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Effects of inflammatory cytokines IFN-γ, TNF-α and IL-6 on the viability and functionality of human pluripotent stem cell-derived neural cells

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ABSTRACT

Multiple Sclerosis (MS) is an inflammatory neurodegenerative disease, where neural progenitor cell (NPC) transplantation has been suggested as a potential neuroprotective therapeutic strategy. Since the effect of inflammation on NPCs is poorly known, their effect on the survival and functionality of human NPCs were studied. Treatment with interleukin (IL)-6, tumor necrosis factor (TNF)-α and interferon (IFN)-γ did not induced cytotoxicity, but IFN-γ treatment showed decreased proliferation and neuronal migration. By contrast, increased proliferation and inhibition of electrical activity were detected after TNF-α treatment. Treatments induced secretion of inflammatory factors. Inflammatory cytokines appear to modulate proliferation as well as the cellular and functional properties of human NPCs.

1. Introduction

Multiple sclerosis (MS) is an inflammatory disease of the central nervous system (CNS) characterized by presence of focal inflammatory lesions associated with oligodendrocyte and neuron damage, astrogliosis, demyelination and axonal degeneration (Weissert, 2013). In MS lesions, the predominant cell types are T-lymphocytes and macrophages which are involved in the development of tissue damage. Additionally, activated astrocytes and microglia contribute to lesion development by secreting cytokines and other inflammatory mediators (Weissert, 2013). The demyelinated areas can be partly repaired by remyelination in the early stage of the disease, but this mechanism is impaired in the later stage of the disease (Kremer et al., 2015). As damage to neurons and oligodendrocytes and failure of the remyelination process are the neuropathological hallmarks of MS, replacement therapies using exogenous neural progenitor cells (NPC) have been suggested to be a promising approach to promote neuroprotection (Pluchino et al., 2009). In fact, multiple preclinical studies in animal models of MS have shown a beneficial effect of on the disease course (Giusto et al., 2014). One of the main obstacles in designing transplantation therapies for MS is the inflammatory nature of the disease, which has been shown to hinder endogenous remyelination (Pluchino et al., 2009). On the other hand, it has become evident that some degree of neuroinflammation is necessary for remyelination (Yong and Marks, 2010), since activated immune cells present at the site of damage may produce neurotrophic factors as well as stimulate their production in neurons and glial cells (Dooley et al., 2014). The transplanted cells will face the same environmental challenge when entering the damaged brain area.

In MS, lymphocytes and macrophages as well as CNS-resident cells such as astrocytes and microglia secrete cytokines and chemokines that may promote the inflammation but are also able to induce direct neuronal damage (Lassmann, 2014). Interferon (IFN)-γ, tumor necrosis factor (TNF)-α and interleukin (IL)-6 are classical proinflammatory cytokines that play a major role in the pathogenesis of MS by promoting neuroinflammation as well as inducing neurotoxicity (Ellwardt and Zipp, 2014; Janssens et al., 2015). All of these cytokines are detected in perivascular lesions of the MS patients (Cannella and Raine, 1995; Schönrock et al., 2000; McFarland and Martin, 2007). TNF-α and IFN-γ is secreted from the T helper (Th) 1 and Th17 cells, while secretion of IL-6 is associated to astrocytes. Their levels have been shown to increase in the blood and cerebrospinal fluid especially during the active phase of the disease (Martins et al., 2011; Becher et al., 2016). Previous preclinical and clinical studies of MS have also shown that proinflammatory cytokines may have beneficial roles in MS, since administration of monoclonal antibodies against IFN-γ to mice with experimental autoimmune encephalomyelitis, an animal model of MS,

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increased the morbidity rate (Billiau et al., 1988). Additionally, a clinical trial of anti-TNF-α targeted therapy showed significantly more aggressive course among the treated patients than among the placebo-treated patients Neurology, 1999.

There are several reports on the *in vitro* effects of cytokine stimulation on the viability, proliferation, and differentiation of human and rodent NPCs (Ben-Hur et al., 2003; Wong et al., 2004; Sheng et al., 2005; Widera et al., 2006; Johansson et al., 2008; Walter et al., 2011; Ye et al., 2013). These studies show very contradictory results because of the different study designs, the origin and type of used NPC cultures and the outcome measures used. It is, however, reasonable to conclude that the detrimental inflammatory effects of cytokines on NPCs studied *in vitro* are in line with the low success of the transplantation experiments as relatively poor cell survival, migration, differentiation, and maturation have been reported *in vivo* (Ben-Hur et al., 2003; Wong et al., 2004; Liu et al., 2005; Sheng et al., 2005). Even though these challenges exist, reported alleviation of functional deficits and delayed disease progression in animal experiments (Giusto et al., 2014) encourage continuation these *in vitro* studies.

The effects of cytokines on clinically relevant cell types should be studied in detail as there are differences in cellular responses between human and rodent NPCs (Sheng et al., 2005; Widera et al., 2006). For transplantation therapies, human-derived NPCs are needed. These can be obtained from adult nervous tissue, fetal tissue, or pluripotent stem cell origins (Kim and de Vellis, 2009). Adult and fetal cell sources are, however, difficult to harvest in large amounts. Thus, pluripotent cells, including both induced pluripotent stem (iPS) cells and embryonic stem cells (ESCs), are seen as a potential cell source for transplantation therapies because of their upscaling and differentiation properties (Erceg et al., 2011; Mattis and Svendsen, 2011). Prior to transplantation experiments, it is vital to study the effects of inflammation *in vitro* on the survival, proliferation, and maturation on the selected cell type. Importantly, the effects of cytokines should also be studied on a functional level as detrimental effects can compromise cell signaling before any effect on the genotype or phenotype is detectable (Ylä-Outinen et al., 2010).

Previous studies have focused on the cytokine mediated effect on cellular level, but as far as we know, no studies are available that shows also their effect on the neuronal network activity. Here, we studied the effects of three major inflammatory cytokines, namely, IFN- γ, TNF-α, and IL-6, on the viability and functionality of human pluripotent stem cell (hPSC)-derived neural cells *in vitro*. In addition, we aimed to determine how these cytokines affect differentiation, proliferation, expression of cytokine receptors, and secretion of different growth factors, chemokines, and cytokines. The effect of the cytokines was studied at wide concentration range (1, 10 and 100 ng/ml) to reveal their effect in the low and high concentrations. Especially, the lower concentrations are of interest, since cytokines are detected in the blood and CSF of MS patient at the pg/ml level (Cannella and Raine, 1995; Schönrock et al., 2000; Martins et al., 2011). Moreover, as most of the *in vitro* studies of NPCs have been carried out with NPC aggregates, comparison of the cytokine effect between in singe cell and aggregate culture were also included. According to our results, the present study bring novel information on how these cytokines modulates proliferation and both cellular and functional properties of human NPCs *in vitro*.

