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Unveiling skin macrophage dynamics explains both tattoo persistence and strenuous removal

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Here we describe a new mouse model that exploits the pattern of expression of the high-affinity IgG receptor (CD64) and allows diphtheria toxin (DT)–mediated ablation of tissue-resident macrophages and monocyte-derived cells. We found that the myeloid cells of the ear skin dermis are dominated by DT-sensitive, melanin-laden cells that have been missed in previous studies and correspond to macrophages that have ingested melanosomes from neighboring melanocytes. Those cells have been referred to as melanophages in humans. We also identified melanophages in melanocytic melanoma. Benefiting of our knowledge on melanophage dynamics, we determined the identity, origin, and dynamics of the skin myeloid cells that capture and retain tattoo pigment particles. We showed that they are exclusively made of dermal macrophages. Using the possibility to delete them, we further demonstrated that tattoo pigment particles can undergo successive cycles of capture–release–recapture without any tattoo vanishing. Therefore, congruent with dermal macrophage dynamics, long-term tattoo persistence likely relies on macrophage renewal rather than on macrophage longevity.

Introduction

Recent studies, based on ontogeny and transcriptomic analysis, have succeeded in discriminating conventional dendritic cells (cDCs) from monocyte-derived cells. cDCs comprise two lineages known as cDC1 and cDC2 (Guilliams et al., 2016), have a short life span, and are continually replenished from committed cDC precursors that originate in the bone marrow (BM). Under steady-state conditions, BM-derived blood Ly-6Chigh monocytes extravasate at a slow pace into nonlymphoid tissues, such as the intestinal lamina propria and the skin dermis, where they give rise to two developmental streams (Tamoutounour et al., 2012, 2013; Bain et al., 2013; Jakubzick et al., 2013). Progression through the first developmental stream is associated with the preservation of CC-chemokine receptor 2 (CCR2) expression, the acquisition of MHCII molecules, and a diminution of Ly-6C expression. It can be visualized in Ly-6C–MHCII dot plots as a “monocyte waterfall,” the final product of which share features with cDC2 and is called monocyte-derived DC (mo-DCs; Tamoutounour et al., 2013). Blood Ly-6C– monocytes extravasate at a higher pace into inflamed tissues (Tamoutounour et al., 2012, 2013; Bain et al., 2013) and give rise to cells that, because of their ability to produce TNFα and inducible NO synthase, are denoted as Tip-DCs (Serbina et al., 2003; De Trez et al., 2009; Tamoutounour et al., 2012). Under steady-state conditions, extravasated Ly-6Chigh monocytes also proceed through a second developmental stream yielding tissue-resident macrophages (mo-Mac) that have lost expression of CCR2 and Ly-6C and express or not MHCII molecules (Jakubzick et al., 2013; Tamoutounour et al., 2013; Yona et al., 2013; Bain et al., 2014; McGovern et al., 2014). In the intestine and dermis, such mo-Mac replace in part or in totality a pool of macrophages that develops before the establishment of definitive hematopoiesis in a CCR2-independent manner (Tamoutounour et al., 2013; Bain et al., 2014; Sheng et al., 2015). Monocyte fate toward mo-Mac or mo-DCs is specified by microenvironmental cues (Goudot et al., 2017).

