Low-frequency Electromagnetic Fields do not Alter Responses of Inflammatory Genes and Proteins in Human Monocytes and Immune Cell Lines

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The effects of low frequency electromagnetic fields (LF EMF) on human health are the subject of on-going research and serious public concern. These fields potentially elicit small effects that have been proposed to have consequences, either positive or negative, for biological systems. To reveal potentially weak but biologically relevant effects, we chose to extensively examine exposure of immune cells to two different signals, namely a complex multiple waveform field, and a 50 Hz sine wave. These immune cells are highly responsive and, in vivo, modulation of cytokine expression responses can result in systemic health effects. Using time course experiments, we determined kinetics of cytokine and other inflammation-related genes in a human monocytic leukemia cell line, THP-1, and primary monocytes and macrophages. Moreover, cytokine protein levels in THP-1 monocytes were determined. Exposure to either of the two signals did not result in a significant effect on gene and protein expression in the studied immune cells. Also, additional experiments using non-immune cells showed no effects of the signals on cytokine gene expression. We therefore conclude that these LF EMF exposure conditions are not expected to significantly modulate innate immune signaling. Bioelectromagnetics

Key words: LF EMF; cytokines; THP-1; monocytes; macrophages

INTRODUCTION

Low frequency electromagnetic fields (LF EMF) are abundantly present in modern day society. These fields, ranging from 0 to 300 kHz in frequency, are produced by electrical devices and power lines and are capable of penetrating deep into tissue. Their potential harmful effects on biological systems, such as leukemia, breast cancer, and cardiovascular disease, are under constant debate [Ahlbom et al., 2008]. In contrast, beneficial effects of LF EMF have been reported, including decreased severity of intestinal lesions in coccidian-infected chickens [Elmusharaf et al., 2007], accelerated wound healing [Callaghan et al., 2008; Goudarzi et al., 2010] and accelerated bone healing in rats and humans [Grana et al., 2008; Griffin et al., 2008], and augmented response of macrophages to bacterial challenges in humans [Akan et al., 2010]. To date, no mechanism of action has been elucidated, although several possible mechanisms have been suggested, including

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Disclaimer: Dr. Cuppen owns stock in and is employed by Immunent. Immunent 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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changes in signal transduction pathways and modulation of oxygen radicals [Liburdy et al., 1993; Blank and Soo 2001; Rollwitz et al., 2004; Frahm et al., 2010]. A large variation exists in the use of signals in scientific studies for effects of LF EMF on human health. The fact that daily exposure to EMF is also highly variable makes the choice of which LF EMF signals to study both important and difficult in order to draw any conclusions on human health effects.

In our study, we used a signal that has already been shown to affect biological systems by reducing mortality in fish and improving feed conversion and health in broiler chickens [Cuppen et al., 2007; Elmusharaf et al., 2007]. This signal, denoted Immunent (IM) signal, consists of multiple waveforms and creates a complex, continuously changing field with steep rise times, and exponential decays. It has been suggested that these characteristics of an EMF signal may be the causal factor for biological responses [Pilla, 2006]. Additionally, we used a more frequently studied 50 Hz sine wave signal that resembles the household alternating current (AC) electrical power supply in a large part of the world.

Cells of the immune system are plausible study targets since these cells regulate health on a systemic level. In response to a pathogen challenge they must respond in a very sensitive, swift, and effective way. Immune cells produce important signaling molecules called cytokines, which are key regulators of cell activation and inhibition. In this study we chose to examine the effects of LF EMF on monocytes and macrophages because these cells are highly reactive and have an important function as the first line of defense against pathogens. They can act as antigen-presenting cells to trigger a specific response from lymphocytes, and are capable of producing several cytokines including interleukin-1 beta (IL-1β), tumor necrosis factor-alpha (TNF-α), and interleukin-10 (IL-10). Upon activation they induce a wide range of intracellular signaling pathways. A subtle disruption of one of these processes will ultimately have consequences for the immune defense.

Cytokine production and secretion patterns are altered upon differentiation of monocytes into macrophages. We therefore used the human monocytic cell line THP-1 as a model to study the function of both monocytes and macrophages in vitro. This cell line has been well characterized and is frequently used to simulate monocyte as well as macrophage responses [Tsuchiya et al., 1980; Tsuchiya et al., 1982; Stokes and Doxsee, 1999]. Kinetics of expression of cytokine and transcription factors has been described recently [Chanput et al., 2010]. To test the effects of LF EMF ex vivo, we used freshly isolated human monocytes from several healthy individuals. Moreover, to determine if potential effects were comparable in other immunologically relevant cell types, we also tested the LF EMF signals on a pharyngeal epithelial cell line, which is capable of producing cytokines in a similar fashion as immune cells.

For detection of subtle modulatory effects of LF EMF on the production of cytokines we chose to determine changes of expression in genes that code for cytokines. Changes in gene expression can be very precisely measured. Because gene expression changes take place within hours, detection can occur rapidly. Moreover, to assess potential post-transcriptional, post-translational, and modulatory effects on the level of protein production and secretion, we also determined LF EMF effects on cytokine protein secretion.

MATERIALS AND METHODS

Cell Lines

The human acute monocytic leukemia cell line THP-1 [Tsuchiya et al., 1980] and human pharyngeal Detroit 562 cells were obtained from the American Type Culture Collection (Rockville, MD, USA). The THP-1 cells were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum and the antibiotics penicillin (100 U/ml) and streptomycin (100 μg/ml), in a humidified incubator at 37 °C with 5% CO_2. The THP-1 cells were differentiated by the addition of 100 ng/ml phorbol 12-myristate 13-acetate (PMA) for 48 h to obtain macrophages. The Detroit 562 cells were maintained in RPMI-1640 medium supplemented with 1 mM sodium pyruvate, 10% fetal bovine serum and the antibiotics penicillin (100 U/ml) and streptomycin (100 μg/ml), in a humidified incubator at 37 °C with 5% CO_2. All experiments were performed with cells from the same batch.

Monocyte Isolation

Fresh, whole blood was drawn with written informed consent from healthy blood donors at the research department of the blood bank (Sanquin, Nijmegen, The Netherlands). Blood withdrawal for research purposes was approved by the medical ethics committee of the blood bank. Peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll-Paque PLUS density gradient centrifugation (Amersham Biosciences, Roosendaal, The Netherlands). $CD14^+$ monocytes were isolated from PBMCs by positive selection using a magnetic-activated cell

sorting (MACS) system (Miltenyi Biotech, Bergisch Gladbach, Germany) according to the manufacturer's protocol.