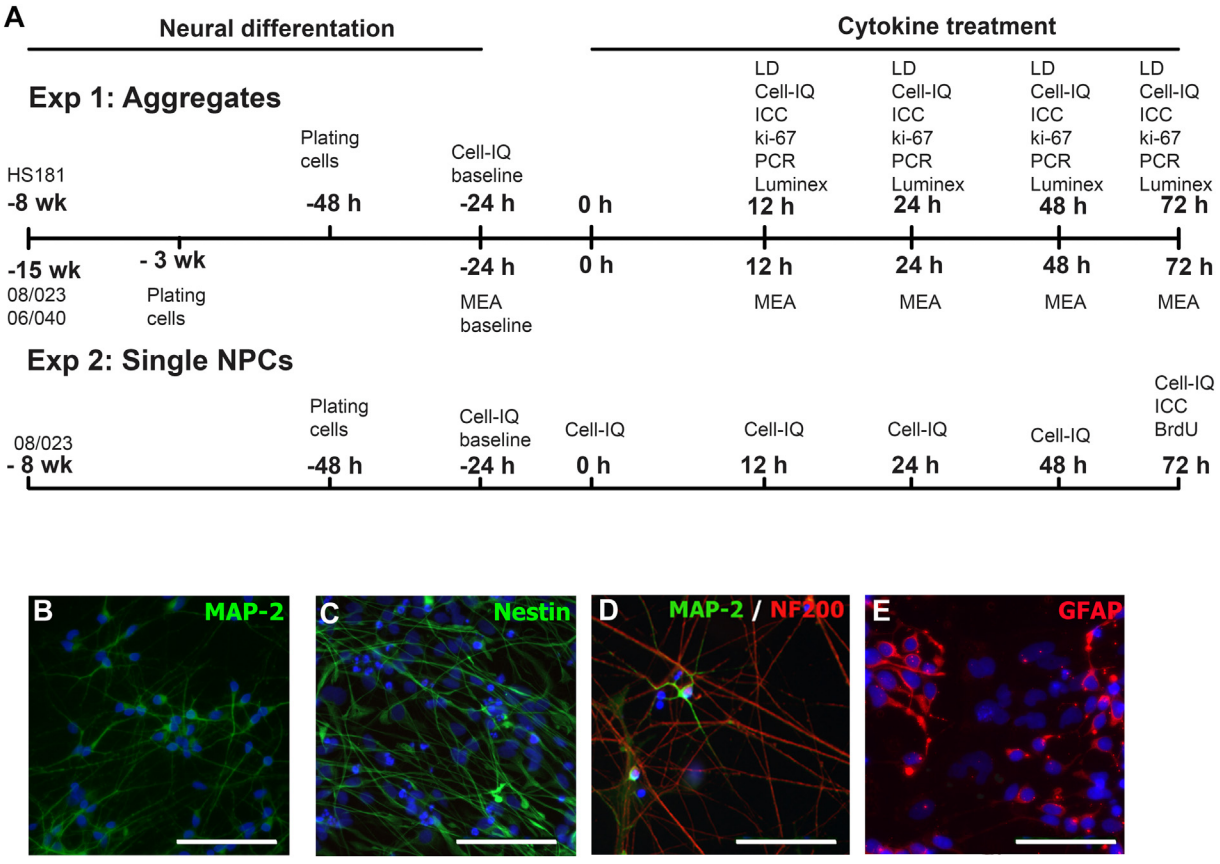

Fig. 1. Experimental design of the study (A) and characterization of NPCs (B-E). (A). hESCs were differentiated toward a neural lineage for 8–15 weeks and plated thereafter. In experiment 1 (Exp 1), NPCs (cell lines HS181, 08/023, 06/040) were studied as aggregates, and in Exp2, the cells (cell line 08/023) were dissociated into single cells before plating (A). NPCs were then treated with one of the cytokines IFN-γ, TNF-α, or IL-6 for 12, 24, 48, or 72 h. (B-E) Immunostaining of NPCs against MAP-2 (B), Nestin (C), MAP-2/NF-200 (D), GFAP (E) shows the representative figures of the Exp 1. DAPI stains nuclei blue. Scale bar 100 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

2. Materials and methods

2.1. Experimental design of the study

This study was composed of two sets of experiments, Exp1 and Exp2, in which the responses of human embryonic stem cell (hESC)-derived NPCs to the cytokines IFN-γ, TNF-α and IL-6 were studied both in aggregate and in single cell culture (Fig. 1A). In all experiments, hESCs were differentiated toward a neural lineage for 8–18 weeks, resulting in cultures containing both neurons and astrocytes (Fig. 1B-E) (Lappalainen et al., 2010). In experiments with aggregates, the effects of cytokines on cell viability and neuronal characteristics were studied through an analysis of LIVE/DEAD cell viability (LD), time-lapse imaging (Cell-IQ), immunocytochemistry (ICC), proliferation measurements with an immunocytochemical staining procedure (ki-67), gene expression of cytokine receptors and protein levels of secreted growth factors, cytokines, and chemokines (Exp 1). Additionally, the effects of cytokines on spontaneously functional neuronal networks were studied using microelectrode arrays (MEA). In single NPC studies, the effects of cytokines on cell viability and neuronal characteristics were studied using time-lapse imaging and analysis, immunocytochemistry, and proliferation measurements with an ELISA-based BrdU proliferation analysis (Exp 2).

2.2. Neuronal cell differentiation of hESC

The hESC lines HS181, derived at Karolinska Institute (Hospital Huddinge, Stockholm, Sweden, kindly provided by Prof. Hovatta), and 08/023, 06/040, 08/023, derived at Regea (University of Tampere, Tampere, Finland), were used for neuronal differentiation. The study has ethical approval from the ethics committee of the Karolinska Institute to derive, characterize, and differentiate the hESC lines, and from the ethics committee of the Pirkanmaa Hospital District to culture the hESC lines derived at the Karolinska Institute and to derive and culture new hESC lines. Furthermore, a supportive statement from the ethical committee of the Hospital District of Pirkanmaa allows derivation, culturing, and differentiation of hESC lines (R05116).

hESC lines culturing and neural differentiation was performed as previously described (Hovatta et al., 2003; Lappalainen et al., 2010; Rajala et al., 2010; Skottman, 2010). Briefly, hESC colonies were plated on 6-well Low Cell Bind plates (NuncTM, Thermo Fisher Scientific) and cultured in suspension culture in neural differentiation medium (NDM) including DMEM F12 Neurobasal medium, 2 mM Glutamax, 20 µl/ml B-27 supplement, 10 µl/ml N2 supplement (all from Gibco Invitrogen), and 0.25% penicillin/streptomycin (Lonza Group Ltd.), supplemented with 20 ng/ml basic fibroblast growth factor (bFGF) (R&D Systems).

2.3. Cell plating and cytokine stimulation

NPCs were plated on human laminin (10 µg/ml, Sigma-Aldrich, St. Louis, MO, USA)-coated 24- or 48-well plates (NuncTM, Thermo Fisher Scientific) as aggregates (Experiment 1) or single NPCs (Experiment 2). For aggregate culture, neurospheres were mechanically cut into small 50–200 m in diameter clusters, while for single cells cultures, neurospheres were dissociated with TrypLE Select enzyme (Invitrogen). The cells were allowed to grow for two days before cytokine stimulation. The adherent NPCs were treated with 0, 1, 10, or 100 ng/ml *E. coli*-derived recombinant human TNF-α, IFN-γ, or IL-6 (R&D systems, Minneapolis, MN, USA) for 12 to 72 h before the cells were collected or fixed for analysis.

