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Macrophages and Fibroblasts Differentially Contribute to Tattoo Stability

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Keywords

Tattoo · Macrophage · Fibroblast · Tattoo maintenance

Abstract

Background: Little information is available about the complexity and function of skin cells contributing to the high stability of tattoos. It has been shown that dermal macrophages play an important role in the storage and maintenance of pigment particles. By contrast, the impact of dermal fibroblasts, forming the connective tissue of the skin, on the stability of the tattoo is not known. **Method:** In this study, we compared the cell number and the particle load in dermal macrophages versus dermal fibroblasts, isolated from tail skin of tattooed mice. **Results:** Microscopic analysis revealed that both cell populations contained the tattoo particles, although in largely different amounts. A small number of macrophages with high side scatter intensity contained a large quantity of pigment particles, whereas a high number of dermal fibroblasts harbored only a few pigment particles. Using the CD64^{dtr} mouse model that allows for selective, diphtheria toxin-mediated depletion of macrophages, we have pre-

viously shown that macrophages hold the tattoo in place by capture-release and recapture cycles. In the tattooed skin of macrophage-depleted mice, the content of pigment particles in fibroblasts did not change; however, the total number of fibroblasts carrying particles increased. **Conclusion:** The present study demonstrates that dermal macrophages and fibroblasts contribute in different ways to the tattoo stability and further improves our knowledge on tattoo persistence.

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Introduction

In the course of the tattooing process, tattoo ink is injected through the epidermis into the dermal layer. The dermal compartment contains a broad variety of different immune cells, including macrophages. Dermal macrophages serve as guardians of the skin by ingesting pathogens, dead cells and also any type of particle [1], thereby preventing the spread of potentially harmful substances through our body. Pigmented skin hosts a special type of melanin-containing macrophages, designated as melano-

phages. Originally described in human skin [2], melanophages have recently been described in mouse skin as well [3]. Moreover, this last study showed that dermal macrophages in tattooed mouse skin are able to store pigment particles, hence being responsible for the maintenance of the tattoo in the dermis. By using the CD64^{dtr} mouse model for the diphtheria toxin (DT)-mediated depletion of macrophages, it has been demonstrated that dying macrophages release the pigment particles into the surrounding tissue. Interestingly, these particles remained in place in the dermis, and were subsequently recaptured by macrophages, which replaced the depleted macrophages [3].

Together with immune cells, the dermis also contains numerous nonimmune cells such as fibroblasts, which have an essential function for the stability and homeostasis of the skin by producing and degrading extracellular matrix proteins. Moreover, fibroblasts are involved in wound healing and scar formation. As such, these cells are activated through tissue damage, remove degraded collagen and build up a new collagen fibril network [4].

Beside their specific roles on skin stability, fibroblasts have been associated with the storage of pigment particles, as electron microscopic analysis of tattooed human

skin showed granules of pigment particles bound to cell membranes of fibroblasts [5, 6].

Therefore, we hypothesized that dermal fibroblasts might also be important for the maintenance and stability of tattoos. In the present study, we compared the total numbers of particle-storing macrophages and fibroblasts, as well as their pigment particle load. For this purpose, we isolated and characterized macrophages and fibroblasts from tattooed mouse tail skin. We carefully assessed the number of pigment particle-containing cells and the intensity of the incorporated green particles to test the current view that considers macrophages as the main cell type responsible for storing the pigment particles [3]. Taking advantage of the CD64^{dtr} mouse model, which allows for the DT-mediated depletion of macrophages and monocyte-derived cells, we further investigated the role of fibroblasts in macrophage-ablated tattooed skin. Indeed, as reported recently [3], after macrophage depletion, particles were released in the dermis, and during the time window of macrophage depletion, the appearance of the tattoo did not change. This leaves the question open whether fibroblasts eventually have some role in these processes. Therefore, with the present study, we aim at extending the current understanding of the cellular distribution of pigment particles.