Exposure System

Cells were exposed in 12-well plates placed in the middle of an exposure (coil) system designed to be used inside a standard cell culture incubator (Immunent, Veldhoven, The Netherlands). The system is composed of two concentric cylinders made of polymethyl methacrylate (PMMA). The inner cylinder supports two copper wire solenoid coils. One inner coil has windings across the whole length of the cylinder and generates most of the exposure field. The second inner coil has windings at both ends of the cylinder and ensures a high field homogeneity in the exposure area in the center of the coil (<0.4% deviation). The outer cylinder supports a third coil with windings at both ends to reduce fringe fields. All coils are connected in series; therefore, the complete system can be viewed as a single exposure coil. The coil is connected with a signal generator with preprogrammed signals. Basic voltage signals $V(t)$ are produced by adding a maximum of four block or sine waves together with individual amplitude, frequency, and phase set in software. These are then shaped in an analog circuit that standardizes the effect of the impedance of the attached coil, resulting in a magnetic signal $B(t)$ that would be generated in a coil with characteristic frequency of 300 Hz by the basic voltage IM signal (for details, see patent number EP2020250; http://ep.espacenet.com).

In this experiment we used a basic voltage signal of either 50 Hz sine wave, or four combined block waves with equal relative amplitude and phase 0 at $t = 0$, and frequencies 320, 730, 880, and 2600 Hz (Fig. 1). The block wave signal was shown to produce biological effects with 30 min exposure [Cuppen et al., 2007; Elmusharaf et al., 2007], and is also representative of so called "dirty electricity" because it contains sharp edges and peaks. The field strength for all experiments used was 5 μT at the position of the cells and was monitored by continuous real-time sampling of the coil current $I(t)$ that was calibrated against $B(t)$ using a Gauss/Tesla meter with sensitive probe (Model 5180, F.W. Bell, Rochester, NY, USA).

Shielding of the incubator reduced the ambient (Earth's magnetic) DC field to 10 from 47 μT. Background AC fields were determined to be less than 0.2 μT at the experimental area in the incubator. Temperature, humidity, and carbon dioxide concentrations were kept constant during each experiment.

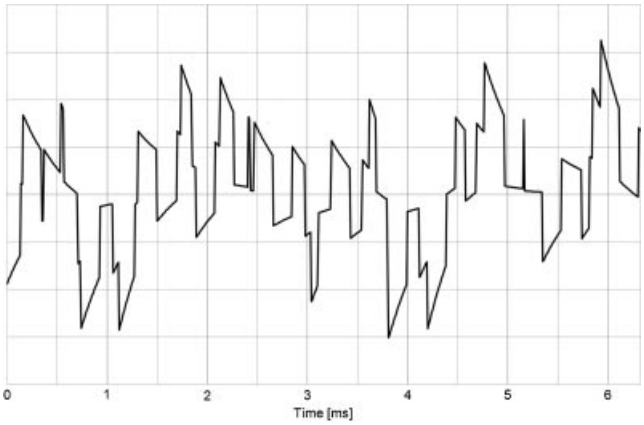

Fig. 1. Immunent signal shape when adapted for a coil with resistance R being 400 times the self-inductance L of the coil. The time given at the x-axis (in ms) starts 1 ms after the four signal components start all in phase. The signal has a non-repetitive character and sharp edges. The scale of the y-axis is arbitrary.

Experimental Design

The various cell types used were exposed for 30 min to either the exposure signal or no signal (unexposed control, inside the exposure system) in a standard incubator at 37 °C with 5% CO_2. This time period was chosen based on previously found results with the IM signal [Cuppen et al., 2007; Elmusharaf et al., 2007]. Experimental conditions were run randomized, in series and under the same conditions. Cells were plated in 12-well plates with 10^6 cells per well in 1 ml RPMI medium with 10% fetal bovine serum and 1% penicillin and streptomycin, with or without a suboptimal concentration of 1 μg/ml lipopolysaccharide (LPS) from *E. coli*. 0111:B4 (Sigma, St. Louis, MO, USA). Cell viability was verified before and after each experiment using trypan blue exclusion.

Gene Expression Analysis

RNA was isolated from the cells using the Qiagen RNeasy Mini Kit (Qiagen, Leusden, The Netherlands). RNA yield was quantified on a Nanodrop ND 1000 spectrophotometer (Thermo Scientific, Breda, The Netherlands). RNA quality criteria were set at optical density (OD) 260/280 ratio >1.95, and all samples met these criteria. RNA was reverse transcribed using Invitrogen's SuperScript III First Strand Synthesis System for RT-PCR Systems (Invitrogen, Breda, The Netherlands) according to the manufacturer's instructions. Standard quantitative real-time polymerase chain reaction (Q-PCR) was performed in a 72-well Rotor-Gene 2000 standard Q-PCR

machine (Corbett Research, Mortlake, Sydney, Australia) with Brilliant SYBR Green QPCR (Stratagene, La Jolla, CA, USA) for chemistry detection. Primer sequences used in the Q-PCRs were chosen based on PrimerBank sequences [Spandidos et al., 2010]. Q-PCR data were normalized by measuring cycle threshold (Ct) ratios between candidate genes and the housekeeping genes glyceraldehyde 3-phosphate dehydrogenase (GAPDH) for THP-1 monocytes, fresh monocytes and Detroit 562 cells, and β-actin for THP-1 macrophages. Fluorescence data from Q-PCR experiments were analyzed using Rotor-Gene version 6.0.21 software (Corbett Research, Mortlake, Sydney, Australia) and exported to Microsoft Excel.

Cytokine Production

Cytokines IL-1β, interleukin-6 (IL-6), interleukin-8 (IL-8), IL-10, interleukin-12 (IL-12), and TNF-α were determined using the cytometric bead array (CBA) Human Immunoglobulin Flex-Set cytokine assay (BD Biosciences, Franklin Lakes, NJ, USA) according to the manufacturer's instructions. The Flex-Set cytokine assay allows analysis of multiple cytokines in a small volume of cell culture supernatant. Cytokine production was measured by flow cytometry (FACS Canto, BD Biosciences), and the data were analyzed using BD FACSDiva software (BD Biosciences) and flow cytometric analysis program (FCAP) array software (Soft-Flow, Burnsville, MN, USA).

