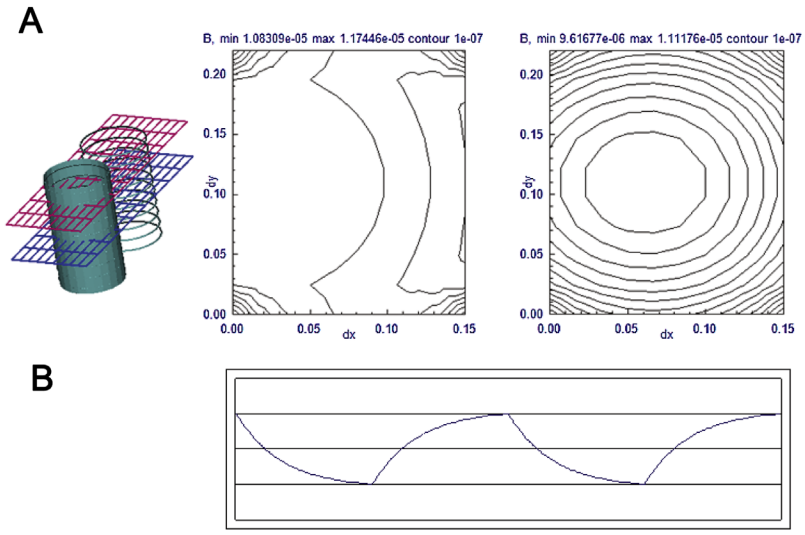


Fig. 1. Field characteristics. **A**: In the drawing, one coil is shown as a solid, and its neighbor as a set of windings. Coils were placed next to each other with opposite current direction to reduce distant stray fields. A central plane ($z = 0$) and a higher plane ($z = 0.2$ m) are indicated, $x$ direction is from center of one coil to center of the next coil, along the long side of plane indicators; the $y$ direction is the short side. Cages are placed central in the coil and 0.2 m above and below. Short side of the cage (0.15 m) is along the $x$ direction, long side (0.25 m) along the $y$ direction. Homogeneity in the cage on central plane (left graph) and in cages placed above and below (right graph) is given as contours are spaced 0.5 μT (5% of central value). $dx$ and $dy$ measure from the left front corner of each cage. The four contours visible indicate $20\% = \pm 10\%$ inhomogeneity. **B:** Graphical representation of two periods of one component of signal. Signal is summation of four such components with periodicity: 401, 432, 700, and 750 Hz, respectively. Horizontal axis for each component, therefore, has a total width of 4.99, 4.63, 2.86, and 2.67 ms, respectively.

that produces the right $B(t)$ in the actual coils. For full details about the signal, see patent number EP2020250 [Cuppen, 2009]. Amplitude of $V(t)$ was set to achieve an rms value for $B(t)$ of 10 μT.

## Animals

The study was approved by the animal ethics committee of the University of the Free State, Bloemfontein, South Africa. BalB/c mice, males 6 weeks old, were obtained from own breeding facility and housed in small plastic cages of 150 × 250 mm with three or four animals.

Mean mass of the mice per cage was kept within the range of $23 \pm 1.5$ g. Food and water were provided ad libitum. During exposure with different exposure regimes, cages were circulated every 3 days to ensure equal light for all animals, as light within the exposure system was inhomogeneous. Day/night rhythm was 12–12 h. In the animal rooms, music was played via a radio continuously to familiarize animals with sounds. Coils for the exposure groups were all connected to the same signal source. This system ran at 24 h continuous exposure. Based on time switches, 24 h/day group was continuously exposed, 4 h/day group for 4 h during the morning, and 1 h/day group for 1 h during the morning. Experimental groups were exposed in a blind setting. Standard animal behavior and health were monitored on a daily basis in the short-term experiment and twice a week in the long-term experiment. At the endpoint of all experiments, mice were sacrificed in the morning (between 9 and 11 A.M.). Animal handling before anesthesia was kept to a minimum to prevent induction of stress.