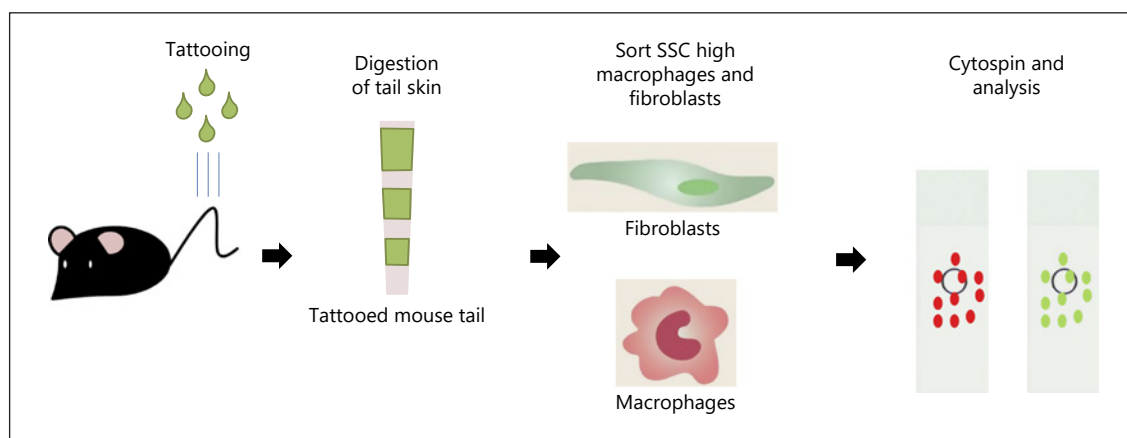
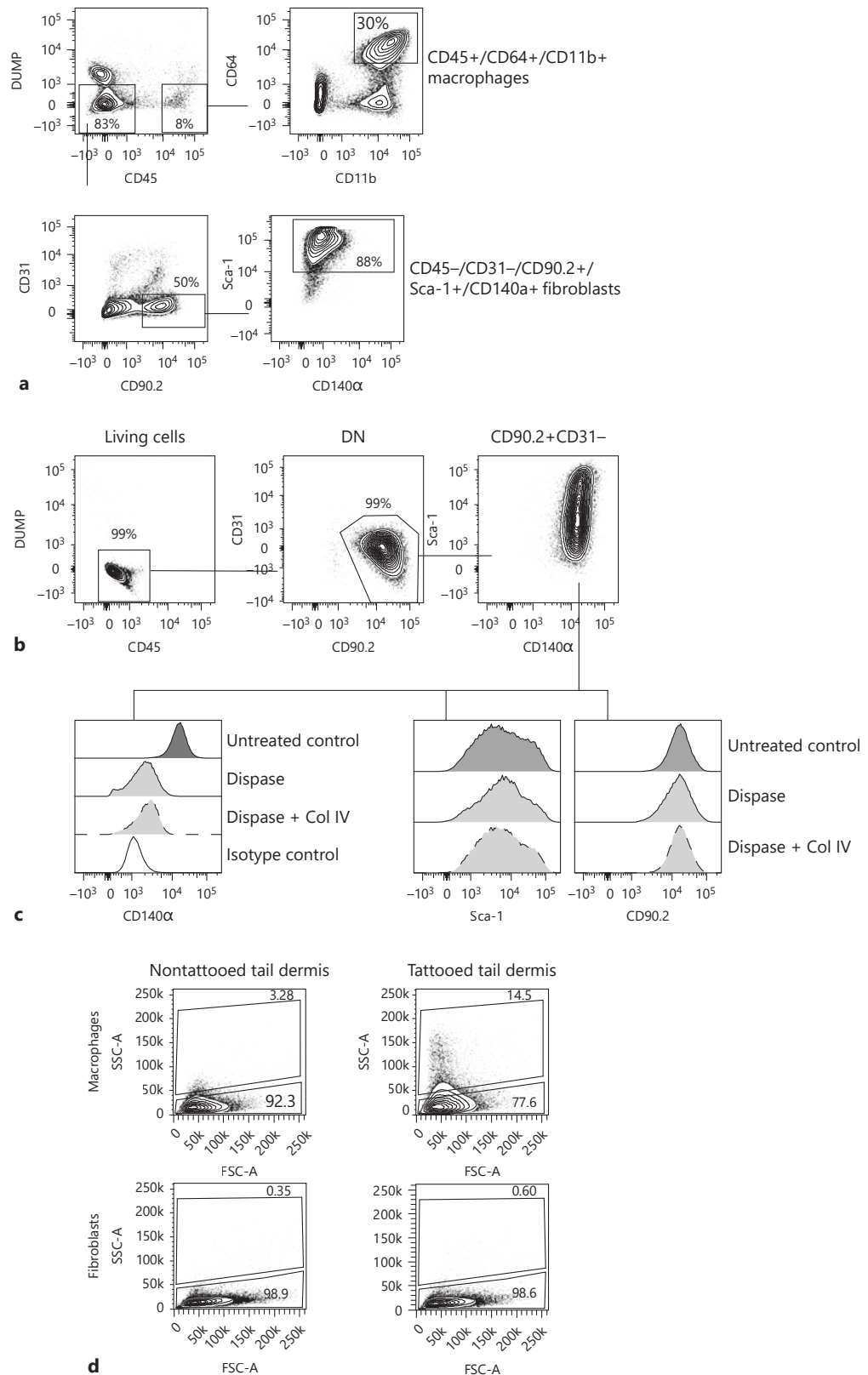


