Biofabrication

PAPER

Sensor macrophages derived from human induced pluripotent stem cells to assess pyrogenic contaminations in parenteral drugs

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Abstract

Ensuring the safety of parenteral drugs before injection into patients is of utmost importance. New regulations around the globe and the need to refrain from using animals however, have highlighted the need for new cell sources to be used in next-generation bioassays to detect the entire spectrum of possible contaminating pyrogens. Given the current drawbacks of the Monocyte-Activation-Test (MAT) with respect to the use of primary peripheral blood mono-nuclear cells or the use of monocytic cell lines, we here demonstrate the manufacturing of sensor monocytes/macrophages from human induced pluripotent stem cells (iMonoMac), which are fully defined and superior to current cell products. Using a modern and scalable manufacturing platform, iMonoMac showed typical macrophage-like morphology and stained positive for several Toll like receptor (TLRs) such as TLR-2, TLR-5, TLR-4. Furthermore, iMonoMac derived from the same donor were sensitive to endotoxins, non-endotoxins, and process related pyrogens at a high dynamic range and across different cellular densities. Of note, iMonoMac showed increased sensitivity and reactivity to a broad range of pyrogens, demonstrated by the detection of interleukin-6 at low concentrations of LPS and MALP-2 which could not be reached using the current MAT cell sources. To further advance the system, iMonoMac or genetically engineered iMonoMac with NF-κB-luciferase reporter cassette could reveal a specific activation response while correlating to the classical detection method employing enzyme-linked immunosorbent assay to measure cytokine secretion. Thus, we present a valuable cellular tool to assess parenteral drugs safety, facilitating the future acceptance and design of regulatory-approved bioassays.

1. Introduction

Human induced pluripotent stem cells (hiPSCs) have been a game-changing technology in the medical field. The iPSC technology paved the way for deriving new advantageous cells for modern cell-based therapies [1–3], or for a variety of non-therapeutic applications including drug discovery and/or potency [4, 5]. In fact, the constant development of superior stem cell-based human *in vitro* bioassays and the global change to refrain from using animals has changed the regulators' point of view to accept stem cell-based assays stepwise to release new drugs into the clinics (e.g. FDA Modernization Act 2.0) [6]. While iPSCs are now frequently used in pre-clinical bioassays, iPSC-derivatives have not yet been introduced in a bioassay, which is approved by different pharmacopeias and regularly requested by regulatory authorities for drug batch release [7]. The Monocyte-Activation-Test (MAT) [8] is one example of such bioassays that is approved and mandatorily requested by regulators to ensure the safety of parenteral drugs prior to drug batch release. While standard safety screening- and batch release- tests heavily rely either on animals (such as the rabbit pyrogen test; RPT), or insufficient formats which can detect only endotoxins but not non-endotoxins (such as the Limulus amoebocyte lysate (LAL) or Recombinant factor C assay; rFc test) [8], the MAT aims to replace such sub-optimal and animal based safety testing with a more physiological and human *in vitro* test. Indeed, the MAT relies on the sensitivity of human monocytes/macrophages to respond to contaminating pyrogens in drug formulations, providing a more accurate and reliable tool. As such, the pyrogenicity and thus safety of the formulation will be assessed based on the detection of pro-inflammatory cytokines such as interleukin-6 (IL-6), IL-1β, and TNFα that typically mediate a pyrogen fever reaction in the human organism. Current efforts by the European Pharmacopeia (EP), the European Medical Agency, the World health organization (WHO), and several National Release authorities like German Paul Ehrlich-Institute (PEI, Germany), NIBSC (UK), NOMA (Norway), ISS (Italy) and others, favor the future use of the MAT for drug release and to ban the use of animals. The EP decided to erase the RPT by 2026 [9], replacing it with the MAT. Current sources of macrophages however, challenge the standardization and broad industrial applicability of this *in vitro* test. In fact, the use of primary monocyte-derived macrophages (MDM) is limited by donor availability, low isolation yields, and in particular donor-to-donor variability [10]. As an alternative, the immature neoplastic monocyte cell line (Mono-Mac-6; MM6) has been introduced, which is however, highly prone to genetic mutation upon long-term passaging [11], while not reflecting the physiological innate immune response. Given the insufficient cell sources available for the MAT, we here introduce the beneficial and superior use of highly standardized human iPSC-derived sensor macrophages (iMonoMac) in the context of MAT for drug safety evaluation.

Our work highlights not only the beneficial use of the iPSC technology for MAT but also extends the use of iPSC in the context of regulatory-approved assays in general.

2. Materials and methods

2.1. iPSC cultivation and differentiation to iMonoMac

The present study utilized six different human iPSC cell lines that were previously generated and fully characterized, as follows: iPSC line#1: MSN01-02S (https://hpscreg.eu/cell-line/ISMMSi004-A) [12], iPSC line#2: LiPSC-GR1.1(https://hpscreg.eu/cell-line/RUCDRi002-A) [13], iPSC line#3:CD34^{+}hPBHSC_GMPDU_SeViPS8(https://hpscreg.eu/cell-line/MHHi008-A) [14], iPSC line#4: hCD34iPSC16 (https://hpscreg.eu/cell-line/MHHi015-A) [15], iPSC line#5: MSN19-07S (https://hpscreg.eu/cell-line/ISMMSi022-A) [12], iPSC line#6: iPAP5.2 [15]. To induce the hematopoietic differentiation from iPSC lines #3, 4 and 6 the previously published differentiation protocol was used [16]. In brief, iPSC cells were expanded in 6-well plates, and after reaching almost 70% confluency the iPSCs were collected to start the embryoid body formation (EB). EBs were maintained on orbital shaker in knockout DMEM medium (Gibco, Thermo Scientific, USA, catalogue# 10829-018), supplemented with 20% knockout serum replacement (Gibco,Thermo Scientific, USA; catalogue# 10828-028), 1 mM L-glutamine (Invitrogen, ThermoFisher Scientific, USA, catalogue# 25030-024), 1% NEAA(Invitrogen, catalogue# 11140-035), 1% penicillin/streptomycin (Gibco,Thermo Scientific, USA, catalogue# P11-010PAA), 0.1 mM β-mercaptoethanol (Thermo Scientific, USA, catalogue# 31350-010), and 10 μM Y-27632 (Tocris, catalogue# 1254/50). On the fifth day of EB culture, EBs were selected with a binocular, and were placed in a new 6-well plate containing X-VIVO 15 medium (Lonza, Germany, catalogue# BE02-060F), supplied with 1% penicillin-streptomycin (P/S), 1 mM L-glutamine, 0.05 mM β-mercaptoethanol (Sigma-Aldrich, ThermoFisher Scientific, USA), 25 ng ml^{-1} hIL-3 (Peprotech, UK, catalogue# 200-03), and 50 ng ml^{-1} hM-CSF (Peprotech, UK, catalogue# 300-25). This mixture constituted the differentiation medium I. The medium was refreshed once per week, and after 7–10 days from seeding, the EBs continuously produced iMonoMac, which were collected on a weekly schedule. The collected iMonoMac were seeded in terminal differentiation medium II composed of RPMI 1640