Statistical Analysis

The significance of the differences between cytokine profiles with and without LF EMF exposure were assessed using repeated-measures analysis of variance (ANOVA) with the Bonferroni correction. A one-factor ANOVA was used to determine significant differences between LF EMF-exposed and unexposed controls in freshly isolated monocytes. Statistical significance was accepted at $P < 0.05$. All calculations were performed by using the SPSS software package 15.0.1 (SPSS, Chicago, IL, USA).

RESULTS

We examined the effects of two LF EMF, a complex, continuously changing field, and a commonly applied 50 Hz sine wave signal, on gene expression of cytokines in THP-1 monocytic cells, freshly isolated human monocytes, and the epithelial cell line Detroit 562.

As each gene has its own specific pattern of expression, gene expression kinetics of each cytokine, the nuclear factor NF-κB, and the enzyme cyclooxygenase 2 (COX-2), were determined after LPS stimulation of THP-1 monocytes and macrophages (Fig. 2). A suboptimal LPS concentration of 1 μg/ml was chosen based on concentration-effect experiments (data not shown). Suboptimal LPS stimulation showed that in THP-1 monocytes, the pro-inflammatory genes IL-1β, TNF-α, IL-8, and COX-2 were induced early (2–3 h after stimulation), varying from 30-fold (TNF-α) to 200-fold (IL-1β) compared to baseline, and then tended to decrease from 6 h after LPS stimulation onward. The expression of the anti-inflammatory cytokine IL-10 showed a later peak of expression (at 6 h) which declined 12 h after LPS stimulation. IL-6, which is known to have both pro- and anti-inflammatory properties, increased in gene expression response from 1 h and continued to increase up to 24 h (at least) after LPS stimulation. The induction of IL-12 gene expression reached peak values around 100-fold at 6 h after LPS stimulation and then decreased again. Finally, gene expression induction of nuclear factor-kappa B (NF-κB) increased from 2 h after LPS stimulation up to 24 h, with a peak increase in response at 6 h after stimulation.

Macrophage gene expression differed from monocyte gene expression in both kinetics and response. Patterns of the pro-inflammatory cytokines IL-1β, TNF-α, and IL-8 were shifted and showed their peak response at 6 h after LPS stimulation, while the anti-inflammatory IL-10 kinetic pattern was similar to that present in monocytes. When absolute values of expression were examined, in the form of average quantitative real-time PCR Ct values, IL-1β was shown to be expressed at higher levels and IL-10 was expressed at lower levels in macrophages (Table 1). At baseline (T0) this was also the case for IL-6, but absolute expression of IL-6 after LPS stimulation was higher in macrophages compared to monocytes. Therefore, IL-6 gene expression responses in macrophages were much stronger than in monocytes.

Experiments were performed with the IM signal and the 50 Hz signal using both THP-1 monocytes and THP-1 macrophages. Exposure to the IM signal (Fig. 2 and Online Supplementary Material) or the 50 Hz signal (Online Supplementary Material) for 30 min, directly after LPS addition to the medium, did not alter the kinetics of gene expression in monocytes or macrophages. Also, average quantitative real-time PCR Ct values showed that when examining absolute values, no large variation between the exposures was present (Table 1). At 2, 3, and 6 h after LPS stimulation, relative gene expression

