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Assessment of cytotoxicity and sensitization potential of intradermally injected tattoo inks in reconstructed human skin

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Abstract

Background: The number of people within the European population having at least one tattoo has increased notably, and with it the number of tattoo-associated clinical complications. Despite this, safety information and testing regarding tattoo inks remain limited.

Objective: To assess cytotoxicity and sensitization potential of 16 tattoo inks after intradermal injection into reconstructed human skin (RHS).

Methods: Commercially available tattoo inks were injected intradermally into RHS (reconstructed epidermis on a fibroblast-populated collagen hydrogel) using a permanent makeup device. RHS biopsies, tissue sections, and culture medium were assessed for cytotoxicity (thiazolyl blue tetrazolium bromide assay [MTT assay]), detrimental histological changes (haematoxylin and eosin staining), and the presence of inflammatory and sensitization cytokines (interleukin [IL]-1α, IL-8, IL-18; enzyme-linked immunosorbent assay).

Results: Varying degrees of reduced metabolic activity and histopathological cytotoxic effects were observed in RHS after ink injection. Five inks showed significantly reduced metabolic activity and enhanced sensitization potential compared with negative controls.

Discussion: Using the RHS model system, four tattoo inks were identified as highly cytotoxic and classified as potential sensitizers, suggesting that allergic contact dermatitis could emerge in individuals carrying these inks. These results indicate that an RHS-based assessment of cytotoxicity and sensitization potential by intradermal tattoo ink injection is a useful analytical tool to determine ink-induced deleterious effects.

KEYWORDS
allergic contact dermatitis, allergy, cytotoxicity, IL-18, in vitro, reconstructed human skin, sensitization, tattoo ink

Abbreviations: ACD, allergic contact dermatitis; ELISA, enzyme-linked immunosorbent assay; H&E, haematoxylin and eosin staining; KGF, keratinocyte growth factor; LC, Langerhans cell; MTT, thiazolyl blue tetrazolium bromide assay; P/S, penicillin/streptomycin; PMU, permanent makeup; RHS, reconstructed human skin.

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1 | INTRODUCTION

The art of tattooing has been practiced for millennia and is at least as old as Ötzi, the Tyrolean Iceman, who lived around 3250 BC, and is the earliest example of humans having tattoos to date.[1] Now, more than 5250 years later, many Europeans have followed his example: 14% of the European population had one or more tattoos in 2016, and the number is increasing.[2] This development is accompanied by a rise in associated clinical complications, including infections, allergic reactions, autoimmune diseases, scars, keloids, and various pigment changes.[3,4] Some of these complications may be partly attributed to the mechanical aspect of tattooing, which has remained largely undefined.[5,6] By repetitively inserting a needle into the skin, microtrauma is introduced which in itself may cause inflammation, but which also creates ports of entry for potential pathogens.[7] However, other complications are caused by the injected tattoo-ink itself, for example, allergic contact dermatitis (ACD) to tattoo pigment.

ACD is a type IV delayed hypersensitivity reaction of the skin which is characterized by symptoms such as pruritus, erythema, and oedema.[8] Symptom severity ranges from mild to intense, potentially causing considerable chronic discomfort for the patient resulting in decreased quality of life.[9] When left untended, ACD can eventually lead to ulceration, hyperkeratosis, and necrosis of the skin.[7,10] Previous research showed that tattoo inks can induce ACD, with disease onset varying from within 1 month after tattoo application to several years later.[11,12] In particular, allergic reactions were predominantly found in tattoos containing red ink.[13] Whereas allergic reactions to tattoo ink were once primarily linked to inorganic impurities such as nickel, cadmium, and mercury, research expanded towards organic pigments as the culprit of the pathogenesis as well.[14,15]

An important reason why specifically red tattoo inks appear to cause sensitization is that these inks contain high amounts of potentially hazardous organic pigments, a variety of which were identified by Serup et al[16] after analysing 104 dermatome biopsies derived from patients exhibiting allergic reactions to their tattoos. This group of harmful organic pigments was found to mainly consist of azo pigments. As the name suggests, these pigments contain azo groups (—N=N—), mostly bound to benzene or naphthalene rings.[17] Over the last few decades these pigments have been increasingly used in tattoo inks due to their vivid colour and longevity. However, azo pigments are principally produced for applications such as printing, painting cars, and colouring toys and have not been adequately tested for use in humans.[18] Therefore, azo pigments have been hypothesized to cause the described adverse health effects, claims that are supported by clinical and toxicological data. Various pigment molecules were found to be apt for photochemical cleavage into carcinogens (polycyclic aromatic hydrocarbons and aromatic amines) and potential haptens, possibly eliciting an allergic response.[16,19–21]

Therefore, there are a number of toxicological effects to be considered regarding tattoo inks. Although organic pigments have been replacing inorganic pigments in commercial inks, tattoo inks still contain heavy metals as either additives, chromophores, or contaminants.[22] Titanium, barium, and aluminium were found as common colourants while antimony, arsenic, cadmium, lead, chromium, and nickel were identified as contaminants after chemical analysis of commercially available inks.[22,23] While all of these metals possess toxic properties, some extremely toxic metals such as chromium, nickel, and lead have also been found to be highly concentrated in some tattoo inks.[22,24] Taken together with their sensitization capacity, these findings have led to concern about the additional health hazards tattoos impose and urge for thorough safety assessment of tattoo inks. Research uncovered a wide range of sensitizing azo pigments in US-manufactured tattoo ink.[25] Alarmingly, while an estimated 70%-80% of all tattoo inks are produced in the United States, these tattoo pigments are poorly tested on potential adverse health effects as no Food and Drug Administration (FDA) regulations exist for their skin-injected use.[26,27] In fact, the FDA did not authorize the use of any ink for tattooing, and most European countries lack effective regulations on tattooing in general.[21,28] The primary reason for this is that tattoo inks are not covered by the European Cosmetic Products Regulation or the Medical Devices Directive.[29,30] Laux and Luch[28] report that this in turn is mainly due to insufficient analytical methods and missing data on toxicity of tattoo inks and their constituents. Thus, in order to create adequate regulations on tattooing and tattoo inks, it is of vital importance that effective analytical methods producing accurate toxicological data on tattoo inks are developed and validated first.

Seeking to design such a method, we have previously investigated cytotoxicity and sensitization potential of five tattoo inks in an organotypic 3D reconstructed human skin (RHS) model. RHS consists of air-exposed epidermis attached to a fibroblast-populated collagen hydrogel. Culture at the air–liquid interface stimulates epidermal differentiation and stratification.[31] Tattoo ink was added to the culture medium to facilitate direct exposure to the fibroblast-populated hydrogel that mimicked the dermis, which was then screened for release of interleukin (IL)-18, a key cytokine involved in the initiation of ACD.[32,33] IL-18 is a proinflammatory cytokine that promotes Langerhans cell migration and stimulates the release of interferon-γ, tumour necrosis factor-α, and IL-8, all involved in the T helper 1 cell–type immune response.[33,34] Release of IL-18 by keratinocytes is inflammasome mediated and contributes to an individual becoming sensitized and developing type IV delayed hypersensitivity, leading to ACD.[35] The role of IL-18 in ACD pathogenesis was thoroughly investigated in reconstructed human epidermis and NCTC 2544 (a human skin keratinocyte cell line) and was found to distinguish potential contact sensitizers from irritants and respiratory sensitizers.[33,36,37] Using this principle in RHS, a red ink named Eternal Light Red (ELR), supplemented in the culture medium, was shown to increase IL-18 release, thus classifying it as a potential sensitizer. It was also established that the assessed red and black inks induce varying levels of cytotoxicity, after analysis of postexposure metabolic activity and

histology.[32] However, the inks were not directly applied intradermally and because most inks are suspensions, their validity is limited compared with tattoo application in vivo, where the injected ink comes in direct contact with the cells and extracellular matrix. Besides, addition of ink in the culture medium affects the entire RHS, whereas effects of real-life intradermal ink injection are localized to the injection site. Furthermore, only IL-18 was measured as a sensitization marker, leaving out additional inflammatory markers.