medium (ThermoFisher Scientific, USA, catalogue# 21875-034) supplemented with 10% fetal calf serum (FCS), 1% P/S, and 50 ng ml^{-1} of hM-CSF. The iPSC lines #1, 2, 5 were subjected to feeder free culture and differentiation generating macrophages using the previously published protocol [17]. Briefly, after expanding iPSCs on Geltrex™ coated flasks, the cells were collected and re-seeded at 500 000 cell per well of a 6- well CELLSTAR plate. For the first day, the cells were cultured on an orbital shaker at 70 rpm in mesoderm priming I medium composed of E8 medium (prepared as DMEM/F-12 (Gibco, Life Technologies, catalogue# 12634-010), with 64 mg l^{-1} ascorbic acid 2-phosphate, (Sigma-Aldrich, catalogue# A8960-5G), 14 μg l^{-1} sodium selenite(Sigma-Aldrich, catalogue# S5261-10G), 543 mg l^{-1} NaHCO3 and 20 mg l^{-1} insulin (Sigma-Aldrich, catalogue# I2643), 10.7 mg l^{-1} human recombinant transferrin (Sigma Aldrich, catalogue# T4132), 100 ng ml^{-1} human basic fibroblast growth factor (Peprotech catalogue# 100-18B), 2 ng ml^{-1} human transforming growth factor beta (TGFß) (Peprotech catalogue# 100-21)) supplemented with 10 mM Y-27632 (Tocris, catalogue# 1254/5), 50 ng ml^{-1} hVEGF (Peprotech, catalogue# AF-100-20A) and hBMP4 (Peprotech, catalogue# AF-120-05ET), and 20 ng ml^{-1} hSCF (Miltenyi, catalogue# 130-093-991) to allow for the formation of EBs. On day 2, the medium was changed to E6 medium (containing only hVEGF, hBMP4 and hSCF), while the cells were still shacking on an orbital shaker at 70 rpm. On day 4 after mesoderm priming I, the media was changed to mesoderm priming II medium composed of E6 medium supplemented with 50 ng ml^{-1} hVEGF and hBMP4, 20 ng ml^{-1} hSCF and 25 ng ml^{-1} hIL-3 (Peprotech, UK, catalogue# 200-03), and the shaking speed was increased to 85 rpm. Mesoderm priming medium II was refreshed at day 7 before macrophage differentiation starts at day 10. On day 10, the medium was exchanged to the previously mentioned differentiation medium I, and from that point onward weekly production of iMonoMac is expected that can be later terminally differentiated using differentiation medium II.

2.2. Generation of MDM

Monocytes were isolated from the buffy coat blood by two steps of different density gradient centrifugation. In the first step, peripheral blood mononuclear cells (PBMCs) were separated using Biocoll solution (Biosell, Feucht, Germany, catalogue# BS.L6115) at a centrifugation speed of 460×g for 45 min. Thereafter, a second gradient was performed using Percoll solution (Sigma-Aldrich, St. Louis, MO, USA, catalogue# 17-0891-01) at a centrifugation speed of 800×g for 20 min, which allowed to further sperate the monocytes from the PBMCs fraction. Following isolation, monocytes were further matured by culturing them in RPMI 1640 medium (ThermoFisher Scientific, USA, catalogue# 21875-034) supplemented with 10% FCS, 1.5 mM NEAA, 2 mM L-glutamine, 1 mM sodium pyruvate, 0.05 mM b-mercaptoethanol, 1% P/S. Additionally, 10 ng ml^{-1} hM-CSF, and 10 ng ml^{-1} hIL-3 were added for seven days. Following that monocytes were cultured with the same RPMI medium but with only 30 ng ml^{-1} hM-CSF for an additional period of 7 days.

2.3. Culturing the monocytic cell line MM6

The human monocytic cell line MM6 was purchased from the Leibniz Institute DSMZ German collection of microorganisms and cell culture GmbH (DSMZ no ACC 124). The suspension cell line was passaged by dilution and cultured in 12-well suspension plates using RPMI 1640 medium (ThermoFisher Scientific, USA, catalogue# 21875-034) supplemented with 10% FCS, 1% P/S, and 10 μg ml^{-1} human insulin (Sigma Aldrich, Thermo Scientific, USA, catalogue# 19278).

2.4. Stimulation of monocytes with pyrogens or spiked pharmaceutical preparation

Different pyrogens were used to stimulate the monocytes, including the WHO 3rd international Endotoxin standard (WHO-LPS); NIBSC (London, UK, catalogue# 10/178), MALP-2 (ENZO, New York, USA; catalogue# ALX-162-027), rek. Flagellin (Biomol, Taiwan catalogue# AG-40B-0126-C010), Heat killed staphylococcus aureus (Invivogen, San Diego, USA catalogue# tlrl-hksa). Additionally, material-mediated pyrogen such as non-functionalized polystyrene nanospheres (Lab201, Cambridge, Uk, catalogue# PST100/PST1000) were used. Reconstitution of the pyrogens was performed according to the manufacturer's recommendation for every product. Serial dilutions of pyrogens were prepared in sterile water for injection. MDM, and iMonoMac were seeded at different cellular densities in wells of a 96-well plate and terminally differentiated with RPMI 1640 medium (supplied with 10% FCS, 1% P/S) and hM-CSF (50 ng ml^{-1}) for 6 days. MM6 were seeded with the aforementioned culture medium. On the test day, the medium was completely removed from the wells seeded with the monocytes to be replaced with 20 μl of the respective pyrogen concentration and 240 μl of the respective culture medium. After different incubation periods with the pyrogen, the supernatants were collected and stored at −80 °C for further cytokine analysis assays. To determine if iMonoMac can be used to screen for the contamination of pharmaceutical preparations, iMonoMac were seeded at 10 000 cell per 96 well plate and incubated either with culture medium alone as the control condition, or with WHO LPS alone at 200 pg ml^{-1}, or with Sterofundin® ISO (B. Braun Melsungen AG, catalogue# 234618143) alone, or with Sterofundin® ISO spiked with WHO

LPS at 200 pg ml^{-1}. The supernatants were collected after overnight incubation, and IL-6 levels were quantified with an enzyme-linked immunosorbent assay (ELISA).