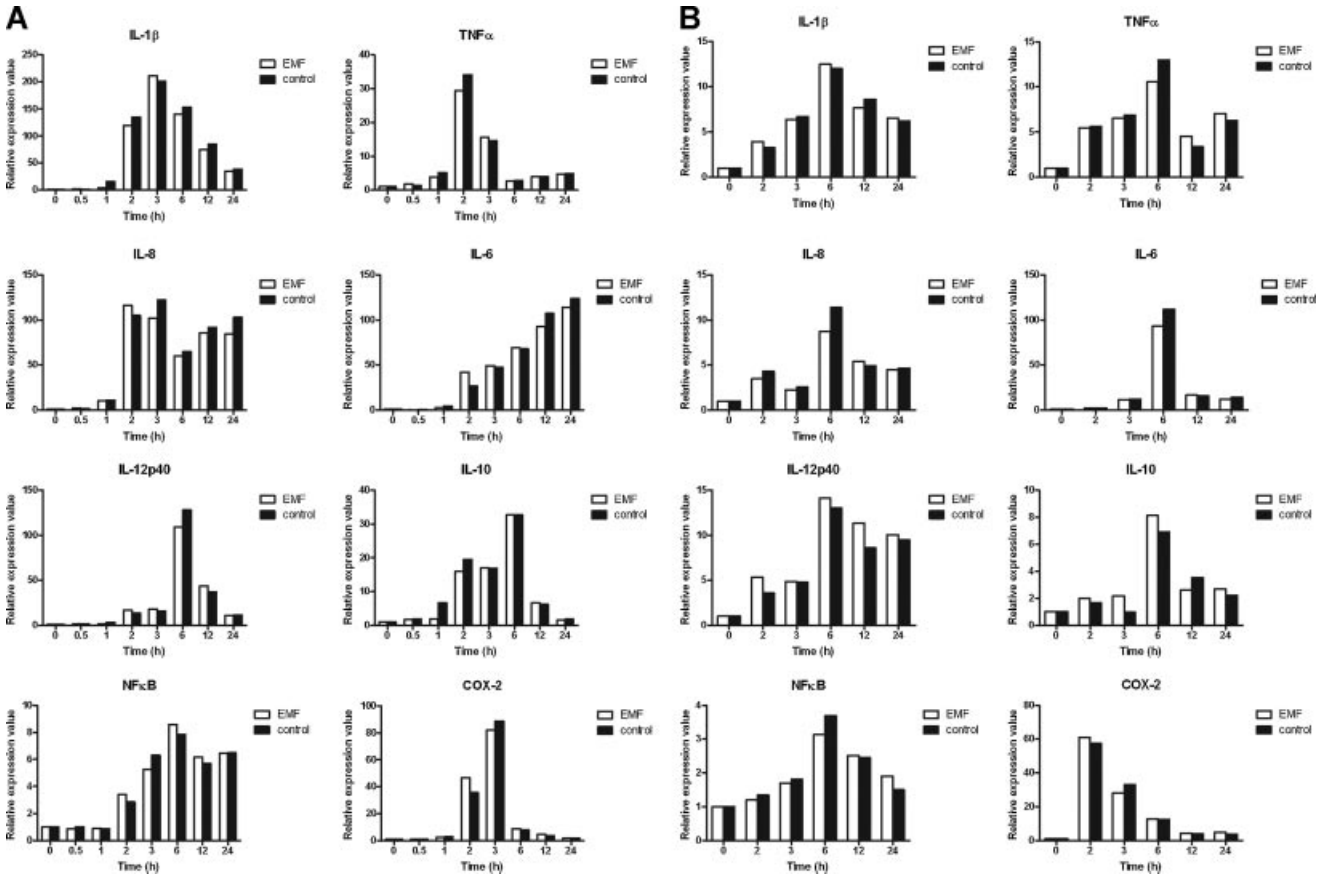

Fig. 2. Effects of LPS (1 μg/ml) and LF EMF (IM signal, 30 min, 5 μT) on gene expression kinetics of IL-1β, IL-6, IL-8, TNF-α, IL-12p40, IL-10, NF-κB, and COX-2 in THP-1 monocytes (A) and macrophages (B).

differences were determined between controls and exposures. No differences were found between exposure and control both after exposure to the IM signal and the 50 Hz sine wave signal at all three time points in monocytes (Fig. 3) and macrophages (Fig. 4).

To determine if LF EMF affects post-transcriptional processes, secreted cytokine protein concentrations were determined 6 h after LPS stimulation. IL-10 and IL-12 concentrations were too low to detect at this time point. Concentrations of the other four cytokines strongly increased after LPS stimulation, ranging from 13-fold (IL-6) to 631-fold (TNF-α) compared to controls (data not shown). Results did not show any differences between controls and LF EMF-exposed cells (IM signal or 50 Hz) 6 h after stimulation with LPS (Fig. 5).

Fresh monocytes were isolated from four blood donors to determine ex vivo responses of these cells to LF EMF exposure. Because of limited monocyte cell availability, one time point was chosen to determine LF EMF effects. Gene expression kinetics were determined, which showed that patterns of gene expression in freshly isolated monocytes were similar to those of THP-1 monocytes (Fig. 6). Also, pro-inflammatory genes IL-1β, TNF-α, and IL-8 showed early gene expression responses, while the anti-inflammatory gene IL-10 responded later. Based on these results, 3 h after LPS stimulation was chosen as an optimal time point to determine the influences of LF EMF. Gene expression of all previously mentioned genes was determined but no differences were detected in either kinetics or response between control and LF EMF-exposed monocytes (Fig. 7). Finally, another cytokine producing cell line, Detroit 562, which is derived from pharyngeal epithelial cells, was stimulated with LPS and exposed to either control or LF EMF (IM signal). These cells also showed no difference in gene expression kinetics and response to LF EMF (Fig. 8).

DISCUSSION

LF EMF presumably induce subtle effects in the immune system and we therefore chose to study

TABLE 1. Cytokine Gene Expression of THP-1 Cells in Response to LF EMF

A	IL-1ß	TNFα	IL-6	IL-8	IL-10	NF-κB	COX-2	IL-12p40	GAPDH
T0	27 ± 1.6	22.3 ± 1.4	26.8 ± 2.3	24.6 ± 1.5	29.4 ± 1.3	22.6 ± 1.2	30.1 ± 0.7	33.8 ± 2.1	14.7 ± 0.9
T2									
Control	16.5 ± 1.8	17.1 ± 1.6	24.8 ± 1.3	16 ± 1.4	24.2 ± 1.9	19.9 ± 1.9	25.1 ± 1.2	27 ± 3.1	14.7 ± 1.2
IM	16.2 ± 1.6	16.8 ± 1.4	24.2 ± 1.6	15.7 ± 1.2	23.6 ± 2.2	19.6 ± 1.8	24.6 ± 1.1	26.7 ± 3	14.4 ± 1
50 Hz	15.5 ± 2.3	17.2 ± 2.3	25.5 ± 1.8	14.9 ± 1.3	25.1 ± 2.9	20.2 ± 2.9	25.5 ± 1.8	25.1 ± 4.8	13.5 ± 0.7
T3									
Control	17.1 ± 2.3	17.7 ± 1.7	24.1 ± 1.5	15.9 ± 1.2	23.5 ± 2.1	19.1 ± 2	25.9 ± 1.9	26.2 ± 3.1	14.7 ± 1
IM	16.4 ± 1.7	17.4 ± 1.6	24.2 ± 1.5	15.6 ± 1.4	23.5 ± 2.2	18.9 ± 1.9	25.2 ± 1.3	26.4 ± 3	14.5 ± 1
50 Hz	16 ± 2.5	18.1 ± 2.7	24.5 ± 2.5	15.1 ± 1.4	24.9 ± 3.4	19.6 ± 3.1	26.5 ± 2.2	24.9 ± 4.7	13.7 ± 1
T6									
Control	17.6 ± 2.1	20.9 ± 1.5	23.6 ± 1.4	16.6 ± 1.6	23.9 ± 2.1	19.3 ± 1.8	27 ± 1.5	24.4 ± 3	14.7 ± 1
IM	17 ± 1.6	20.8 ± 1.4	23.2 ± 1.5	16.6 ± 1.6	23.8 ± 2.4	19.1 ± 1.7	26.5 ± 1.1	24.7 ± 3.1	14.5 ± 0.9
50 Hz	16.5 ± 2.2	20.7 ± 1.8	23.6 ± 2.3	15.3 ± 1.2	25.4 ± 3.3	20 ± 2.8	27.1 ± 1.9	23.8 ± 5.5	13.6 ± 0.9