## Blood Cell Counts and Immune Phenotyping

At experiment endpoint, blood was collected in Ethylenediaminetetraacetic acid anticoagulation tubes by either orbital bleeding or decapitation, both under inhaled halothane (3–5%) anesthesia. Between 0.5 and 1 ml blood was recovered from mice. Whole blood was used for blood cell counts on an automated leukocyte analyzer (ABX Pentra 60, Horiba, Kyoto, Japan) and subsequent immunephenotyping on Facscalibur (Becton Dickonson, Franklin Lakes, NJ) by measurement of CD3, CD4, CD8, and CD19 (Becton Dickonson). A complete list of antibodies used for flow cytometry is shown in Table 1. Remaining blood was centrifuged and plasma was stored at −80°C for ACTH quantification.

**TABLE 1. List of Antibodies Used for Flow Cytometry Analysis**

| Antibody | Supplier | Cat no. |
|---|---|---|
| CD3 FITC/IgG2a | Becton Dickonson | 731992 |
| CD4 PE/IgG2b | Becton Dickonson | 733259 |
| CD8 PE/IgG2a | Becton Dickonson | 733264 |
| CD19 FITC/IgG2a | Becton Dickonson | 732058 |
| Mouse IgG2a FITC control | Becton Dickonson | A12690 |
| Mouse IgG2a PE control | Becton Dickonson | A09141 |
| Mouse IgG2b PE (clone A-1) control | Becton Dickonson | 731601 |

### Material Extraction and Spleen Cell Stimulation

After sacrificing mice by either cervical dislocation (after orbital bleeding) or decapitation, brain was removed from the mouse skull and snap-frozen in liquid nitrogen. Pituitary was dissected from the base of the skull and also snap-frozen. Furthermore, adrenal glands were removed and snap frozen in liquid nitrogen. Upon Ribonucleic acid (RNA) isolation, brains were sliced in 300 μm thick slices with a cryomicrotome (Microm, Walldorf, Germany). The paraventricular nucleus was carefully punched out and kept frozen. Spleens were extracted and kept on ice. Spleens were homogenized using a cell strainer (70 μm) (Corning, New York, NY) and plunger (Becton Dickonson). Cells were washed in phosphate buffered saline and suspended in Roswell Park Memorial Institute (RPMI) medium (Gibco, Waltham, MA). Subsequently, spleen cells were stimulated with Lipopolysaccharide (LPS) (1 μg/ml) (Sigma–Aldrich, St Louis, MO), PAM3cys (100 ng/ml) (EMC, Tubingen, Germany), or Zymosan (1 μg/ml) (Sigma–Aldrich) and incubated at 37°C with 5% $CO_2$ for 6 h. After stimulation, spleen cells were washed and suspended in RNA later (Sigma–Aldrich). All samples were kept coded and blinded until after analysis.

### RNA Isolation and q-PCR

For stress axis analysis: adrenal gland, pituitary, and paraventricular nuclear tissue were homogenized by frozen mortaring and suspended in RLT RNA cell lysis buffer (Qiagen, Venlo, the Netherlands) containing 1% beta mercaptoethanol (Sigma–Aldrich). After centrifugation, the supernatant was taken and processed following the manufacturer's protocol of Qiagen RNeasy mini kit (Qiagen). In addition, RNA was treated with on column DNase 1 treatment (Qiagen). The RNA was then reverse transcribed by superscript III reverse transcriptase (Life Technologies, Waltham, MA). The cDNA was used in taqman gene expression assays (Life Technologies) for CRH (NM_205769.2, Mm01293920_s1), proopiomelanocortin (POMC) (NM_001278581.1, Mm00435874_m1), and Cyp11a1 (NM_019779.3, Mm00490735_m1). PPIA (NM_008907.1, Mm02342430_g1) was used as reference gene.

For stimulated spleen cells: cells in RNAlater were centrifuged and the pellet was suspended in RLT buffer containing 1% beta mercaptoethanol (Sigma–Aldrich). RNA was isolated following manufacturer's protocol with Qiagen RNeasy mini spin columns. The RNA was then reverse transcribed by superscript III reverse transcriptase (Invitrogen, Waltham, MA). The cDNA was used in taqman gene expression assays (Life Technologies) for TNFA (NM_001278601.1, Mm01300094_m1), interleukin 10 (IL10) (NM_010548.2, Mm01288386_m1), IFNG (NM_008337.3, Mm01168134_m1), and ACTB (NM_007393.3, Mm04394036_g1) as reference gene.