2.4. Gene expression analyses of cytokine receptors

The gene expression levels of cytokine receptors for IFN-γ (IFNGR1, IFNGR2), TNF-α (TNFRSF1A, TNFRSF1B), and IL-6 (GP130, IL6R) and the housekeeping gene β-actin in NPCs (Experiment 1) were studied with reverse transcription PCR. RNA was isolated from pooled samples of two wells and analyses were done from single sample. Total cellular RNA was isolated from stored cell lysates using an RNeasy Micro Kit (Qiagen, Germany) according to the manufacturer's instructions. DNA was removed with a Qiagen RNase-Free DNase set. The total RNA was eluted with nuclease-free water, and the samples were stored at −70°C. The purity and concentration of the RNA were measured with a NanoDrop (Thermo Scientific, USA).

A total of 100 ng of RNA was reverse transcribed to complementary DNA (cDNA) using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA) with random hexamer primers, following the standard protocol. Ten nanograms of cDNA was used in a 20 µl total reaction volume for the PCR reactions. The final concentrations of the reagents were as follows: 1 × reaction buffer (10 mM Tris-HCl, 1.5 mM $MgCl_2$, 50 mM KCl, 0.1% Triton-X-100), dNTP mix (400 nm), DyNAzyme™ II DNA Polymerase (0.08 U/ml) (Finnzymes Oy, Finland) and reverse and forward primers (200 nM, TAGC, Copenhagen, Denmark). The amplification was performed in a Biometra T-Personal thermal cycler using the following cycling conditions: denaturation, 95 °C for 10 min, amplification 35 cycles: 95 °C for 1 min, 55–60 °C (annealing temperature) for 1 min and 72 °C for 1 min. The amplified samples were characterized with 1.5–2.5% agarose gel. The primers and annealing temperatures used are listed in Supplementary Table 1.

2.5. Cell viability analysis

A LIVE/DEAD® Cell Viability/Cytotoxicity Kit for mammalian cells (Molecular Probes, Invitrogen) was used to directly measure the cell viability of NPCs (Experiment 1). Calcein-AM and ethidium homodimer-1 were diluted in NDM and incubated with cells for 30 min at room temperature (RT) in the dark. The cells were imaged immediately under a fluorescence microscope (IX51, Olympus, Finland) and photographed (DP71 camera, Olympus). Calcein-AM stains living cells and emits green light at 488 nm, whereas ethidium homodimer-1 stains dead cells and emits red light at 568 nm. The cells were imaged with a 20× objective in at least 4 non-overlapping fields for each of two duplicate wells.

NPCs (Experiment 1 and 2) were monitored with time-lapse imaging using the Cell-IQ cell culturing platform (ChipMan Technologies, Finland), which allows continuous monitoring of selected cells as described previously (Supplementary Fig. 1.) (Narkilahti et al., 2007; Nat et al., 2007; Huttunen et al., 2011). One day after the cells were plated, the 24-well plates were transferred into a time-lapse imaging system and the cells were imaged for 24 h to obtain a baseline recording. The cells of interest were selected for monitoring in 500 µm × 670 µm areas using an all-in-focus imaging system, and all areas of interest were imaged at 45-min intervals. After the baseline measurement and the start of cytokine stimulation, the plates were measured for an additional 72 h. The captured images were stored in JPEG format for later use. The alterations in NPC number and neuronal length were analyzed with Cell-IQ Analyzer software. In total, 27 images per cytokine per concentration were used in the analysis over 72 h follow-up.

2.6. Neuronal network functionality analysis

The effects of cytokines on spontaneously functional networks were studied using MEA technology. First neuronal cells were plated in MEA dishes (200/30iR-Ti, Multichannel Systems, Reutlingen, Germany, MCS), as described earlier (Heikkilä et al., 2009). In this experiment, the plated cells were allowed to grow and develop into functional networks for three weeks before cytokine stimulation. Recordings were obtained with an MEA60 amplifier (MCS), and the detection of spikes was performed with MC_Rack software (MCS). The MEA measurements were performed one day before cytokine stimulation and 12, 24, 48, and 72 h thereafter without changing the culture media between measurements. The number of MEA wells per cytokine per cytokine

concentration was 3–4. At each timepoint, the neuronal networks were measured for 5 min.

2.7. Immunocytochemistry

The effects of cytokines on neural differentiation were characterized immunocytochemically by staining cells positive for microtubule-associated protein-2 (MAP-2), nestin, and glial fibrillary acidic protein (GFAP) after cytokine stimulation (Experiments 1 and 2). Briefly, the cytokine-treated or control cells were fixed with 4% paraformaldehyde (PFA) for 15 min at RT and washed twice with phosphate-buffered saline (PBS). For blocking, PBS with 10% normal donkey serum (NDS), 1% bovine serum albumin (BSA), and 0.1% Triton X-100 was used. Primary antibodies were diluted in a fresh washing solution of 1% NDS, 1% BSA and 0.1% Triton X-100 in PBS and incubated with the cells overnight at +4 °C. Secondary antibodies were diluted with fresh 1% BSA in PBS and incubated with the cells for 1 h at RT in the dark. The cells were then washed with PBS and phosphate buffer (PB) solution, dried, and mounted with Vectashield® (Vector Laboratories Inc., UK) containing diamidino-2-phenylindole (DAPI).

The primary antibodies for NPCs were rabbit anti-MAP-2 IgG (1:400, Millipore), mouse anti-nestin IgG (1:100, Chemicon-Millipore), and sheep anti-GFAP IgG (1:1000, R&D Systems). The secondary antibodies used were Alexa Fluor 488 donkey anti-rabbit IgG (1:400, Invitrogen), Alexa Fluor 488 goat anti-mouse IgG (1:400, Invitrogen), Alexa Fluor 568 donkey anti-sheep IgG (1:400, Invitrogen), and Alexa Fluor 568 donkey anti-mouse IgG (1:400; Invitrogen). The cells were imaged using an Olympus IX51 phase-contrast microscope equipped with fluorescence optics and an Olympus DP71 camera. The cells were counted in at least from 4 non-overlapping fields of one well obtained with a 20× objective. The positive cells were counted as a percentage of all DAPI-positive cells, and > 100 DAPI-positive cells were counted from each group.

2.8. Cell proliferation analysis

Cell proliferation analysis for aggregate culture was performed using a Ki-67 immunocytochemical staining procedure (experiment 1), while single cell culture analysis (experiment 2) was performed using an ELISA-based BrdU kit (Roche Applied Science).

Proliferation in the aggregate culture was studied immunocytochemically using the proliferation marker rabbit anti-Ki-67 IgG (1:400, Chemicon International) and mouse anti-nestin IgG (1:100, Chemicon-Millipore). Staining and imaging were performed as described in the immunocytochemistry section.