B	IL-1ß	TNFα	IL-6	IL-8	IL-10	NF-κB	COX-2	IL-12p40	β-actin
T0	23.8 ± 2.3	23.2 ± 4	30.7 ± 3.2	20.4 ± 2.7	32.3 ± 1.6	24.5 ± 2.7	27.8 ± 1.5	33.2 ± 0.7	13.1 ± 1
T2									
Control	15.7 ± 2.2	16.2 ± 3.9	20.2 ± 3.2	14.6 ± 2.6	30.3 ± 1.6	20.9 ± 2.6	20.7 ± 1.5	30.6 ± 0.7	13.3 ± 1
IM	15.2 ± 2.1	15.8 ± 4.1	20.1 ± 2.8	14.1 ± 2.9	30 ± 0.9	20.4 ± 2.6	19.9 ± 1.3	29.8 ± 0.7	12.9 ± 1.2
50 Hz	17.2 ± 0.4	19.2 ± 0.4	22.9 ± 0.3	11.5 ± 0.4	28.6 ± 0.5	18.2 ± 0.1	19.1 ± 0.4	29.3 ± 0.3	12.5 ± 0.5
T3									
Control	15.2 ± 2.1	16.4 ± 4.2	18.7 ± 2.9	13.5 ± 3.1	30.7 ± 1	20.1 ± 2.5	20.6 ± 1.8	29 ± 0.8	13.1 ± 1.1
IM	14.8 ± 1.8	16.7 ± 4.2	18.6 ± 2.5	13.3 ± 3.7	30.2 ± 0.9	20.2 ± 2.7	20.1 ± 2	29 ± 0.8	13.2 ± 1.3
50 Hz	16.7 ± 0.2	20 ± 0.2	20.3 ± 0.4	10.3 ± 0.6	29.5 ± 0	17.6 ± 0.4	18.4 ± 0.5	28.6 ± 0.2	12 ± 0.2
T6									
Control	15.8 ± 2.1	18.2 ± 4.3	19.4 ± 3.2	12.9 ± 2.6	28.2 ± 0.7	20.3 ± 2.3	22.6 ± 2.4	25.9 ± 0.6	13.5 ± 1
IM	15.8 ± 1.8	18.6 ± 3.9	19.5 ± 2.6	12.9 ± 3.4	28.1 ± 0.8	20.5 ± 2.8	22.9 ± 2.4	25.5 ± 0.5	13.6 ± 1.4
50 Hz	17.6 ± 0.2	22.1 ± 0.2	21.8 ± 0.2	10.2 ± 0.8	27.3 ± 0.6	18.1 ± 0	20.6 ± 0.7	25.7 ± 1.3	12.5 ± 0.1

Mean quantitative real-time PCR Ct values ± 95% confidence intervals of all genes, including housekeeping genes GAPDH and β-actin, at baseline and 2, 3, and 6 h after stimulation with LPS (1 μg/ml) in THP-1 monocytes (A) and macrophages (B). IM, complex immunent signal.

the highly reactive and subtly regulated process of inflammation in various human cells including monocytes and macrophages. Upon infection these immune cells are capable of rapidly responding in a subtle, regulated, and strong manner, whereby both kinetics and level of expression of the inflammatory signals are crucial for the outcome of the infection. By determining kinetics of gene expression of pro- and anti-inflammatory cytokines, potential modulation by LF EMF, even if very subtle, should be detectable. Also, by determining the released cytokine proteins of these cells, the cytokine gene expression in freshly isolated monocytes, and the cytokine gene expression kinetics in epithelial cells, we attempted to illustrate a comprehensive view on pro- and anti-inflammatory cytokine modulation by our two chosen LF EMF signals.

Increased production of pro-inflammatory cytokines such as TNF-α and IL-1β activates the inflammatory reaction, resulting in local effects including chemotaxis, leukocyte adherence and prostaglandin production, and systemic effects including loss of appetite and increased heart rate. Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), a protein complex that controls the transcription of DNA, is an important cell-signaling molecule for inflammation. NF-κB activation induces the expression of many crucial pro-inflammatory genes including COX-2, which is responsible for the production of prostaglandins at inflammatory sites [D'Acquisto et al., 2002]. Anti-inflammatory cytokines such as IL-10 are produced several hours after initiation of inflammation, and regulate a coordinated program that controls inflammatory responses. This is reflected in our results, which show that gene expression kinetics followed highly consistent patterns. Moreover, these were comparable to previously determined kinetics in THP-1 monocytes and macrophages [Chanput et al., 2010].

We found no modulatory effects of the LF EMF signals on all cytokines tested at all time points both on gene and protein expression level, while variation

