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Received: 20 August 2023 | Accepted: 29 October 2023

DOI: 10.1111/imm.13714

ORIGINAL ARTICLE

immunology
Insights into immunity in health and disease

Macrophage-like rapid uptake and toxicity of tattoo ink in human monocytes

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Funding information
Bayer Vital; Bundesministerium für Bildung und Forschung, Grant/Award Number: 16LW0143; Deutsche Forschungsgemeinschaft, Grant/Award Number: BA6226/2-1; European Cooperation in Science and Technology, Grant/Award Number: CA20117; RWTH Aachen University, Grant/Award Number: G:(DE-82)EXS-SF-OPSF732; The State of NRW, Grant/Award Number: ZM-1-27B; Wilhelm Sander-Stiftung, Grant/Award Number: 2018.129.1; China Scholarship Commission, Grant/Award Numbers: 202206170024, 202008320329; EU; Federal State of North Rhine-Westphalia, Grant/Award Number: 30 00 883 02

Abstract

Macrophages play a critical role for the persistence of tattoo ink in human skin. However, a comparison to other skin-resident and blood circulating immune cells and a profound analysis of REACH-compliant tattoo ink are unmet medical needs. We hence characterized the size distribution of ink particles using physico-chemical methods. We studied the uptake of tattoo ink by key human skin cells and blood-derived immune cells using optical and electron microscopy as well as flow cytometry. Scanning electron microscopy of ink revealed its crystalline structure, and a tendency towards aggregations was indicated by size changes upon diluting it. Flow cytometric analyses of skin and immune cells after incubation with tattoo ink demonstrated an increase in cellular granularity upon uptake and red ink additionally evoked fluorescent signals. Human macrophages were most potent in internalizing ink in full thickness 3D skin models. Macrophage cultures demonstrated that the ink did not lead to elevated inflammatory mediators, and showed no indications for toxicity, even after nice days. Strikingly, monocytes were most efficient in ink uptake, but displayed reduced viability, whereas granulocytes and lymphocytes showed only temporary ink uptake with flow cytometric signals declining after 1 day. Mechanistic studies on ink retention by corticosteroids or dexpanthenol in macrophage cultures demonstrated that these compounds do not lead to ink excretion, but even slightly increase the ink load in macrophages. The highly motile monocytes, precursors of macrophages, may play

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an underrated role for tattoo ink translocation from dermal blood vessels into internal organs.

KEYWORDS
cytotoxicity, flow cytometry, macrophage, neutrophil, skin

INTRODUCTION

Humans use tattoos since ancient cultures, with the earliest documentation of root-based tattoo 5300 years ago in the Tyrolean Iceman Ötzi [1]. Despite millions of people being tattooed, analyses of tattoo ink are relatively scarce. The first published use of ink for staining macrophages appeared in 2007 when black ink was used to stain hepatic macrophages [2]. The most extensive study done in mice in vivo suggests that macrophages maintain tattoos by their constant renewal but not by their longevity [3]. In human skin, tattoo the ink was located in the dermis near vessel walls, but it remained unclear if the ink was inside or attached to fibroblasts [4]. Thus, it remains unclear if tattoo ink is exclusively internalized by macrophages or if fibroblasts might contribute to ink deposition in the skin.

Owed by reports on toxic effects of tattoo ink [5, 6], a new REACH guideline from 3rd of August 2020 has defined new safety standards for substances or mixtures for tattooing purposes in the entry 75 of Annex XVII (Regulation (EC) No. 1907/2006) [7]. Safety investigations should therefore include, but are not limited to physicochemical analytics. Additionally, studying the fate of tattoo ink in the human body at the level of cells is important because it translocates to internal organs such as the lymph nodes [8], or to the liver [9]. Phagocytic immune cells of the blood such as granulocytes or monocytes might function in this translocation from the skin to internal organs since they are motile and known to take up various types of nanoparticles such as those made of gold, silver, or iron [10]. Potential toxic effects of REACH-compliant tattoo ink on human primary circulating immune cells have not been studied before.

Importantly, models for human skin can replace animal models [11]. Treatment of tattooed skin using dexpanthenol aims to reduce the inflammatory reactions and to improve wound healing following the insertion of tattoo ink into the human skin by micro-needling. The reactions of inflammatory cells, particularly macrophages, are key factors that predict also the reaction of the skin. Inhibition of the mechanisms that may lead to a re-uptake of ink by surrounding macrophages may lead to improved techniques for tattoo ink removal.

Therefore, we characterized a REACH-compliant tattoo ink using physicochemical methodology such as electron microscopy, energy dispersive x-ray spectroscopy (EDX), and dynamic light scattering. We used six different types of human primary cells that may contribute to tattoo ink uptake in the body. We further generated 3D skin replacement models and applied tattoo ink to these using a common tattoo needle. Light microscopic analysis in monolayer cell culture, and electron microscopy were utilized to study the uptake by the different cell types. We further used corticosteroids and dexpanthenol to study their impact on tattoo ink retention. We analysed the inflammatory potency of ink on macrophages by quantitative Realtime-PCR and quantified the cellular uptake using flow cytometry.

MATERIALS AND METHODS

Tattoo ink

The first batch of blazing red Sailor Jerry ink was obtained from INKgrafiX in 2020 (27 June 2020). This ink was very viscous and did not include a mixing help. It was used in one experiment with macrophages where it showed toxic effects and hence was not used any more (data not shown). The second type of ink was ordered on 4 January 2022 from the same company and this batch was declared as REACH-conform (used for the experiments shown for the flow cytometry). This ink included a mixing helper to increase solubility of the ink. A black ink was used to tattoo the models (Sailor Jerry Magic Black 22 991-010, Lot: D14382).

Electron microscopy and energy-dispersive x-ray spectroscopy of tattoo ink

Transmission electron microscopy of the ink was done using a Zeiss Libra 120 (Oberkochen, Germany) microscope operated at 120 kV. The ink dispersion was put on a silicon monoxide TEM grid using the drop-on-grid procedure. A Hitachi UHR-FESEM SU9000 SEM (UHR FE-SEM SU9000, lateral resolution 0.4 nm at 30 kV, 1.2 nm at 1 kV) equipped with an EDX spectrometer (Oxford Instruments) X-max 80 M detectors was used for Scanning electron microscopy (SEM) and Energy-dispersive x-ray spectroscopy (EDX). In brief, a droplet of the ink dispersion was put on a silicon wafer and dried under

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ambient conditions. In the figures, only the six most abundant elements are shown in the graphs.

Dynamic light scattering analysis of tattoo ink

Two methods were chosen for dynamic light scattering (DLS). The first method was based on an angle of 173.5° using a Malvern Zetasizer. Tattoo ink was diluted in PBS and equilibrated for 30 s at 25°C prior to analysis. Measurements were done using a semi-micro cuvette (Polystyrene, SARSTEDT) and each measurement was repeated three times.

The second method was exerted using an ALV/CGS3 compact goniometer, ALV/LSE-7004 Tau Digital Correlator and a JDE Uniphase HeNe laser (wavelength 632.8 nm) in toluene as index matching fluid. Data were collected in automatic mode at 90° scattering angle for 120 s. The data was fit using an excessive range of hydrodynamic radii (100 data points along a logarithmic scale between 1 nm and 1 mm) and a modified CONTIN algorithm selecting the best fit with the minimal stabilizing parameter ($\alpha = 5 \times 10^{-3} - 50$, 50 points again along a logarithmic scale) similar to that published before [12]. The analysis was performed using R (https://www.R-project.org). The data of both methods were used to calculate the size distribution of the ink.