The proliferation rate of single NPCs was measured 72 h after cytokine stimulation using a colorimetric enzyme-linked immunosorbent assay (ELISA)-based BrdU kit (Roche, Basel, Switzerland). Briefly, single NPCs were incubated with BrdU (final concentration 10 μM) in basic NDM for 24 h. On the following day, the cells were trypsinized and transferred to a 96-well plate. The plate was centrifuged, after which the cells were dried and fixed with FixDenat solution. The cells were incubated with anti-BrdU-peroxidase for 90 min at RT. Thereafter, substrate solution was added and the colorimetric reactions were stopped with a stop solution of 1 M H_2SO_4. Finally, the absorbance was measured at 450 nm in a Victor2 1420 Multilabel Counter ELISA reader (PerkinElmer-Wallac). The cells were collected from one well and 6 technical replicates were used.

2.9. Culture medium analysis by Luminex

The culture media from treated and control cells were collected and stored at −70 ° > *C*. Medium samples pooled together from two wells and in the analysis two technical analyses were used. The levels of the cytokines IL-4, IL-6 and IL-10; the chemokines interferon gamma-induced protein 10 (IP-10) and monocyte chemotactic protein-1 (MCP-1); and the growth factors epidermal growth factor (EGF), bFGF, nerve growth factor (NGF), platelet-derived growth factor subunit BB (PDGF-BB), and vascular endothelial growth factor (VEGF) in the cell culture supernatant were analyzed with a Procarta® Cytokine Human Assay Kit (Panomics, Affymetrix Inc., Santa Clara, USA). Briefly, mixed antibody beads were added to each well as well as standards and medium and incubated for 60 min at RT. Detection antibody solution was added, and the plate was incubated for 30 min. Thereafter, streptavidin-PE solution was added and incubated for 30 min. Finally, reading buffer was added and the samples were run with a Luminex instrument (Bio- PlexTM 200 System, based on Luminex® xMAP Technology). All the data were collected and analyzed using Bio-Plex manager software 4.1 (Bio-Rad laboratories). Five-parameter regression models were used to calculate the concentrations of the molecules.

2.10. Statistical analysis

Data of the cell number analysis from live imaging were conducted with STATA program (StataCorp LP, College station, USA), while neurite length data were analyzed with SPSS version 21.0 (Armonk, NY: IBM Corp). The cell number data from time-lapse imaging were analyzed with a Poisson regression model with covariates which were determined from the baseline measurement (24 h before cytokine addition) using a linear regression model. Neurite length data were

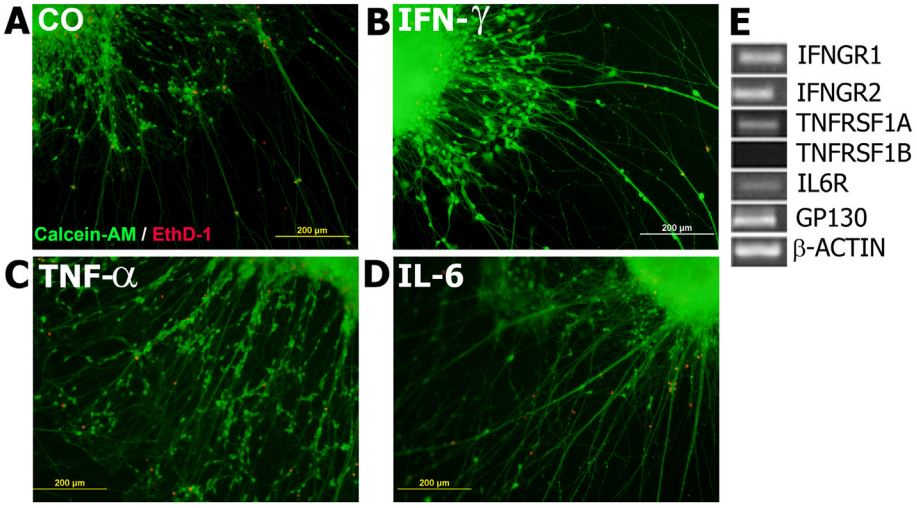

Fig. 2. IFN-γ, TNF-α, and IL-6 did not affect the viability of the NPCs. (A-D) The effects of the cytokines IFN-γ (B), TNF-α (C), and IL-6 (D) and in the control conditions (A) on the viability of NPC aggregates were analyzed with a LIVE/DEAD® viability/cytotoxicity kit. Calcein-AM (green color) stains living cells, and ethidium homodimer-1 (EthD-1, red color) stains dead cells. Each sample shown here was treated with 100 ng/ml of a particular cytokine for 72 h (A-D). Scale bar 200 μm. (E) The gene expression levels of the cytokine receptors IFNGR1, IFNGR2, TNFRSF1A, TNFRSF1B, GP130 and IL6R were studied with RT-PCR. The figure illustrates the gene expression of cytokine receptors under the control conditions. RT-PCR analyses were performed from one sample per cytokine per cytokine concentration, while LIVE/DEAD® cell viability analyses were performed in at least 4 non-overlapping fields for each of two duplicate wells. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

analyzed with ANOVA. Owing to the unequal variance of the neurite lengths, the length data were transformed to the log scale. Covariates were calculated as in the cell number analysis, with a linear regression model using transformed log values.

Group comparisons of proliferation analysis, immunocytochemistry, and active channels in the MEA analysis were performed with a Mann-Whitney *U* test. Bonferroni's correction was performed in the case of multiple comparisons, and *p*-values < .05 were considered significant. All the values were expressed as mean ± SD.

3. Results

3.1. IFN-γ and TNF-α did not induce cell death, but prevented migration of human NPCs

The effects of inflammatory cytokines on the viability and neural characteristics of NPCs were studied with a LIVE/DEAD cell viability assay, time-lapse imaging using Cell-IQ and immunocytochemistry (Fig. 1, Exp 1). Also the gene expression of cytokine receptors IFNGR1, IFNGR2, TNFRSF1A, TNFRSF1B, IL-6R, and GP130 was analyzed in the cultured NPCs. All the cytokine receptors except TNFRSF1B were detected in control and cytokine-treated cells. (Fig. 2E).

