Extremely low frequency electromagnetic field exposure does not modulate toll-like receptor signaling in human peripheral blood mononuclear cells

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The effects of extremely low frequency electromagnetic fields (ELF-EMF) on human health remain unclear. It has been reported that ELF-EMF may modulate the innate immune response to microorganisms in animal models and mammalian cell-lines. With the recently gained insight in innate immune signal- ing and the discovery of pattern recognition, we aim to study whether ELF-EMF modulates innate inflammatory signaling pathways. We used human peripheral blood mononuclear cells (PBMCs), isolated from blood of healthy volunteers, which we stimulated with specific TLR2 and TLR4 ligands, and with several microorganisms. The cells were subsequently exposed in $B_{ac}=5 \mu T$ and Alternating current electromagnetic field component; $B_{dc}=3 \mu T$, for 30 min. Cytokine production was measured at different time points after stimulation. No significant difference in immune response, as reflected by IL-1β, IL-6, TNFα, IL-8 and IL-10 production, could be detected after stimulation with LPS (TLR4 ligand), Pam3Cys (TLR2 ligand) or a panel of heat killed microorganisms: Mycobacterium tuberculosis, Salmonella typhimurium, Candida albicans, Aspergillus fumigatus and Staphylococcus aureus (multiple TLR ligands). We therefore conclude that under our experimental conditions, ELF-EMF does not modulate the innate immune response of human primary cells after TLR stimulation in vitro.

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1. Introduction

Electromagnetic fields (EMF) are continuously present in the environment of modern society. We are increasingly exposed to EMFs from wireless and mobile telecommunication and power lines, but this is not accompanied by sufficient knowledge of effects on public health. Various epidemiological studies suggest a relationship between living close to power lines or UMTS base stations and the occurrence of e.g. childhood leukemia and brain tumors [1–5]. However, insufficient biological evidence has been presented. In fact, there is a controversy as some scientists claim serious health risks of EMF whereas others claim that these fields do not interact with the human body [6–9].

Abbreviations: ELF-EMF, extremely low frequency electromagnetic field; $B_{ac}$, direct current electromagnetic field component; $B_{dc}$, alternating current electromagnetic field component.

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Nowadays, especially extremely low frequency electromagnetic fields (ELF-EMF), which are produced by power lines and electrical devices, obtain increased interest because we are continuously exposed to these fields. These fields are non-ionizing and unable to induce thermal effects (heating of the body). Because of their large wavelength, they can penetrate deep into tissue. It has been suggested that these ELF-EMFs can influence ion- and membrane potential dependent processes such as Ca2+ influx and effects on membrane based enzyme linked transport molecules [10,11].

It has been shown that ELF-EMF has an effect on components of the immune system in vivo by inducing changes in blood cell levels in both mice and rats, probably caused by affected lymphocyte proliferation, the underlying effects of which are unclear [12,13]. Furthermore, ELF-EMF might influence macrophage functioning in vitro by increasing free radical production and stimulating phagocytic activity [14–16]. This suggests an influence of ELF-EMF on innate immunity. Because the innate immune system is the fast and effective first line of defense mechanism, which is essential for further effective immune functioning, subtle effects of ELF-EMF exposure as a co-stimulator may have relevant consequences for the progression of inflammation and host defense. Since experimental evidence is scarce, there is a need for a good controllable model to study the effects of ELF-EMF on innate immune responses.
Interleukin 1
immune system activation is characterized by the production of a factor in the fast first response to invading pathogens [17]. Innate, in particular by the Toll-like receptor (TLR) family, is an important immune stimulation. 