Cell isolation

Blood was obtained from healthy anonymized donors from the Transfusion medicine of the RWTH University Hospital. Human primary leukocytes were isolated from fresh human whole blood using dextran-based sedimentation. Briefly, 20 mL of human blood was collected in a syringe filled with 50 µL of heparin and mixed with 2.5 mL 5% dextran, resulting in 1% dextran in PBS. Leukocytes were separated by transferring the upper layer containing the leukocytes. To eliminate erythrocytes, an osmotic shock procedure was carried out by introducing 40 mL of distilled water for a duration of 40 s. Following this, 5 mL of 10× PBS was carefully added to the mixture. The suspension was then centrifuged at a speed of 1200 rpm for a duration of 10 min at a temperature of 20°C. The resulting solution was then dissolved in RPMI1640 supplemented with 5% human autologous serum.

To generate macrophages, peripheral blood mononuclear cells (PBMC) were isolated from blood using gradient centrifugation based on Ficoll of a density of 1.077 g/mL. PBMC were washed with 4°C PBS for thrombocyte depletion. Cells were suspended in RPMI1640 containing 1.5% human autologous serum and cultured on petri dishes (Greiner Bio-one 35 × 10 mm, 627 161) at a cell density of 3 million/mL. Cells were left to attach for 35 min at 37°C in a humidified incubator with 5% CO_2 until monocytes became adherent. Non-adherent cells were removed with the supernatant after four times of washing with RPMI1640 medium. Monocytes were cultured for 7 days in RPMI1640 medium supplemented with 5% human serum to obtain macrophages. For usage in the 3D skin models, monocytes were cultured for 2 days and then transferred into the models in which they differentiate into macrophages. NHDF and normal human epidermal keratinocytes (NHEK, keratinocytes) were isolated and cultivated as described previously in detail [11].

Human skin model

Full-thickness 3D skin models were prepared as described previously [13, 14]. In brief, the dermal part of the skin models was constructed by merging 2×10^5 Normal human dermal fibroblasts (NHDF, simply called fibroblasts) and 1.35×10^6 macrophages into bovine collagen I solution (Vitrogen, Cohesion Technologies, Palo Alto, CA, USA). Fibroblast/macrophage collagen gels were poured into polycarbonate cell culture inserts in a 6 well cell culture plate and cultured at 37°C and 5% CO_2. At the second day of culture, two million NHEK were seeded on top of the dermal equivalent and cultured submerged in equal parts of medium mixture DMEM, Keratinocyte Medium and RPMI +5% human serum. Skin models were lifted to the air-liquid interface (ALI) after 1 day and cultured for up to 7 days. The concentration of calcium in the medium was raised to 0.5 mM. After tattooing with black ink, skin models were continuously cultured in the serum free medium Derma Life K without TGFα at 37°C and 5% CO_2. Investigation of the experiments was done on day five of culture. Experiments were performed in triplicates.

INCUBATION OF CELL CULTURES AND SKIN MODELS WITH TATTOO INK AND DRUGS

Tattooing of skin models

One set of skin models was tattooed with Magic Black Sailor Jerry at day seven after air liquid interphase using undiluted black Tattoo Ink (Sailor+Jerry SJ-Magicschwarz-10 [Deep Colours GmbH, Neuburg, Germany]). The ink was carefully infused into the dermal layer of the skin models using sterile tattoo needles of 0.3 mm diameter (Round Liner, No. 1001RL, Pain-is-Art, China). Using a standardized grid, lines were meticulously etched at

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1 mm intervals across a 1 cm^2 area. After a period of 5 days, the skin models were carefully collected for an in-depth immunohistological analysis to examine the absorption of the ink.

An additional set of skin models was meticulously tattooed with undiluted red ink (Sailor Jerry, SJ-Dukelrot-10). Post-tattooing, the ink was allowed to settle in the skin models for 24 h before cells were collected.

Treatment of human primary cell cultures with tattoo ink

Keratinocytes and fibroblasts were differentiated in special media, but in order to study the uptake of ink, the special media were washed off and all cells received RPMI1640 with 5% human serum and the ink was added at the designated concentrations and for the periods indicated, diluted in phosphate-buffered saline (PBS) at amounts in µL per mL of cell culture medium. To study the effects of corticosteroids and Pantothenate on macrophage ink uptake, injectable Dexamethasone (Fortecortin) was added to the cells on day 0, directly after starting the cultures, at a concentration of 10^{-7} M and the supplemented medium remained unchanged until day seven. Pantothenate (Sigmaaldrich) was administered to the cells daily at a concentration of 10 µg/mL of Dexpanthenol-free cell culture medium DMEM AS 61965 (Gibco), because of the instability of Pantothenate. Long-term treatment of macrophages for two weeks was done by adding the ink one time on day six of culture. For the two week-experiment, medium was replaced completely at day seven (RPMI1640 with 5% human serum).

Electron microscopy of cells

Following the incubation with tattoo ink, cell pellets were harvested by centrifuging at 400*g* for a span of 10 min. This process was conducted using at least one million cells. These were dispersed and fixed in 3% glutaraldehyde (GA) solution. The fixed samples were transferred to 1.5 mL reaction tubes (Eppendorf), centrifuged for 10 min at 400*g*, then the supernatants were discarded. The pellet was dispersed in B2 buffer and incubated for 5 min. The supernatant was removed after centrifugation (see above). 0.5 mL of 60°C agarose was pipetted onto the pellet and gently stirred with a metal rod to ensure complete coverage of the pellet. The tube was then stored at 4°C for 10 min for polymerization of agarose. The resulting agarose block containing the embedded cell pellet was cut into pieces <1 mm^3 and stored in 3% GA solution at 4°C for 16 h. The samples were washed in 0.1 M B2 buffer for 60 min (or up to 16 h) for embedding, then incubated with 1% OsO_4 solution for 60 min on a rotator. The samples were then washed with an equal mixture of distilled water and sucrose buffer for 10 min on a rotator.

This was followed by dehydration in a series of ethanol solutions of increasing concentration, including 30%, 50%, 70%, 90%, and 100% ethanol, with each solution being incubated for 10 min on a rotator. The samples were then incubated twice in 100% ethanol for 10 min each. The samples were further processed in propylene oxide for 30 min on a rotator with closed glass containers. The samples were then infiltrated with a 1:1 mixture of Epon and propylene oxide for 60 min on a rotator. This was followed by incubation in pure Epon resin on a rotator for 60 min at 37°C. The samples were then placed in a resin-filled embedding mould and polymerized for 2 h at 90°C. Ultrathin sections (80–100 nm) were cut using an ultramicrotome and collected on copper grids. The sections were then contrasted with uranyl acetate for 12 min and lead citrate for 1 min. The grids were examined under a transmission electron microscope. The fibroblast and KC cells were imaged using a Libra 120 (Zeiss) electron microscope operated at 120 kV and Microphages using Hitachi SU7800 operated at 100 kV.

Light microscopy of ultrathin sections

The same ultrathin sections that were studied in TEM were analysed for ink-derived signals using an Axioplan 2 motorized video microscope from Zeiss and a 20x EC Epiplan Neofluar objective with a numerical aperture of 0.5 was used. The attached Axiocam Mrc camera recorded images comprised of 1330 × 1300 pixels with a resolution of 0.74 µm/pixel.