2.5. Production of viral vector particles

A third-generation lentiviral vector of pCDH-MCS-NFKBIA-Luc-EF1 containing the NF-κB responsive promoter and the Firefly reporter gene was kindly provided by the Institute of Microbiology, University of Jena, Jena, Germany [18]. For lentivirus production, HEK293T cells (Clontech catalogue# 632180) were used. These cells were cultured in DMEM with 10% FBS, 1% P/S, 20 mM HEPES (PAA Laboratories, Austria, catalogue# S11-001), and 25 μM chloroquine (Sigma-Aldrich, USA, catalogue# C6628-25G). The transfection of HEK293T was done following the calcium phosphate precipitation method with 8 μg ml^{-1} of pcDNA3.GP.4xCTE (gag/-pol) (Plasmid factory, catalogue# PF895-120 306), 5 μg ml^{-1} of pRSV-Rev (Plasmid factory, catalogue# PF898-140718), 5 μg ml^{-1} of lentiviral vector plasmid, and 2 μg ml^{-1} of PM.DG vesicular stomatitis virus glycoprotein plasmid (Plasmid factory, catalogue# PF1000-121009). Viral supernatants were collected after 24 and 48 h of transfection, and were concentrated by 6 h centrifugation at a speed of 14000 × g at 4 °C.

2.6. Generation of reporter iPSC line

8.0 × 10^4 cells of hCD34iPSC16 line per well were seeded in Geltrex^{TM} (ThermoFisher Scientific, USA, catalogue# A1413201) coated 12-well plates. Afterwards, cells were transduced with the third-generation lentiviral vector harboring the NF-κB-Luciferase expression cassette, in the presence of 4 μg ml^{-1} of protamine sulfate (Sigma-Aldrich, USA, catalogue# P4020-5G) and 10 mM of Y-27632 in 500 μl of iPSC medium. The culture volume was increased to 1 ml of iPSC medium 24 h post transduction, and the medium was completely refreshed 48 h post transduction. Four days post transduction, when the well reached 80% confluency, the transduced iPSCs were collected and expanded to initiate hematopoietic differentiation and iMonoMac production as described above.

2.7. NF-κB-luciferase gene reporter assay

The NF-κB luciferase reporter monocytes were seeded in wells of a 96-well plate in the respective terminal differentiation medium II for iMonoMac. At day 6 post terminal differentiation, the cells were stimulated with WHO-LPS as described earlier for 6 h. At the endpoint, the supernatants were collected for possible ELISA assays, and 80 μl of Bright-Glo luciferaseassay reagent (Promega, Wisconsin, United States, catalogue# E2610) was added and mixed briefly with the cells, followed by measuring the luminescent signal by a plate reader (Paradigm Detection Platform by Beckman Coulter).

2.8. Phagocytosis assay using pH Rodo bioparticles

To determine the phagocytic activity of the monocytes, 2 × 10^5 of MM6, MDM, or iMonoMac cells per well were seeded in adherent or suspension 12-well culture plates in the regular culturing medium of the respective monocyte subtype. 10 μl of pHrodo-conjugated E.coli particles (ThermoFisher Scientific, catalogue# P35361) was added and cells were incubated either at 37 °C or at 4 °C as phagocytosis control condition. After 6 h of incubation with the bioparticles, the cells were collected, and the phagocytic events were quantified by flow cytometry.

2.9. Reactive oxygen species (ROS) assay

A total number of 7 × 10^5 MM6, MDM, and iMonoMac cells were used to evaluate ROS production. For every cell type, three conditions were prepared. A control condition composed of unstained cells, cells stained with 0.25 μg Dihydrorhodamine 123 (Invitrogen, ThermoFisher Scientific, USA, catalogue# D23806; second condition), and cells stained with DHR followed by stimulation with a ROS inducer (0.2 μM of phorbol myristate acetate (PMA)) for 15 min (third condition). The cells were prepared for the assay as follows, after collection in 500 μl of HBSS (Invitrogen, ThermoFisher Scientific, Massachusetts, USA, catalogue# 030m169S), incubation followed at 37 °C for 5 min. After that, DHR staining and PMA stimulation was carried out to the respective conditions for a total of 20 min while shaking at 37 °C in the dark at 175 rpm. Lastly, ROS production was quantified by measuring the fluorescent signal of the oxidized rhodamine dye using flow cytometry.

2.10. Cytospins

3 × 10^4 cells were centrifuged to be spotted on glass slides using a Cytofuge® (Medite) for 10 min at 700 ×g. Following that, the slides were air dried and stained with 0.25% (w/v) of May–Grünwald solution (Roth, catalogue# T863.3) for 5 min. After the first staining, the slides were washed thoroughly with distilled water and then stained with 5% GIEMSA solution (Roth, catalogue# T862.1) for 20 min. Following the second round of washing and drying, the slides were imaged using Olympus IX71 and the CellSens Dimension imaging software (Olympus).

2.11. Flow cytometry

A minimum of 5 × 10^5 cells were collected, washed with cold PBS and filtered through a 100 μm strainer to prepare a single cell suspension. Following that, nonspecific antibody binding was blocked by adding human FcR Blocking Reagent (Miltenyi Biotec, Bergisch Gladbach, Germany; catalogue#

130059-901) to the re-suspended cells in FACS buffer constituted of EDTA (2 mM) and FCS (2%), for 10 min on ice. Thereafter, the cells were stained with the recommended dilution of fluorescently labelled antibodies for 30 min on ice in the dark. The following antibodies and their respective isotypes were used: hCD45-ef450 (catalogue# 48-0459-42), hCD11b-APC(catalogue# 17-0118-42),hCD14-PE(catalogue# 12-0149-42), hCD163-APC (catalogue# 17-1639-41), hCD86-PE (catalogue# 12-0149-42), hCD16-FITC (catalogue# 302005), hTLR2-FITC (catalogue# 11-9922-42), hTLR5-PE (catalogue# MA5-16236), hDectin1-PE (catalogue# 12-9856-42), hTLR4- APC. (catalogue# 17-9917-42). The isotypes used were as follows: mouse IgG1 kappa isotype control (P3.6.2.8.1)-APC, (catalogue# 17-4714-81) mouse IgG1 kappa isotype control (P3.6.2.8.1)-PE (catalogue# 12-4714-42), mouse IgG1 kappa isotype control (P3.6.2.8.1)- FITC (catalogue# 11-4714-4181), mouse IgG2a kappa isotype control (eBM2a)-FITC (catalogue# 11-4724-42), mouse IgG2a kappa isotype control (eBM2a)-APC (catalogue# 17-4724-81), mouse IgG2a kappa isotype control (eBM2a)-PE(catalogue# 12-4724-42). All antibodies were from (eBioscience, Thermo Fisher Scientific, California, USA). All flow cytometry experiments were performed using a CytoFLEX S (Beckman Coulter, Krefeld, Germany), and data were analyzed with Flowjo v10.

2.12. Cytokine secretion

The fresh or frozen supernatants were assayed for human IL-6 secretion using ELISA Ready-Set-Go! Kit (R&D Systems, Minneapolis, USA, catalogue# DY206) following the manufacturer's instructions.