Recent discoveries in innate immune signaling revealed that the recognition of pathogen-associated molecular patterns (PAMPs), in particular by the Toll-like receptor (TLR) family, is an important factor in the fast first response to invading pathogens [17]. Innate immune system activation is characterized by the production of inflammatory cytokines. These are either pro-inflammatory e.g. interleukin 1β (IL-1β), interleukin 6 (IL-6) and tumor necrosis factor α (TNFα), anti-inflammatory e.g. interleukin 10 (IL-10), or chemotactic e.g. interleukin 8 (IL-8). More important, these cytokines act as key regulators in innate immunity and have a crucial role in differentiation of T lymphocytes as part of the adaptive immune response as well as regulation of other innate immune cells such as neutrophils. Peripheral blood mononuclear cells (PBMCs) have already been used frequently, to study effects of ELF-EMF on the inflammatory response and cytokine production. Some studies show an increase in IL-1β, IL-2, IL-6 and TNFα [18,19], whilst others have contradicted this or have shown a decrease in cytokine production reflected by IL-2, Interferon γ, IL-10 and TNFα [20,21]. Monocytes, which express a wide variety of TLRs, constitute 10–25% of PBMCs, and are a suitable model to study cytokine production after TLR stimulation [22].

Since ELF-EMFs are low in energy, they are not likely to induce large effects on human immunity. However, they may modulate ongoing inflammatory responses (Fig. 1). It has been previously proposed, that a signal that comprises continuous multiple waveforms (20–5000 Hz), reduces mortality in fish and improves feed conversion and health in broiler chickens i.e. reduced coccidiosis [23,24]. This signal may induce a change in the innate immune response. In this study, we investigated the effect of this specific ELF-EMF signal on a variety of components of the human innate immune system in a standardized in vitro cellular model.

2. Methods

2.1. Exposure system

For the purpose of studying dedicated effects of specific low frequency electromagnetic fields on cellular components of the innate immune system in vitro, a special exposure system was designed. The system fits in a standard cell culture incubator that guarantees 37 °C and 5% CO2 culture conditions. The system exists of a double cylinder made of PMMA (Poly methyl methacrylate). The inner cylinder has a double copper wire solenoid coil. One inner coil with windings across the whole length of the cylinder generates the exposure field. The second inner coil has windings at both ends of the cylinder and assures a high homogeneity in the exposure area of <0.4%. The outer cylinder contains windings at both ends to reduce fringe fields. The coil consists of one continuous copper conductor, ensuring equal current in different coil components.

The coil is connected to a signal generator with preprogrammed signals, which supply multiple simultaneously shaped waveforms between 20 and 5000 Hz to the cells (Immunent BV, Veldhoven, The Netherlands). The system is able to create both ac and dc vertical field components with field strengths Bac and Bdc in the range from 0.1 μT to 1 μT. Field strengths were calibrated by F.W. Bell Gauss meters and continuous digital monitoring of the coil current.

2.2. Human peripheral blood mononuclear cell (PBMC) isolation

After informed consent, blood was drawn from healthy volunteers and collected in EDTA tubes. Blood was diluted 1:1 with pyrogen-free PBS (Lonza, Basel, Switzerland) and then used for isolation by gradient centrifugation with lymphoprep (Axis Shield, Oslo, Norway). The upper buffycoat layer with the PBMC fraction was taken off and washed three times with ice cold PBS and brought at a concentration of 5 x 10⁶ cells/ml in serum free RPMI (Gibco, Invitrogen, Paisley, United Kingdom) with 100 U/ml of penicillin/streptavidin (Gibco, Invitrogen, Paisley, United Kingdom).

2.3. PBMC stimulation

Cells were stimulated with either LPS (Escherichia coli serotype 055:B5, Sigma Aldrich, purified as described previously [25]), Pam3Cys (EMC microcollections, Tubingen, Germany) or a panel of heat killed pathogens containing Mycobacterium tuberculosis, Salmonella typhimurium, Candida albicans, Aspergillus fumigatus and Staphylococcus aureus [26]. In addition, the cells were exposed to a 5 μT Immunent ELF-EMF signal for 30 min at 37 °C and 5% CO2. After incubation, at different time points, the cells were centrifuged and supernatant was stored at –20 °C until cytokine measurement.

2.4. ELISA

Cytokine analysis of supernatants was done by ELISA (Sanquin, Amsterdam, the Netherlands) with IL-1β, IL-6, IL-8, IL-10 and TNFα, according to the manufacturer’s protocol.