Immunohistochemical staining of skin model samples

Cryosections 4 µm in height were stained with the antibody CD163 (mouse monoclonal, AbD Serotec) 1:100 in antibody diluent (DAKO, Glostrup, Denmark) for 1 h at room temperature. For detection, the LSAB system was used (Dako REAL Detection System, Alkaline Phosphatase/RED, Rabbit/Mouse, Agilent). Sections were counterstained with haematoxylin to get an overview of the tattooed areas in the skin models.

Flow cytometric analysis of cells from the skin model and monocultures

Cells were isolated from the skin models by removing the cells from the culture plates and mincing the skin models

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with scissors. After homogenization, cells were digested with collagenase type IV. More details can be found in previous studies where the method was used to isolate cells from liver [15]. Cells from the skin models or cells from the monocultures were collected and homogenized. Cells were washed twice with PBS to remove the residual phenol red after 10 min of centrifugation at 400*g*. To study blood cells and macrophage uptake of ink by flow cytometry, the ink uptake was quantified in the channel normally used to quantify the dye red fluorochrome Allophycocyanin (APC), a red dye. Viability of cells was analysed by using Hoechst 33258, as described before [16]. Flow cytometry was done using a BD FACS Canto II and analysed using the FlowJo 8.8.6 software (La Jolla, CA, USA).

Quantitative Realtime-PCR of cell cultures

Total RNA was isolated from at least one million cells which were detached using a cell scraper. Then cells were centrifuged and an RNeasy Mini kit (Qiagen) was used for isolation of total RNA, according to the instructions of the manufacturer. 500 ng of total RNA were reverse-transcribed by the Applied Biosystems™ High-Capacity cDNA Reverse Transcription Kit from Thermo Fisher Scientific. Applied Biosystems™ RNase inhibitor (20 U/μL) from Thermo Fisher Scientific was employed. Quantitative real-time polymerase chain reaction was conducted using a QuantStudio® 5 Real-Time PCR System (Applied Biosystems, Foster City, CA). Reactions were executed in triplicates using Applied Biosystems™ PowerUp™ SYBR™ Green Master Mix from Thermo Fisher Scientific. The thermocycler parameters for the amplification of these genes were as follows: UDG activation at 50°C for 2 min, followed by Dual-Lock DNA polymerase at 95°C for 2 min, then 40 cycles started to denature at 95°C for 1 s then anneal/extend at 60°C for 30 s. The thermocycler parameters for the amplification of these genes were as follows: UDG activation at 50°C for 2 min, followed by Dual-Lock DNA polymerase at 95°C for 2 min, then 40 cycles started to denature at 95°C for 1 s then anneal/extend at 60°C for 30 s. β-actin (*ACTB*) was used as an endogenous control gene for gene expression normalization. The comparative $^{\Delta\Delta}C_t$ method was used to calculate relative quantification of gene expression. Primer sequences are provided in the supporting information (Table S2).

Statistical analysis

Differences between groups with $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ were considered significant. We utilized One way ANOVA for multiple group comparisons. Statistical analysis of data was done using GraphPad Prism 9.0 (GraphPad software, La Jolla, USA).

RESULTS

We had initially analysed a non-REACH compliant tattoo ink and observed a toxicity for macrophages at concentrations above 1 μL of ink per mL of cell culture medium. Statistical analyses demonstrated a significant impact on macrophage viability (data not shown). We therefore continued our study with a REACH-compliant version of the same red ink that was available after the REACH reform—this ink did not show such toxic reactions on macrophages and was used for all upcoming experiments.

Due to a missing physicochemical characterization of REACH-compliant tattoo ink, we performed a comprehensive analysis to determine the size and composition of tattoo ink. The morphological properties of the ink were investigated using transmission electron microscopy (TEM) where we observed that the tattoo ink contained organic pigments that formed aggregates (Figure 1a,b). Scanning electron microscopy (SEM) revealed that the tattoo ink was a colloidal dispersion comprised of rod-like faceted nanoparticles, with an average size of approximately 200 nm (Figure 1c,d). Owed to the aggregations of the tattoo ink particles and the resulting difficulties to obtain the size distribution by electron microscopy, we performed dynamic light scattering (DLS) analysis using two different methods. The first method employed DLS settings that we have used in earlier studies. Routinely we use DLS as it is done in the first method where we characterize for instance lipid nanoparticles for siRNA, at a DLS angle of 173.5° [15]. This method revealed that at a concentration of 1:10 diluted in phosphate-buffered saline (PBS), the ink particles sized 1080 nm, but when they were diluted 1:100 in PBS, the size increased to 5615 nm (Table S1). We then used a second sophisticated type of DLS settings with an angle of 90°, systematically explored different dilutions, and additionally we filtered water or PBS before using it for ink dilution (to exclude for instance dust-derived data peaks in DLS). We noted that the sizes of the ink recorded changed with different concentrations (Figures S1–S6). EDX demonstrated that there were no unexpected chemical elements that could affect cell viability (Figure S7).

Owed to the fact that macrophages are known as key cells of tattoo ink uptake in the body, but since there are also reports on a potential uptake by fibroblasts [4], we studied 3D models of human skin composed of skin-derived dermal primary keratinocytes, fibroblasts, and macrophages. The cells self-assemble and form a skin

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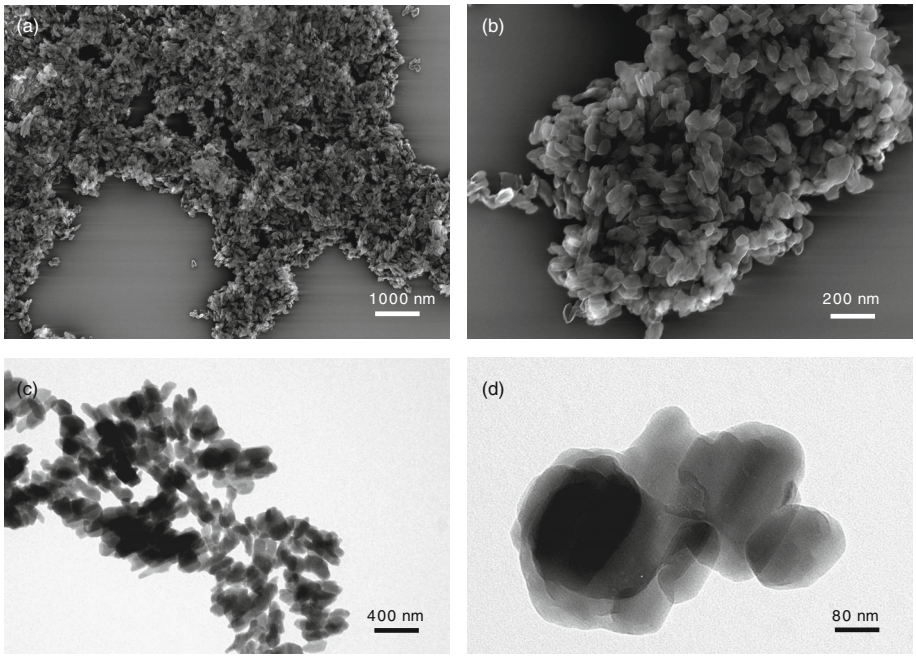

FIGURE 1 Electron microscopical analysis of tattoo ink. (a and b) Transmission electron microscopy of tattoo ink and (c and d) scanning electron microscopy of Tattoo ink.