The possibility of discriminating cDCs from mo-DCs and macrophages emphasized their distinct functional specialization. Upon infection, antigen-laden cDCs migrate to draining LNs and trigger the conversion of antigen-specific naive T cells into effector T cells. In contrast, most mo-DCs remain in tissue parenchyma where they contribute to local immune responses and provide potent antimicrobial function during infection. Macrophages contribute to tissue homeostasis and wound repair because of their capacity to phagocytose cellular debris, invading organisms, and apoptotic cells. Owing to their strategic positioning at body barriers, macrophages capture a wide range of exogenous particulates. They also ingest self-derived particulates, as...
Figure 1. Replenishment kinetics of monocytes, mo-DCs, and macrophages in the dermis of CD64dtr mice after DT treatment. CD64dtr mice were left untreated (-DT) or treated twice and 24 h apart with DT. Analysis of the CD64+CCR2+ monocytes and mo-DCs and CD64+CCR2- macrophages found in the ear dermis (Fig. S1A) was performed before (-DT) and 1, 2, 3, 4, 5, 10, 20, and 90 d after the last DT injection. (A) CD64+CCR2+ dermal cells were analyzed at the specified time points by using Ly-6C–MHCII dot plots to define the percentages of Ly-6C+MHCII– (P1) dermal monocytes, Ly-6C+MHCII+ (P2) mo-DCs, and Ly-6C–MHCII+ (P3) mo-DCs (Tamoutounour et al., 2013). (B) Quantification of the results in A showing the absolute number of cells obtained from two ears of each mouse. Each symbol corresponds to a mouse and the mean (horizontal bar) is indicated. (C) CD64+CCR2+ dermal cells were analyzed at the specified time points by using Ly-6C–MHCII dot plots to define the percentages of Ly-6C+MHCII– (P4) dermal macrophages and Ly-6C+MHCII+ (P5) dermal macrophages (Tamoutounour et al., 2013). A Ly-6C+MHCII– to high cell population transiently developed 2–5 d after the last DT treatment and is denoted as Px cells. (D)
exemplified by the melanin-laden macrophages that have been sporadically observed in the normal human dermis and referred to as “melanophages” (Haniffa et al., 2009, 2012).

To study the functional specialization that exists among cells of the mononuclear phagocyte system, several strains of gene-targeted mice have been developed to ablate a given cell subset by placing the human diphtheria toxin receptor (hDTR) under the control of a gene promoter specific for the subset of interest. Those models are sometimes associated with drawbacks (van Blijswijk et al., 2015; Durai and Murphy, 2016). For instance, in CD11b-DTR mice, cDC2s, macrophages, and mo-DCs were depleted, and because of off-target DTR expression, those mice died after a few diphtheria toxin (DT) injections (Duffield et al., 2005). Although CCR2-DTR mice were not prone to DT-induced lethality (Hohl et al., 2009), some of the phenotypes observed by using those mice likely resulted from the depletion of CCR2+ cDC2s rather than of monocytes and monocyte-derived cells as generally assumed (Mallisien et al., 2014).

In contrast to cDCs, both the mo-DCs and mo-Mac, including their precursors, and the macrophages of embryonic origin that are found in tissues such as the skin, the lung, and the gut express the high-affinity IgG receptor FcγRI (CD64; Bain et al., 2013; Plantinga et al., 2013; Tamoutounour et al., 2013). Here, we developed mice in which a hDTR gene was placed under the control of the FcγRI gene that codes for CD64. This model has a tighter ablation specificity compared with previous models in that it permitted specifically ablating macrophages, monocytes, and monocyte-derived cells in vivo. It allowed us to identify in the ear skin dermis of C57BL/6 (B6) mice a population of melanophages that constitutes a quantitatively major subset of dermal myeloid cells and has been missed in previous studies. By characterizing their phenotype, origin, and dynamics, we showed that they corresponded to dermal macrophages that have ingested melanin granules. Benefiting from the knowledge gained on melanophage dynamics, we further elucidated the identity, origin, and dynamics of the myeloid cells that are found in the mouse skin and capable of capturing and retaining tattoo pigment particles.

**Results**

**Mice permitting ablation of macrophages and monocyte-derived cells**

To assess the dynamics and functional role of CD64+ macrophages and monocyte-derived cells in vivo, we generated a line of gene-targeted mice containing in the 3′-UTR of the FcγRI gene a cassette composed of an internal ribosomal entry site, an enhanced green fluorescent protein (EGFP), a 2A cleavage sequence, and an hDTR. To demonstrate that the mo-Mac and monocyte-derived cells found in mice homozygous for the FcγRI-IRES-EGFP-hDTR allele (also known as B6-FcγRIimCiphe and called CD64dr mice here) developed normally and expressed normal levels of CD64, we analyzed their skin dermis because it contains a full complement of such cells (Fig. S1 A; Tamoutounour et al., 2013). Among dermal CD11b+ cells, cDC2s can be distinguished from non-cDC2s cells by their Ly-6C CD64+ phenotype. Dermal CD11b+ CD64+ non-cDC2 cells can be further resolved into CCR2+ and CCR2− cells. CCR2+ cells comprise Ly-6ChighMHCII+ dermal monocytes (P1), Ly-6Chigh (P2), and Ly-6C− (P3) MHCII+ mo-DCs that constitute a P1 → P2 → P3 developmental series. CCR2− cells comprise MHCIIlow (P4) and MHCIIhigh (P5) dermal macrophages. The ear skin dermis of DT-untreated CD64dr mice showed ratios and numbers of monocytes, mo-DCs, and macrophages comparable to those of WT mice (Fig. S1 B), and all those cells expressed normal levels of CD64 (Fig. S1 C). Similar results were obtained when CD64+ cells found in the lamina propria of the large intestine were analyzed (not depicted).