Fig. 1. Flowchart of the Materials and Methods.

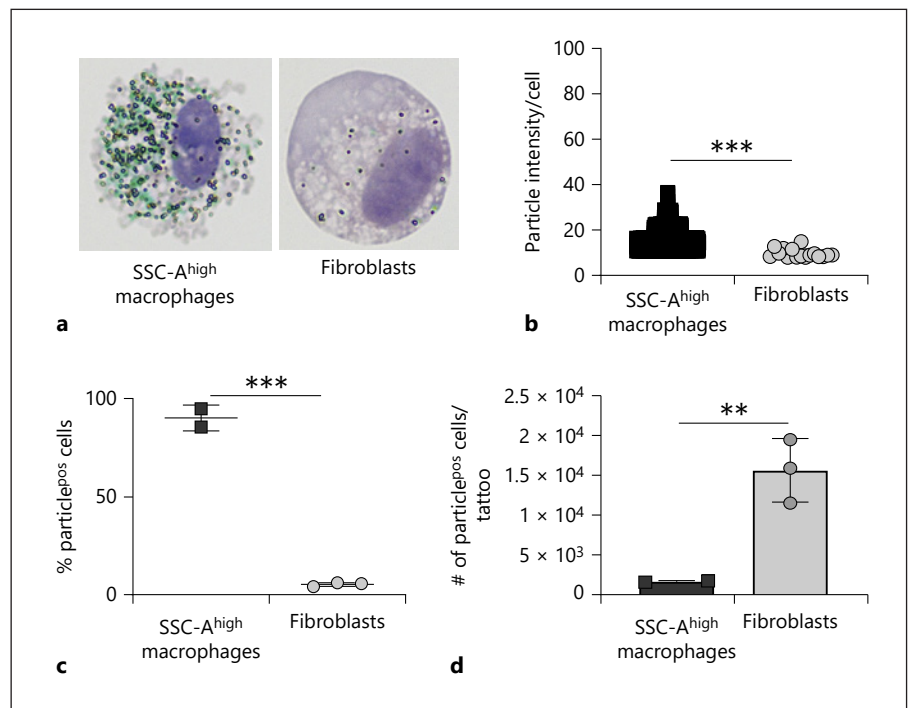
Fig. 2. a Identification of macrophages and fibroblasts from mouse tail skin after extraction using an enzymatic digestion cocktail (dispase and collagenase IV, col IV) and staining with CD45, DUMP (CD3, CD19, CD45R, CD161, NK1.1, Ter-119), CD64, CD11b, CD31, CD90.2, Sca-1 and CD140a. DN, double negative population. **b** NIH3T3, a mouse embryonic cell line, was stained with the same antibody panel as in **a**. **c** Expression of CD140a, Sca-1 and CD90.2 are shown on NIH3T3 incubated with dispase (for 2 h at 37 °C) or dispase followed by collagenase IV (2 h at 37 °C). Untreated cells served as control. **d** BL/6 mice were tattooed on their tails with green tattoo paste. After 4 weeks, dermal skin was harvested, and a single cell suspension was stained for fibroblasts and macrophages. Side scatter profiles of macrophages and fibroblasts were compared between tattooed and nontattooed mice.

(For Figure see next page.)



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Fig. 3. Pigment particle-containing dermal macrophages and fibroblasts. BL/6 mice were tattooed with green tattoo paste on the tail. After a healing phase, SSC-A^{high} macrophages and fibroblasts were isolated from the pooled dermal cell suspensions of 4–5 mice. **a** Representative microscopic pictures of a typical tattoo particle-containing macrophage and a fibroblast. **b** Pigment particle intensity per cell assessed after TissueFAXS acquisition followed by Strataquest software analysis. **c** Frequency of pigment particle-positive cells (% particle^{pos} cells) from sorted and analyzed SSC-A^{high} macrophages and fibroblasts. **d** Absolute numbers of pigment particle-containing SSC-A^{high} macrophages and fibroblasts in the tattooed area. Data represent means \pm SD of 2–3 independent experiments with 4–5 mice per group.