The read out was performed in duplicate in an ELISA plate reader at a wavelength of 450 nm.

2.5. Statistics

A minimum of three biological replicates was used for analysis. Statistical analysis was performed by using Wilcoxon signed-rank or one sample t-test when appropriate. Values of <0.05 were considered statistically significant. In case of multiple comparisons, we applied Bonferroni correction for multiple testing on p-values.

3. Results

3.1. ELF-EMF has no effect on IL-6 release after stimulation with different doses of specific TLR2 and TLR4 ligands

We studied whether ELF-EMF is able to modulate the innate cytokine response to different doses of the TLR ligands LPS...
For this purpose, PBMCs were exposed to ELF-EMF at 5 µT for 30 min following stimulation. IL-6 was chosen as read out for this experiment, because its response is sensitive and fast. IL-6 levels were measured in the cell culture medium supernatant at 24 h post stimulation. PBMCs stimulated with either LPS (0.01–100 ng/ml) or Pam3Cys (0.01–100 µg/ml) showed normal dose response curves for IL-6. Sensitivity to both LPS as well as Pam3Cys varied between test subjects but was not significantly different between ELF-EMF treated cells and control cells. Highest variation between individuals was observed at lower concentrations of stimulus (Fig. 2A and B). Interindividual differences in sensitivity to TLR stimulation were compensated by calculating the Experiment/Control (E/C) ratio for each individual (Fig. 2C and D). Dosages of 1 ng/ml (LPS) and 1 µg/ml (Pam3Cys) were considered to be in mid range and to allow maximal modulation by ELF-EMF as is suggested in Fig. 1.

### 3.3. ELF-EMF exposure has no effect on transcriptional and translational processes reflected in cytokine production

To study the possible effect of ELF-EMF on early ongoing cellular processes like gene transcription and translation, we applied ELF-EMF at different time points after TLR stimulation. When cells were exposed to 30 min of ELF-EMF at 4 h after TLR4 stimulation with LPS, an increased IL-6 concentration was observed after 24 h, but not after 8 h (Fig. 4A, C and E). This difference after 24 h was however not statistically significant. In addition, no significant difference was observed after TLR2 stimulation with Pam3Cys (Fig. 4B, D and F). To further investigate these observations, other cytokines were also measured. Induction of IL-1β, TNFα, IL-10 and IL-8 showed a similar pattern compared to IL-6 (Table 1). ELF-EMF exposure at t = 2 h resulted in a significant reduced IL-10 release after TLR2 stimulation with Pam3Cys (p ≈ 0.0004) and exposure at t = 6 h resulted in a significant reduced IL-8 release after TLR2 stimulation with Pam3Cys (p ≈ 0.04). However, these differences were not significant after the Bonferroni correction for multiple comparisons (cutoff p ≈ 0.0017).
3.4. ELF-EMF exposure does not influence cytokine production induced by activation of multiple TLR signaling pathways

To include TLR stimulation via different receptors for extra- and intracellular pathogens, a panel of heat killed microorganisms was used to stimulate cells. Stimulation of PBMCs with these pathogens resulted in a clear increase of IL-6, TNFα and IL-1β except with Aspergillus fumigatus (Fig. 5A, C and E). Again, there was variation between test subjects but no significant difference was found between ELF-EMF exposure and control (Fig. 5B, D and F).

4. Discussion

By using cytokine secretion as a read out, we included the whole chain of TLR signaling, from receptor binding to protein secretion. We hypothesized that if ELF-EMF induces significant changes in any of these cellular processes, it is reflected in the cytokine secretion. Here, we show that the ELF-EMF signal used in this study does not modulate the TLR induced innate inflammatory cytokine response of human PBMCs.