CD64dr mice were treated with DT twice and 24 h apart without lethality, and the myeloid cells present in the ear skin were analyzed at various time points after the last DT injection. DT treatment was not associated with overt inflammation as documented by the lack of skin infiltrating neutrophils (not depicted). CD64−CCR2− cells were all ablated within 24 h (Fig. 1, A and B), demonstrating that the hDTR was functional. 48 h after the last DT injection, Ly-6ChighMHCII+ dermal monocytes started to return, and Ly-6Chigh and Ly-6C− MHCIImo-DCs were replenished in sequence over the next 5 d. A transient rebound in their numbers occurred around day 4, suggesting that their rate of generation can increase in response to acute, DT-induced loss. Both MHCIIlow and MHCIIhigh CCR2+ CD64+ dermal macrophages showed protracted reconstitution kinetics compared with CD64−CCR2− cells in that it took up to 10 d to fully reconstitute their pools (Fig. 1, C and D). Analysis of the gate corresponding to CCR2−CD64− cells at days 2–3 revealed a population of Ly-6ChighMHCIIlow to high cells (denoted as Px cells in Fig. 1, C and D) that started losing Ly-6c expression on day 4 and thus progressively merged within gates corresponding to MHCIIlow and MHCIIhigh dermal macrophages. The phenotype, kinetics of reappearance of Px cells, and the occurrence at days 4 and 5 of cells straddling the Px and P4/P5 gates suggest that they correspond to intermediaries between Ly-6Chigh blood monocytes and MHCIIlow and MHCIIhigh dermal macrophages. The percentage of Px cells among CCR2+ CD64+ cells reached pre-DT-treatment levels upon replenishment of the MHCIIlow and MHCIIhigh dermal macrophage compartments (Fig. 1, C and D).

In the lamina propria of the large intestine of CD64dr mice, CD64+ cells were also ablated within 24 h after the last DT injection and reconstituted from 48 h onward akin to dermal CD64+ cells (Fig. S2). Therefore, by allowing to conditionally empty the niche occupied in the skin dermis and intestinal lamina propria by CD64+ myeloid cells, the CD64dr mouse model demonstrated that all the mo-DC and macrophage subsets that were present in adult skin and intestine before DT treatment can be readily

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Quantification of the results in C showing the absolute number of cells obtained from two ears of each mouse. Each symbol corresponds to a mouse and the mean (horizontal bar) is indicated. Data are representative of three independent experiments involving three to six animals per group. *, P ≤ 0.05; **, P ≤ 0.01; ***, P ≤ 0.001; unpaired Student’s t test.
Figure 2. Replenishment kinetics of blood monocytes of CD64<sup>−</sup> mice after DT treatment. (A) Gating strategy used to identify CD11b<sup>+</sup>Lin<sup>−</sup> cell subsets within the blood. Red blood cells were lysed before analysis, and the absolute number of a given cell type present in a given blood volume was determined by staining samples in Trucount Absolute Counting Tubes. Beads were gated out on the basis of their FSC−A–SSC-A profile, and after excluding CD5<sup>+</sup> T cells, CD19<sup>+</sup> B cells, CD161<sup>+</sup> NK cells, as well as SSC-A<sup>int</sup> eosinophils, and Ly-6G<sup>+</sup> neutrophils, the majority of the remaining CD11b<sup>+</sup>Lin<sup>−</sup> cells were CD45<sup>+</sup>CD115<sup>+</sup>; when analyzed on CD115−CD43 dot plots, they comprise CD115<sup>+</sup>CD43<sup>−</sup> and CD115<sup>+</sup>CD43<sup>+</sup> subsets. As expected, CD115<sup>+</sup>CD43<sup>−</sup> cells corresponded to Ly-6Chigh blood monocytes, whereas most CD115<sup>+</sup>CD43<sup>+</sup> corresponded to Ly-6C<sup>low</sup> blood monocytes. (B) Absolute number (mean values ± SD) of the specified cells in the...
reconstituted in normal ratios and numbers and with kinetics consistent with their expected precursor-product relationship.