2.13. Preparation of blinded samples using DNA-origami nanocarrier

The preparation of the blinded samples as a challenge for the Monocyte activation assay, as shown in (figure 2(f)) was conducted following the procedures described in the supplementary information of the publication by Wagenbauer *et al* [19]. Briefly, DNA origami nanostructures were folded using a scaffold DNA concentration of 50 nM and oligonucleotide strands at 200 nM. The thermal annealing ramp was performed using TETRAD (MJ Research, now Biorad) thermal cycling devices. The oligonucleotides were obtained from IDT, and the DNA scaffolds were produced in-house, as previously described by Engelhardt *et al* [20].

To ensure the *in vivo* viability of the produced scaffold ssDNA, endotoxin purification was carried out using Triton X-114, as described by Hahn [21]. To concentrate DNA origami objects such as chassis or programmable T cell engagers, PEG precipitation or ultrafiltration was employed, as previously described [22]. For protein conjugation, maleimide-modified DNA strands were either purchased from Biomers or prepared in-house by mixing amine-modified DNA with an SMCC crosslinker followed by ultra-centrifugation (using Amicon 10k filters). The scFv proteins, which contained an N-terminal free cysteine and were obtained from Genscript or Icosagen, were reduced with 5 mM TCEP for 30 min. Excess TCEP was removed using ultra-centrifugation (Amicon 10k filters). The reduced scFv proteins were then mixed with maleimide-modified DNA strands in 50 mM HEPES with 200 mM NaCl at pH 6.7 and incubated overnight at room temperature. The resulting conjugates were purified using ion exchange chromatography with a NaCl gradient of 150-1000 mM in PBS (pH 7.2) (proFIRE, Dynamic Biosensors). The purified oligo-antibody conjugates were then analyzed using SDS-PAGE and agarose gel electrophoresis. For binding of the antibody-DNA conjugates and DNA-origami objects, the corresponding binding sites were incubated in equimolar ratios for 1 h at 37 °C. Before conducting *in vivo* experiments, samples were stabilized for use in low-ionic strength buffers and in the presence of nucleases using the protocol described by Ponnuswamy *et al* [21]. All structures were coated with K10-PEG oligolysine (Alamanda Polymers, USA). To assess the endotoxin concentration of the in-vivo ready constructs, and all controls for the Monocyte Activation Assay, we used a Charles River nexgen PTS150 V10.2.3 instrument and cartridges with a range between 5–0.05 EU ml^{-1}. Samples were diluted to fit into the sensitive range of the cartridges, if necessary.

2.14. Statistics

The statistical tests were performed using Prism7 (GraphPad). The plotted data represent the mean ± SD, and the number of biological repeats are stated in the figure legend as the value of n. For a comparison between two groups unpaired student's t test was conducted. For reporting data comparing the effect of one independent variable across three groups one way ANOVA was used, while two way ANOVA was performed for evaluating the effect of two independent variables. A p-value of 0.05 or less was considered as statistically significant. Every statistical test performed is reported in the respective figure.

3. Results

3.1. Human iPSC-derived macrophages (iMonoMac) share phenotypic and functional similarities to primary MDM

The current format of the MAT is based on classical ELISA and has not been changed since its first introduction in 1995 or its addition to the general chapter 2.6.30 of the EP in 2010 (figure 1(a)). Since then, the MAT has been performed with either whole