This research elaborates on our previous study, aiming on assessment of cytotoxicity and sensitization potential of intradermally injected tattoo inks. Intradermal injection, rather than medium supplementation, was achieved using an actual permanent makeup (PMU) device, which for the first time enabled direct embedding of tattoo inks into the reconstructed dermis of RHS. The use of this device closely approached in vivo tattooing and ensured that RHS cells and the dermal matrix were directly exposed to the inks. A panel of 16 differently coloured inks was injected into RHS in a standardized 12 × 12 dotted square of 0.7 cm^2, thus mimicking real-life tattooing. The inks were obtained from the Academic Tattoo Clinic Amsterdam and from various tattoo shops. Inks were selected based on reported adverse reactions (allergy, inflammation, or irritation), supplemented with random inks for experimental comparison. As most chronic tattoo reactions are caused by red tattoo inks, a variety of red inks comprised the majority of selected inks.[13] Cytotoxicity, metabolic activity, inflammation, and sensitization biomarkers were investigated to further validate the RHS-based assay as an effective and accurate analytical method to evaluate tattoo inks.

2 | MATERIALS AND METHODS

2.1 | RHS culture

Human neonatal foreskin was obtained from healthy donors, in accordance with the VU University Medical Center, Amsterdam UMC's ethical guidelines and the "Code for Proper Use of Human Tissues," devised by the Foundation Federation of Dutch Medical Scientific Societies (see www.federa.org). RHS was cultured as described in previous research.[38] All culture media additives were obtained from Sigma-Aldrich (Merck, Darmstadt, Germany) unless stated otherwise. In short, dermal fibroblasts were isolated, cultured in fibroblast medium, Dulbecco's Modified Eagle's medium (DMEM; Lonza, Basel, Switzerland) containing 1% UltroserG serum substitute (BioSepra, Cergy–Saint-Christophe, France) and 1% penicillin/streptomycin (P/S) (Thermo Fisher [Invitrogen], Paisley, UK), and incubated at 37°C, 5% CO_2 until 90% confluency was reached over 6 to 8 days. Medium was refreshed every 3 to 4 days. Passage 1 fibroblasts were incorporated into RHS. Epidermal keratinocytes, isolated from foreskin, were cultured in KC1 (keratinocyte medium one), DMEM/Ham F-12 3:1 (Lonza; Gibco, Grand Island, Nebraska) supplemented with 1% UltroserG serum substitute, 1% P/S, 1 μM isoproterenol, 1 μM hydrocortisone, 0.1 μM insulin, and 1 ng/mL keratinocyte growth factor. Keratinocytes were incubated at 37°C, 5% CO_2, to 75% to 85% confluency in 6 to 8 days. Medium was refreshed every 3 to 4 days. Passage 1 keratinocytes were incorporated into RHS.

RHS: Fibroblasts were integrated into the collagen hydrogel at a concentration of 0.8 × 10^6 cells/gel. Then, 2 mL of this fibroblast gel was poured into 24-mm diameter transwell inserts (pore size of 0.4 μm; Corning, NY, USA) and allowed to solidify for 2 hours before being submerged in the fibroblast medium. After 24 hours, keratinocytes were seeded on the fibroblast-populated gels (5 × 10^6 cells/gel) and cultured submerged for 3 to 4 days in KC1. Hereafter, RHS were cultured at the air–liquid interface: reconstructed epidermis was deprived of medium and exposed to air to promote epidermal differentiation, while the underlying fibroblast gels remained exposed to fresh KC2 from underneath (DMEM/Ham F-12 (3:1), containing 0.2% UltroserG serum substitute, 1% P/S, 1 μM isoproterenol, 1 μM hydrocortisone, 0.1 μM insulin, 10 μM L-carnitine, 10 μM L-serine supplemented with 4 ng/mL keratinocyte growth factor, 1.0 ng/mL epidermal growth factor, 50 μg/mL vitamin C, 1.0 μM vitamin E, and a lipid mixture consisting of 25 μM palmitic acid, 15 μM linoleic acid, and 7 μM arachidonic acid. During ink exposure, cultures were incubated overnight in hydrocortisone-deprived KC2. To reduce donor variability, cells from an average of three donors were pooled and processed into each batch of RHS.

2.2 | Tattooing

The LaBina PL-1000 Mobil PMU device (LaBina, Bad Arolsen, Germany) was used to apply the ink intradermally mimicking in vivo tattooing. Amiea Creatip one-liner needle cartridges with a 0.4-mm diameter (Amiea, Berlin, Germany) were attached to the PL-1000 Mobil. A standardized square of 12 × 12 dots (about 0.7 cm^2) was injected into the centre of RHS at a frequency of 150 strokes per second, to decrease the chance of ink leaching out from the periphery of the RHS construct (Figure 1). Each dot was injected with a contact duration of 0.5 seconds at an angle of 85° and a needle depth of 1 mm. Pilot experiments were performed in which contact duration, angle, and needle depth until maximum 2 mm were investigated to optimize this procedure (data not shown). Excess ink was carefully removed from the epidermis using a sterile swab. Tattooing occurred under sterile conditions.

2.3 | Ink exposure

RHS were exposed to 16 differently coloured inks for 24 hours at 37°C after intradermal injection. An unexposed RHS was included in each experiment as a negative control. As a vehicle control, *Hamamelis virginiana* extract was included to compensate for any deleterious effects of repetitive needle insertion. Inks were derived from the

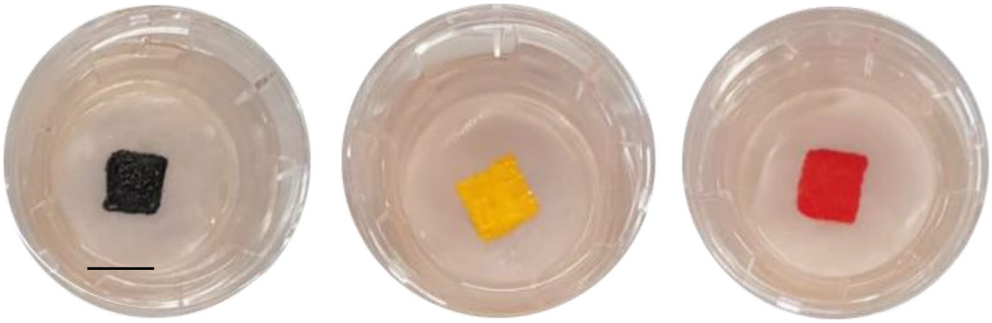

FIGURE 1 Transwell inserts containing tattooed reconstructed human skin (RHS). Tattoos were applied at the centre of each RHS to prevent ink leaching out from the periphery, in a standardized square (~0.7 cm^2) at a frequency of 150 strokes/second. Scale bar: 80 mm

following different manufacturers: Cheyenne (Berlin, Germany), Eternal Ink Inc. (Brighton, USA), Intenze Products (Kalsdorf, Austria), Kuro Sumi (Fort Mill, USA), H-A-N (Esslingen, Germany), and StarBrite Colors (Somers, USA), while the origin of one ink remained elusive due to missing details on the label. The various chemicals and (azo) pigments that appear in the assessed inks are listed in Table 1, supplemented with administrative details and respective hazard identification.[29,30,39,40] All ink types were applied undiluted. After a 24-hour exposure time, RHS was harvested and processed as described below. Culture supernatant was directly used to perform the lactate dehydrogenase assay to estimate cytotoxicity. Remaining culture supernatant was stored at −20°C for cytokine analysis using the enzyme-linked immunosorbent assay (ELISA).