**Kinetics of replenishment of blood monocytes after DT treatment**

Adult steady-state monopoiesis occurs in BM, where monocyte–macrophage DC progenitors (MDPs) give rise to common monocyte progenitors (cMoP) that generate two main blood monocyte subsets that are both CD115 (CSF1R)⁺ and can be distinguished on the basis of CD43 expression (Fig. 2 A). CD43⁻ cells are CX3CR1intCCR2⁻CD64⁺Ly-6Chigh and CD43⁺ cells are mostly CX3CR1highCCR2⁺CD64⁺Ly-6Clow (Sunderkötter et al., 2004). They are denoted here as Ly-6Chigh and Ly-6Clow monocytes, respectively. Ly-6Clow monocytes remain within blood vessels (Carlin et al., 2013) whereas Ly-6Chigh monocytes contribute to the replenishment of mo-DCs and mo-Mac in tissues, including the skin and the intestine (Jakubzick et al., 2013; Tamoutouneour et al., 2013; Yona et al., 2013; Bain et al., 2014; McGovern et al., 2014). Intraperitoneal injection of DT twice and 24 h apart resulted within 24 h in the complete ablation of the MDPs and cMoPs and Ly-6Chigh, Ly-6Cint, and Ly-6Clow monocytes found in the BM (Fig. S3) and of the Ly-6Chigh and Ly-6Clow blood monocytes (Fig. 2, B–D). Analysis of the blood and BM of CD64dtr mice at various time points after the last DT injection showed that blood Ly-6Chigh and Ly-6Clow monocytes were rapidly replenished and that a transient rebound in their numbers occurred (Fig. 2, B–D; and Fig. S3). Consistent with the observation that CD115 is dramatically down-regulated in response to CSF-1 (Yu et al., 2012), the earliest BM-derived monocytes reappearing in the blood expressed lower levels of CD115 than CD115⁺ steady-state monocytes and are denoted here as CD115int cells (Fig. 2, A, B, and E). Because of their CD115int phenotype, those Ly-6Chigh monocytes were not recorded in the CD115⁺CD43⁻ gate used in Fig. 2 C. Their numbers peaked at day 3 after DT treatment (Fig. 2, B and E), and when blood monocytes were fully replenished, their relative representation dramatically decreased. Congruent with the view that circulating CD43⁻Ly-6Chigh monocytes give rise to CD43⁺Ly-6Clow monocytes (Varol et al., 2007; Thomas et al., 2016; Mildner et al., 2017), the first CD43⁺ monocytes reappeared at days 3–4 after the last DT treatment and gradually lost Ly-6C expression (Fig. 2 D). An approximately fourfold increase in blood neutrophils occurred in CD64dr mice and reached 3 d after DT treatment (Fig. 2 B). It occurred, however, with a far lower magnitude and shorter duration than that observed upon DT administration in CD11c-DTR mice (Autenrieth et al., 2012; Tittel et al., 2012).

**DT treatment of CD64dr mice spares skin cDCs**

We analyzed next whether DT treatment of CD64dr mice affects the cDCs found in the skin. Langerhans cells (LCs; CD24hiCD11b⁻), cDCIs (CD11b⁻CD24⁺), cDCIs (CD11b⁻CD24⁻), and double-negative (DN) cDCs (CD11b⁻CD24⁻) were identified as described in Fig. S4 A. Although the absolute numbers of LCs, cDCIs, and DN cells were not affected by DT treatment, a 1.3-fold reduction in cDC2 absolute number was observed 1 d after DT treatment (Fig. S4 B). Therefore, our regimen of DT administration had a rather minimal impact on the cDC subsets found in the skin of CD64dr mice. CD64dr mice can thus be used to monitor the dynamics and function of macrophages and monocyte-derived cells without major collateral effects or lethality.