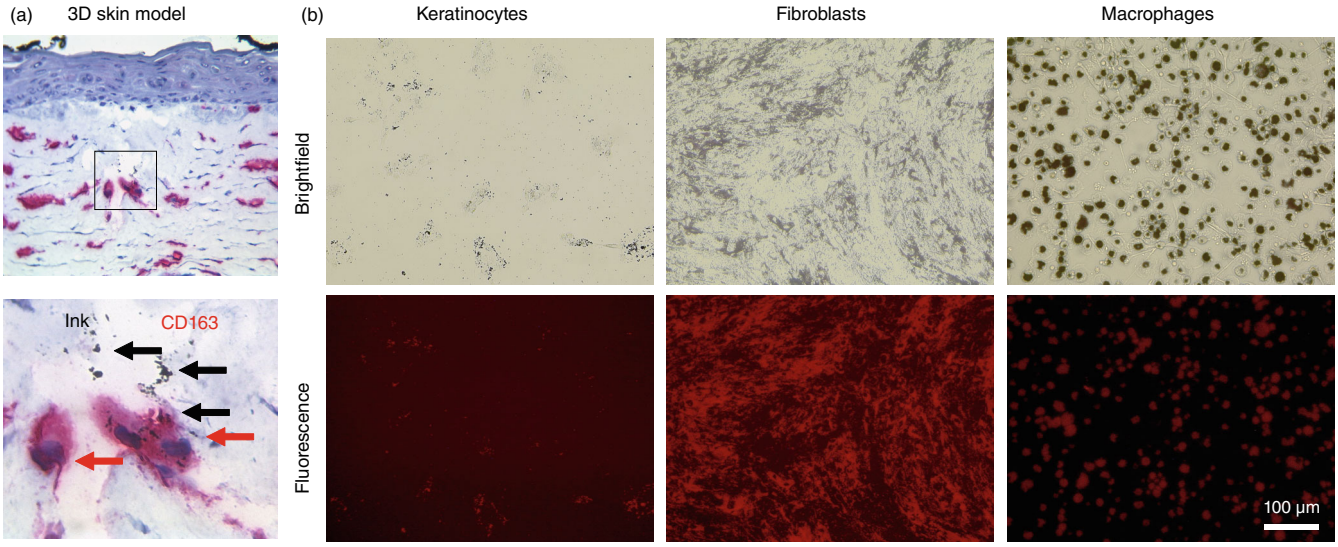

FIGURE 2 Section and cell culture analysis of tattoo ink uptake. (a) Black tattoo ink was added to skin models and macrophages were stained using CD163 immunohistochemistry. (b) Human primary keratinocytes, fibroblasts, and macrophages were incubated with 2 µL of red tattoo ink in RPMI1640 medium with 5% human serum. After 24 h the fluorescent signal was detected using fluorescence microscopy.

equivalent that can replace animal experiments. We brought the ink into the skin models by tattooing them using a commonly used sterile tattoo needle with a diameter of 0.3 mm. After 24 h, cryosections were generated from the models to stain the macrophages. We observed a co-localization of black tattoo ink and macrophages (Figure 2a). In order to further clarify the uptake capabilities of the different types of skin cells, we used monocultures of each cell type and incubated these for 24 h with the red ink at the concentration of 2 µL ink/mL in RPMI1640 medium supplemented with 5% human serum, which we used for the upcoming experiments. We found that keratinocytes did not show uptake, whereas the ink generally sticked to fibroblasts without distinct cellular inclusions. It was apparent that macrophages were the most efficient cell type for tattoo ink uptake with the ink being located inside endosomes (Figure 2b).

After observing the fluorescent signals derived from the red tattoo ink, we opted to use flow cytometry to compare the absorption rates in a comprehensive skin model versus monocultures of distinct cell types. Flow cytometric analysis demonstrated that the red ink

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resulted in dose-dependent fluorescent signals by macrophages and fibroblasts whereby the latter ones showed a much lower uptake. We noted only slightly altered signals in the samples of the 3D skin model and the keratinocytes, as shown in representative flow cytometric plots (Figure 3a). Quantifications of the data demonstrated that significantly elevated signals of more than 80% of the macrophages were obtained compared to only 2% of the fibroblasts. Only the uptake by macrophages was also dose-dependent regarding the mean fluorescence intensity (MFI) (Figure 3b). We further studied the longitudinal uptake by macrophages which showed up to be time dependent as suggested by flow cytometric plots (Figure 3c), and as evident by signal quantifications thereof (Figure 3d).

Transmission electron microscopy (TEM) confirmed that the ink was present in endosomes of macrophages whereas in the fibroblasts, it occurred rather extracellularly (Figure 4). An investigation of the same ultrathin sections that were studied by TEM using light microscopy confirmed the finding that the ink was most clearly enriched in macrophages (Figure S8).

The next aim was to study potential drugs that might affect ink persistence in macrophages. Thus, we studied the impact of dexpanthenol (administered in the form of pantothenate to the cell cultures), and of dexamethasone. These drugs are routinely utilized in the topical aftercare treatment after the tattoo procedure and they are hypothesized to decrease the amount of ink in macrophages. We found that both drugs unexpectedly led to a similarly strong uptake of ink (Figure 5a). Data evaluation revealed statistical significance for all three conditions (Figure 5b). Pantothenate led to a slight elevation of dead cells as indicated by flow cytometric plots (Figure 5c), and signal quantifications revealed that as few as 2.7% cells showed decreased viability (Figure 5d).

Tattoo ink may exhibit a potential immunogenicity and since macrophages are clearly the most important cell type for tattoo ink accumulation, we incubated human primary macrophages with ink at four different concentrations (2 μL ink/mL medium) and studied their expression of inflammatory mediators such as tumour necrosis factor (Tnf), Interleukin 1β (Il1β), and the CC chemokine ligand 22 (Ccl22) after 24 h. We noted that there was no significant elevation of any of the mediators (Figure 6).

In order to study the long-term fate of tattoo ink in macrophages, we prolonged the incubation after washing off the ink after 6 days and replaced the medium after 24 h of incubation. Interestingly, even after 9 days of culture, the ink was still present in the cells (Figure 7a), and there was no indication for toxicity, with most macrophages being viable (Figure 7b).

Blood cells can play an important role for the distribution of tattoo ink in the body but have not been studied in this context. Thus, the next step was to study the uptake of the ink by human primary blood immune cells. We therefore incubated blood-derived leukocytes for 1 or 24 h with the ink at 2 μL/mL or left them untreated, and studied the uptake of ink by flow cytometry. We found that the ink profoundly increased the granularity of monocytes, so that they could not be studied by plots on forward and sideward scattering light. The granularity of granulocytes and lymphocytes was much less affected (Figure 8a). We then studied the ink uptake based on its fluorescence in flow cytometry and noted a low uptake by granulocytes and no effects on their viability (Figure 8b). Similar findings were obtained in data analysis of lymphocytes (Figure 8c).

Human monocytes, the precursors of macrophages, appeared to strongly interact with the ink what was indicated by strong reductions of their numbers. Hence, monocytes could not be analysed from this experiment. We henceforth isolated monocytes from PBMC and studied them in monocultures at an amount of ink of 2 μL/mL. We noted a very strong uptake of the ink after 1, 12, and 24 h. Monocyte cell numbers on cell culture plates were significantly reduced after 24 h (Figure 9a). Flow cytometric analyses outlined that the ink led to a translocation of the monocytes in the FSC-SSC plot from the lower right to the upper left section (Figure 9b). The uptake of ink into monocytes was significantly elevated after one and 24 h (Figure 9c). Furthermore, their viability was found to also be significantly reduced as noted from statistical quantifications of flow cytometric signals (Figure 9d).