2.4 | MTT assay

Metabolic activity was measured by the colorimetric conversion of thiazolyl blue tetrazolium bromide (MTT assay) to purple formazan crystals by NAD(P)H-dependent oxidoreductases present in functional mitochondria exactly as previously described.[36,41] In short, two 3-mm punch biopsies were taken from each RHS; the first from an ink-exposed area (except for unexposed RHS) and the second from an unexposed area as an internal RHS control. Biopsies were placed in individual wells on a F96 MaxiSorp Elisa Plate (Thermo Fisher) containing 200 μL phosphate-buffered saline–diluted MTT (2 mg/mL) per well. Plates were incubated at 37°C in the dark for 2 hours. Hereafter, biopsies were transferred into 200 μL isopropanol and hydrochloric acid (3:1) and incubated overnight at room temperature in the dark. On the next day, biopsies were removed and the colour intensity of 100 μL of each resuspended sample solution was quantified spectrophotometrically at 570 nm on a Mithras LB 940 Multimode Microplate Reader (Berthold Technologies, Bad Wildbad, Germany). Data were processed as a percentage relative to unexposed RHS. To determine whether the tattoo inks were able to interfere with the MTT assay, 10% of each tattoo ink was tested in the absence of RHS. No colour change at 570 nm was observed and therefore it was concluded that the inks did not interfere with the MTT assay at concentrations used for RHS exposure. This is the method recommended in the OECD TG 431 and 439 for in vitro skin corrosion test (epiCS 2012).[42,43]

2.5 | Tissue histology

A 2-mm broad transversal segment was taken from the centre of each RHS, containing unexposed areas on both sides and an ink-exposed region in the middle. The tissue was fixed in 4% formaldehyde (Klinipath VWR, Amsterdam, Netherlands) and embedded in paraffin. Tissue sections (6 μm) were stained with haematoxylin and eosin staining (H&E). Sections were visualized and examined using light microscopy (Nikon Eclipse 80i; Nikon Instruments Inc., Melville, USA). Images were obtained from ink-exposed regions and from unexposed areas as control.

2.6 | ELISA

Commercially available ELISA kits for IL-1α (R&D, Minneapolis, Minnesota, USA), IL-8 (Sanquin, Amsterdam, Netherlands) and IL-18 (MBL, Nagoya, Japan) were used as described by the supplier. Cytokine levels determined in the exposed RHS are expressed as fold increase relative to the vehicle or unexposed RHS.

2.7 | Data analysis

Data were obtained from three separate experiments each with an intraexperimental duplicate per condition. To minimize the effects of single-donor variability, skin cells from a minimum of three donors were pooled and used to construct each batch of RHS. Significance for reduction of metabolic activity and cytokine Stimulation Index was calculated using a nonparametric Friedman test for multiple comparisons (GraphPad Prism, version 8.2.1). Mean values per test condition were compared with the mean rank of the unexposed controls. Statistically significant differences compared with RHS samples which have not been tattooed were defined as $^{*}P \leq .05$ and $^{**}P \leq .01$. A cut-off value of a fivefold IL-18 increase compared with vehicle-tattooed RHS was used to classify sensitization capacity, as described in our previous study.[32] Four histopathological cytotoxic effects were defined, namely, cellular swelling, loss of laminar organization, karyorrhexis, and karyolysis. H&E-stained cross sections of tattoo-exposed RHS biopsies were imaged and analysed for the presence of

TABLE 1 Commercially available tattoo inks and the relevant hazard identification of these substances

Tattoo ink	Batch number	Chemicals listed in ink	CI no.	Pigment	CAS no.	Hazard identification
Cheyenne ink pure red (CPR)	NA	1,4-Bis(4-chlorophenyl)-3-hydroxy-2*H*-pyrrolo [3,4-c]pyrrol-6-1	CI 56110	Pigment Red 254	84 632–65-5	NI
		Propylene glycol	NA	NA	57–55-6	NI
		Isopropanol	NA	NA	67–63-0	Eye irritant
		Polyethylene glycol	NA	NA	2532-68-3	NA
		Calendula officinalis extract	NA	NA	84 776–23-8	NI
		Hamamelis virginiana extract	NA	NA	84 696–19-5	NI
Eternal light red (ELR)[a]	NA	4-[(4-Carbamoylphenyl)diazenyl]-*N*-(2-ethoxyphenyl)-3-hydroxynaphthalene-2-carboxamide	CI 12475	Pigment Red 170	2786-76-7	Skin sensitizer
		Isopropanol	NA	NA	67–63-0	Eye irritant
		Hamamelis virginiana extract	NA	NA	84 696–19-5	NI
Intenze American Rose[a] (IAR)	RD70W125O76IMX40	2,9-Dichloro-5,12-dihydroquinolino[2,3-b]acridine-7,14-dione	CI 12477	Pigment Red 210	61 932–63-6	NA
		Titanium dioxide	CI 77891	NA	13 463–67-7	Suspected carcinogen, eye irritant, organ damage upon prolonged/repeated exposure
		4-[[2-Chloro-4-[3-chloro-4-[(5-hydroxy-3-methyl-1-phenylpyrazol-4-yl)diazenyl]phenyl]phenyl]diazenyl]-5-methyl-2-phenylpyrazol-3-ol	CI 21110	Pigment Orange 13	3520-72-7	Skin sensitizer, eye irritant, respiratory toxicity
		Glycerin	NA	NA	56–81-5	NI
		Hamamelis virginiana extract	NA	NA	84 696–19-5	NI
Intenze Bright Red (IBR)[a]	HCY003RD72O79IMX40	2,9-Dichloro-5,12-dihydroquinolino[2,3-b]acridine-7,14-dione	CI 12477	Pigment Red 210	61 932–63-6	NA
		2-[(4-Methoxy-2-nitrophenyl)diazenyl]-*N*-(2-methoxyphenyl)-3-oxobutanamide	CI 11740	Pigment Yellow 65	6528-34-3	NI
		4-[[2-Chloro-4-[3-chloro-4-[(5-hydroxy-3-methyl-1-phenylpyrazol-4-yl)diazenyl]phenyl]phenyl]diazenyl]-5-methyl-2-phenylpyrazol-3-ol	CI 21110	Pigment Orange 13	3520-72-7	Skin sensitizer, eye irritant, respiratory toxicity
		Glycerin	NA	NA	56–81-5	NI
		Hamamelis virginiana extract	NA	NA	84 696–19-5	NI

TABLE 1 (Continued)

Tattoo ink	Batch number	Chemicals listed in ink	CI no.	Pigment	CAS no.	Hazard identification
Intenze Dragon Red (IDR)	21C06316E07130141	2,9-Dichloro-5,12-dihydroquinolino[2,3-b]acridine-7,14-dione	CI 12477	Pigment Red 210	61 932–63-6	NA
		2-[(4-Methoxy-2-nitrophenyl) diazenyl]-*N*-(2-methoxyphenyl)-3-oxobutanamide	CI 11740	Pigment Yellow 65	6528-34-3	NI
		4-[[2-Chloro-4-[3-chloro-4-[(5-hydroxy-3-methyl-1-phenylpyrazol-4-yl)diazenyl] phenyl]phenyl]diazenyl]-5-methyl-2-phenylpyrazol-3-ol	CI 21110	Pigment Orange 13	3520-72-7	Skin sensitizer, eye irritant, respiratory toxicity
Intenze Red Cherry (IRC)[a]	RD68B79IMX40	2,9-Dichloro-5,12-dihydroquinolino[2,3-b]acridine-7,14-dione	CI 12477	Pigment Red 210	61 932–63-6	NA
		Copper; 2,11,20,29,37,38,39,40-octazanonacyclo [28.6.1.13,10.112,19.121, 28.04,9.013,18.022,27.031,36] tetraconta-1(37),2,4,6,8,10(40), 11,13,15,17,19(39), 20,22,24,26,28(38),29,31,33,35-icosaene	CI 74160	Pigment Blue 15	147–14-8	NI
		Glycerin	NA	NA	56–81-5	NI
		Hamamelis virginiana extract	NA	NA	84 696–19-5	NI
	RD76B90IMX40[b]	*o*-Anisidine[b]	NA	NA	90-04-0	Carcinogenic, suspected mutagen[b]
"John Doe" Red (JDR)[c]	NA	NA	NA	NA	NA	NA
StarBrite Colors Light Red[c] (SLR)	NA	1,4-Bis(4-chlorophenyl)-3-hydroxy-2*H*-pyrrolo[3,4-c]pyrrol-6-1	CI 56110	Pigment Red 254	84 632–65-5	NA
		Titanium dioxide	CI 77891	NA	13 463–67-7	Suspected carcinogen, eye irritant, organ damage upon prolonged/repeated exposure
Intenze True Magenta[a] (ITM)	Q23IMX40	[9-(2-Carboxyphenyl)-6-(diethylamino)xanthen-3-ylidene]-diethylazanium	CI 45170	Pigment Violet 1	81–88-9	Eye irritant, oral/enteral toxicity
		Glycerin	NA	NA	56–81-5	NI
		Hamamelis virginiana extract	NA	NA	84 696–19-5	NI