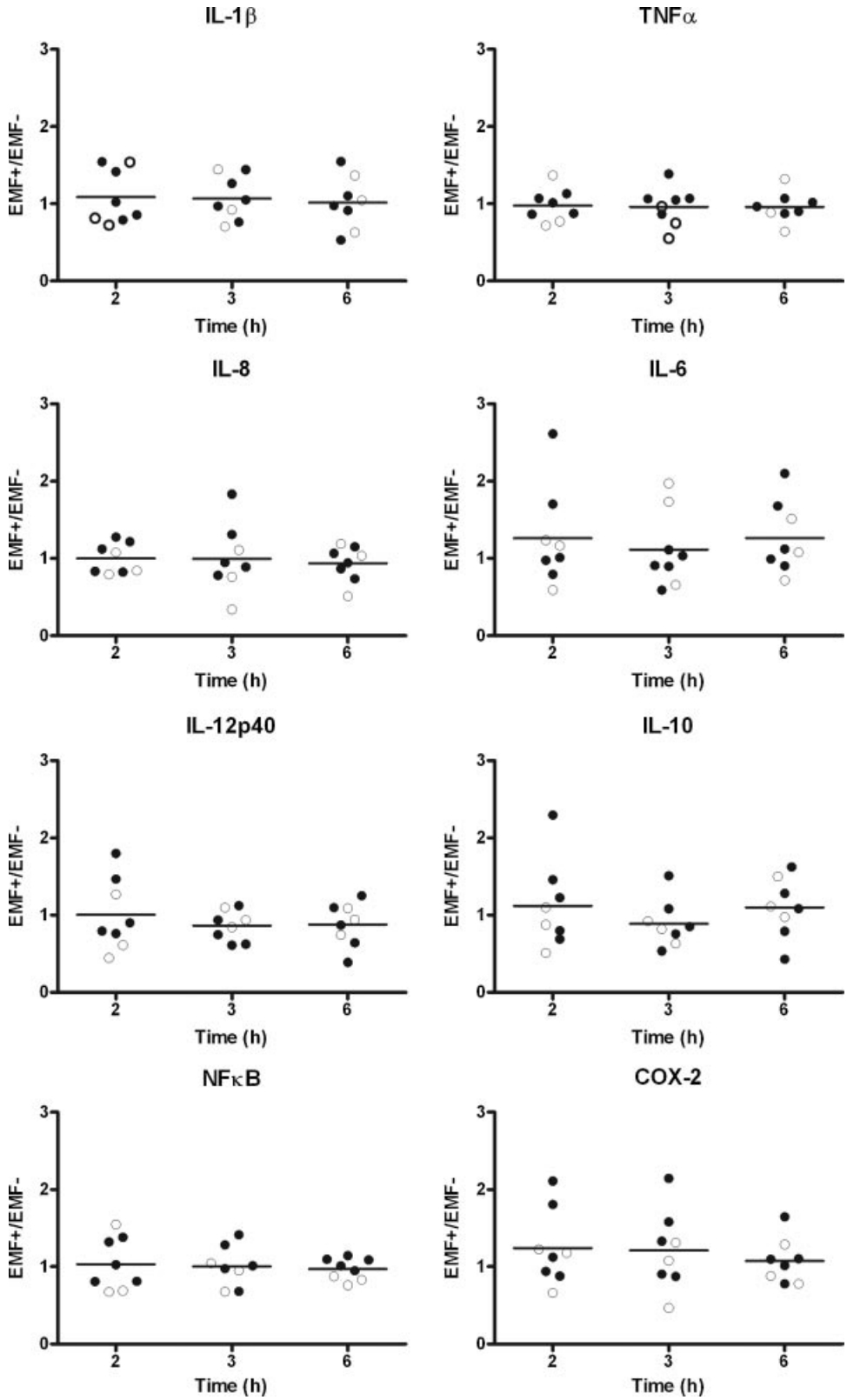

Fig. 3. Differences in gene expression between control and LF EMF (IM signal and 50 Hz sine wave, 30 min, 5 μT) on LPS-stimulated THP-1 monocytes at 2, 3, and 6 h after stimulation. Filled symbols represent IM signal, open symbols represent 50 Hz signal. Lines represent means.

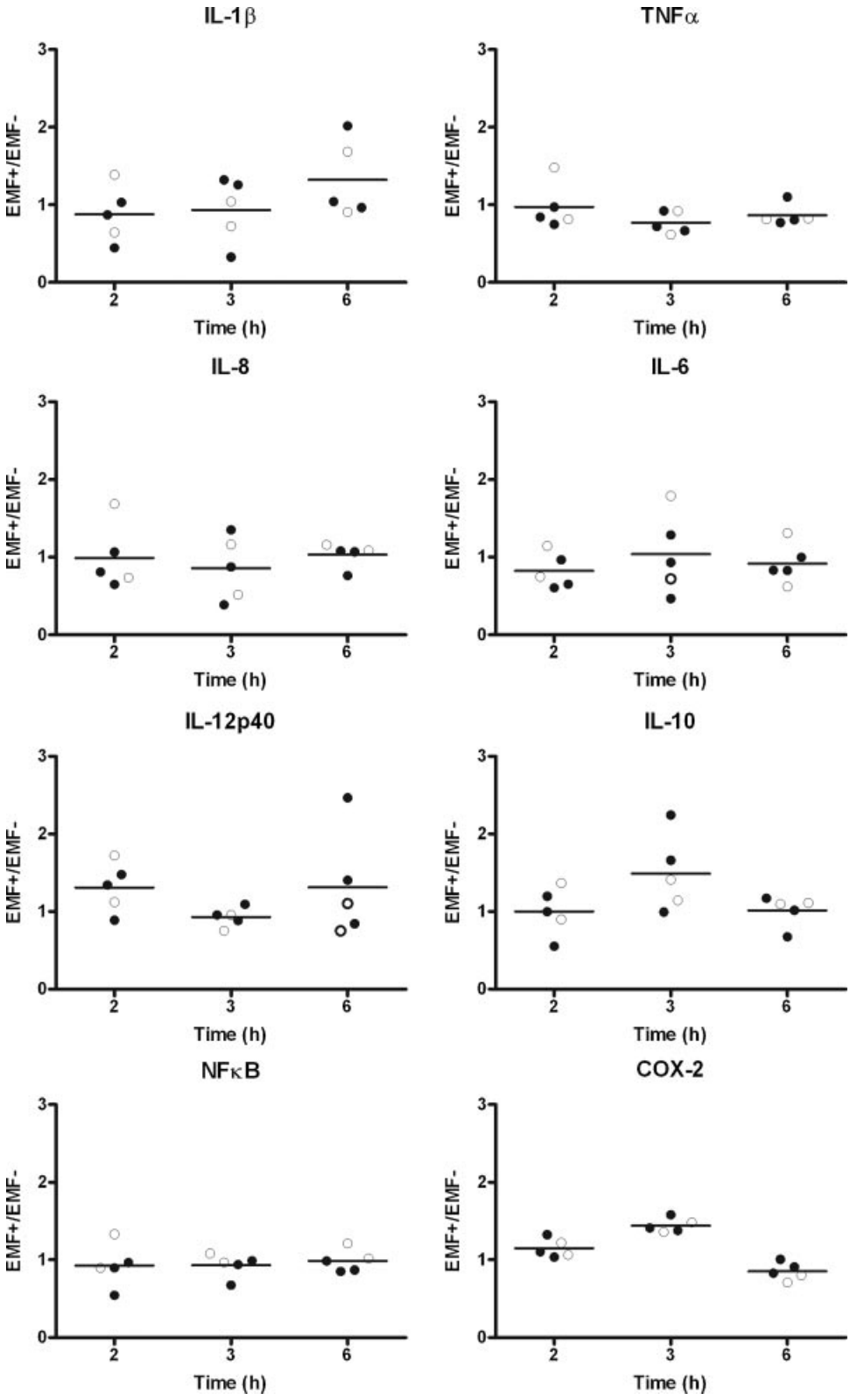

Fig. 4. Differences in gene expression between control and LF EMF (IM signal and 50 Hz sine wave, 30 min, 5 μT) on LPS-stimulated THP-1 macrophages at 2, 3, and 6 h after stimulation. Filled symbols represent IM signal, open symbols represent 50 Hz signal. Lines represent means.