### ACTH ELISA

ACTH concentrations in mouse plasma were measured with the mouse/rat EIA kit (Phoenix Pharmaceuticals, Burlingame, CA). Assay was performed according to manufacturer's instructions. Sensitivity of the assay was 0.08 ng/ml and range 0–25 ng/ml.

### Data Analysis

Quantitative Polymerase Chain Reaction (qPCR) data were analyzed in two different ways. For stimulated spleen cells, gene expression ratios were calculated using the Pfaffl method [Pfaffl, 2001]. For genes in the HPA axis, the gene expression was determined relative to the housekeeping gene.

### Statistics

Differences were statistically tested by either a 1-way analysis of variance (ANOVA, Becton Dickinson, Franklin Lakes, NJ) for cell count measurements (one variable, normally distributed) and HPA-axis parameters or 2-way ANOVA for spleen restimulation (two variables, normally distributed) with Tukey (1-way ANOVA) or Bonferroni (2-way ANOVA) post hoc test. $P < 0.05$ was considered statistically significant.

## RESULTS

General mouse behavior during the 1-week short-term exposure experiment was normal without any signs of illness or discomfort during the entire experiment. There were no signs of fighting or other stress behavior observed in all test cages. This was similar during the 15-week exposure experiment,

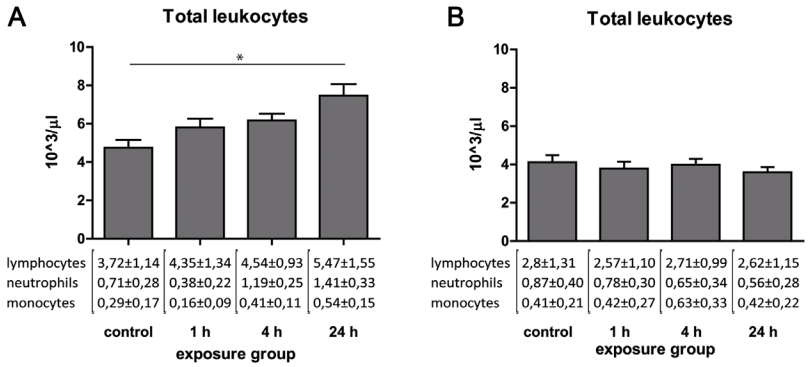


Fig. 2. Leukocyte counts in whole blood. **A**: Absolute number of total leukocytes after 1-week short-term exposure. Number of major subsets is shown at the bottom of graph. $N = 12$ animals per group. **B:** Absolute number of total leukocytes after 15-week long-term exposure. Number of major subsets is shown at the bottom of graph. $N = 20$ animals per group. Mice were exposed for 1, 4, or 24 h a day or not exposed as control. Values are depicted as amount of cells $\times 10^3/\mu$l with SEM. $^*P < 0.05$.

although from 12 weeks onward, mice seemed to be a bit more violent (signs of fighting).

First, we determined the effect of 1-week short-term ELF-EMF exposure and 15-week long-term ELF-EMF exposure (in regimes of 1, 4, or 24 h/day) on numbers and composition of circulating leukocytes (Fig. 2A). After the 1-week exposure, total amount of circulating leukocytes increased in a dose dependent way (up to 50%), being statistically significant in the 24 h/day exposure group. Increase in circulating leukocytes was represented by lymphocytes, monocytes, and neutrophils. Proportional increase was the largest for neutrophils (Fig. 2A). After 15-week exposure, total amount of leukocytes was overall lower compared with 1-week exposure, with highest numbers in the control group and no significant differences in composition of leukocyte subsets (Fig. 2B).

Lymphocyte phenotypes were further characterized based on their specific surface marker expression. The 1 and 24 h/day exposure groups showed a significant increase in CD3+/CD4+ T-lymphocytes compared with control mice (Fig. 3B). No significant differences in lymphocyte subsets (T-lymphocytes, B-lymphocytes) were found in the blood of long-term exposed mice (Fig. 3C).