(Continues)

TABLE 1 (Continued)

Tattoo ink	Batch number	Chemicals listed in ink	CI no.	Pigment	CAS no.	Hazard identification
Intenze Dragon Yellow (IDY)	W126Y78IMX40	2-[(4-Methoxy-2-nitrophenyl) diazenyl]-*N*-(2-methoxyphenyl)-3-oxobutanamide	CI 11740	Pigment Yellow 65	6528-34-3	NI
		Titanium dioxide	CI 77891	NA	13 463–67-7	Suspected carcinogen, eye irritant, organ damage upon prolonged/repeated exposure
		Barium sulfate	CI 77120	NA	7727-43-7	Eye irritant, respiratory toxicity
		Glycerin	NA	NA	56–81-5	NI
		Hamamelis virginiana extract	NA	NA	84 696–19-5	NI
Mario Barth Light Green (MBLG)	Y78W124G88	2-[(4-Methoxy-2-nitrophenyl) diazenyl]-N-(2-methoxyphenyl)-3-oxobutanamide	CI 11740	Pigment Yellow 65	6528-34-3	NI
		Titanium dioxide	CI 77891	NA	13 463–67-7	Suspected carcinogen, eye irritant, organ damage upon prolonged/repeated exposure
		Copper; 2,11,20,29,37,38,39,40-octazanonacyclo [28.6.1.13,10.112,19.121, 28.04,9.013,18.022,27.031,36] tetraconta-1(37),2,4,6,8,10(40), 11,13,15,17,19(39), 20,22,24,26,28(38),29,31,33,35-icosaene	CI 74160	Pigment Blue 15	147–14-8	NI
		Diazolidinyl urea	NA	NA	78 491–02-8	Skin sensitizer, eye irritant
Intenze Mario's Blue (IMB)	21C01316D26090433	Copper; 2,11,20,29,37,38,39,40-octazanonacyclo [28.6.1.13,10.112,19.121, 28.04,9.013,18.022,27.031,36] tetraconta-1(37),2,4,6,8,10(40), 11,13,15,17,19(39), 20,22,24,26,28(38),29,31,33,35-icosaene	CI 74160	Pigment Blue 15	147–14-8	NI
		Glycerin	NA	NA	56–81-5	NI
		Hamamelis virginiana extract	NA	NA	84 696–19-5	NI
Intenze Sculpting Black (ISB)	BK76DIS	Carbon	CI 77226	Pigment Carbon Black	1333-86-4	Carcinogen
		Proprietary ingredient(s)	NA	NA	NA	NA
		Glycerin	NA	NA	56–81-5	NI
		Isopropanol	NA	NA	67–63-0	Eye irritant

TABLE 1 (Continued)

Tattoo ink	Batch number	Chemicals listed in ink	CI no.	Pigment	CAS no.	Hazard identification
Intenze True Black (ITB)	BLK1301MX40-GE	Carbon	CI 77226	Pigment Carbon Black	1333-86-4	Carcinogen
		Ammonium acrylate copolymer	NA	NA	26 100–47-0	NI
		Ethylpropandiol	NA	NA	115–84-4	Eye irritant
		Glycerin	NA	NA	56–81-5	NI
		Polyethylene glycol	NA	NA	57–55-6	NA
		Capryl glycol	NA	NA	1117-86-8	Corrosive, eye irritant, enteral toxicity
		Simethicone	NA	NA	8050-81-5	Eye irritant
		Phenylpropanol	NA	NA	93–54-9	Skin + eye irritant, oral toxicity
		Benzoic acid	NA	NA	65–85-0	Corrosive, skin + eye irritant, organ damage upon prolonged/ repeated exposure
		Hamamelis virginiana extract	NA	NA	84 696–19-5	NI
No. 13 Blackout (no. 13)	NA	Pigment Carbon Black	CI 77226	Pigment Carbon Black	1333-86-4	Carcinogen
		Ammonium acrylate copolymer	NA	NA	26 100–47-0	NI
		Propylene glycol	NA	NA	57–55-6	NI
		Poloxamer 331	NA	NA	9003-11-6	Eye irritant, respiratory toxicity
		Poloxamer 188	NA	NA	9003-11-6	Eye irritant, respiratory toxicity
		Isopropanol	NA	NA	67–63-0	Eye irritant
Kuro Sumi Glow (KSG)	NA	Titanium dioxide	CI 77891	NA	13 463–67-7	Suspected carcinogen, eye irritant, organ damage upon prolonged/ repeated exposure
		Glycerin	NA	NA	56–81-5	NA
		Hamamelis virginiana extract	NA	NA	84 696–19-5	NA

Note: The 16 inks were commercially available from Cheyenne (Berlin, Germany), Eternal Ink Inc. (Brighton, USA), H-A-N (Esslingen, Germany), INTENZE Products (Kalsdorf, Austria), Kuro Sumi (Fort Mill, USA), and StarBrite Colors (Somers, New York, USA), while the origin of one ink remained elusive due to missing details on the label ("John Doe" Red). Tattoo ink chemical constituents and accompanying hazard identification were based on database information obtained from the Classification, Labelling and Packaging of Substances and Mixtures Regulation (CLP, Regulation [EC] No. 1272/2008), PubChem, the European Chemicals Agency Base (ECHA), the Dutch Federation of University Medical Center's database for hazardous chemicals (NFU) and the European Commission Rapid Alert System, Non-Food (RAPEX). *Hamamelis virginiana* extract, an inert solvent commonly used in tattoo inks, was applied as vehicle control in all experiments.

Abbreviations: CAS no, Chemical Abstracts Service number; CI, colour index; NA, not available; NI, none identified.

[a]Inks to which adverse reactions were observed in the Academic Tattoo Clinic Amsterdam.[8]

[b]Chemical hazard identified by RAPEX.

[c]Inks to which suspected adverse reactions were observed in the Academic Tattoo Clinic Amsterdam.[8]

any of these cytotoxic effects and compared with control sites, consisting of unexposed areas on the same RHS biopsy.

3 | RESULTS

3.1 | Tattoo inks exhibit localized cytotoxicity when injected into reconstructed human skin

Sixteen tattoo inks were selected for testing in RHS (Table 1). An unexposed RHS was included as a negative control, and *H virginiana* extract was included as vehicle control to compensate for any deleterious effects of repetitive needle insertion. The substances were intradermally injected into RHS and cultures were harvested 24 hours later (Figure 1). In unexposed RHS and control areas of RHS distant from the injection site, a stratified, differentiated epidermis was observed on the dermis consisting of a fibroblast-populated collagen hydrogel (Figure 2). After injection of the *H virginiana* extract, epidermal ingrowth was observed in the lesions created by the needle action to repair the damaged epidermis (Figure 2).