In treated samples (Fig. 2B, C, D), an increased number of dead cells

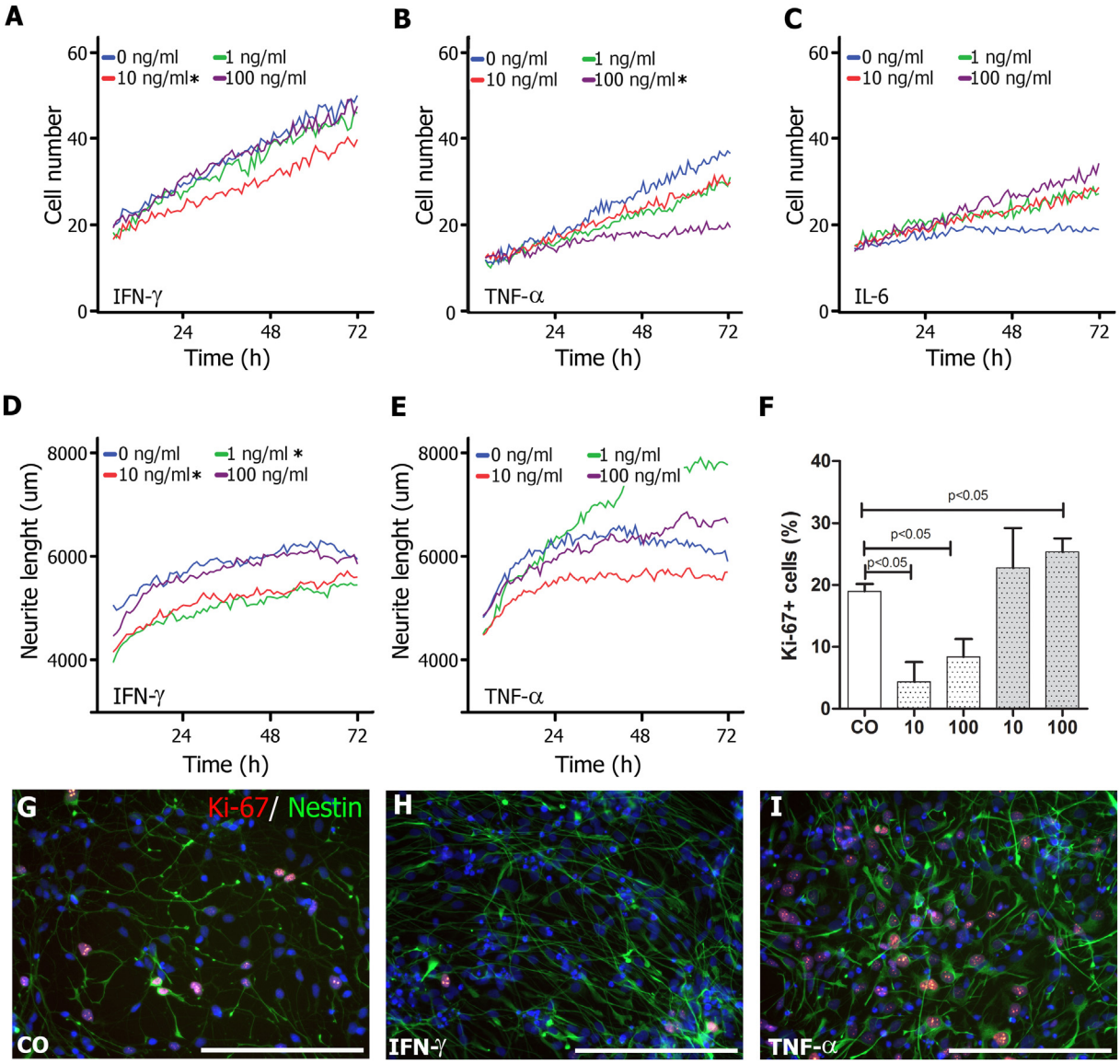

Fig. 3. IFN-γ and TNF-α had effects on neuronal migration, neurite lengths, and proliferation. The effects of cytokines on neuronal migration (A-E) and NPC proliferation (F—I) were studied using time-lapse imaging (Cell-IQ) and immunocytochemistry, respectively. The NPCs were treated with IFN-γ, TNF-α, or IL-6 at concentrations 0, 1, 10, and 100 ng/ml for 72 h. (A-E) In the Cell-IQ analyses, the cells were imaged continuously 24 h before cytokine addition and for 72 h thereafter. Neuronal migration was analyzed with a Poisson regression model (A-C), and neurite lengths with an ANOVA model (D-E). In the analyses, total 27 images per cytokine per concentration were used in the analysis over 72 h follow-up. Figures of cell number (A-C) and neurite length (D-E) were statistical model based that were adjusted with baseline measurement. (F-I) For cell proliferation analysis, NPCs were double labeled for the proliferation marker Ki-67 and neural stem and the progenitor cell marker nestin at 72 h timepoint. The cells were imaged and counted in at least from 4 non-overlapping fields of one well obtained with a 20× objective. The bar graph displays the mean (± SD) number of Ki-67+ cells out of all DAPI-positive cells (F). Representative figures of Ki-67+ (red color) co-labeling with nestin (green color, G-I) shows the 100 ng/ml cytokine treatments at 72 h timepoint. DAPI stains nuclei blue. Scale bar 200 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

(red color) compared with untreated controls was not detected (Fig. 2A). In all the samples, most of the cells were viable (green color, Fig. 2A-D, Supplementary Fig. 2). Since the cytokines did not affect the viability of the NPCs, the effect of the cytokines on neuronal migration from the attached aggregates was determined next using time-lapse imaging (Fig. 3A-E; Supplementary Video 1 and Supplementary Video 2). Cell number analyses (Fig. 3A-C) showed that IFN-γ at concentration of 10 ng/ml (coefficient − 0.4, p = 0.040) and TNF-α at concentration 100 ng/ml (coefficient − 0.6, p = 0.005) decreased the neuronal migration in comparison to untreated samples. IL-6 did not have any effect on the neuronal migration, and therefore IL-6 was excluded from the subsequent analyses. Neurite length analyses (Fig. 3D-E) showed that IFN-γ at concentrations of 1 ng/ml and 10 ng/ml (p = 0.005 and p = 0.010, respectively) inhibited the growth of the neurites, whereas TNF-α did not have any impact ($p > 0.05$ in all cases).

To determine whether cytokines promote NPC proliferation, cultures were double labeled for the proliferation marker Ki-67 and the neural stem/progenitor cell marker Nestin at the 72 h timepoint (Fig. 3G-I). The number of Ki-67-positive cells was counted as a percentage of DAPI-positive cells (Fig. 3F). IFN-γ at concentrations of 10 ng/ml (4.4 ± 6.4 *vs.* 19.0 ± 2.3%, p = 0.021) and 100 ng/ml (8.4 ± 2.9 *vs.* 19.0 ± 2.3%, p = 0.021) significantly decreased, whereas TNF-α at concentration 100 ng/ml (25.4 ± 4.4 *vs.* 19.0 ± 2.3%, p = 0.021) significantly increased, the proliferation of the NPCs compared with the control.

Immunocytochemical staining was performed on treated and untreated samples in order to characterize the effects of cytokines on neural differentiation. In the control conditions, NPCs, Nestin