The cytokine IL-6, which has both pro- and anti-inflammatory function, is very sensitive to stimulation, making it an ideal cytokine to screen different parameters of both TLR stimulation and ELF-EMF exposure. Furthermore, IL-6 is released very quickly after stimulation. IL-6 has been described to play a role in lymphocyte proliferation and bone healing. These both have been suggested to be affected by low frequency EMF exposure, although these were applied at higher field strengths [27,28]. Since, our data do not show changes in IL-6 secretion and assuming that it plays a role in these processes, we conclude that, under the conditions tested, this effect is not explained by an altered cytokine secretion from PBMCs.

It has been suggested that exposure to ELF-EMF can change gene transcription which precedes cytokine production and release [29]. For that reason ELF-EMF exposure was also applied at time points with high induced transcriptional activity. Some modulations at specific time points seemed to be significant, such as IL-8 and IL-10 (Table 1), however, this could not be confirmed by other cytokines induced by similar transcriptional pathways. Indeed, after application of Bonferroni correction, these differences were not significant and therefore most likely caused by coincidence. Although we did not measure transcriptional processes directly, our data indicates that it is unlikely that ELF-EMF modulates transcription of cytokine genes.

Most pro-inflammatory cytokines are transcribed via NF-κB dependent processes. One of the important regulators of NF-κB is Ca2+/calmodulin dependent kinase 2 [30]. Ca2+/Calmodulin binding potentially provides the strongest interaction of EMF in a biological mechanism, reflected by Myosin light chain kinase activity and Actin polymerization [31]. Our findings argue against a comparable mechanism in human PBMCs, as shown...
by the inability of ELF-EMF to notably modulate the cytokine response when applied at later time-points during the transcription of cytokine genes.

The pro-inflammatory cytokines TNFα and IL-1β are both important in antimicrobial responses. IL-1β requires cleavage by caspase-1 to become active and is therefore of interest for studying ELF-EMF effects at the level of post translational modification. During IL-1β secretion, which is ATP dependent, potassium efflux and interaction with calcium are essential [32]. Both potassium and calcium are important signal molecules and targets for ELF-EMF interaction [33]. However, with our experiments we could not detect any modulatory effect of ELF-EMF on IL-1β secretion; this suggests that this signal is not influencing potassium and calcium dependent signaling in PBMCs.

Stimulation of TLR2 and TLR4 is representative for recognition of both gram positive and gram negative extracellular bacterial pathogens. A wide range of these bacteria is involved in infectious diseases. However, the innate immune response is not limited to

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**Fig. 4.** Secretion of IL-6, after TLR2 and TLR4 stimulation followed by ELF-EMF exposure at different time points. Fresh PBMCs (N = 6) were stimulated with LPS (0.1 ng/ml) or Pam3Cys (0.1 μg/ml) followed by 30 min of ELF-EMF exposure (5 μT) at time points t = 2, t = 4 and t = 6 h after stimulation. At t = 8 and t = 24 h, the production of IL-6 was determined in the supernatant. (A) Absolute amounts of IL-6 in pg/ml after LPS stimulation measured at t = 8. (B) Absolute amounts of IL-6 in pg/ml after Pam3Cys stimulation measured at t = 8. (C) Absolute amounts of IL-6 in pg/ml after LPS stimulation measured at t = 24. (D) Absolute amounts of IL-6 in pg/ml after Pam3Cys stimulation measured at t = 24. (E) E/C ratio between EMF treated cells and control cells after LPS stimulation measured at t = 24. (F) E/C ratio between EMF treated cells and control cells after Pam3Cys stimulation measured at t = 24.
One sample was used, applied higher intensities [6,7]. However, these exceed standards. The majority of studies in which an exposure systems these very low intensity fields remains below public exposure obtained in animal studies [23,24]. Furthermore, application of significant results, with respect to feed conversion and health, were in vitro with other decide not to shield for this noise to maintain comparability sequences for the outcome of the experiments [35]. However, we is very different from the exposure signal, it may have conse-
quences for the outcome of the experiments [36]. In this way we included a large spec-
trum of TLR signaling in the context of ELF-EMF. Our results show, as expected, that there are differences in response between the six pathogens, and variation in personal sensitivity. Importantly, with this experiment, a wide spectrum of pattern recognition signaling pathways was covered. Thus, signaling via these pathways comprises an important part of innate immune activation.