Next, we determined whether this increase in leukocytes after 1-week short-term exposure was associated with changes in stress hormone levels of ACTH and corticosterone. Plasma concentration of ACTH was overall lower in exposure groups compared with control (Fig. 4D). There was a significant difference between 4 h/day-exposed and control mice. Although mice were handled carefully and killed and bled within 1 min of capture, it was not possible to collect corticosterone samples without a risk of interference due to final handling of the animals. We therefore investigated stress hormone regulation at a more upstream level in the HPA axis by measuring gene expression of three important regulator proteins in the HPA axis. CRH gene expression was determined in the paraventricular nuclear region of the hypothalamus. POMC, the precursor protein for ACTH, was measured in the mouse pituitary and Cyp11a1, which encodes an enzyme that cleaves cholesterol and forms the rate-limiting step in corticosterone synthesis, was measured in the adrenal gland.

Gene expression of POMC was lower in a dose-dependent way, reaching statistical significance for 24 h/day exposure. No significant changes were observed for CRH and Cyp11a1 expression (Fig. 4A–C).

To study whether ELF-EMF exposure of 1 week can directly influence immune activation of immune cells, isolated mouse splenocytes (containing T-lymphocytes, B-lymphocytes, monocytes, and neutrophils) were stimulated with pattern recognition receptor ligands LPS (Toll-like receptor 4), Pam3Cys (Toll-like receptor 2), or Zymosan (Toll-like receptor 2, Dectin-1). These ligands are well-recognized immune activators targeting major immune activation pathways. At 6 h, gene expression of inflammatory cytokines tumor necrosis factor alpha (TNF), IL10, and Interferon-gamma (IFNG), was determined relative to unstimulated cells (Fig. 5). Upon stimulation with LPS, Pam3Cys, or Zymosan, expression of TNF was not induced. IL10 expression increased with the highest induction by Zymosan. IFNG expression was also increased after stimulation with all ligands. There was no significant difference in gene expression in splenocytes of exposed and non-exposed mice.