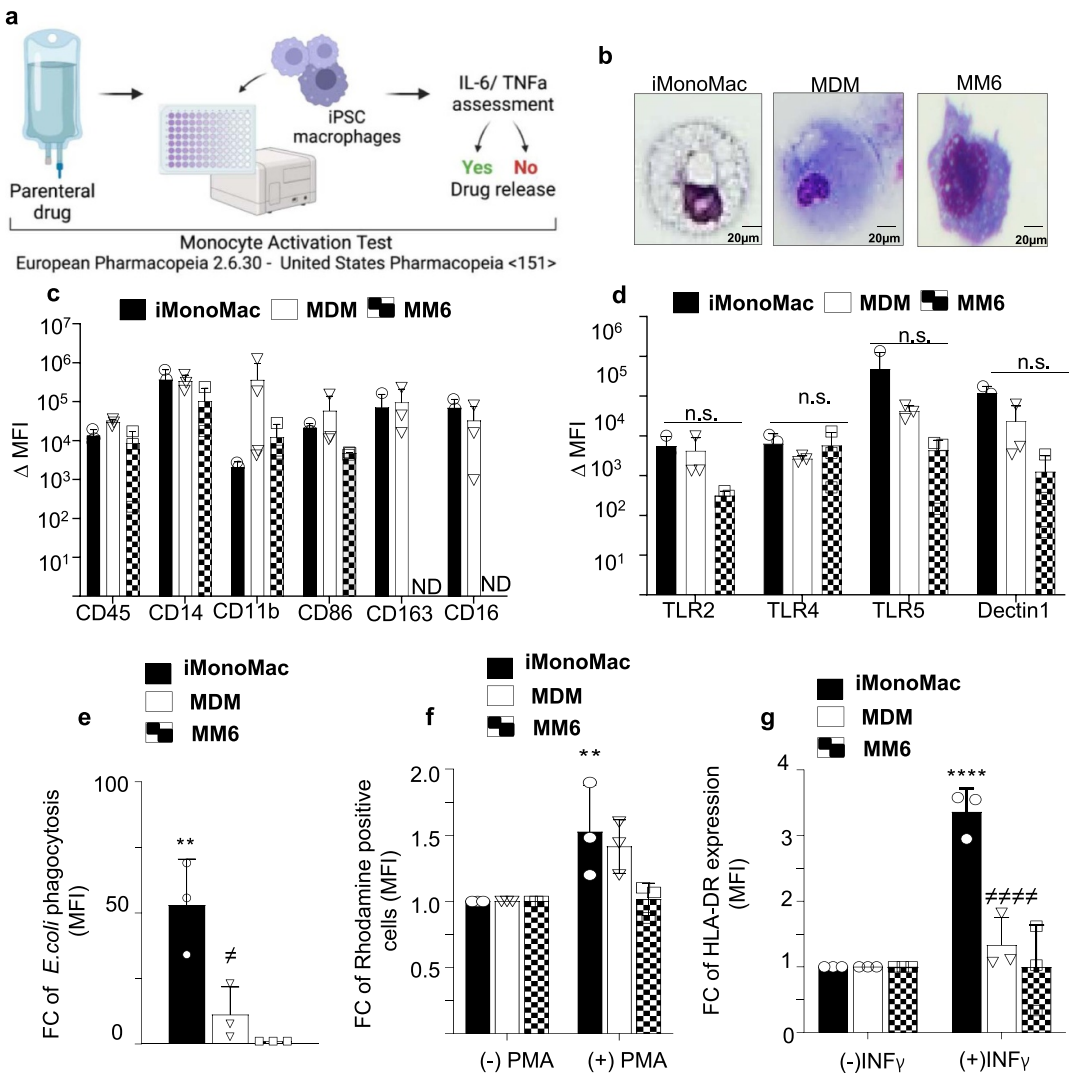

Figure 1. Phenotypic and functional characterization of iMonoMac compared to standard monocytes. (a) Schematic overview of the Monocyte Activation test application. (b) Representative pictures of cytospin preparations of the different monocytes, iMonoMac, MDM, MM6 (scale bar 20 μm). (c) Fold change of the mean fluorescent intensity (MFI) of typical macrophage surface markers expressed on the indicated monocytes cells, acquired by flow cytometry. ($n = 3$ of biological replicates, mean ± SD). (d) Fold change of the mean fluorescent intensity (MFI) of different pathogen recognition receptors including Toll like receptors and Dectin-1, cells acquired by flow cytometry. ($n = 3$ of biological repeats, mean ± SD, two-way ANOVA with Tukey's multiple comparisons test). (e) Bar chart quantifying the fold change of the mean fluorescent intensity (MFI) of the fluorescently labeled E. coli bioparticles phagocytosed by the different monocytes at 37 °C in comparison to the MFI of the bioparticles phagocytosed at 4 °C as control ($n = 3$ of biological repeats, mean ± SD, one-way ANOVA with Tukey's multiple comparisons test). (f) Bar chart quantifying the fold change of the MFI of rhodamine positive cells with phorbol myristate acetate (PMA) treatment to the untreated cells acquired by flow cytometry ($n = 3$ of biological repeats, mean ± SD, biological repeats, two-way ANOVA with Tukey's multiple comparisons test). (g) Quantification of the fold change of HLA-DR MFI expression in the treated cells with 25 ng μl^{-1} of INFγ in comparison to the untreated ($n = 3$ of biological repeats, mean ± SD, biological repeats, two way ANOVA with Šídák's multiple comparisons test). (h) ELISA analysis of IL-6 secretion of the stimulated versus non-stimulated different monocytes at 10 000 cells per well after overnight incubation with WHO LPS at different concentrations (3.3, 100 pg ml^{-1}). ($n = 4$ of biological repeats, mean ± SD, two way ANOVA with Tukey's multiple comparisons test). (i) IL-6 secretion of iMonoMac post overnight incubation with WHO-LPS (100 pg ml^{-1}) with or without the addition of 10 μg ml^{-1} of monoclonal antibodies against CD14, and TLR4. ($n = 3$ of biological repeats, mean ± SD, unpaired t-test) measured with ELISA. (j) ELISA analysis of IL-6 release from iMonoMac and MDM after WHO-LPS (100 pg ml^{-1}) stimulation at different time points. iMonoMac: iPSC derived macrophages, MDM: monocyte derived macrophages, MM6: Mono-Mac-6, ND: not detected, FC: fold change, n.s.: not significant.*Indicates significant difference between iMonoMac and MM6, ≠ indicates significant difference between iMonoMac and MDM.*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$,≠ $p < 0.05$, ≠,≠ $p < 0.01$,≠,≠,≠ $p< 0.001$,≠,≠,≠,≠ $p < 0.0001$.

blood samples, PBMCs, MDM or the MM6 cell line. In our study, we aimed to benchmark iMonoMac to either primary MDM or MM6 cells, as these two cell types represent the two most commonly used cell sources so far in the MAT. Of note, all studies have been performed by harmonizing defined test parameters such as duration of the test or cell density. To derive iMonoMac from human iPSC, we initially started by producing macrophages from a single iPSC line#4 (hCD34iPSC16//MHHi015-A) [15], using a

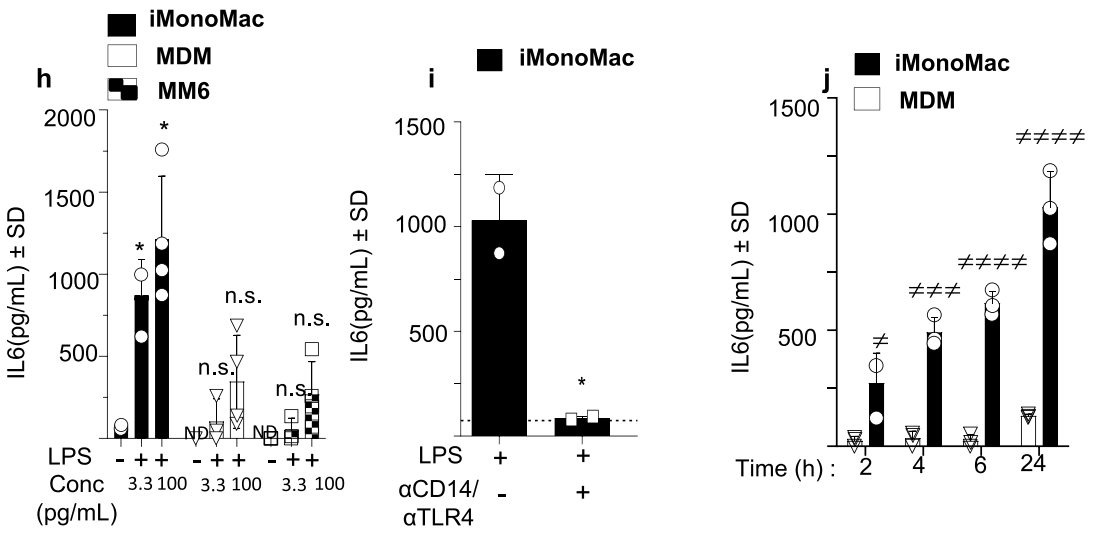

Figure 1. (Continued.)