The ELF-EMF signal used in this study contains different frequencies in the very low frequency range. This signal was especially designed to induce stimulating effects for improving immune function and health. In vitro experiments indicated such effects [23,24], but these had not been confirmed by specific in vivo stimulations. The signal, by its irregular shape, is likely to result in a consistent reduction or increase of innate immune activity. In summary, we show that stimulation of PBMCs induced by TLR signaling. This forms the major starting point of host defense, and therefore ELF-EMF exposure is not likely to result in a consistent reduction or increase of innate immune activity.

Table 1

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Time point of ELF-EMF exposure (hours)</th>
<th>LPS mean ± SEM</th>
<th>Pam3Cys mean ± SEM</th>
<th>Replicates</th>
<th>P value LPS</th>
<th>P value</th>
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<tbody>
<tr>
<td>IL-6</td>
<td>t = 2</td>
<td>0.89 ± 0.34</td>
<td>1.32 ± 0.69</td>
<td>N = 6</td>
<td>0.4572</td>
<td>0.3051</td>
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<tr>
<td></td>
<td>t = 4</td>
<td>1.53 ± 0.55</td>
<td>0.97 ± 0.30</td>
<td></td>
<td>0.0672</td>
<td>0.8352</td>
</tr>
<tr>
<td></td>
<td>t = 6</td>
<td>1.07 ± 0.51</td>
<td>0.98 ± 0.33</td>
<td></td>
<td>0.7640</td>
<td>0.8805</td>
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<tr>
<td>IL-1β</td>
<td>t = 2</td>
<td>0.85 ± 0.09</td>
<td>1.18 ± 0.14</td>
<td>N = 6</td>
<td>0.1776</td>
<td>0.2615</td>
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<tr>
<td></td>
<td>t = 4</td>
<td>1.08 ± 0.15</td>
<td>1.21 ± 0.15</td>
<td></td>
<td>0.6213</td>
<td>0.6149</td>
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<tr>
<td></td>
<td>t = 6</td>
<td>0.95 ± 0.21</td>
<td>1.37 ± 0.21</td>
<td></td>
<td>0.8146</td>
<td>0.4949</td>
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<tr>
<td>TNFα</td>
<td>t = 2</td>
<td>0.77 ± 0.15</td>
<td>1.00 ± 0.55</td>
<td>N = 3</td>
<td>0.2694</td>
<td>0.9957</td>
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<tr>
<td></td>
<td>t = 4</td>
<td>0.95 ± 0.21</td>
<td>1.54 ± 0.96</td>
<td></td>
<td>0.8206</td>
<td>0.6735</td>
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<tr>
<td></td>
<td>t = 6</td>
<td>0.79 ± 0.42</td>
<td>0.95 ± 0.16</td>
<td></td>
<td>0.6624</td>
<td>0.8050</td>
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<tr>
<td>IL-10</td>
<td>t = 2</td>
<td>1.46 ± 0.54</td>
<td>0.61 ± 0.18</td>
<td>N = 3</td>
<td>0.3839</td>
<td>0.0041*</td>
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<tr>
<td></td>
<td>t = 4</td>
<td>1.30 ± 0.42</td>
<td>1.11 ± 0.45</td>
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<td>0.3087</td>
<td>0.8062</td>
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<tr>
<td></td>
<td>t = 6</td>
<td>1.27 ± 0.40</td>
<td>0.92 ± 0.27</td>
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<td>0.2747</td>
<td>0.2100</td>
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<tr>
<td>IL-8</td>
<td>t = 2</td>
<td>1.04 ± 0.17</td>
<td>0.97 ± 0.16</td>
<td>N = 6</td>
<td>0.8237</td>
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<tr>
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<td>t = 4</td>
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<td>0.69 ± 0.14</td>
<td></td>
<td>0.0815</td>
<td>0.0765</td>
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<tr>
<td></td>
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<td>1.05 ± 0.40</td>
<td>0.66 ± 0.27</td>
<td></td>
<td>0.7338</td>
<td>0.0381*</td>
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</table>

* One sample t-test statistically significant, after Bonferroni correction statistically not significant.