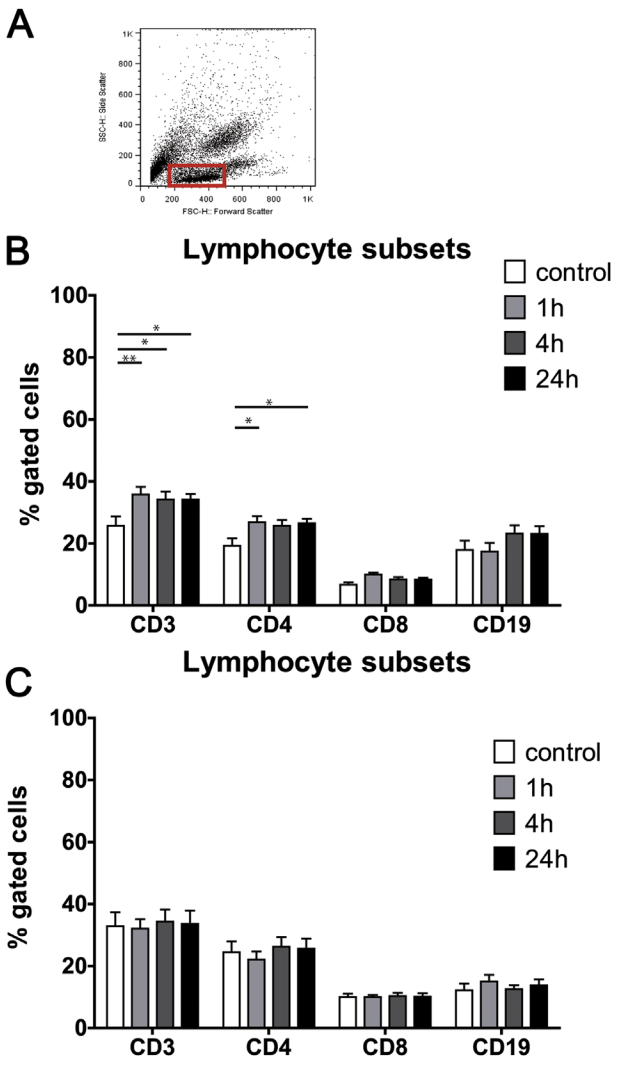


Fig. 3. Lymphocyte subsets in whole blood. Blood samples were analyzed by flow cytometry. **A**: Based on forward sideward scatter, lymphocyte fraction was selected as part of total population of viable cells. **B**: Lymphocyte subsets after 1-week exposure as percentage of total lymphocyte population. $N = 20$ animals per group. CD3 T-cell marker, CD4T helper cell marker, CD8 cytotoxic T-cell marker, and CD19 B-cell marker. **C**: Lymphocyte subsets after 15 weeks exposure as percentage of total lymphocyte population. $N = 20$ animals per group. Error bars represent SEM, $^{*}P < 0.05$, $^{**}P < 0.01$.

## DISCUSSION

For our experimental exposure conditions, we demonstrated that 1-week ELF-EMF exposure of 24 h/day increased circulating leukocyte numbers. Furthermore, mice showed signs of altered stress response as determined by measuring HPA axis parameters. No differences were observed in a 15-week exposure experiment and in ex vivo stimulated immune responses in spleen cells.

We showed that after 1-week 24 h/day exposure, amount of total circulating leukocytes was significantly increased. This was in line with results from an independent follow-up study under identical exposure conditions (Supplemental Fig. S1). Interestingly, our data showed a clear proportional increase in circulating granulocytes. Within the selected mononuclear cell population, we showed a relative increase in circulating CD4+ T cells after 1-week exposure. Percentage of lymphocytes (CD3 or CD19 positive) was relatively low [Kishimoto et al., 1987]. This suggests that a relative large number of monocytes and NK cells were present in this gated mononuclear cell fraction. The low level of lymphocytes could be explained by increased levels of corticosterone that can cause apoptosis in these cells [Sato et al., 2010].

In humans, increased leukocyte numbers, in particular neutrophils, are a sign of infection and increased immune system activation, but these may also be induced by altered stress responses [Zieziulewicz et al., 2012]. Mice that were used in this study were considered clean and specific pathogen free, and exposure of mice to microbes was limited and equal in all groups. Although an altered immune response induced by ELF-EMF interacting with microbiome at the mucosa could not be ruled out, the consistent change in leukocyte composition was most likely caused by other factors than infection, because increased neutrophils in general reflect invasive disease of which no signs were present.

Adaptation to potentially stressful circumstances involves activation of neural, neuroendocrine, and neuroendocrine-immune mechanisms. An adaptive system of allostasis (stability through change) is pivotal to keep a homeostatic balance. In healthy individuals, the body is able to cope with moderate challenges, but if the allostatic system is overactivated or maladaptive, the "allostatic load" may induce a vulnerability to disease [McEwen, 2000].

Acute stress is characterized by a fast adrenalin release resulting in strong leukocyte mobilization that is composed of lymphocytes, monocytes, NK-cells, and granulocytes. With a small delay, this response is directly followed by an increase in circulating glucocorticoid hormone levels, resulting in clear changes in the composition of leukocyte subtypes. Glucocorticoids decrease mononuclear leukocyte types (in circulation represented by monocytes, lymphocytes, NK cells) and further increase the amount of neutrophils [Dhabhar and McEwen, 1997; Dhabhar et al., 2012]. In mice, chronic activation of the HPA axis in a genetic stress model and in a restraint stress model leads to decreased leukocyte numbers but relatively more neutrophils appear [Murray et al., 2001; Bowers