DISCUSSION

The physicochemical analyses of the tattoo ink partly support the hypothesis that tattoo ink or rather the organic pigments that make up the main constituent of tattoo ink should be considered as nanoparticles [4]. However, we further detected larger particles depending on defined dilutions of the ink. This corroborates to an earlier study of another group where the authors reported that organic pigments from tattoo ink exhibit a broad size distribution [8]. In particular, this is in line with our second DLS method. Notably, in in vivo studies of humans, only smaller nano-sized ink pigments were found in the lymph nodes, likely because the larger particles cannot be transported by the lymphatic system [8]. It might thus be the case that the larger particles are translocated into the liver, as suggested by another study [9].

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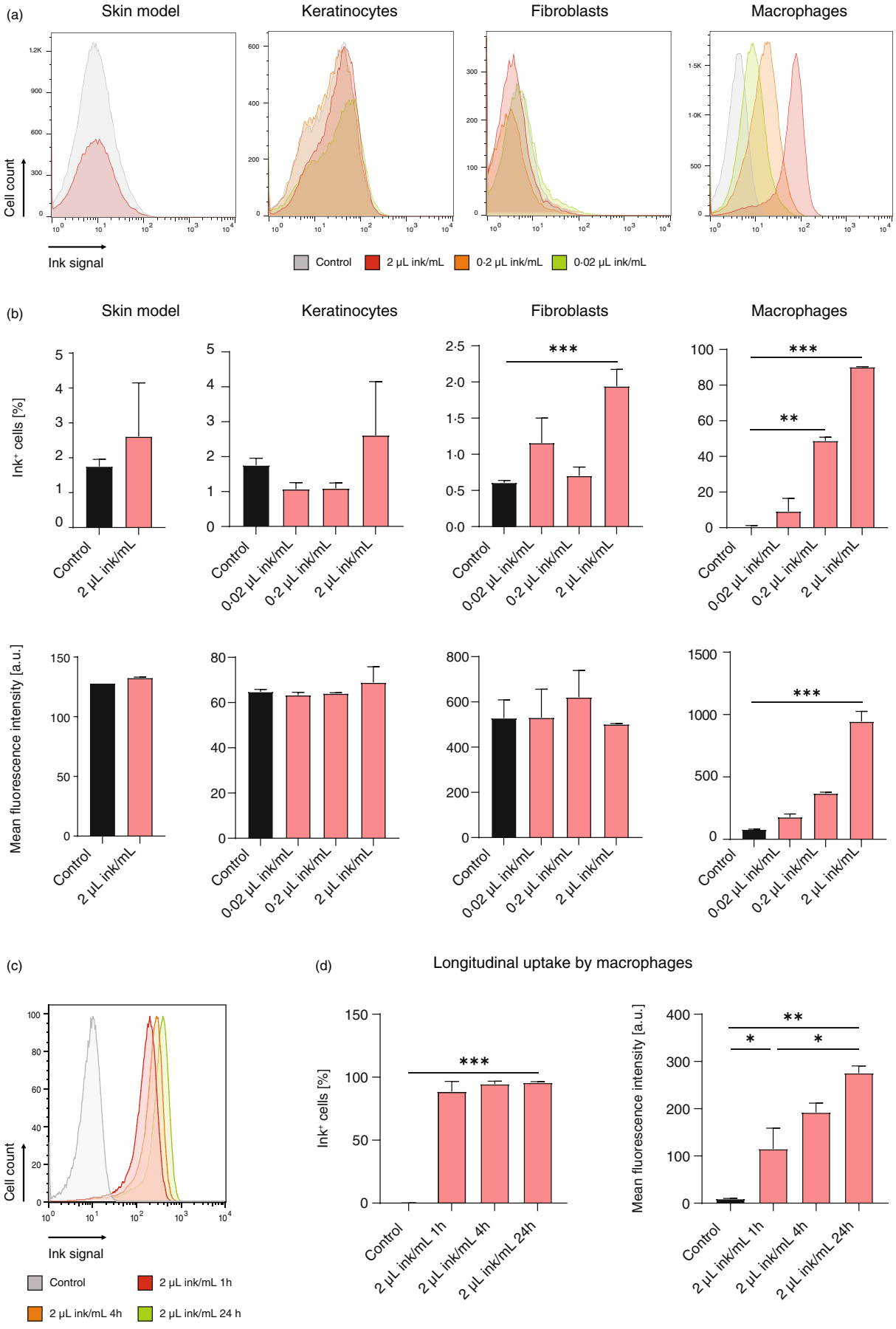

FIGURE 3 Legend on next page.

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Importantly, the changes in size that occur during different dilutions of the ink in our analyses indicate that tattoo ink is different from nanoparticles desired for medical applications—these are desired to keep their size constant. Medical nanoparticles (nanomedicines) are often coated with polymers such as polyethylene glycol, what inhibits unspecific protein adsorption, increases their stability, and also reduces uptake by immune cells [17]. Upon intravenous injection, nanoparticles are taken up by blood circulating immune cells, particularly monocytes that constantly migrate into the liver [16]. The physicochemical instability of tattoo ink that leads to its elevated size may form the base for the rapid uptake by macrophages and monocytes; very likely, a PEGylated type of tattoo ink would not be usable for tattooing as it would likely lead to a lower uptake by macrophages.

Our data indicate that monocytes may undergo important changes in their differentiation process into macrophages that critically affects that sensitivity for tattoo ink. Upon tattooing, portions of the tattoo ink may diffuse into dermal blood vessels. Inside the blood, monocytes hence are the phagocytic carriers of tattoo ink. Monocytes were very likely overseen in tattoo ink translocation in the body, despite the fact that they are precursors of macrophages and also of dendritic cells that are known to migrate into the lymph nodes [18]. Monocytes hence function in transporting tattoo ink upon uptake and very likely transport it into the liver, while they differentiate into macrophages [9]. Improved understanding of the uptake of tattoo ink by monocytes could therefore be a key factor for novel tattoo removal therapies—monocytes might be engaged for a tattoo removal therapy.

Interestingly, we also confirmed an uptake of tattoo ink by lymphocytes as reported before by other groups where the authors demonstrated that although tattoo ink was located in lymph nodes, it did not affect the responses of B and T cells [19]. Moreover, the uptake by