Materials and Methods

For further details, see the supplementary material (for all online suppl. material, see www.karger.com/doi/10.1159/000506540) (Fig. 1).

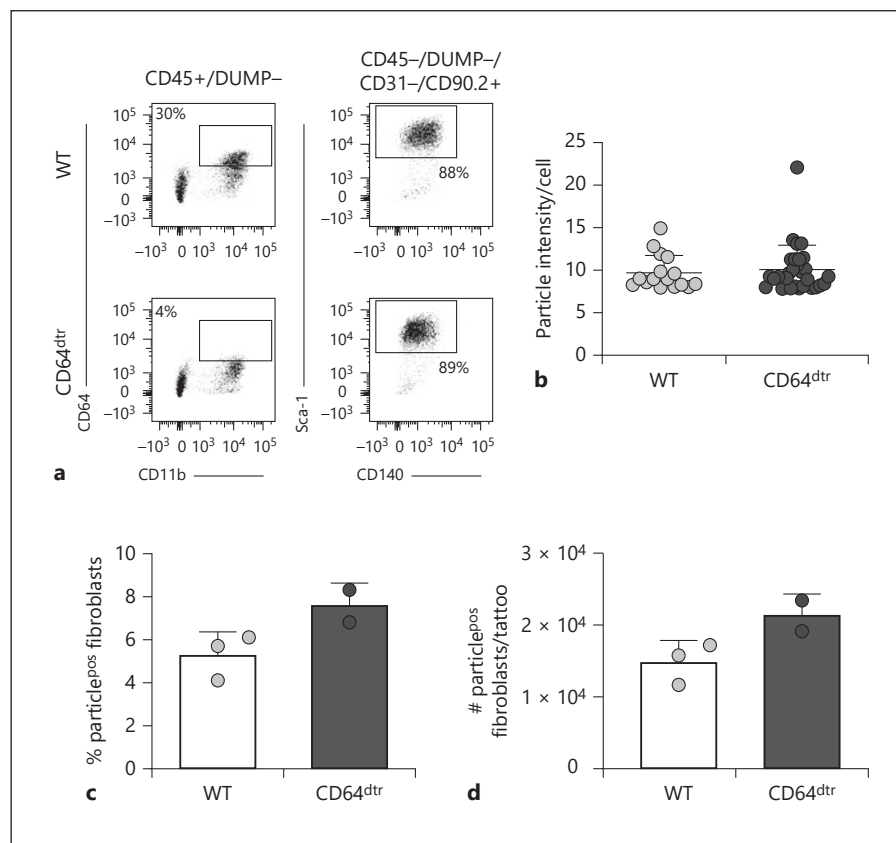
Results

Identification of Macrophages and Dermal Fibroblasts from Adult Mouse Tail Skin

First, we established a protocol for the isolation of macrophages and fibroblasts from adult mouse tail skin for subsequent flow cytometry analysis. For all experiments, the dermis was separated from the epidermis after incubation with dispase, followed by digestion of the dermis with DNase I and collagenase IV. This protocol yielded a high number of fibroblasts and macrophages. For the identification of macrophages in tail dermis, we used a simplified version of the gating strategy as described in a previous study [3]. Here, macrophages were identified as living CD45⁺, DUMP[−] (CD3, CD19, CD45R, CD161, Ly-6G, Ter-119), CD11b⁺, CD64⁺ cells (Fig. 2a). Based on the literature [7, 8], a surface marker panel was set up to allow the identification of fibroblasts. As shown in Figure 2a, we identi-