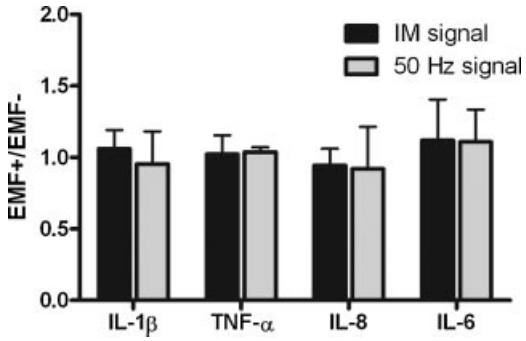

Fig. 5. Mean (+SD) differences in cytokine production between control and LF EMF (IM signal and 50 Hz sine wave, 30 min, 5 μT) on LPS-stimulated THP-1 monocytes at 6 h after stimulation. $n = 3$ for IM signal, $n = 3$ for 50 Hz signal. IL-10 and IL-12 were not expressed high enough to be detected.

in both absolute and relative gene expression values between experimental samples did not exceed normal ranges [Barksby et al., 2009]. Gene expression patterns were not altered by exposure to either of the two LF EMF signals used, in kinetics or absolute change. These findings indicate that the examined LF EMF signals do not, in a 10 μT ambient DC field, elicit effects strong enough to surmount the normal cellular variations. Although this does not necessarily mean that LF EMF do not affect cellular processes, it indicates that LF EMF are not sufficiently altering cellular signaling pathways that define cellular functioning. As a measure for cellular functioning in monocytes, expression of the studied pro- and anti-inflammatory cytokines represent important signal transduction pathways. The lack of effects of the LF

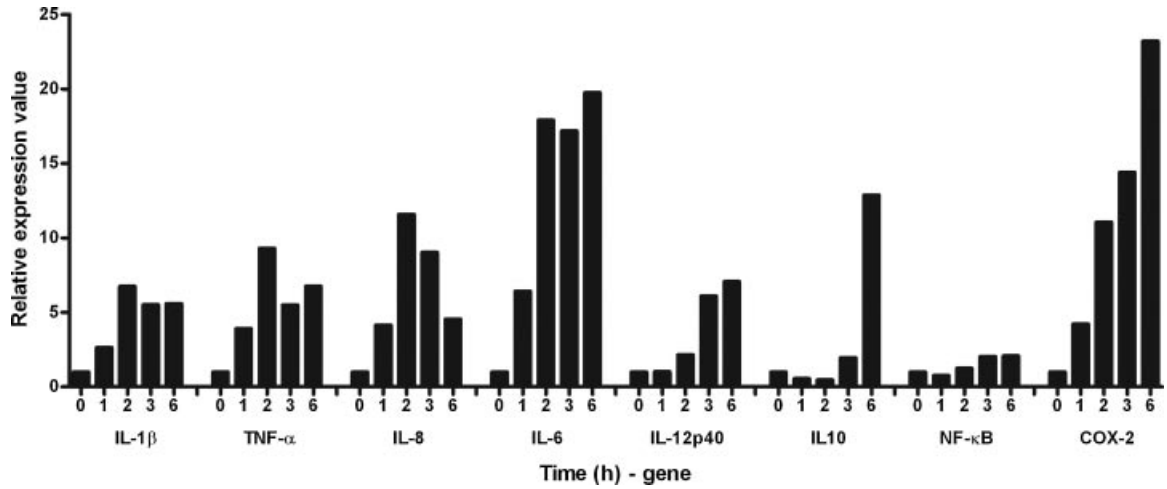

Fig. 6. Effects of LPS (1 μg/ml) on gene expression kinetics of IL-1β, IL-6, IL-8, TNF-α, IL-12p40, IL-10, NF-κB, and COX-2 in freshly isolated monocytes. A representative experiment is shown.

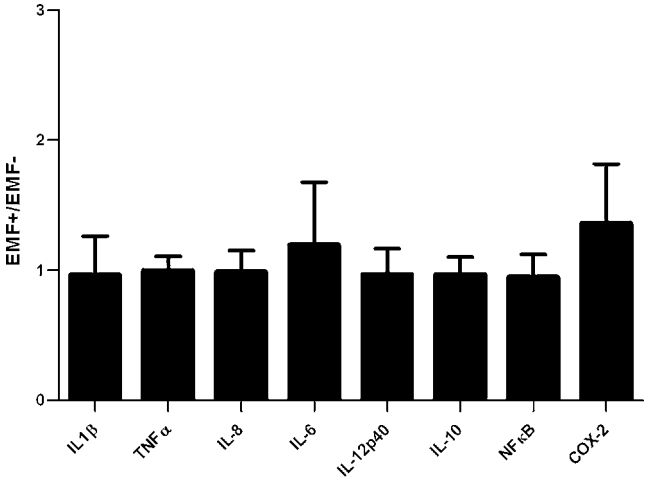

Fig. 7. Mean (+SD) differences in gene expression between control and LF EMF (IM signal, 30 min, 5 μT) on LPS-stimulated monocytes from four different individuals at 3 h after stimulation.

EMF on expression of both the pro-inflammatory cytokines IL-1β and TNFα, and the anti-inflammatory cytokine IL-10 indicates that these inflammatory pathways were not influenced by the exposure. In addition, since IL-12 is an important regulator of T lymphocyte differentiation, COX-2 is a key regulatory enzyme involved in the production of inflammatory prostaglandins, and the chemokine IL-8 is an important pro-inflammatory mediator, we can conclude that inflammatory immune processes are not affected by our signals in ways strong enough to be detected at gene or protein levels. Moreover, COX-2 and most pro-inflammatory cytokines are transcribed via NF-κB-dependent processes. We showed that NF-κB gene expression was not affected by our signals, nor were any of the NF-κB-regulated genes modulated in any of the used cell types.