TLR2 and TLR4. We therefore also stimulated PBMCs with additional TLR ligands. By using a panel of heat killed pathogens at optimized concentrations, a much wider variety of PAMPs is presented to the PBMCs [26]. In this way we included a large spectrum of TLR signaling in the context of ELF-EMF. Our results show, as expected, that there are differences in response between the six pathogens, and variation in personal sensitivity. Importantly, with this experiment, a wide spectrum of pattern recognition signaling pathways was covered. Thus, signaling via these pathways comprises an important part of innate immune activation.

The ELF-EMF signal used in this study contains different frequencies in the very low frequency range. This signal was especially designed to induce stimulating effects for improving immune function and health. In vitro experiments indicated such effects [23,24], but these had not been confirmed by specific in vivo stimulations. The signal, by its irregular shape, is likely to result in a consistent reduction or increase of innate immune activity. In summary, we show that stimulation of PBMCs reflected by inflammatory cytokine production. Sensitivity for TLR ligands varied between individuals. Although variation in sensitivity is dependent on various causes, like TLR polymorphisms, it normally has no major consequences for the efficiency of innate immune responses to pathogens [36].

The previous in vivo studies suggest effects of ELF-EMF, however these were difficult to control. In this study we focused on a specific part of the immune system that we can control experimentally. Our results did not reveal an explaining mechanism for the effects that were observed in vivo. This suggests the involvement of other mechanisms. Innate immunity is not limited to TLR signaling and cytokine production. Besides recognition, the effectiveness of the anti-microbial response is also important. Effectiveness of mechanisms like binding, phagocytosis and killing directly modifies the outcome of immune efficiency. It is conceivable to study these processes during ELF-EMF exposure as well [37]. Furthermore, it would be highly relevant to compare these specific signals with standard 50 Hz exposure. This frequency is most common in human environment because it is produced by power lines and common electrical devices. Some studies already used a frequency of 50 Hz to study immune effects, but did not focus on innate immunity on the whole system level. Eventually, this will give better insight in the effect of weak low frequency electromagnetic fields in our environment, on the functioning of the innate immune system.

In summary, we show that stimulation of PBMCs with standardized TLR ligands resulted in normal dose dependent cytokine production and therefore is a useful method to study the effect of ELF-EMF. In this model we show that additive ELF-EMF exposure at a weak 5 µT is not sufficiently potent to consistently modulate the innate immune response via TLR signaling. The signal may affect the gene transcription of inflammatory cytokines, but this has to be further elucidated. The obtained in vitro results do not support a modulation of the innate immune response in human PBMCs induced by TLR signaling. This forms the major starting point of host defense, and therefore ELF-EMF exposure is not likely to result in a consistent reduction or increase of innate immune activity.
Fig. 5. Cytokine responses after stimulation with different microorganisms. Fresh PBMCs from healthy volunteers (N = 6) were stimulated with heat killed *Mycobacterium tuberculosis* (MTB), *Salmonella typhimurium* LPS (SAL LPS), heat killed *Salmonella typhimurium* (SAL HK), heat killed *Candida albicans* (CAND HK), heat killed *Aspergillus fumigatus* (ASP HK) or heat killed *Staphylococcus aureus* (SA HK), followed by 30 min of ELF-EMF exposure at 5 µT. After 24 h incubation, IL-1β, IL-6 and TNFα cytokine release was determined by ELISA. (A) Absolute amounts of IL-6 in pg/ml, error bars with SEM (B) E/C ratio between EMF treated cells and control cells. (C) Absolute amounts of TNFα in pg/ml, error bars with SEM (D) E/C ratio between EMF treated cells and control cells. (E) Absolute amounts of IL-1β in pg/ml, error bars with SEM (F) E/C ratio between EMF treated cells and control cells.
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