**Melanophages predominate among dermal myeloid cells of B6 ear skin**

Former analyses of CD45⁺Lin⁺ myeloid cells present in the ear skin dermis focused on SSC-Alow-FSC-Alow to high (SSC-Ahigh in short) cells, leaving aside an uncharacterized population of SSC-Ahigh-FSC-Alow to int (SSC-Ahigh in short) cells (Tamoutouneour et al., 2013). After treatment of CD64dr mice with DT twice and 24 h apart, all those SSC-Ahigh cells were unexpectedly ablated (Fig. 3 A). Further characterization of such DT-sensitive, SSC-Ahigh cells showed that they had a CD24lowCD11b⁻CD64⁺CCR2⁻MHCIİ⁺ or - phenotype identical to that of SSC-Alow dermal macrophages and that CD64 expression accounted for their DT sensitivity (Fig. 3 B).

In the almost hairless skin of mouse ears, melanocytes are primarily found in the dermis (Fig. 3 C; Aoki et al., 2009) and synthesize melamin pigment within specialized lysosome-related structures known as melanosomes. Considering that the SSC-A parameter used in cytometry is proportional to cell granularity and that CD64dr mice were of B6 origin and have a black coat, we surmised that the high granularity of the SSC-Ahigh myeloid cells found in the ear dermis was due to their capture of melanin granules from neighboring melanocytes. Hematoxylin and eosin staining of cytoplasmic preparations showed that the SSC-Alow—CCR2⁻—CD64⁺ cells and the SSC-Ahigh cells sorted from the ear dermis have typical features of tissue macrophages; they were large with prominent cytoplasmic vacuoles (Fig. 3 D). The vacuoles of SSC-Ahigh cells differed, however, from those of SSC-Alow—CCR2⁻—CD64⁺ cells in that they were loaded with dark material (Fig. 3 D). Upon analysis by electron microscopy, such dark vacuolar material had an extremely electron-dense appearance and an ellipsoid shape (~0.2 μm × 0.7 μm), two hallmarks of melanosomes (Fig. 3 E). All the ingested melanosomes were in a mature stage, showing a uniformly electron-dense granule without discernable internal structure (Fig. 3 E).

In contrast to their precursors, dermal macrophages do not express CX3CR1 (Tamoutouneour et al., 2012). Consistent with our hypothesis that melanophages correspond to macrophages that ingested melanosomes, they were CX3CR1⁻ (not depicted)

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**Baranska et al.**

**Skin and melanoma melanophages**

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Figure 3. CD64<sup>dtr</sup> mice unveil the existence of a novel CD64<sup>+</sup> cell population in the skin that is highly granular and DT sensitive and corresponds to melanophages. (A) Cell suspensions from the ear skin of CD64<sup>dtr</sup> mice were analyzed by flow cytometry before (-DT) or 48 h after (DT + 48 h) DT treatment. After excluding CD3<sup>+</sup> T cells, CD19<sup>+</sup> B cells, CD161<sup>+</sup> NK cells, and Ly-6G<sup>+</sup> neutrophils, the remaining lineage-negative (Lin<sup>−</sup>) CD45<sup>+</sup> live cells were analyzed by using FSC-A–SSC-A dot plots to define the percentages of SSC-A<sub>low</sub> and SSC-A<sub>high</sub> cells. (B) Comparative analysis of the SSC-A<sub>low</sub> and SSC-A<sub>high</sub> cells found in the ear skin by using a gating strategy resolving the cDC2s, monocytes, mo-DCs and macrophages present among CD45<sup>+</sup>Lin<sup>−</sup>CD11b<sup>+</sup> cells (Fig. S1 A). The percentage of cells found in each of the specified gate is indicated. (C) Transversal tissue sections of B6 ear or trunk skin (at telogen and anagen phases) were stained with...
and identified as melanin ‘tdTomato’CD64+ cells in the ear section of CX3CR1Cre/+ Rosa26td-Tomato mice that fate map cells that have expressed CX3CR1 during their development (Fig. 3 F). In the densely haired mouse trunk, melanocytes are absent from the dermis and confined to the hair follicles where melanocytes are transferred from melanocytes to matrix keratinocytes to give rise to melanized hairs (Fig. 3 C). Such transfer is strictly coupled to the anagen phase of hair growth, and melanin is not formed during the telogen phase (Fig. 3 C; Slominski et al., 2005). Consistent with these anatomical features, the CD11b+CD64+CCR2− macrophages isolated from the trunk skin during both the anagen and telogen phases lacked melanophages (Fig. 3 G). Likewise, the ear skin of B6-albino and BALB/c mice, which is deprived of melanin pigment, also lacked SSC-Ahigh melanophages (Fig. 4, A and B). Therefore, the presence of dermal melanophages correlates with the presence of dermal melanocytes. Although they represent 55±10% of the monocyte-derived cells present in the B6 ear dermis (Fig. 4, A and B), it is likely that melanophages have been missed in previous studies because of their unusual SSC-Ahigh phenotype.