Many of the tattoo inks showed clear detrimental effects on tissue histology (Figure 2). Only injection of Cheyenne Pure Red (CPR), Intenze True Black (ITB), and No. 13 Blackout (no. 13) resulted in no detrimental effects. For the other inks, cytotoxicity was observed as dermal fibroblast karyolysis and/or epidermal loss of laminar organization, karyorrhexis, and cellular swelling. These adverse effects ranged from minor to extensive. ELR, StarBrite Light Red (SLR), and Kuro Sumi Glow (KSG) can be considered to induce acute cell death due to the high levels of cytotoxicity observed. A remarkable finding is that some inks exerted cytotoxicity in both the epidermis and dermis in the location of the injected ink (eg, Intenze Dragon Red [IDR], Intenze Red Cherry [IRC]), whereas other inks effected predominantly either epidermis ("John Doe" Red [JDR]) or dermis (Intenze American Rose [IAR]) of RHS. Notably, intradermal injection of IDR and Mario Barth Light Green (MBLG) resulted in the complete destruction of the basement membrane zone as well as being cytotoxic to the epidermis and fibroblasts. KSG contained fatty droplets (Figure 2, arrows), implying improper emulsification. The yellow Intenze Dragon Yellow (IDY) became blackened after H&E staining, suggesting that pigments within this ink react chemically with some of the chemicals or solvents involved in the H&E staining.

3.2 | Tattoo inks influence metabolic activity of reconstructed human skin

Next, the influence of tattoo inks on mitochondrial metabolic activity (which correlates with cell viability) was assessed (Figure 3). The vehicle, *H virginiana* extract, had no effect on metabolic activity, indicating that the experimental procedure of repetitive needle insertion was not detrimental to RHS in line with histological assessment shown in Figure 2. By contrast, injection of most inks resulted in varying degrees of decreased metabolic activity of RHS, with ELR (decrease 89.9% ± 0.8% standard error of the mean [SEM]), SLR (decrease 88.7% ± 1.9% SEM), KSG (decrease 86.6% ± 0.7% SEM), Intenze Sculpting Black (ISB; decrease 65.0% ± 14.1% SEM) and IDR (decrease 56.4% ± 7.6% SEM) exhibiting a significant decrease, indicating these inks are highly cytotoxic, again in line with our histological assessments.

3.3 | Tattoo inks result in inflammatory cytokine release

In order to determine whether tattoo inks are potential sensitizers, IL-18 release into RHS culture supernatants was determined (Figure 4). Five of the 16 tattoo inks resulted in increased IL-18 release compared with the vehicle *H virginiana* extract (fold increase: KSG: 15.5 ± 3.2; ELR: 8.6 ± 3.1; SLR: 6.1 ± 1.6; ISB: 5.5 ± 2.2; IDR: 2.67 ± 0.2). In particular, the fivefold increase in IL-18 secretion in RHS exposed to KSG, ELR, SLR, and ISB indicates that these inks are potential sensitizers.[32]

The general inflammatory cytokine release, which corresponds to skin trauma and an irritant response, was assessed by determining IL-1α and IL-8 release into RHS culture supernatants (Figure 4). Notably, IL-1α release greatly increased after tattooing with ELR (fold increase: 6.9 ± 1.8), SLR (5.2 ± 1.5), and KSG (3.3 ± 0.6) compared with unexposed and vehicle-exposed RHS, indicating that these tattoo inks were able to mediate an inflammatory reaction greater than that caused by the repetitive needle insertion alone. However, a 2.8 ± 0.4-fold increase in IL-8 release was already observed after injection of the *H virginiana* extract compared with RHS that had not been tattooed. Considering that *H virginiana* extract has no known irritant properties, the repetitive needle insertion, resulting in mechanical stress and microtrauma, can be considered to be sufficient to trigger this IL-8 release. The tattoo inks did not result in more IL-8 release than that observed with the vehicle *H virginiana* extract.

4 | DISCUSSION

Research and clinical data both indicate that tattoos can lead to clinical complications such as ACD.[7,12] Although it is unclear which molecules are responsible for skin sensitization, various azo pigments have been identified to cause tattoo-related allergies.[16,20] The intradermal use of tattoo inks, including azo pigments, is poorly regulated by the European Medicines Agency (EMA), while the FDA has formulated no specific regulations at all.[27,28] To improve user safety and reduce the prevalence of ACD, among other complications in tattooed patients, tattoo inks should be thoroughly tested for adverse medical reactions. In this study, for the first time, an actual PMU device was used to tattoo an organotypic 3D RHS model and we demonstrate that injection of 5 of the 16 inks (three red, one black, and one white) resulted in significant IL-18 release, with four inks resulting in a fivefold increase in IL-18 release (proposed sensitizer cut-off level),[32,36] indicating that they are potential sensitizers. Furthermore, 13 inks resulted in cytotoxicity as observed by detrimental effects in tissue histology and inflammatory cytokine release (IL-1α, IL-8).