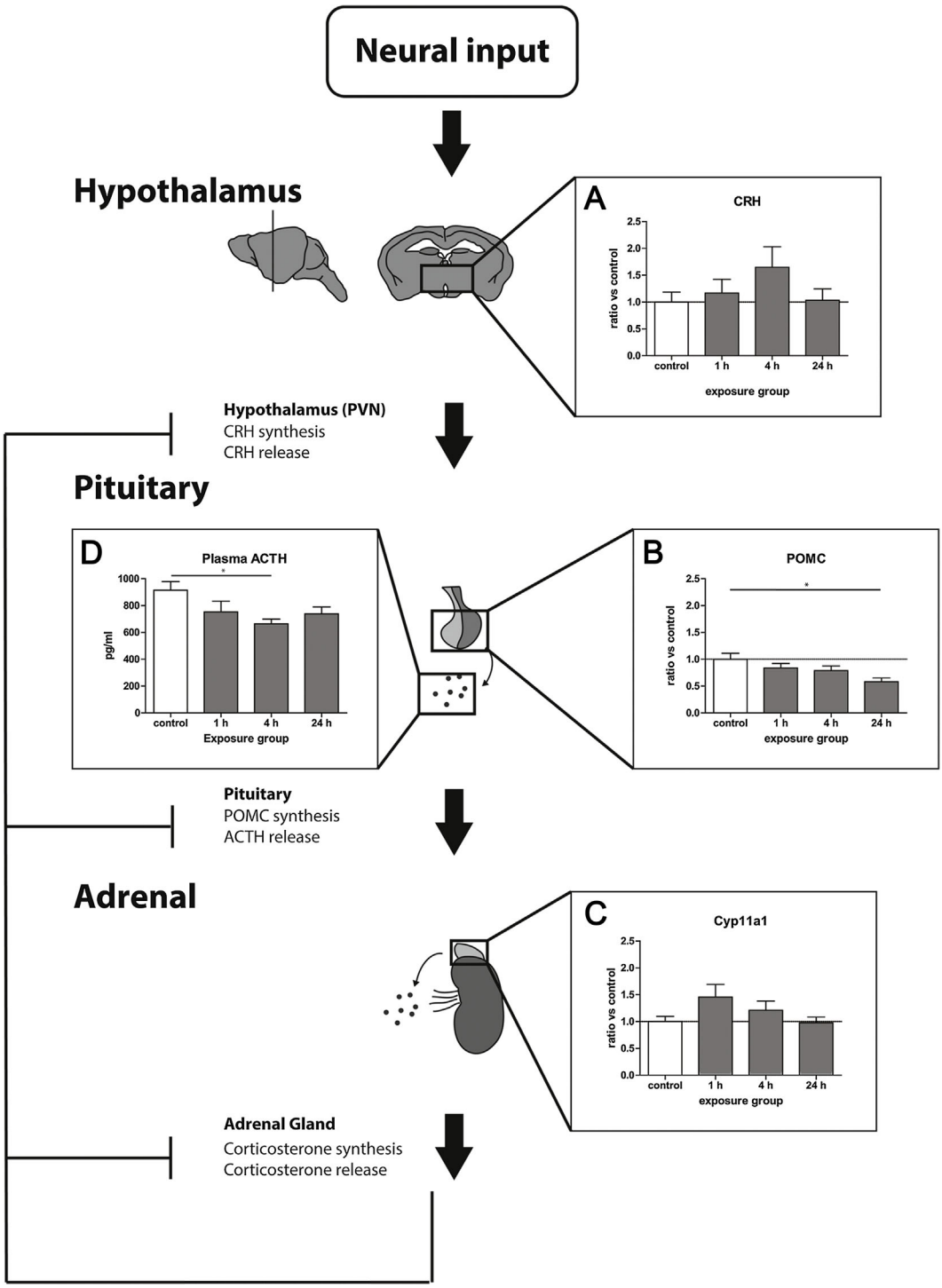


Fig. 4. HPA axis parameters in 1-week exposure model. Neural input is transduced to stress hormone release via PVN in hypothalamus, pituitary gland, and adrenal gland. **A**: Gene expression of CRH measured in 1 mm punches of hypothalamic region. **B**: Gene expression of POMC in pituitary. **C**: Gene expression of Cyp11a1 in adrenal gland. All expression values are depicted relative to mean control. Data are a combination of two independent experiments with a total of $N = 20$ animals per group. Gene expression of genes was calculated by subtracting gene of interest Ct values from reference gene Ct values. Gene expression ratios are relative to mean of control values. **D**: Plasma ACTH levels in pg/ml measured by ELISA, $N = 18$ animals per group. Error bars represent SEM $^{*}P < 0.05$.