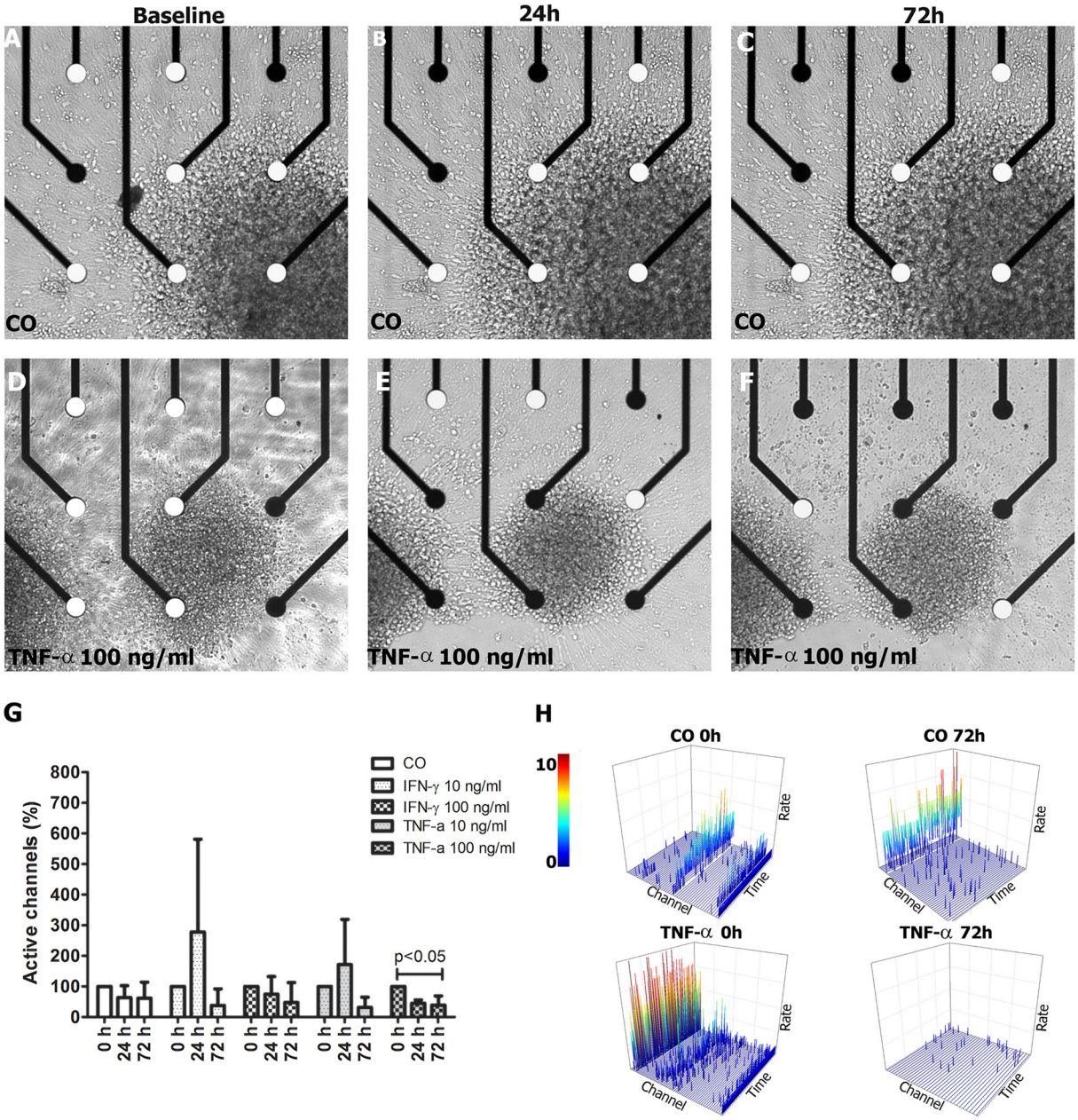

Fig. 4. The effects of IFN-γ and TNF-α on the electrical activity of neural networks were studied using MEA. (A-F) The figure illustrates a functional neuronal network and active electrodes. White electrodes represent actively signaling sites, and black electrodes detect no electrical signals. (G) Normalized percentages of active channels for controls, IFN-γ-treated, and TNF-α-treated neuronal networks on MEA. TNF- α (100 ng/ml) inhibited neuronal network activity at the 72 h timepoint. (H) Representative examples of activity on MEA for control (0 h and 72 h) and TNF-α-treated (100 ng/ml, 0 h and 72 h) neuronal networks. * $p < 0.05$ compared with controls. The number of MEA wells per cytokine per concentration was 3–4.

(62.5 + 5.0%) and MAP-2 (29.2 + 12.8%)-positive neuronal cells were detected in all the samples studied (Fig. 1B-D). Additionally, some GFAP-positive astrocytes (3.4 + 4.7%) were detected in the untreated samples (Fig. 1E). No obvious difference of neural differentiation among controls and cytokine treated NPCs was detected (Supplementary Fig. 3).

3.2. The cytokine treatments caused inhibition of spontaneous neuronal network activity

The effects of IFN-γ and TNF-α on spontaneous neuronal network activity were studied using microelectrode arrays (MEA). For that purpose, differentiated NPCs were cultured for three weeks on MEA dishes to allow them to develop into functional networks, and thereafter they were treated with cytokines. TNF-α (100 ng/ml)-treated cultures showed decreased activity after 72 h (Fig. 4G), but IFN-γ did not showed any statistical significance. A representative example of control (Fig. 4A-C) *vs.* 100 ng/ml TNF-α (Fig. 4D-F) is shown as reduction of white-labeled active electrodes and as a representative activity recording (Fig. 4H).

3.3. Cytokine stimulates secretion of inflammatory molecules from NPCs

To understand whether cytokines can influence the immunomodulatory responses of NPCs, the secretion of cytokines, chemokines, and growth factors was analyzed at the protein level from