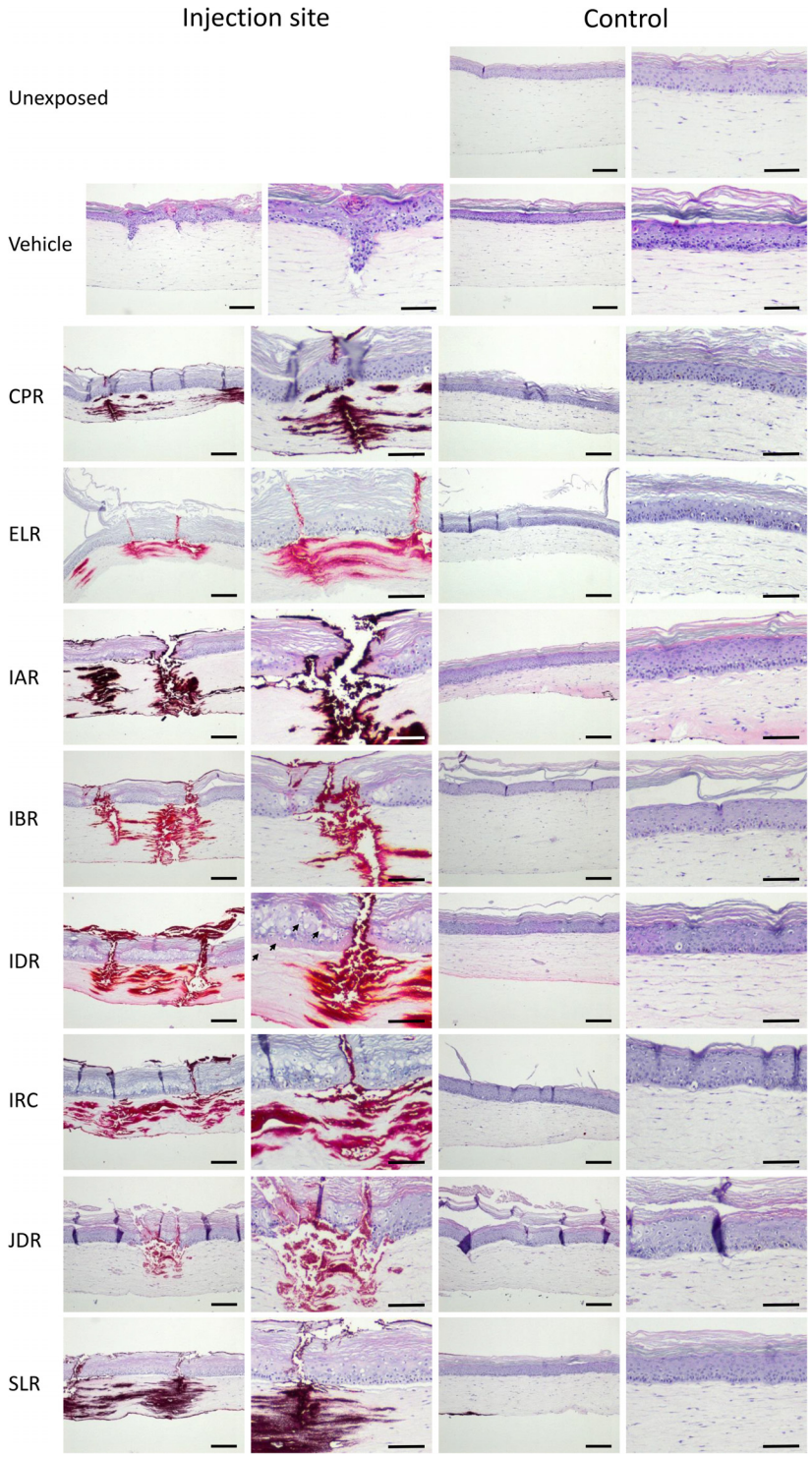

FIGURE 2 Representative haematoxylin and eosin staining images of the entire ink panel, including unexposed and vehicle *Hamamelis virginiana* controls. The first two columns show images of the ink-injection sites. The third and fourth columns show images of unexposed control sites adjacent to the tattoo in the same reconstructed human skin (RHS). Second and fourth columns show higher magnification of needle insertions and control sites, respectively. The different cytotoxic effects are illustrated by the arrows in Intenze Dragon Red (IDR; second column). From left to right, these arrows indicate karyolysis, karyorrhexis, loss of laminar organization, and cellular swelling. These effects are observed in varying measures throughout the different conditions, with exception of vehicle, Cheyenne Pure Red (CPR), Intenze True Black (ITB), and no. 13. Kuro Sumi Glow (KSG) contains fatty droplets (arrow), implying improper emulsification. Magnification: ×10. Scale bar for first and third columns: 100 μm. Scale bar for second and fourth columns: 50 μm. Representative of N = 3 with intraexperiment duplicate. See Table 1 for full ink names and details. ELR, Eternal Light Red; IAR, Intenze American Rose; IBR, Intenze Bright Red; IRC, Intenze Red Cherry; IDY, Intenze Dragon Yellow; IMB, Intenze Mario's Blue; ISB, Intenze Sculpting Black; ITM, Intenze True Magenta; JDR, "John Doe" Red; MBLG, Mario Barth Light Green; SLR, StarBrite Light Red

The application of an actual PMU device for intradermal injection of tattoo inks into the RHS simulated real-life effects of tattooing. This allowed for direct embedding of the tattoo ink into the dermis, enabling the ink to come into direct contact with skin cells and extracellular matrix. Another important advantage of this approach is that effects are localized to the injection site, facilitating intraexperimental

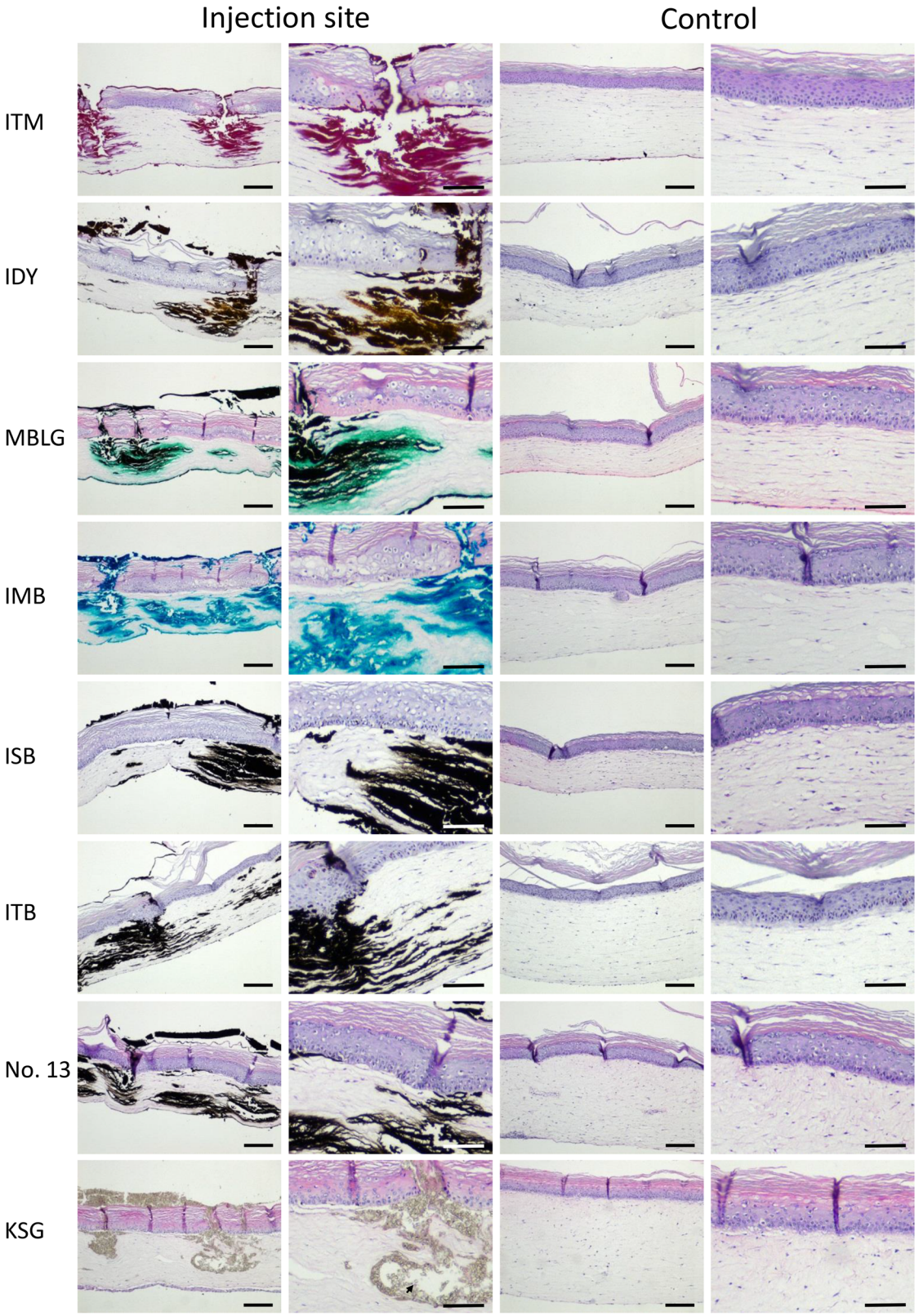

FIGURE 2 (Continued)

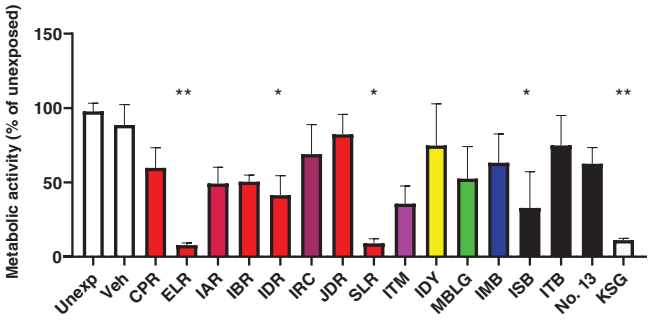

FIGURE 3 Metabolic activity (correlating with viability) of reconstructed human skin (RHS) biopsies after 24-hour ink exposure, expressed as a percentage relative to unexposed RHS. Bar colours match ink colours. A reduction of metabolic activity is interpreted as a measure of cytotoxicity. Although most inks show varying degrees of cytotoxicity, Eternal Light Red (ELR), Intenze Dragon Red (IDR), StarBrite Light Red (SLR), Intenze Sculpting Black (ISB), and Kuro Sumi Glow (KSG) are significantly cytotoxic relative to unexposed (*$P \leq .05$ and **$P \leq .01$). N = 3 ± standard error of the mean independent experiments each with an intraexperimental duplicate. CPR, Cheyenne Pure Red; ELR, Eternal Light Red; IAR, Intenze American Rose; IBR, Intenze Bright Red; IRC, Intenze Red Cherry; IDR, Intenze Dragon Red; IDY, Intenze Dragon Yellow; IMB, Intenze Mario's Blue; ISB, Intenze Sculpting Black; ITB, Intenze True Black; ITM, Intenze True Magenta; JDR, "John Doe" Red; KSG, Kuro Sumi Glow; MBLG, Mario Barth Light Green; No. 13, No. 13 Blackout; SLR, StarBrite Light Red; Veh, vehicle