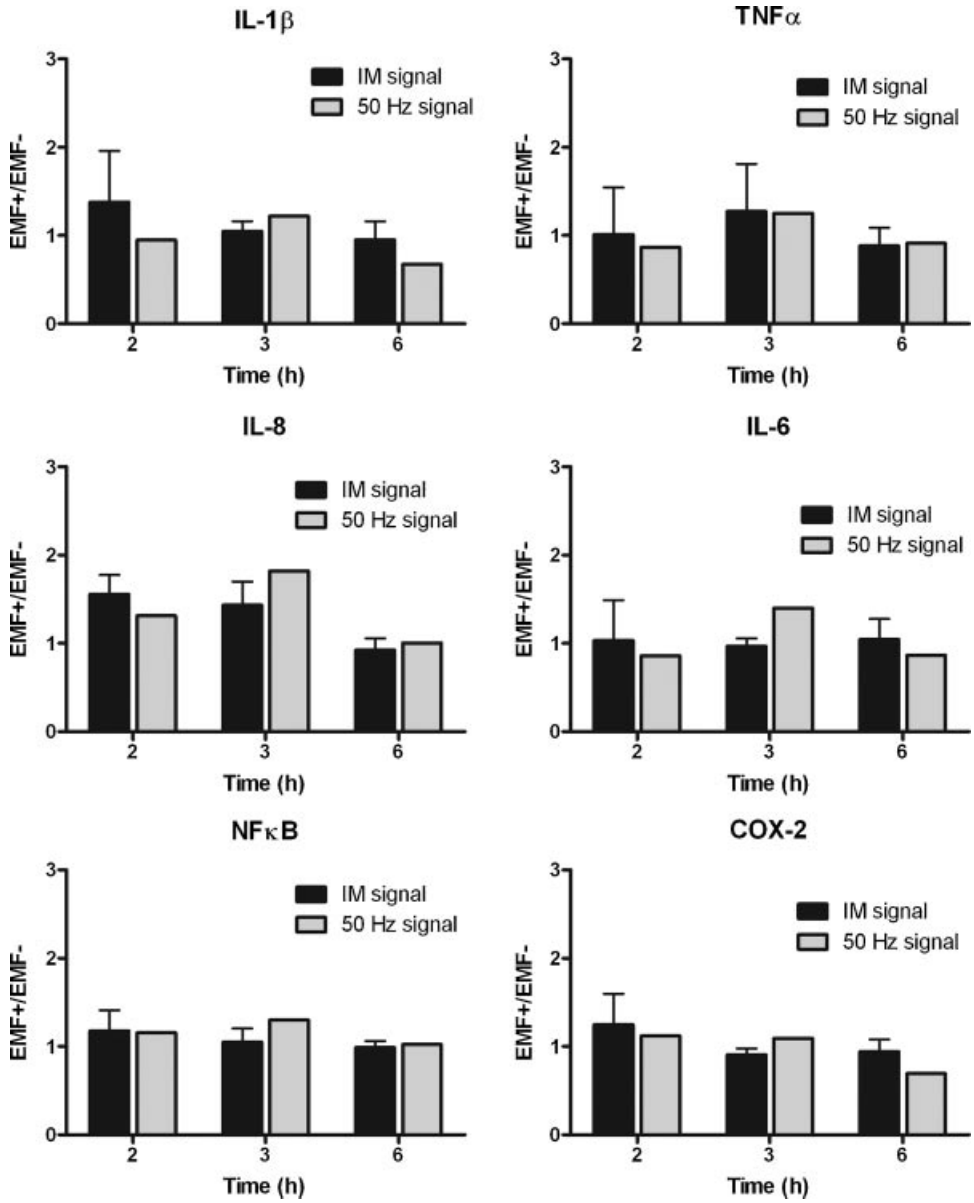

Fig. 8. Mean (+SD) differences in gene expression between control and LF EMF (IM signal and 50 Hz sine wave, 30 min, 5 μT) on LPS-stimulated detroit 562 cells at 2, 3, and 6 h after stimulation. $n = 3$ for IM signal, $n = 1$ for 50 Hz signal.

These results are in line with previously found outcomes in several studies on cytokine expression and production after LF EMF exposure. The cytokines IL-1β, IL-6, IL-10, and TNF-α were found unchanged in gene expression and protein production in LF EMF-exposed PBMCs [Pessina and Aldinucci, 1997; Ikeda et al., 2003] and rat and human lymphocytes [Jasti et al., 2001; Luceri et al., 2005]. NF-κB gene expression was also found unchanged in human monocytic cells in two separate studies [Miller et al., 1999; Natarajan et al., 2006]. Although the setup of the experimental and exposure conditions differed greatly from our study, most of these studies used a 50 Hz signal. Conversely, other studies did find differences in cytokine production after LF EMF exposure in serum [Boscolo et al., 2001], PBMCs [Cossarizza et al., 1989; Cossarizza et al., 1993; Jonai et al., 1996; Pessina and Aldinucci, 1998] and lymphocytes [Murabayashi et al., 2004], or gene expression changes in human monocytes [Lupke

et al., 2006; Reale et al., 2006] or HL-60 myeloid leukemia cells [Tokalov and Gutzeit, 2004]. Changes in the gene expression of pro-inflammatory genes were also found in skin cells [Vianale et al., 2008]. In these studies, the experimental setup and exposure conditions differed strongly from our study, but all studies used a 50 Hz signal. Interestingly, other immune cell-related responses were found to be modulated by 50 Hz LF EMF, including reactive oxygen species production in macrophages [Akan et al., 2010; Frahm et al., 2010]. The differences in the findings between these studies and our study can partially be explained by our use of 5 μT signals, compared to the milliTesla (mT) ranges most studies employ. We chose 5 μT because common daily exposure to LF EMF will mainly be experienced in comparable field strengths, ranging from 0.07 μT for average residential power-frequency magnetic fields in homes in Europe, to about 20 μT under power lines [WHO, 2007]. Moreover, effects of fields with similar strengths have been found, such as differentiation in adult cardiac progenitor cells exposed to 2.5 and 10 μT fields [Gaetani et al., 2009]. The complex IM signal has shown interesting results using this low magnetic field strength. Although our results cannot explain the previously found effects of this complex IM signal on animal health [Cuppen et al., 2007; Elmusharaf et al., 2007], they are in line with recent results showing no significant difference in immune responses, as reflected by cytokine production in PBMCs stimulated with Toll-like receptor (TLR) 2 and TLR4 stimuli and with several microorganisms, after exposure to the complex IM signal [Kleijn et al., 2011].

CONCLUSION

This study shows that neither 30 min exposure to a complex, multiple waveform signal, nor 30 min exposure to a 50 Hz sine wave signal induces any modulatory effects in cytokine gene or protein expression in an LPS-stimulated monocyte/macrophage cell line, freshly isolated monocytes, or a pharyngeal epithelial cell line. Therefore, we do not expect that these exposure conditions will modulate immune signaling pathways. Because these are crucial parameters for immune functioning, we find no evidence that these LF EMF will alter human innate immune defenses.

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