previously published differentiation technique [16], which enables the derivation of iMonoMac in scalable quantity. Terminally differentiated iMonoMac exhibited a more similar morphology to the primary MDM compared to MM6 cells. As expected, iMonoMac and MDM are adherent cells displaying a typical macrophage-like morphology with extending filopodia, vacuoles, and irregular cell surface, as illustrated by the cytospins (figure 1(b)). The close phenotypical proximity of iMonoMac to MDM was further confirmed by the positive expression of CD45, CD14, CD163, CD11b, CD86, and CD16 that are used to mark macrophages (figure 1(c)). In contrast, the monocytic cell line MM6 demonstrated a more immature profile, as seen by cytospin images and a lack of CD163 and CD16 expression (figures 1(b) and (c)). To provide early evidence that iMonoMac can respond to a variety of pyrogens (endotoxins and non-endotoxins), the expression of different pattern recognition receptors, including the different Toll-like receptors (TLRs), were evaluated. Assessing the expression levels of TLR2, TLR4, TLR5, and Dectin1 demonstrated no statistical difference in the expression pattern across all three different monocyte cell types. However, iMonoMac exhibited expression levels of TLR2, TLR5, and Dectin 1 more similar to MDM as compared to the lower expression levels observed on MM6 cells (figure 1(d)). This points to a more immature phenotype and a limited capacity of the MM6 cells to detect a broad spectrum of pyrogens. To mimic the *in vivo* pyrogen reaction elicited by the drug contaminants, cells that resemble the *in vivo* myeloid counterpart the most would be ideal for the MAT assay. Thus, we next assessed the general capacity of phagocytosis, induction of ROS, and the response to inflammatory stimulation (upregulation of HLA-DR). Upon six hours of incubation with E. coli bioparticles conjugated to a pH-sensitive fluorescent dye, both iMonoMac and MDM showed a significant phagocytic activity compared to control samples, while MM6 showed no sign of phagocytosis (figure 1(e)). A similar finding was made for the induction of ROS and upregulation of HLA-DR. Both iMonoMac and MDM were able to respond to PMA or interferon gamma (IFN-γ) respectively, while MM6 showed no sign of activation upon exposure to these pro-inflammatory stimuli (figures 1(f) and (g)). In contrast to the poor performance of MM6 in the functional assays, iMonoMac closely resembled the phenotype and function of primary MDM. This observation goes in line with other findings using the iPSC technology to generate macrophages [23], further underlining the suitability of these cells to detect pyrogen contamination. After confirming the maturity and functionality of iMonoMac, the next step was to evaluate their sensitivity to a broad spectrum of pyrogens typically applied in the MAT framework using the classical IL-6 ELISA readout. Upon challenge with 100 pg ml^{-1} of WHO-LPS as an endotoxin-based pyrogen, iMonoMac produced the highest level of IL-6 compared to MDM and MM6. Of note, the secreted IL-6 with iMonoMac was approx.15-fold higher than the basal level (figure 1(h)). To prove the specific detection of LPS via TLR4/CD14, which is also an important hallmark for regulators, we applied anti-CD14/TLR4 antibodies to block this signaling pathway. The addition of blocking antibodies and WHO-LPS to iMonoMac cells resulted in only baseline levels of IL-6 secretion, whereas in the absence of CD14/TLR4 blocking antibodies, significant levels of IL-6 were detected (figure 1(i)). This underscores the specificity of the system. Of note, the higher induction of IL-6 secretion by iMonoMac was also correlated

with a faster inflammatory reaction upon pyrogen contact. While the standard MAT format requires an incubation period of 22 ± 2 h irrespective of the cell type used, time-dependent stimulation of iMonoMac with WHO-LPS for two hours was sufficient to induce the pro-inflammatory response with significant secretion of IL-6, whereas MDM failed to show a similar response (figure 1(j)). This data suggests the beneficial use of iMonoMac compared to current cell types used in the MAT framework, while introducing at the same time crucial advantages of iMonoMac, making the MAT even more attractive for regulators and industrial users.

3.2. iMonoMac displayed superior sensitivity spectrum to endotoxin and non-endotoxin-based pyrogens with a broad dynamic range

The ability of monocytes to respond to a variety of different pyrogens [24] is a crucial feature for their use in MAT for parenteral drugs safety testing. Ideally, monocytes should be able to detect contaminants at low concentrations that have the potential to induce a pyrogenic reaction. Thus, as a next step, we first analyzed the dynamic range of iMonoMac derived from a defined iPSC line#4 against a variety of pyrogens, including endotoxin and non-endotoxins. To explore the limit of detection (LOD) of iMonoMac against endotoxins, a dose-dependent challenge of WHO-LPS was performed for 24 h. Already at a seeding density of 10 000 cells per well of a 96-well plate, iMonoMac showed an acceptable sensitivity for WHO-LPS, with 3.3 pg ml^{-1} as the lowest detected concentration (figure 2(a)). Of note, commercially available MAT assays provide a LOD of 2 or 5 pg ml^{-1} for PBMCs or MM6, respectively, using cell densities that are at least 10-fold higher than the 10 000 cells used with iMonoMac. One reason why regulators favor the future use of the MAT is the detection of non-endotoxin (NEPs) based contaminants, which is not possible using current alternatives such as the bacterial endotoxin test (BET(LAL), and rFC). Hence, we examined the ability of iMonoMac to detect broad classes of NEPs based contaminants. The sensitivity of iMonoMac allowed for the detection of a synthetic TLR2/6 MALP-2 agonist with a starting concentration of 0.005 μg ml^{-1} (figure 2(b)). Additionally, the detection of a TLR5 agonist like Flagellin (Flg) was achieved with 3.12 ng ml^{-1} as a starting concentration (figure S1(a)). Besides testing fully or semi-synthetic pyrogens, our cells were also used to detect more natural preparations. Of note, iMonoMac were able to detect heat-killed *Staphylococcus aureus* (HKSA) at concentrations of 10^6 cell ml^{-1} (figure S1(b)). As an important hallmark for the MAT assay, the acquired data were of better reproducibility with lower standard deviations and a broader dynamic range using iMonoMac compared to the primary MDM, reflecting potential donor-dependent variations of the isolated MDMs. Besides the sensitivity of iMonoMac to detect the biological non-endotoxins mentioned above, iMonoMac also showed activity to detect material-mediated or process-related pyrogens. The incubation with polystyrene nanospheres elicited a significantly higher IL-6 secretion level than the secretion levels generated by MDM, while the IL-6 release showed a trend towards increased secretion of IL-6 with the increased size of the nanoparticles (figure 2(c)).

While the displayed results so far are pertaining to iMonoMac derived from the same, single, parental iPSC line/donor, we further addressed whether the reactivity of iMonoMac to pyrogen stimulation is generally similar across various iPSC lines or different, i.e. dependent on the selected iPSC line/donor. Therefore, we expanded the analyses to iMonoMacs derived from a total of six different iPSC lines generated from three male donors and three female donors (figure S2(a)). Irrespective of the iPSC line background, all of the tested lines were successfully differentiated to mature macrophages expressing the typical macrophage surface markers, highlighting the robustness and the reproducibility of our hematopoietic differentiation protocol (figure S2(b)). The generated iMonoMac from the six donors were efficiently responding to a dynamic range of concentrations of LPS stimulation in a reproducible manner (figures S2(c) and (d)). However, while the secreted IL-6 levels were variable between the different donors, the gender of the parental iPSC line could not be associated with the iMonoMac sensitivity (figure S2(e)). Interestingly, the analysis demonstrated that on average iMonoMac IL-6 secretion levels from the six different donors (mean ± SD, 869.4 ± 658.3) were higher than that of MDMs from four different donors (mean ± SD 340.7 ± 373.9) at a relatively low LPS concentration (50 pg ml^{-1}) that can still mediate meaningful biological responses across all donors (figure S3(f)). This confirm the previously described results comparing the sensitivity of iMonoMac derived from the defined female iPSC line to MDM, and MM6 (figure 2). Despite the observed differences attributed to the use of different iPSC lines, the possibility to pre-screen the iPSC line/donor of choice, along with lifelong access to the same iPSC line/iMonoMac, provides numerous options to promote the standardized use of iMonoMac in the MAT context.