comparison of ink-exposed tissue with unexposed tissue. Previously, we reported a medium-based method for tattoo-ink exposure to RHS. While this created a direct dermal exposure route, it exposed the entire reconstructed dermis to tattoo inks, without the added trauma resulting from the mechanical stress of repetitive needle insertion.[32] The novel approach used in this study correlates more with the in vivo tattooing situation, and therefore, gives a more accurate representation of tattoo-related adverse effects than medium-based ink exposure.

Using a broad 16-ink panel allowed us to distinguish harmful (sensitizers) from less harmful (irritant) or harmless (inert) inks. By assessing cell metabolism (related to viability) we showed that ELR, IDR, SLR, ITB, and KSG were very cytotoxic to RHS, which was supported by the histological data, as the reconstructed epidermis in tattoo-exposed regions appeared severely compromised. Histological analysis also suggested that tattoo ink may induce variable modes of cytotoxicity on individual cell types.

The assessed cytokines IL-18, IL-8, and IL-1α are all involved in skin inflammation and ACD. IL-18 is specifically related to ACD, whereas IL-1α and IL-8 are related to general inflammation (eg, irritation and trauma-induced injury).[36,44,45] Previously we have shown that increased IL-18 release from reconstructed human epidermis can be used to identify and label chemicals as potential sensitizers.[36] Furthermore, because sensitizer potency is related to irritant potency and cytotoxicity, we have correlated a decrease in metabolic activity and an increase in IL-1α release from reconstructed human epidermis with sensitizer potency, thus enabling chemicals to be classified.[37] Because

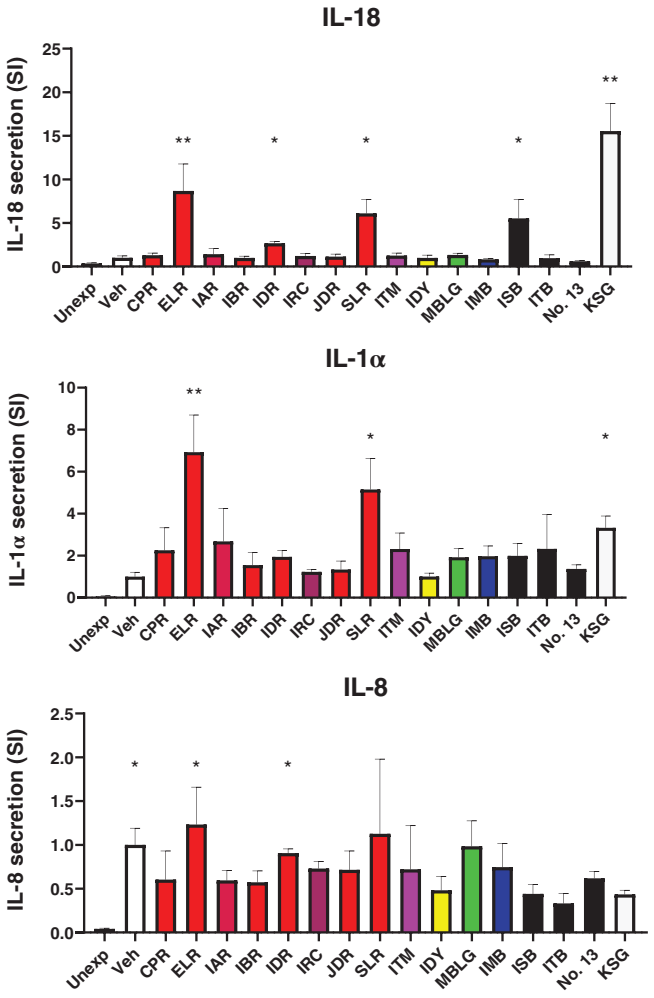

FIGURE 4 Sensitization biomarker interleukin (IL)-18 and inflammatory biomarkers IL-1α and IL-8 released into culture supernatants of reconstructed human skin (RHS) after 24-hour ink exposure. Cytokines were analysed by enzyme-linked immunosorbent assay. Baseline levels for cytokine release of RHS tattooed with vehicle were as follows: IL-18, 220.70 ± 51.68 pg/mL; IL-1-1α, 37.20 ± 8.96 pg/mL; and IL-8, 266.84 ± 59.53 ng/mL. Stimulation Index (SI) relative to vehicle cytokine levels is shown. Statistical significance compared with RHS samples which have not been tattooed (Unexp) was defined as *$P \leq .05$ and **$P \leq .01$. N = 3 ± independent experiments each with an intraexperimental duplicate. CPR, Cheyenne Pure Red; ELR, Eternal Light Red; IAR, Intenze American Rose; IBR, Intenze Bright Red; IDR, Intenze Dragon Red; IDY, Intenze Dragon Yellow; IMB, Intenze Mario's Blue; ISB, Intenze Sculpting Black; ITB, Intenze True Black; ITM, Intenze True Magenta; JDR, "John Doe" Red; KSG, Kuro Sumi Glow; MBLG, Mario Barth Light Green; No. 13, No. 13 Blackout; SLR, StarBrite Light Red; Veh, vehicle

RHS injected with ELR, SLR, ISB, and KSG showed more than a five-fold increase in IL-18, these inks can be labelled as potential sensitizers.[32] Furthermore, significantly increased release of IL-α, decreased metabolic activity, and increased IL-18 release for ELR, SLR, and KSG, respectively, would suggest that these inks can be ranked as potential strong sensitizers in our assay.

IL-1α is associated with skin irritation, apoptosis, and necrosis.[36,46] Therefore, reduced levels of cell metabolism (or cell viability) should enhance IL-1α release and can be used to characterize irritation potency of chemicals. Indeed, IL-1α release was in line with reduced metabolic activity, enabling irritation potency to be ranked as ELR = SLR = KSG = ISB > IDR, with ELR having the greatest irritation potency and IDR having the lowest potency in our assay.

It should be noted that our assay only identifies inks which activate keratinocytes and fibroblasts (key event 2 of the adverse outcome pathway for sensitization), indicating that they may have sensitizing potential.[47] After sensitization, by a continued or repetitive exposure to the same allergen, the full elicitation of ACD, which is an adaptive immune memory T-cell response, may occur. Furthermore, if an ink is a weak sensitizer, our 24-hour exposure time may be insufficient and longer exposure times may be required to activate key event 2 and detect increased IL-18 release. This is illustrated for IDR which caused a significant increase in IL-18 release, but did not reach the fivefold threshold. This may also explain why we did not label IAR, Intenze Bright Red (IBR), and Mario Barth Light Green (MBLG) as sensitizers even though they contain ingredients listed to be sensitizers (Table 1). Furthermore, this would also be in line with the observation that tattoo-mediated ACD is generally a late-onset reaction.