fied fibroblasts extracted from mouse tail skin as CD45[−], DUMP[−], CD31[−], CD90.2⁺ and Sca-1⁺. Unlike the NIH3T3 mouse embryonic fibroblast cell line, fibroblasts extracted from mouse tail skin were expressing low levels of CD140 α /PDGFR α (Fig. 2a, b). Indeed, applying the same staining panel, the in vitro cultured NIH3T3 mouse embryonic fibroblast cell line was CD45[−], DUMP[−], CD31[−], CD90.2⁺, Sca-1⁺ and CD140 α ⁺ (Fig. 2b). As reported [9], CD140 α is highly sensitive to treatment with dispase and collagenase IV. Therefore, when subjecting the NIH3T3 mouse embryonic fibroblast cell line to the same enzymatic treatment as mouse tail skin, we observed by flow cytometric analysis that the CD140 α signal was reduced, whereas other surface markers, such as CD90.2 and Sca-1, were not affected (Fig. 2c). As described previously [3], pigment particle-containing macrophages can be identified through their high SSC-A profile (SSC-A^{high}) compared to normal macrophages. To test whether this was also true for fibroblasts, we tattooed a group of BL/6 mice, and after a healing phase of 4 weeks, a single dermal cell suspension was prepared and analyzed by flow cytometry. SSC-A^{high} macrophages were increased in tattooed tails; however, the SSC-A profile of fibroblasts was not dramatically changed in samples from tattooed compared to nontattooed tails (Fig. 2d) suggesting that fi-

Fig. 4. After macrophage depletion, the number of pigment particle-containing fibroblasts is increased. Wild-type (WT) BL/6 and CD64^{dtr} mice were tattooed on their tails. After a healing phase, CD64^{dtr} and WT mice received 1 µg diphtheria toxin (DT) twice in a 24-h interval for macrophage depletion. **a** Flow cytometric analysis of dermal CD64+ CD11b+ macrophages and Sca-1+ CD140+ fibroblasts of DT-treated CD64^{dtr} and WT mice. Fibroblasts were isolated from the tattooed tail of CD64^{dtr} and WT mice and analyzed with the TissueFAXs and Strataquest software. **b** Green particle intensity per cell. **c, d** Frequency (**c**) and absolute number (**d**) of pigment particle-positive fibroblasts. Data represent means ± SD of 2 independent experiments with 4–5 mice per group.



broblasts did not contain pigment particles at all, or that the pigment particle content was not sufficient to change their SSC-A profile.

Fibroblasts and Macrophages Contain Different Amounts of Tattoo Particles

A possible explanation for the lack of differences in the SSC-A profile of fibroblasts isolated from a tattooed skin versus control could be that fibroblasts stored only minimal amounts of pigment particles, which were not detectable using SSC-A. Therefore, we decided to sort fibroblasts and, as a positive control, SSC-A^{high} macrophages from tattooed tail dermis, and to characterize the cells by microscopic analysis following cytospin and hematoxylin-eosin staining. SSC-A^{high} macrophages had phagocytosed high numbers of green tattoo particles, whereas only few fibroblasts contained few scarcely distributed particles (Fig. 3a). Quantification of the amount of green particles per cell in dermal cell populations with TissueFAXS microscopy and Strataquest computational analysis revealed that the green particle intensity per cell was highest in SSC-A^{high} macrophages, compared to fi-

broblasts (Fig. 3b). Additionally, microscopic analysis showed that almost all SSC-A^{high} macrophages from the tattooed tail were pigment particle positive, whereas only a minority (5.3%) of fibroblasts incorporated green particles (Fig. 3c). By contrast, when we analyzed the absolute numbers of cells in the tattooed area, fibroblasts were the dominant population containing pigment particles (Fig. 3d) indicating that the particles are not only stored in SSC-A^{high} macrophages, but also by significant numbers of dermal fibroblasts. Macrophages in the SSC-A^{high} gate were almost exclusively green particle-containing macrophages and harbored the highest pigment particle intensity, whereas fibroblasts represent the quantitatively dominant population but storing only small amounts of green particles.

Tattoos of Macrophage-Depleted Mice Have a Higher Frequency of Pigment Particle-Positive Fibroblasts

By using a mouse model permitting DT-mediated depletion of macrophages (CD64^{dtr}), it has recently been shown that the overall appearance of the tattoo did not change despite the particle release into the dermis by dy-

ing macrophages [3]. Since the tattoo did not disappear after macrophage depletion, we wondered whether fibroblasts play a role in the maintenance of the tattoo in the skin of these mice. To address this question, we tattooed CD64^{dtr} mice and depleted macrophages after the healing phase. Treatment with DT led to a selective ablation of the SSC-A^{high} macrophage population, while the fibroblasts were not affected by this treatment (Fig. 4a). Fibroblasts were sorted from DT-treated tattooed CD64^{dtr} mouse tails and analyzed after cytopspin with TissueFAXS and the Strataquest software, and did not reveal any difference in the particle intensity compared to fibroblasts from tattooed wild-type tails (Fig. 4b). However, the frequency of fibroblasts containing pigment particles within sorted fibroblasts (Fig. 4c) and the absolute number of particle-containing fibroblasts (Fig. 4d) were slightly increased after macrophage depletion.