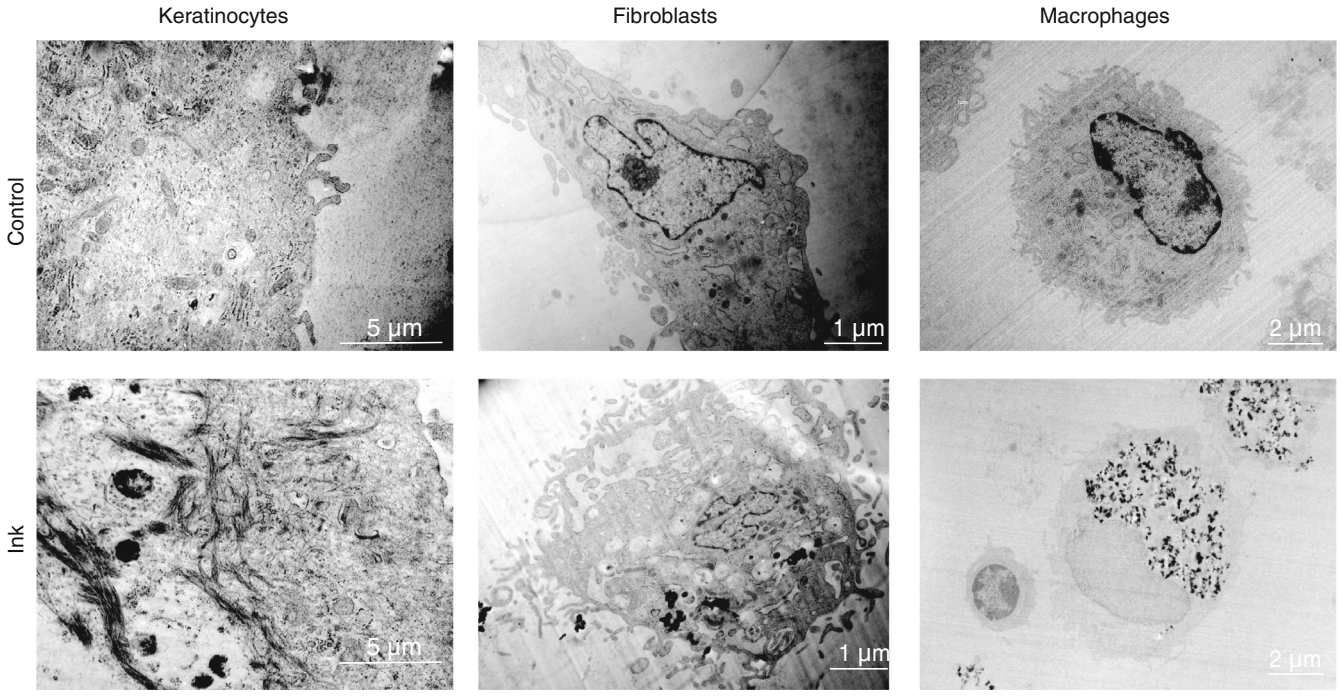

FIGURE 4 Electron microscopical investigation of tattoo ink uptake by human primary skin cell cultures. Ink was added at a concentration of 2 µL/mL of cell culture medium and human keratinocytes, fibroblasts, and macrophages were incubated for 24 h. Ultrathin sections were prepared and analysed by transmission electron microscopy.

FIGURE 3 Flow cytometric analysis of the uptake of tattoo ink in a 3D skin model and by different primary skin cells. Skin models composed of fibroblasts, keratinocytes, and macrophages were generated. (a) Skin models were tattooed with red ink or ink was added to skin monocultures at the indicated concentrations. Cells were isolated from the skin model and subjected to flow cytometry, shown by representative plots. (b) Statistical quantifications of cells scoring positive for ink by the signal in the red channel APC (as percent positive cells and mean fluorescent intensity, MFI). (c) Longitudinal uptake of tattoo ink by macrophages shown by flow cytometric plots and (d) quantifications thereof. Data represent mean of $n = 2–3 \pm$ SD; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ (one-way ANOVA).

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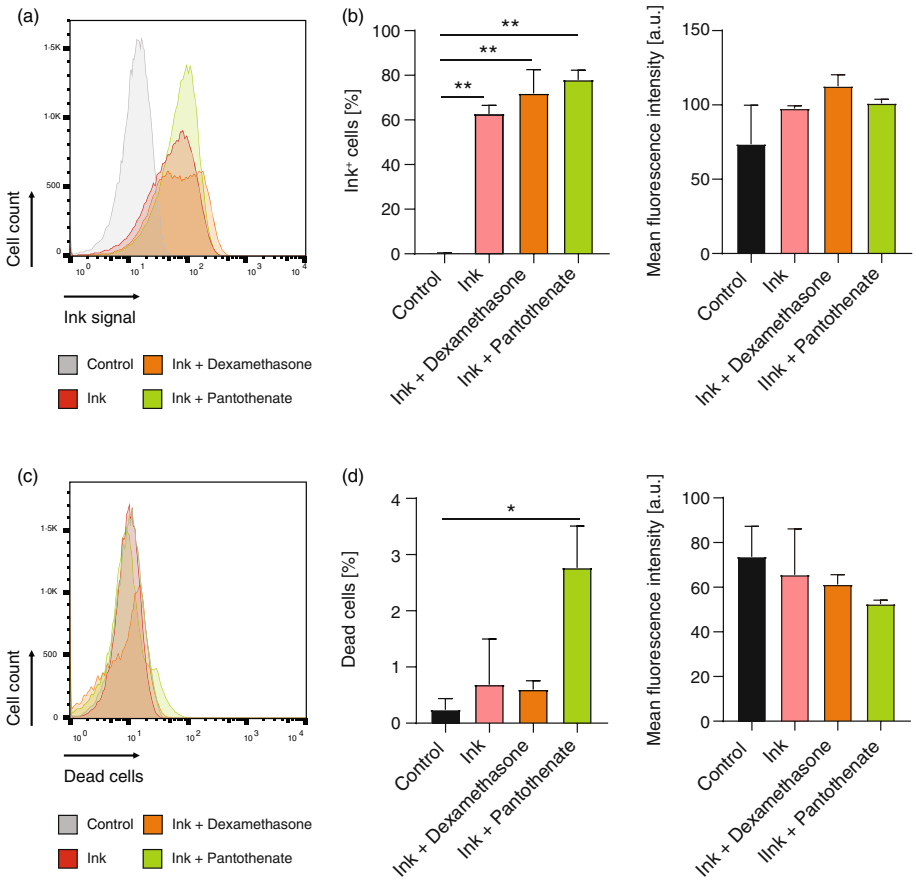

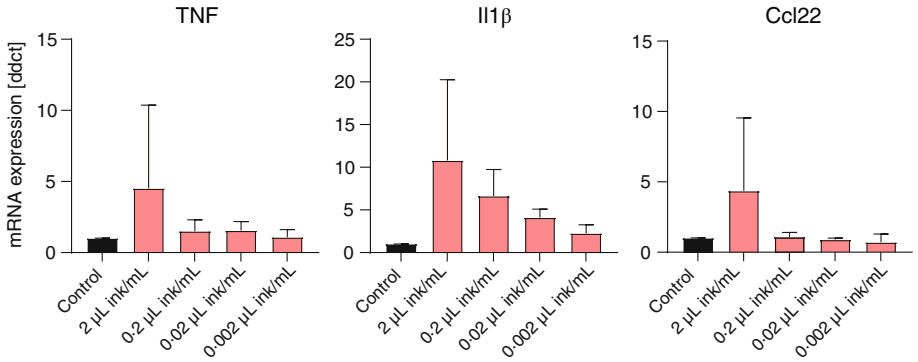

FIGURE 5 Effects of steroids and Pantothenate on the retention of tattoo ink in human macrophages. Human primary macrophages were treated with either 2 µL/mL of tattoo, or in addition with dexamethasone (10^{-7} M from day 0 to day 7, single addition to the medium) or pantothenate at 10 µg/mL (daily supplementation from day 0 to day 7). (a) Flow cytometric analysis of ink uptake, and (b) statistical quantifications thereof. (c) Effects of the drugs on macrophage viability shown in representative flow cytometric plots, and (d) as quantified. Data represent mean of $n = 2 \pm$ SD; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ (one-way ANOVA).

FIGURE 6 Analysis of the inflammatory potency of tattoo ink by macrophages. Human primary macrophages cultured in RPMI1640 with 5% human serum were incubated with different concentration of red tattoo ink for 24 h and the expression of inflammatory mediators was analysed using quantitative Realtime-PCR. Data represent mean of $n = 3 \pm$ SD; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ (one-way ANOVA).

granulocytes might be low, but due to their high numbers, it might be important as well. The initial uptake of ink by lymphocytes and granulocytes, followed by a decline over time, could suggest a dynamic process of ink absorption and potential excretion may occur within these cells.