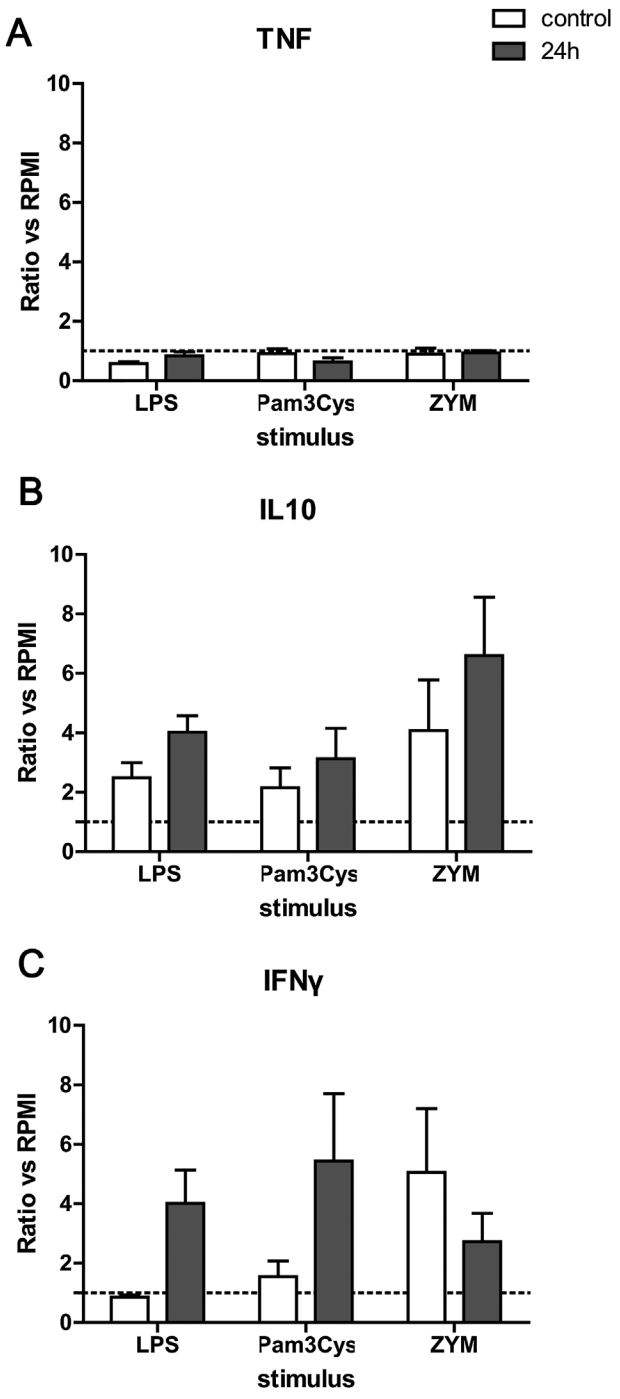


Fig. 5. Stimulation of spleen cells after 1-week exposure. Fold change in gene expression of cytokines in total spleen cells measured by q-PCR. Open bars are control mice and gray bars are 24 h/day exposed mice. Cells were stimulated with RPMI, LPS, Pam3Cys, and Zymosan for 6 h and gene expression is depicted relative to RPMI medium as unstimulated control. **A**: Gene expression of TNF, **B**: gene expression of IL10, **C**: gene expression of IFNγ. Error bars represent SEM, $N = 6$ animals per group, one independent experiment.

et al., 2008]. Although this shift towards high neutrophil numbers is less pronounced than with acute stress, it raised the question whether ELF-EMF exposure is able to induce an allostatic load by chronic activation of the HPA stress axis.

In this study, short-term ELF-EMF exposure of 1 week resulted in a significant increase in neutrophils, but also other leukocyte numbers were elevated. Interestingly, leukocyte distribution showed similarity with acute stress-induced leukocyte mobilization. This is in contrast with profiles observed in chronic stress models where stress decreases leukocyte mobilization [Dhabhar and McEwen, 1997; Bowers et al., 2008]. Although the cause of these differences is currently unclear, our data do suggest a role for stress regulation in observed leukocyte shifts.