Aside from the reliance on the monocytes' pro-inflammatory cytokine secretion as a tedious, time-consuming, and less standardized output for sensing drug contaminates, alternative approach was examined by tapping the NF-κB activation as an earlier step in the inflammatory cascade. A parenteral iPSC line was genetically engineered with a lentiviral vector harboring an NF-κB response element, which mediates the expression of a luciferase

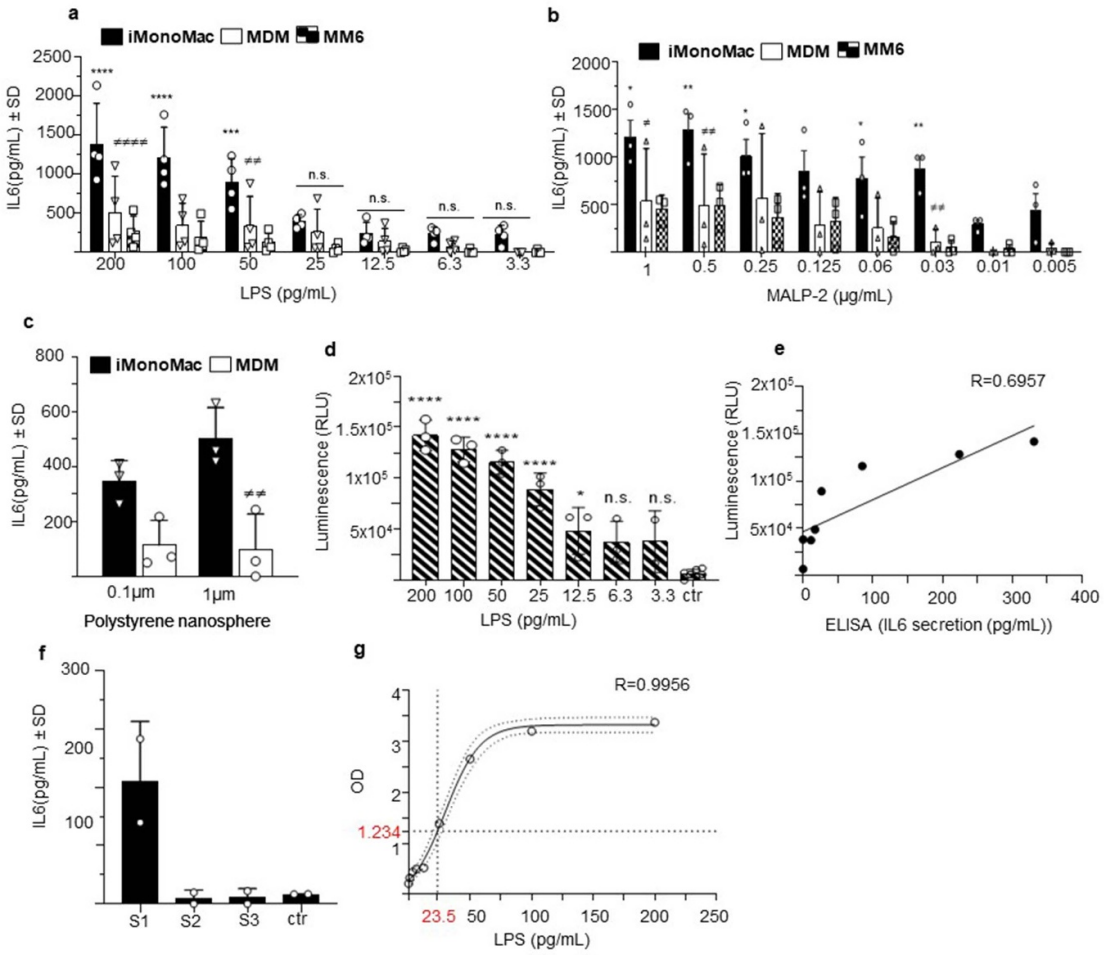

Figure 2. iMonoMac and reporter iMonoMac as an efficient detection tool for endotoxin and non-endotoxin based pyrogens. (a) IL-6 concentrations measured with an ELISA after the incubation of the different monocyte populations with either WHO LPS or a macrophage-activating-lipopeptide-2 (MALP2) (b), or with polystyrene nano sphere (c). ($n = 3-4$ of biological repeats, mean ± SD, biological repeats, two way ANOVA with Tukey's multiple comparisons test). (d) Bar chart quantifying the luminescent signal emitted from the NF-κB luciferase iMonoMac at a density of 50 000 cells per well of 96 well incubated with culturing medium only defining the ctr condition, or incubated with different concentrations of WHO-LPS at 6 h, ($n = 3$ of biological repeats, mean ± SD, biological repeats, one way ANOVA with Tukey's multiple comparisons test) *Indicates significant difference between iMonoMac NF-κB reporter at the indicated LPS concentration compared to the ctr condition.*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$). (e) Correlation graph indicating the Pearson's correlation coefficient between IL-6 ELISA secretion and the luminescent reading upon dose dependent WHO-LPS stimulation of the NF-κB reporter iMonoMac. (f) IL-6 concentrations measured with an ELISA after the incubation of different blinded samples, S1: endotoxin contaminated immune-therapeutic parenteral preparation, S2: DNA-origami nanocarrier, S3: PBS. ($n = 2$ of biological repeats, mean ± SD). (g) Endotoxin calibration curve, plotting the optical density in relation to the WHO-LPS concentration (the plotted values are the average values of $n = 2$).iMonoMac: iPSC derived macrophages, MDM: monocyte derived macrophages, MM6: Mono-Mac-6, ctr: control, n.s.: not significant. *Indicates significant difference between iMonoMac and MM6, ≠ indicates significant difference between iMonoMac and MDM.*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$, ≠$p < 0.05$, ≠,≠ $p < 0.01$,≠,≠,≠ $p < 0.001$,≠,≠,≠,≠ $p < 0.0001$.