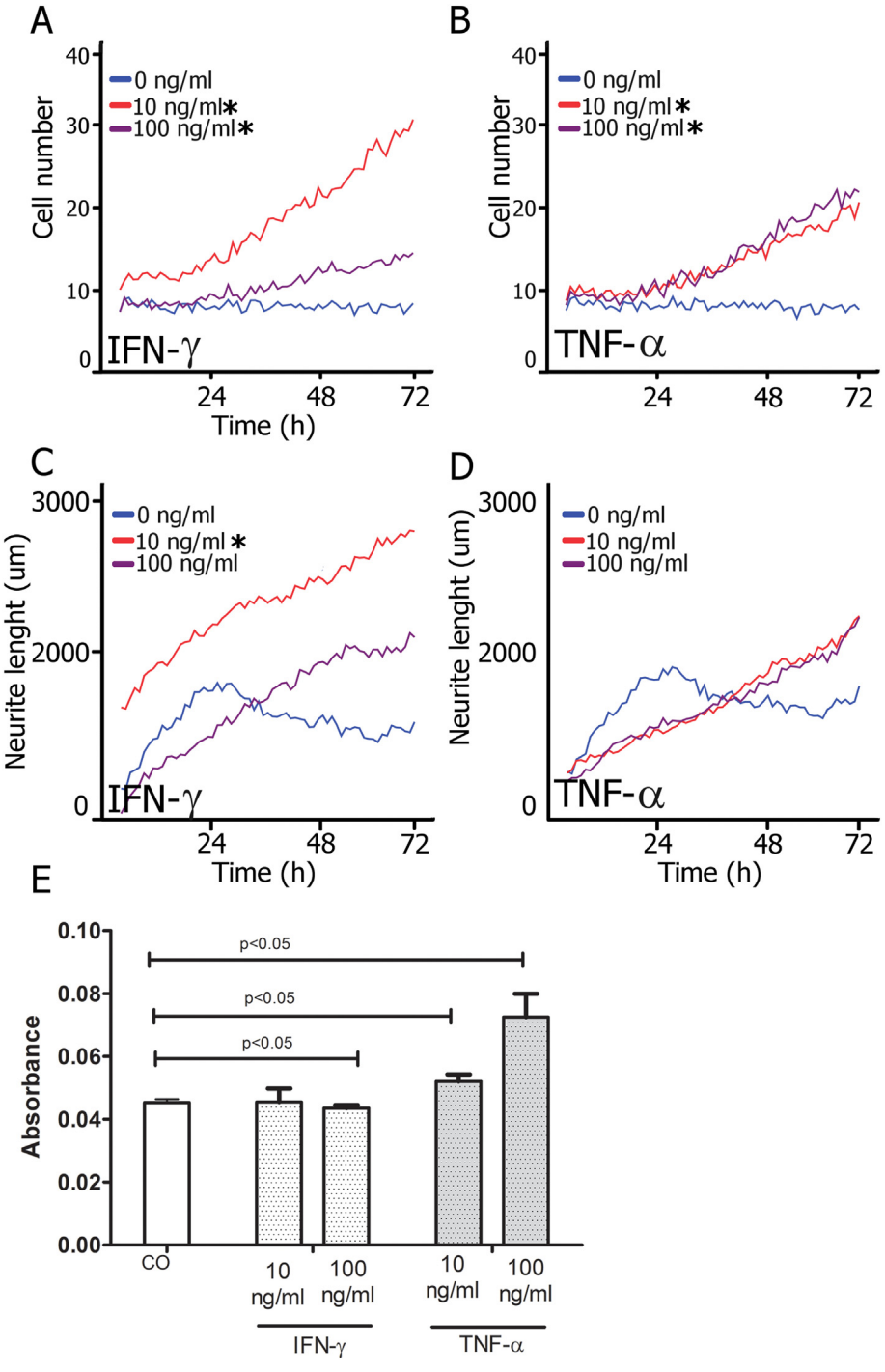

Fig. 5. The effects of IFN-γ and TNF-α on cell growth (A-D) and proliferation (E) in single cell NPC cultures were studied. (A-D) In the Cell-IQ analyses, the cells were imaged continuously for 24 h before the start of cytokine stimulation and for 72 h thereafter. Neuronal migration was analyzed with a Poisson regression model (A-B), and neurite growth with an ANOVA model (C-D). Figures of cell number (A-B) and neurite length (C-D) were statistical model based that were adjusted with baseline measurement. (E) The effects of the cytokines on cell proliferation were determined with ELISA-based BrdU proliferation analysis. Bar graphs display the mean (± SD) absorbance of samples after 72 h. In the live-imaging analysis, 27 images per cytokine per concentration were used in the analysis over 72 h follow-up, while in the BrdU analysis the cells were collected from one well and 6 technical replicates were used.

conditioned medium (Table 1, Supplementary Fig. 4.). Five out of 11 studied molecules (IL-4, bFGF, NGF, and PDGF-BB) were undetectable in both the control and treated conditions. The EGF was already detectable at a low level in the control condition, and cytokine stimulation did not have a significant influence on its secretion. IFN-γ induced secretion of VEGF, IP-10 and MCP-1 within 72 h. TNF-α induced the secretion of IL-6, IP-10 and MCP-1, while IL-6 induced the secretion of VEGF and IL-10.

3.4. IFN-γ and TNF-α stimulate the growth of single NPCs

In addition to the analyses with the aggregate culture, the effects of IFN-γ and TNF-α to NPCs in single cell cultures were also studied (Fig. 1, Exp 2). In single cell analyses, cells were studied with time-lapse imaging (Fig. 5A-D), a BrdU assay (Fig. 5E) and immunocytochemistry after 72 h treatments. Cell number analyses showed that IFN-γ at concentrations of 10 ng/ml and 100 ng/ml (coefficient 0.7, $p < 0.0001$ and coefficient 0.2, $p = 0.048$, respectively) significantly increased the neuronal migration during the 72 h follow-up compared with untreated samples (Fig. 5A). Likewise, TNF-α at concentrations of 10 ng/ml and 100 ng/ml (coefficient 0.5, $p < 0.0001$ and coefficient 0.5, $p < 0.0001$, respectively) significantly stimulated neuronal migration (Fig. 5B). However, only IFN-γ at a concentration of 10 ng/ml increased the neurite length compared with untreated samples ($p < 0.001$, Fig. 5C).

IFN-γ at a concentration of 100 ng/ml slightly decreased the cell proliferation after 72 h ($p = .032$), while TNF-α at concentrations of 10 ng/ml and 100 ng/ml increased it ($p = .008$ in both comparisons), compared with control cells (Fig. 5E). Finally, immunocytochemistry analyses did not show any effect on the number of nestin- or MAP-2-positive neuronal cells or GFAP- positive astrocytes (data not shown).

4. Discussion

In the present study, we were able to show that the proinflammatory cytokines IFN-γ, TNF-α, and IL-6 did not cause extensive cell death in human NPC cultures. In fact, increased proliferation was detected in TNF-α-treated NPCs. The NPCs responded to cytokine treatment by secreting the growth factor VEGF, the chemokines IP-10 and MCP-1, and the cytokines IL-6 and IL-10. The cytokine treatments also caused inhibition of spontaneous neuronal network activity. Thus, it seems that these cytokines modulate proliferation and both cellular and functional properties of human NPCs *in vitro*.

The data about the effects of IFN-γ, TNF-α and IL-6 on neuronal cells is contradictory or has not been studied in details (Ben-Hur et al., 2003; Wong et al., 2004; Sheng et al., 2005; Widera et al., 2006; Walter et al., 2011; Ye et al., 2013; Kulkarni et al., 2016). First, expression of receptors for the cytokines IFN-γ, TNF-α and IL-6 in NPCs were studied; these receptors are necessary to transduce the signals to the cells. All of them except TNFRSF1B were expressed constitutively in NPCs. Previously, it has been reported that both of the TNF receptors are constitutively expressed at the mRNA level in human NPCs, but at the protein level TNFRSF1A is more prominent (Sheng et al., 2005; Guadagno et al., 2013). Also protein expression of IFNGR1 has been reported (Mäkelä et al., 2010). Thereafter, the effects of the cytokines IFN-γ, TNF-α, and IL-6 on the viability of NPCs was studied, revealing that neither of the cytokine caused substantial cell death in human NPC sphere cultures.

However, these cytokines influenced the proliferation of human NPCs. TNF-α increased proliferation, while IFN-γ decreased proliferation, in both single and aggregate cultures. In previous studies, TNF-α has contradictorily been shown to decrease, increase or have no effect on proliferation (Ben-Hur et al., 2003; Wong et al., 2004; Widera et al., 2006; Johansson et al., 2008), while IFN-γ has either decreased or had no effect on proliferation (Ben-Hur et al., 2003; Wong et al., 2004; Widera et al., 2006; Johansson et al., 2008). In consistent with our findings, a recent study showed that IFN-γ signaling *via* IFNGR1 and IFNGR2 receptors activates the JAK-STAT1 pathway, which eventually inhibits the proliferation of NPCs (Kulkarni et al., 2016). TNF-α treatment has been shown to activate the nuclear factor kappa B pathway, which eventually leads to survival of the NPCs (Shih et al., 2015). However, discrepancies between reported results depend on many parameters, such as cell type, origin, media, and differentiation phase. Although the cytokines did not induce cell death of the NPC *in vitro*, *in vivo* situation might be different due to their effect also on the immune cells and CNS-resident cells like microglial cells and astrocytes. These cells may start to produce toxic substances like NO and complement components that are known to be toxic for the neuronal cells (Becher et al., 2016). Moreover, it is noteworthy that used concentrations in this *in vitro* study (ng/ml) varies from the measured ones *in vivo* (pg/ml) and therefore extrapolating results directly to *in vivo* situation should be made with caution. Taken together, our data and those of others imply that IFN-γ and TNF-α can have pleiotropic effects on NPC survival and proliferation *in vitro* depending on their developmental and maturation stage and origin. This poses great challenges for transplantation therapies, as the inflammatory stage in the CNS can alter according to the disease stage, which further complicates the selection of optimal timing for transplantation.