The strong impact of the ink on cellular granularity has to our knowledge not been reported before. We suggest that huge amounts of the ink are stored in vesicles of monocytes and macrophages and that this is owed to the large aggregations of the ink. Clinically usable nanoparticles such as lipid nanoparticles for siRNA [15], or liposomal carriers [20], do not affect the granularity of immune cells, which is likely based on the fact that these nano-sized particles do not lead to large intracellular inclusions.

Macrophages, as part of the immune system, are known to respond to foreign substances in the body. They could be activated by the tattoo ink, and hence may initiate an immune response, as it was reported by non-

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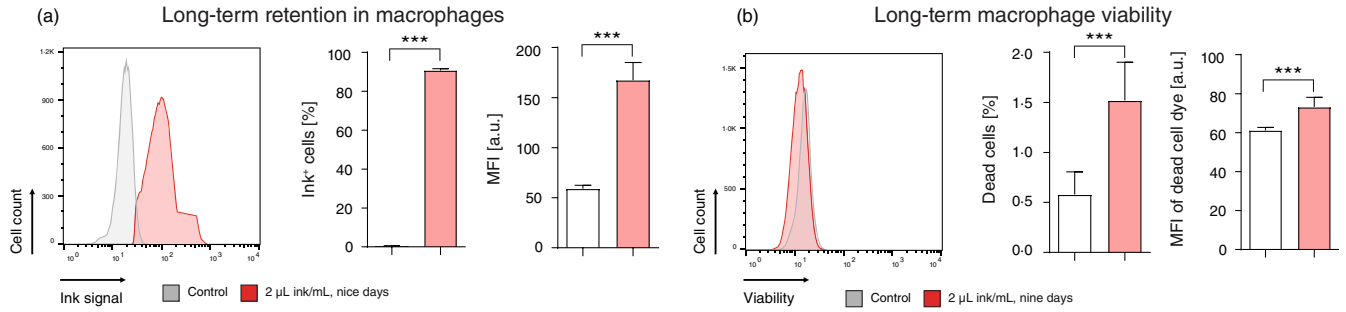

FIGURE 7 Long-term retention of tattoo ink in human primary macrophages. Human primary macrophages cultured in RPMI1640 with 5% human serum were incubated with 2 µL of red tattoo ink/mL a and cultured for 2 weeks. (a) Representative plots of flow cytometric signals after 2 weeks and quantifications thereof. (b) Analysis of cell viability after nine days of culture. Data represent mean of $n = 2 \pm$ SD; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ (one-way ANOVA).

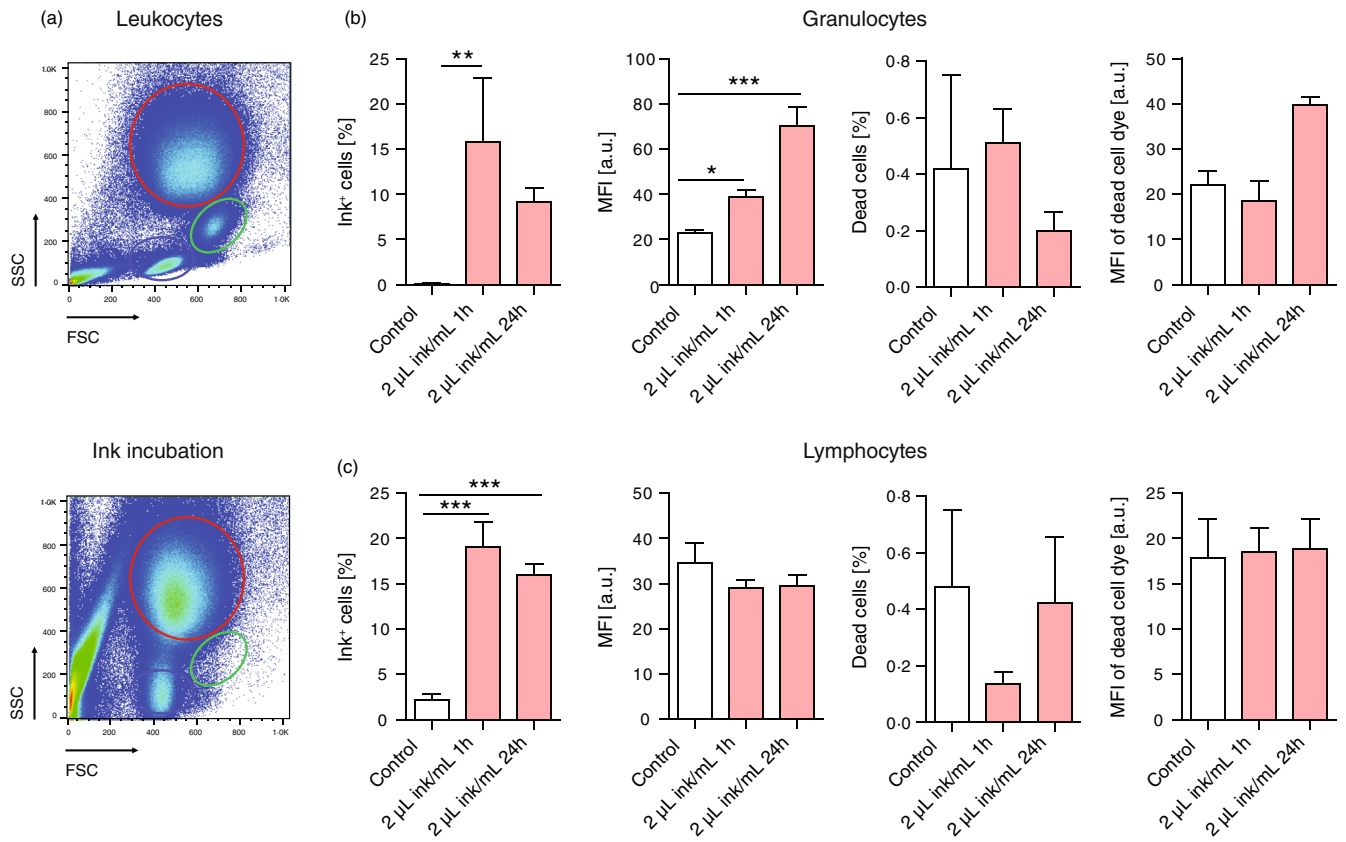

FIGURE 8 Uptake of tattoo ink by human primary blood cells. Human primary immune cells were isolated from blood of healthy human donors and incubated with 2 µL of red tattoo ink/mL and incubated for one or 24 h. (a) Representative Pseudocolor plots from the flow cytometric analysis on the impact of tattoo ink on blood immune cell morphology. (b) Ink uptake and its impact on granulocyte viability. (c) Effects of ink uptake on lymphocytic ink uptake and viability. Data represent mean of $n = 3 \pm$ SD; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ (one-way ANOVA).