reporter in iPSC-derived reporter iMonoMac (NF-κB-Luc iMonoMac). The highest luminescent reading associated with the highest NF-κB activation was achieved already after 6 h of WHO-LPS stimulation of the NF-κB-Luc iMonoMac, giving rise to dose-dependent luminescent reading (figure 2(d)). Furthermore, the retrieved luminescent reading upon WHO-LPS dose titration could correlate with the IL-6 secretion using the standard ELISA assay confirming the fitness of the reporter as an alternative method for MAT, which would, in addition, allow an automated readout (figure 2(e)). While it was shown that iMonoMac could detect various sources of contaminants, it is crucial to demonstrate further the translational use of iMonoMac revealing such contaminations in therapeutic parenteral preparations. In that context, we used blinded samples of an early preclinical immune-therapeutic drug candidate S1 (multi specific DNA origami-based T cell engager [25]), which was endotoxin contaminated and also other control samples were tested (components of the drug candidate: S2: DNA-origami nanocarrier, and S3: PBS). Upon the incubation of the blinded samples with iMonoMac, we could differentiate between the control samples and the contaminated sample (figure 2(f)). Based on the LPS dose-response

curve the retrieved contamination was determined at 23.5 pg ml^{-1} (figure 2(g)), while the predetermined endotoxin contamination load using a bacterial endotoxin test was at 50 pg ml^{-1}. Furthermore, the data indicated that the DNA-origami based nanocarrier (S2) was found not to induce secretion of IL-6, suggesting its potential for parenteral use. While iMonoMac are not yet validated to screen for pharmaceutical preparations pyrogenicity, it is nevertheless of great interest and value to determine if iMonoMac can detect the contamination of a parenterally available drug. To address this aspect, we did contaminate the Sterofundin® ISO with 200 pg ml^{-1} of WHO-LPS. Indeed, iMonoMac were selectively sensitized against the spiked formulation, and the LPS dose titration curve determined the contamination of Sterofundin® ISO at 193.6 pg ml^{-1} (figure S1(c)).

Hence, with this proof of concept with blinded samples, and the spiked parenteral formulation we conclude that iMonoMac can serve as sensors for contamination in parenteral formulations, and highlight the superior use of standardized iMonoMac to assess pyrogenic contaminations in parenteral drugs.

4. Discussion

With increasing frequency, human iPSC-derivatives are nowadays being applied in different therapeutic and non-therapeutic applications. Especially the latter is of great interest, using for example iPSC-derived cardiomyocytes, neurons, beta cells, etc, as standardized cell types in drug discovery, potency, or safety [26]. However, as of yet, iPSCs have not been applied in bioassays which are mandatory by regulators and listed in e.g. the European pharmacopeia, and the United States pharmacopeia, etc. In fact, we here demonstrate the application of iMonoMac as a beneficial cell type for the MAT, a test mandatory for suppliers to release parenteral drugs into the market. Although already accepted by regulators for more than a decade, industry still relies on animals to assess pyrogenic contaminations, as current cell sources for monocyte/macrophages have substantial drawbacks. Using human iPSCs, we demonstrate high phenotypic and functional similarities of iMonoMac to primary monocyte/macrophages, while neglecting all drawbacks of using primary cells, such as donor availability or variability. In fact, other studies have already proven the similarities of iMonoMac to their *in vivo* counterparts utilizing a variety of sophisticated assays [27, 28]. For instance, it was previously shown that iMonoMac have expression of several typical macrophage surface markers comparable to MDM including (CD45, CD11b, CD14, CD86) [16]. Similarly, functional analysis revealed comparable phagocytic capacity of iMonoMac to MDM and denoted a higher release of IL-6 upon LPS stimulation compared to MDMs [16]. These results were further confirmed on a transcriptional level, with iMonoMac clustering closely to MDM with a tendency for a higher expression of pro–inflammatory and macrophage-activation genes [29]. Such findings can indeed justify the observed higher sensitivity of iMonoMac compared to MDM in our study. While the aforementioned studies used LPS and/or Gram-negative bacteria, work performed using gram-positive bacteria such as Staphylococcus aureus highlighted a similar pattern, displaying a faster response to the pathogen with a faster upregulation of macrophage-activation and pro-inflammatory genes compared to MDM [30].

While iMonoMac can in principle be manufactured in scalable quantities [31], in our proof-of-concept study we used iPSC lines from different male and female donors, showing consistent results. Of note, reprogramming of somatic cells towards a pluripotent state goes along with epigenetic reprogramming, and previous publications could demonstrate that residual epigenetic memory is lost upon subsequent differentiation [32]. In fact, iPSC-derived macrophages have been shown to be of primitive origin [33], and it remains elusive whether the reprogramming process would erase the donor-specific fingerprint, which could have a consequence for the activity of iMonoMac against pyrogens. In this line, our study showed that the iPSC line/ donor background can have an influence on the sensitivity of the derived iMonoMac. This suggest that pinpointing the iPSC line of choice is an important factor for the regulator's and MAT user's consideration. Nevertheless, iPSC and iMonoMac have clear advantages due to the possibility of screening for iPSC and thoroughly testing and defining iPSC of choice. In addition, we demonstrate further advancements of the iPSC-based MAT by introducing an NF-κB reporter line, which makes the assessment of drugs faster and more economical, all directed to increase the overall safety of drugs. In fact, the key benefit is to standardize the MAT by using iMonoMac, further fostering the application of this *in vitro* test. The current study extends the use of iPSCs and introduces iPSC-derived macrophages to be used in a bioassay accepted by regulators to assess the safety of parenteral drugs. Given the importance of this bioassay, additional studies will be needed to provide new insights into the impact of the reprogramming process on the epigenetic landscape of somatic cells and its functional consequences on iPSC-derived macrophages in a donor-to-donor dependent manner. Thus, these experiments will broaden our current understanding of how deep the reprogramming process will alter-/reserve the original donor epigenetic or genetic fingerprint with direct impact on the use of iMonoMac in the MAT framework. Along with the immediate use of iMonoMac within the MAT, strategies for an off-the-shelf use must be defined. Clearly, the freshly

generated iMonoMac used in our study would not be applicable to the growing demand of the cells, introducing the need to further elaborate on cryopreservation regimens for iMonoMac and to assess its consequences to recognize a broad variety of pyrogens properly. Hence, the provided solution of iMonoMac for the MAT is already advanced, highlighting additional aspects to derive a cell product that meets industry expectations.

Data availability statement

The data cannot be made publicly available upon publication because no suitable repository exists for hosting data in this field of study. The data that support the findings of this study are available upon reasonable request from the authors.

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Author contribution

Conceptualization, N L, I S, and S A; methodology development, S A, F M, I S, M A, A R, B B, U M, C S, and N L; data acquisition, S A, and F M; analysis and interpretation of data, S A, N L, B K; original draft preparation, S A, and N L; writing—revision, S A, I S, M A, A R, N P H D; study supervision and funding acquisition, N L, and G H All authors have reviewed and commented on the submitted manuscript.

Ethical statement

The generated primary human monocyte derived macrophages (MDM) were isolated from buffy coat blood of anonymous healthy donors. Buffy coat was bought from 'Deutsches Rotes Kreuz, DRK'. The use of human buffy coat blood was approved by the local ethical committee.

Conflict of interest

NL and MA filed and licensed patents in the field of iPSC-derived macrophages outside of the Manuscript. NL, SA and IS have filed IP for the use of human iMonoMac in the MAT framework. All other authors declare not conflict of interest.

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