In contrast with previous findings, where 30 days of ELF-EMF exposure decreases the total amount of circulating leukocytes [Hashish et al., 2007], we observed no significant differences in leukocyte numbers and composition after 15 weeks of exposure. Our data may indicate that longer duration of this exposure period leads to adaptation of mice to environmental ELF-EMF exposure. Overall, cell numbers were lower in these long-term exposed mice and in control mice, which might be explained by batch variation.

For glucocorticoid hormones, it has been demonstrated that they directly can influence immune functioning [Yeager et al., 2009]. Regulation of immune response by HPA-axis activation is mediated through glucocorticoid signaling, either by increased release of corticosterone from the adrenal gland or increased sensitivity of immune cells by high expression of glucocorticoid receptors (GR) [Tait et al., 2008]. We did not measure GR expression and quantification of corticosterone proved difficult since handling of animals, although fast and reproducible, already induced stress resulting in strong instant increases in plasma corticosterone (data not shown). Therefore, we determined upstream regulation of the HPA axis as represented by the expression of CRH in the PVN, POMC in the pituitary, and CYp11a1 in the adrenal cortex. We focused on expression of these genes since they are key in the regulation of stress hormone synthesis [Papadimitriou and Priftis, 2009], and changes at this level in the stress pathway will alter stress hormone synthesis. In our experiments no significant changes were found in the expression of CRH in the PVN and Cyp11a1 in the adrenal. We do report a significant decrease in POMC expression for 4 h/day-exposed animals. Plasma ACTH was high in all tested groups. This may indicate an acute effect caused by the handling of

mice. As with corticosterone, ACTH stores can also be released by acute handling stress [Fujiwara et al., 2011]. In our experiments we measured decreased plasma ACTH and decreased POMC gene expression relative to control mice, although only significant in the 4 h/day exposed group for ACTH and the 24 h/day exposed group for POMC expression. Differences at expression level need a careful interpretation as the final hormone levels in circulation will depend on expression, RNA splicing, protein synthesis of pro-hormones, processing and post-translational modifications, followed by storage and regulated release. High hormone levels in circulation will often result in feedback signaling to reduce expression levels. A decrease in POMC expression and ACTH levels might therefore be the result of a feedback mechanism reacting on high levels of corticosterone or activation in the adrenal. These signals may feedback on different levels in the HPA axis [Papadimitriou and Priftis, 2009]. This implies that ELF-EMF exposure induces changes in HPA signaling. An extensive kinetic analysis of HPA axis regulation during ELF-EMF would be needed to unambiguously reveal its mechanism of action.

In this study, leukocytes from the spleen were stimulated with different toll-like receptor ligands, LPS, Pam3Cys, and Zymosan to activate innate signaling pathways. We show that expression of IL10 and interferon gamma (IFNγ) in splenocytes increased upon stimulation compared with unstimulated cells. TNF expression was not altered. This response might have been slower and not detectable yet at 6 h after stimulation of cells. There was no significant difference in cytokine expression between ELF-EMF exposed and control animals. Whether stress in this case directly influences immune cell activation is still inconclusive. Effects of corticosterone on circulating leukocytes during exposure therefore remain to be investigated.

In conclusion, short-term ELF-EMF exposure with a specific noise signal, similar to fields from environmental sources, did increase total leukocytes, mainly represented by CD4+ T-lymphocytes and neutrophils. We found indications that this increase may be caused by changes in the HPA stress axis. These imply that short-term ELF-EMF exposure may cause small changes in stress regulation, potentially resulting in shifts in different leukocyte subsets. We have not followed up mice after short-term exposure but it appears that observed effects are transient in nature, because the effect was not observed after long-term exposure. Though current data are not sufficient to explain health problems related to ELF-EMF exposure, our ex vivo analysis showed that ELF-EMF exposure altered endocrine and immune parameters in vivo. This approach is therefore suitable to study health consequences of environmental ELF-EMF exposure on the whole body level.

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