Most of the inks investigated in this study contained ingredients which were labelled as skin or eye irritants. However, we only scored five inks with clear irritant properties (reduced metabolic activity and increased IL-1α release). Again, this may be due to the 24-hour exposure time, and longer exposures would be expected to result in more IL-1α release and cytotoxicity. Very elevated levels of IL-8 (2.8 ± 0.4) were detected after injection of the vehicle *H virginiana* extract, even though no decrease in metabolic activity or histological abnormalities was observed. IL-8 is one of many cytokines produced by keratinocytes and fibroblasts in response to skin trauma.[44] Because all of the injected tattoo inks, including the cytotoxic inks, resulted in a similar amount of IL-8 release to the vehicle, this indicates that the microtrauma resulting from the high-frequency needle insertions alone is responsible for the maximum observed IL-8 release. Because tattoos take 1 to 2 weeks to heal, it would be interesting to determine whether the microtrauma-initiated IL-8 release returned to baseline as the tattoo sites healed upon extended culture periods. With regard to MBLG, we observed very clear localized trauma in the histology but only mild effects in the MTT assay and IL-1α release. Notably, MBLG cytotoxicity appears to be limited to cells directly adjacent to the insertion sites, and is observed as localized cellular swelling, which is a reversible damage response and is not necessarily correlated with cell death (MTT assay). Furthermore, because prestored IL-1α is immediately released from the stratum corneum upon skin irritation,[48] it is possible that this is trapped by the localized swollen keratinocytes and therefore not detected in the culture medium. However, this needs further investigation at longer time intervals. Taken together, our combined data show that our RHS model can indeed distinguish sensitizing, irritant, and inert ink types by intradermal injection during a 24-hour exposure period, and is therefore, a useful tool for risk assessment of tattoo inks.

When an individual develops ACD to a tattoo ink, it is common practice for a dermatologist to perform a patch test to identify the harmful tattoo inks and ingredients.[49] However, it is becoming evident that a topical patch test, rather than dermal exposure, may be responsible for false negatives, possibly due to the inability of the ink and ingredients to penetrate the stratum corneum. Serup and Hutton Carlsen[50] investigated tattoo pigment sensitization patch tests in a cohort of 90 patients that had acquired allergic reactions to their tattoos. Only one patient tested positive for Tattoo Pigment Red, even though this pigment is known to be a sensitizer. Gaudron et al[20] performed a similar study, but used patch and prick tests with undiluted ink samples obtained from tattoo shops. Many of these ink types contained the same azo pigments as our tested inks, including Pigment 210, Pigment Yellow 65, Pigment Orange 13, and Pigment Red 170. After 8 days, only one patient patch tested positive for sensitization to a red ink containing Pigment 210, Pigment Yellow 65, and Pigment Orange 13, while the latter pigment is known to cause skin sensitization (Table 1). In our RHS study, tattoo ink IDR, which contains the same pigments, was classified as a potential sensitizer already after only 24 hours of tattooing, which is in line with the hazard identification for Pigment Orange 13. Furthermore, Gaudron et al[20] observed no reaction after patch testing with ELR, even though this ink contains the known sensitizer Pigment Red 170 (Table 1).[32] Of note, in both our current and previous studies, we correctly identified ELR as a potential sensitizer, corresponding with the known hazard label for this chemical.

While the ELR sensitization capacity can be explained by its ingredients, the product labels of SLR, KSG, and ISB, which also scored as sensitizers in our assay, cannot. However, both SLR and KSG do contain the colourant titanium dioxide, which as well as being listed as an eye irritant and a potential carcinogen (Table 1) has recently received attention as being a potential sensitizer.[51] In addition, a study by Smulders et al found that titanium oxide nanoparticles increased chemical-induced skin sensitization.[52] SLR contained only one other (listed) ingredient, (azo-) Pigment Red 254. Whereas various red azo pigments have been confirmed as sensitizers, this particular pigment has no described chemical hazards throughout different chemical databases. Furthermore, it is the only azo pigment listed in CPR, which is one of the least harmful inks found in this study. Based on this, Pigment Red 254 is not likely to be responsible for the increased IL-18 release after tattooing with SLR, suggesting that titanium dioxide may indeed be the culprit, although other proprietary ingredients or chemical impurities may also play a role. This latter result was suggested in our previous study for ISB, where high-performance liquid chromatography analysis identified the presence of benzo[a]pyrene, which is described as a strong sensitizer.[32,53] Therefore, chemical analysis of tattoo inks which are thought to be related to ACD would provide additional insights into the components within the ink which are responsible for the allergic reaction. A final notable aspect is that KSG seems to

contain fatty droplets, whereas all other inks do not, indicative of improper emulsification. This may contribute to the deleterious effects KSG induces on RHS and may contribute to high levels of cytotoxicity, inflammatory, and sensitization capacity observed throughout the experiments.

4.1 | Limitations and future perspectives

As with all complex models, a number of limitations were identified when tattooing RHS. In order to prevent operator technical variation, the PMU device was handled by only one operator who tattooed all RHS. Such challenges have been acknowledged by others as well in different types of studies.[54] Besides, it was not possible to accurately quantify the exact amount of ink injected into RHS: ink could stick to the needle cartridge and ink residue could remain on the topical surface of the RHS. If this occurred, it was carefully removed with a sterile swab. The varying chemical properties of the different inks, such as viscosity and hydrophilicity, also rendered quantification more difficult. Improper quantification of the injected ink could easily cause interexperimental variation, as well as intraexperimental variation between duplicates. Nevertheless, we were still able to identify a number of sensitizing inks from the 16-ink panel, demonstrating that our protocol was sufficient.

Future research could be aimed at introducing immune cells, for example, Langerhans cells and macrophages, into the model in order to gain a more mechanistic understanding of tattoo-mediated ACD.[55–57] In addition, exposure times could be increased because in this study RHS samples were harvested and analysed only 24 hours after receiving the tattoo. An extended exposure time might enable weaker sensitizers to be identified further in line with Table 1 and enable healing of the tattoo site to be further investigated. Studying the effect of bare needle action on RHS could confirm whether needle-induced microtrauma is responsible for generalized IL-8 release throughout the ink panel. While it would mean that IL-8 is hardly useful to distinguish harmful from harmless inks, it would provide insights into non-ink-related consequences of tattooing. Because the model described in this manuscript is quite complex and low throughput, it could be combined with a prior screen of inks using a high-throughput assay (eg, the NCTC 544 keratinocyte IL-18 assay).[33] However, the traditional exposure method used in this 2D submerged cell culture assay has not yet been tested with tattoo inks and would therefore require extensive testing beforehand. Furthermore, the dermal fibroblast is absent in this assay.

To conclude, we have successfully demonstrated intradermal injection of tattoo inks into RHS and used this to assess cytotoxicity and sensitization potential of tattoo inks during a 24-hour exposure period. Intradermal injection allowed for a close approach to in vivo tattooing, yielding more physiologically relevant data than topical and culture medium-based ink exposure, and enabling the identification of four potential sensitizers from a panel of 16 tattoo inks. This method is a valuable tool for studying histological and mechanistic effects of tattoo inks, providing important information for safety regulators, as well as contributing to our understanding of the pathogenesis of tattoo-mediated ACD.

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AUTHOR CONTRIBUTIONS

Joey Karregat: Conceptualization; data curation; formal analysis; investigation; methodology; writing-original draft; writing-review & editing. **Thomas Rustemeyer:** Conceptualization; investigation; methodology; supervision; writing-review & editing. **Sebastiaan A. S. van der Bent:** Conceptualization; investigation; methodology; writing-review & editing. **Sander W. Spiekstra:** Data curation; formal analysis; investigation; methodology; supervision; writing-review & editing. **Maria Thon:** Data curation; methodology; writing-review & editing. **David Fernandez Rivas:** Conceptualization; methodology; writing-review & editing. **Susan Gibbs:** Conceptualization; formal analysis; investigation; methodology; project administration; supervision; writing-original draft; writing-review & editing.

CONFLICTS OF INTEREST

The authors have no conflicts of interest to declare.

DATA AVAILABILITY STATEMENT

Data available on request from the authors

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