REACH compliant tattoo ink [5]. A potential association of skin cancer with tattooed body sites could be accelerated by inflammatory activation of immune cells [6]. Interestingly, even the highest concentration of tattoo ink did not cause inflammatory activation of macrophages. Notably, previous studies have demonstrated that an inflammatory response and granuloma formation were detected in skin tattooed with non-REACH compliant tattoo ink [21]. The absence of elevated inflammatory markers in response to the ink suggests that the REACH-compliant ink which we employed does not instigate an inflammatory activation of macrophages. The mild

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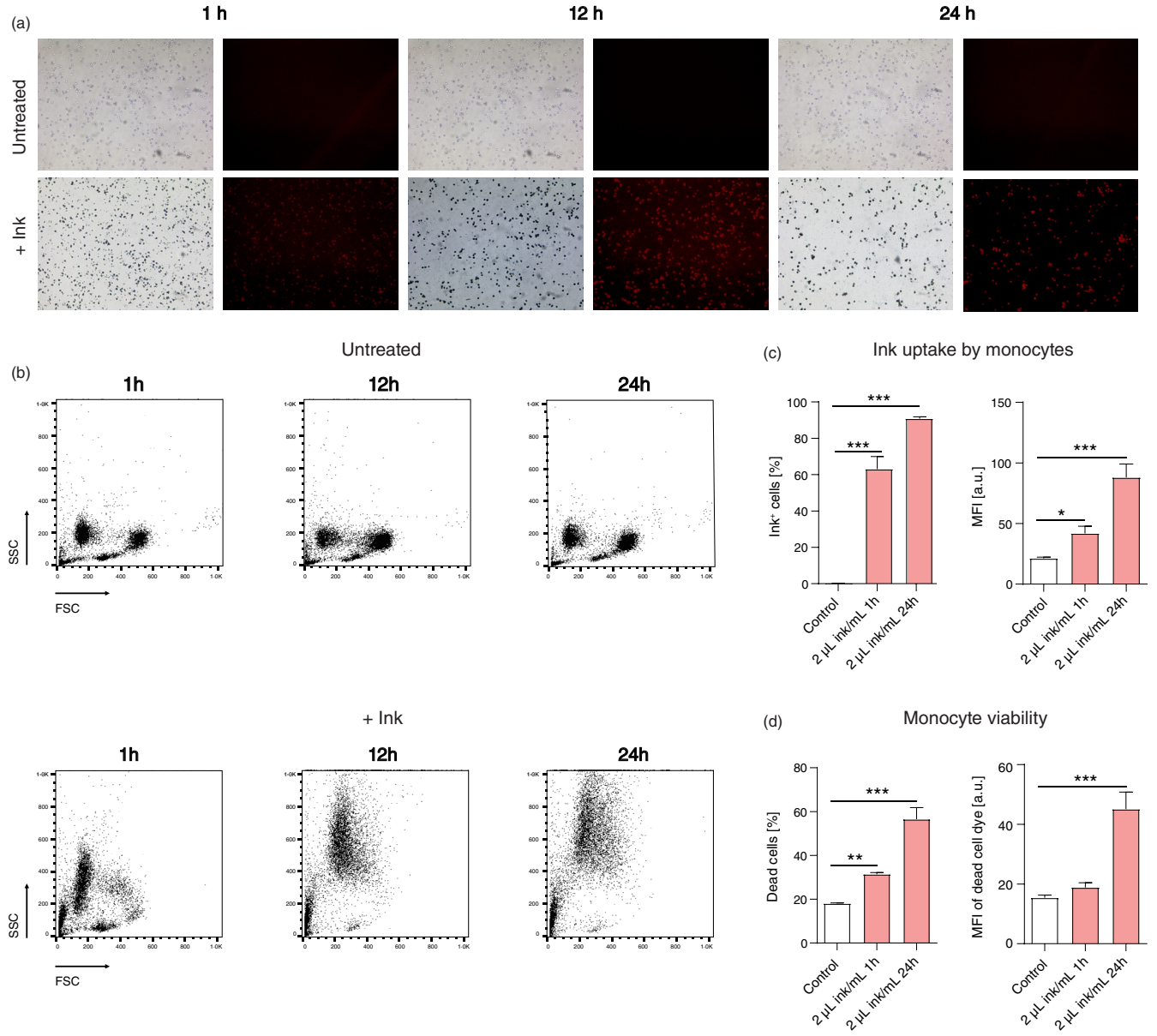

FIGURE 9 Analysis of tattoo ink toxicity for human monocytes. Human primary monocytes were incubated with 2 µL of red tattoo ink/mL for up to 24 h. (a) Cell culture micrographs with brightfield microscopy (left side) and red channel fluorescence microscopy (right had pictures). (b) Flow cytometric analysis of untreated monocytes and of monocytes treated with 2 µL/mL of tattoo ink. (c) Analysis of ink uptake by flow cytometry, and (d) of monocyte viability. Data represent mean of $n = 3 \pm$ SD; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ (one-way ANOVA).

inflammatory response to REACH compliant ink may be owed to the lack of presence of potentially harmful metals such as Co, Fe, Ni and Zn which were shown to be present various different non-REACH compliant inks [22]. Our EDX analyses have not identified metals except for low levels of Al and Cu.

The long-term retention in macrophages, particularly in human primary macrophages, appears to be the likely cause for tattoo persistence and was replicated in our human cell-based 3D skin models, as it was anticipated from in vivo studies of other groups. However, the fact that human macrophages do not proliferate—in contrast to murine macrophages—might represent an important difference between the different species [3, 8]. Our data hence imply that human skin models could potentially serve as suitable substitutes for in vivo studies in the mouse model.

Moreover, our research suggests that the previously reported co-localization of ink with fibroblasts [4] may be more accurately attributed to the ink binding to the

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fibroblasts, rather than a clear uptake by the cells. Indeed, fibroblasts are renowned for their ability to produce extracellular matrix proteins such as collagen [23]. This characteristic could potentially lead to the entrapment of tattoo ink within the tissues during the tattooing process by binding to collagen [4].

Interestingly, neither topical treatment with pantothenate nor corticosteroids negatively interfered with the uptake of ink by monocytes or macrophages. The moderate effect of Pantothenate on macrophage viability that we noted likely does not play a crucial role for tattoo persistence given that less than 3% of the cells were nonviable.

AUTHOR CONTRIBUTIONS

Cheng Lin and Yvonne Marquardt performed the experiments with human primary cells. Yvonne Marquardt prepared the 3D skin models. Matthias Bartneck and Jens Baron planned and funded the study. Cheng Lin, Yvonne Marquardt, Tamas Harazdti, Jens Baron, and Matthias Bartneck evaluated the data. Thomas Rütten and Khosrow Rahimi performed the electron microscopical studies. Cheng Lin and Tamas Harazdti performed DLS measurements. All authors reviewed the final manuscript.

ACKNOWLEDGEMENTS

We are grateful to all people that donated their blood to the Transfusion Medicine of the University Hospital Aachen. We would like to thank Hendrik Schümann and Stefan Hauk for technical assistance with TEM and SEM/EDX, respectively. Open Access funding enabled and organized by Projekt DEAL.

FUNDING INFORMATION

This study was supported by financial support for M.B. from the DFG (BA6226/2-1), the Wilhelm Sander Foundation (Grant No. 2018.129.1), the COST Action Mye-InfoBank 476 (CA20117), a BMBF grant (16LW0143, Mamma-Explant), the State of NRW (ZM-1-27B, NovoKolon), a grant from Bayer Vital, and an ERS grant from RWTH University (G:(DE-82)EXS-SF-OPSF732). C.L. was supported by a CSC China Scholarship Commission stipend (ID: 202008320329) and also L.L. received a China Scholarship Commission stipend (ID: 202206170024). Parts of this study were done at the Center for Chemical Polymer Technology CPT, that was supported by the EU and the Federal State of North Rhine-Westphalia (EFRE grant 30 00 883 02).

CONFLICT OF INTEREST STATEMENT

The authors declare that they have received support for their research. Matthias Bartneck received research support from Bayer Vital company.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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How to cite this article: Lin C, Marquardt Y, Rütten S, Liao L, Rahimi K, Haraszti T, et al. Macrophage-like rapid uptake and toxicity of tattoo ink in human monocytes. Immunology. 2024; 171(3):388–401. https://doi.org/10.1111/imm.13714

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