In addition to cell viability, IFN-γ and TNF-α have been shown to affect neuronal cell migration and neurite outgrowth *in vitro* (Ben-Hur et al., 2003; Wong et al., 2004). In previous studies, neurons and neurites outgrowing from the plated aggregates were counted at a single timepoint. Here, we took advantage of automated time-lapse imaging and analysis (Huttunen et al., 2011) and performed continuous follow-up for 72 h after different cytokine treatments. IFN-γ slightly reduced the neuronal outgrowth rate from aggregates at a concentration of 10 ng/ml, while cell migration was not affected with the highest concentrations. In contrast to our study, Ben-Hur showed increased neuronal outgrowth at a 48 h timepoint after IFN-γ treatment (100 ng/ml) using newborn rat striatal NPCs (Ben-Hur et al., 2003). In addition, the neurite outgrowth was reduced after IFN-γ (1 and 10 ng/ml) treatment, in contrast to a study by Wong and colleagues (Wong et al., 2004) showing increased neurite outgrowth at a 72 h timepoint in IFN-γ-treated NPCs (100 ng/ml) derived from the supraventricular zone of adult mice. Since IFN-γ-treated NPCs inhibited cell proliferation as well as cell migration and neurite outgrowth from the aggregates, it is reasonable to conclude that IFN-γ has an inhibitory role in the cellular functions of NPCs. In addition, TNF-α-treated NPCs (100 ng/ml) showed reduced neuronal outgrowth from aggregates during 72 h follow up but did not show any other significant effects on neurite outgrowth. By contrast, Ben-Hur (Ben-Hur et al., 2003) found increased neuronal outgrowth in aggregates at a 48 h timepoint after TNF-α treatment (100 ng/ml) using newborn rat striatal NPCs. The effect of IL-6 on neuronal migration properties has not been addressed before. According to our results, it does not have any influence on neuronal or neurite outgrowth. In summary, it seems that IFN-γ and TNF-α can have more harmful effects on human neuronal migration properties than previously suggested.

Next, the effects of cytokine stimulation on the functionality of neuronal networks were studied. Previously, Walter and colleagues have shown that IFN-γ pretreatment for 7 days during early neuronal stem cell differentiation impairs the development of functional neuronal networks to a fully mature stage (Walter et al., 2011). In an extension of these results, we report that both IFN-γ and TNF-α treatments tended to have biphasic effects on existing functional networks as they first stimulated and then inhibited the network functionality during the 72 h follow up. In line with our observation, recent reports have suggested that TNF-α enhances glutamate-mediated excitotoxicity of neuronal cells (Ye et al., 2013). In fact, spontaneous neuronal activity is inhibited by high concentrations of glutamate (Tukker et al., 2016). These data would suggest that if the aim of transplantation therapy is regeneration, inflammation needs to be controlled to levels at which the

transplanted cells will be able to rebuild the existing circuits.

NPCs in general are known be able to respond to inflammation not only by interacting with immune cells but also by secreting various factors that contribute to the bystander effects observed after transplantations (Pluchino and Cossetti, 2013). Interestingly, we were able to show that cytokine stimulation induced secretion of certain growth factors, cytokines and chemokines. Secretion of the growth factor VEGF, which is known to contribute to neuroprotection by reducing oxidative stress (Madhavan et al., 2008), was upregulated in IFN-γ- and IL-6-treated NPCs after 72 h. In line with previous studies with human fetal NPCs (Sheng et al., 2005; Turbic et al., 2011; Pugazhenthi et al., 2013), secretion of the chemokines MPC-1 and IP-10 was detected in response to TNF-α and IFN-γ. Both MPC-1 and IP-10 are primarily known to regulate the migration of immune cells to damaged areas, but they also have regulatory effects on neuronal cell proliferation, survival and migration (De Haas et al., 2007). In addition, TNF-α induced IL-6 secretion; this response is most likely related to the activation of the nuclear factor kappa B pathway, which mediates inflammatory events (Pugazhenthi et al., 2013; Cheng et al., 2014) and thereby induces secretion of the proinflammatory cytokine IL-6 (Daniele et al., 2015). Production of the anti-inflammatory cytokine IL-10 was detected in IL-6-treated NPCs. IL-10 has key functions in balancing the pro- and anti-inflammatory responses, but it has also shown to reduce neuronal differentiation and ultimately to impair neurogenesis (Perez-Asensio et al., 2013). Interestingly, the human NPCs seemed to have endogenous EGF secretion that was not affected by the cytokine stimulation, whereas endogenous FGF, PDGF-BB, NGF and IL-4 secretion was not detected within the detection limit of the method. Moreover, it is possible that cytokine-treatment may induce secretion of several molecules at low levels, but they are quickly reabsorbed by the NPCs and were therefore undetectable. Moreover, NPC cultures are never purely neuronal, in our study NPC culture consist 3% of the GFAP- positive astrocytes, therefore, it is likely that some of the cytokine-mediated effect comes from astrocyte activation (Roybon et al., 2013). The secretion of soluble factors by human NPCs most likely explains their good cell survival rates after cytokine treatments.

Inflammatory cytokines did not have any effect on the differentiation of NPCs as determined by percentage of cells labeled against specific markers of neurons and astrocytes. The previous studies have shown contradictory results on whether these cytokines have any effect on differentiation (Ben-Hur et al., 2003; Wong et al., 2004; Widera et al., 2006); the discrepancies are most likely due to the different cells types used.

Among the main challenges in NPC replacement therapies are the route of NPC administration and the form (small cell aggregates *vs.* single cells injections) of the NPCs. Most of the *in vivo* and *in vitro* studies of NPCs have been performed with NPC aggregates, and data about single cell cultures are lacking. Here, opposing effects were found *in vitro* when IFN-γ- and TNF-α-treated NPCs were cultured in aggregates or single cell cultures. The decreased cell number in the NPC sphere study may be explained by the fact that NPCs are drawn back to their central sphere in an inflammatory environment. This might result in decreased cell number, because the total number of neurons could not be measured from the central aggregates. In both experiments, proliferation rates were on the same order of magnitude. To conclude, IFN-γ seems to lack dose-dependent effect in cell migration and neurite outgrowth analysis in both aggregate and single cell cultures, since the most significant results were found with concentration 10 ng/ml in comparison to concentrations of 1 and 100 ng/ml. The observation may be related to biphasic function of IFN-γ, that need to study more detailed in the future studies.

In conclusion, inflammatory cytokines appear to modulate proliferation as well as both cellular and functional properties of human NPCs *in vitro*. These aspects should be taken into account in *in vitro* and *in vivo* studies en route to the design of clinical treatments for MS. Additionally, as profound inflammation is a hallmark of many other neurodegenerative diseases and insults such as stroke, Alzheimer's disease, and Parkinson's disease, these results will also benefit studies in those areas.

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