Discussion/Conclusion

Tattoos have become a must-have over the last decade. However, only few studies focused on skin cells and the poorly understood intricate cellular interactions that occur in the tattooed skin area [3, 6, 10]. Therefore, a more comprehensive knowledge of the cellular distribution of pigment particles will definitely improve current methods of laser-assisted tattoo removal. In the present study, we compared the frequency of pigment particle-positive dermal macrophages and fibroblasts isolated from tattooed mouse tail skin, and their corresponding particle load at a single cell level.

Dermal macrophages were shown to be primarily responsible for assembling and maintaining tattoos due to their unique ability to capture and retain large quantities of pigment particles [3]. Although they do not constitute classical phagocytes and produce the structural framework of the dermis, fibroblasts likely contribute to pigment particle storage [5, 6]. For instance, they are able to phagocytose collagen fibers *in vitro* [11–13], and their phagocytic capacity can increase in the presence of denatured (wound-related) collagens [14]. Along that line, pigment particles have been found associated with collagen fibers [10]. Therefore, it remains possible that during the inflammatory tattooing procedure, fibroblasts might take up the pigment particles in the course of the recycling phase of damaged collagen fibers.

In the present work, we showed that dermal fibroblasts are also capable of capturing pigment particles, however in far smaller amounts than dermal macro-

phages. By using the CD64^{dtr} mouse model for DT-mediated macrophage depletion, we further showed that the release of pigment particles by dying macrophages triggers a rather modest increase in the number of dermal fibroblasts containing particles. It has been suggested that the amount of pigment particles in fibroblasts and macrophages stays constant over the years [6]. Our results are thus consistent with the view that fibroblasts and macrophages keep two independent pools of tattoo particles, and they explain why fibroblasts do not acquire more particles once particle-laden macrophages are killed and, therefore, why most of the particles are available for recapture by the new incoming macrophages. In a study focusing on human skin with aged tattoos, particle agglomerates were found located around collagen fibrils [10], suggesting that in aged tattoos, the particles that are released from dying cells cannot be efficiently recaptured and therefore agglomerate around collagen fibers.

How can we use this knowledge to improve tattoo removal treatment? Laser treatment to date is the gold standard for removing unwanted tattoos. It has been suggested that tattoo removal can be likely improved by combining laser surgery with the transient killing of the dermal macrophages present in the tattoo area [3]. As a result, the fragmented pigment particles generated by using laser pulses will not have the possibility to be immediately recaptured, a condition increasing the probability of having them drained away via the lymphatic vessels. Alternatively, combining laser treatment with cell-specific endocytosis inhibitors could also facilitate the draining of the released and fragmented tattoo particles by preventing their recapture by macrophages or fibroblasts. Along that line, endocytosis is well studied, and various drugs or biopharmaceutical molecules are available to block endocytosis [15].

In conclusion, our study furthers our view on how pigment particles are maintained in the skin dermis following tattoo procedures. Our results demonstrate that in addition to macrophages, fibroblasts are also capable of capturing particles. Although the number of particle-positive fibroblasts found in the dermis is higher than that of particle-positive macrophages, the amount of particles they contain is rather modest, compared to that of macrophages.

Key Message

Tattoo pigments are taken up by dermal macrophages and fibroblasts in different quantities.

Acknowledgment

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Statement of Ethics

Mice were subjected to in vivo procedures that were performed following protocols approved by the Ethics Committee of Marseille in accordance with institutional, national and European directives for animal care (approval APAFIS 779-2015 0605 10534083).

Disclosure Statement

The authors have no conflict of interest to declare.

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Author Contributions

H. Strandt and S. Henri conceived the project. H. Strandt did the experiments with the technical help of O. Voluzan. H. Strandt, J. Thalhamer, A. Stoecklinger and S. Henri wrote the manuscript and prepared the figures. T. Niedermair and U. Ritter performed the acquisition and analysis with TissueFAXS. B. Malissen provided intellectual and financial support and edited the manuscript.