Dynamics and origin of dermal melanophages

To assess the dynamics of dermal melanophages replenishment after DT-induced depletion, CD64+ mice were treated with DT twice and 24 h apart. Melanophages were all ablated within 24 h, and their numbers fully reconstituted ~20 d after the last DT injection (Fig. 5, A and B). The granularity of SSC-Ahigh cells, a proxy for melanin content, increased with time. The upper section (>190 K) of the SSC-Ahigh gate was only filled between days 20 and 90 after DT treatment, and a melanin content distribution identical to pre-DT-treatment levels was regained 90 d after DT treatment (Fig. 5, A and B). Although the dermis of CCR2-deficient mice is only populated by macrophages of prenatal origin that self-maintain without any input from adult monopoiesis (Tamoutounour et al., 2013), it showed a macrophage to macrophage ratio and melanin content distribution identical to that of WT dermis (Fig. 6 A). Therefore, the macrophages found in the ear dermis have the same propensity to capture melanin regardless of their prenatal (Fig. 6 A) or adult (Fig. 5) origin.

To demonstrate without relying on DT ablation that replenishment of the pool of adult dermal macrophages and melanophages relied on continuous input from BM-derived circulating precursors, parabiosis was established between adult mice expressing CD45.1 and CD45.2 (Fig. 6 B). Blood monocytes were allowed to exchange between parabionts for 8 wk before assessing the presence of donor-derived macrophages and melanophages in the ear skin of each parabiont. Dermal MHC II+ macrophages contained ~15% of cells originating from the parabiotic partner (Fig. 6 B), a distribution comparable to that of Ly-6C+ blood monocytes and demonstrating that they continuously develop from BM monocytes (Tamoutounour et al., 2013; Sheng et al., 2015). Consistent with the view that a fraction of dermal MHCII+ macrophages is of embryonic origin and self-renews in situ (Sheng et al., 2015), MHCII+ macrophages showed a twofold reduced rate of exchange with blood-borne precursors compared with dermal MHCII+ macrophages. In parabiosis experiments involving WT and CCR2-deficient mice, the percentages of donor-derived cells found in the CCR2-deficient parabionts reached 80% and 40% of MHCII+ and MHCII+ macrophages, respectively, compared with 80% for Ly-6C+ blood monocytes (Fig. 6 B). As expected for cells linked by a precursor-product relationship, in both types of parabiosis MHCII+ and MHCII+ melanophages achieved a degree of chimerism identical to that of MHCII+ and MHCII+ macrophages (Fig. 6 B). Altogether, these data indicate that dermal macrophages and melanophages are related through a precursor-product relationship and that dying dermal macrophages and melanophages are continuously replenished with new ones via CCR2-dependent recruitment of circulating Ly-6C+ blood precursors.

Comparative transcriptomic analysis of dermal macrophages and melanophages

To assess whether melanophages show a unique transcriptomic signature compared with dermal SSC-Alow macrophages that have not ingested melanin, MHCII+ and MHCII+ melanophages and macrophages from the ear dermis were sorted in triplicates and subjected to gene-expression profiling. Principal component analysis (PCA) on all the genes found to be differentially expressed between at least two of the four subsets showed that the MHCII+ and MHCII+ status accounted for most of the overall variability observed in the dataset (47%) and that differences between melanophages and macrophages was the second component of variability (17%; Fig. 7 A). Insights into the biological pathways underlying these transcriptomic differences were obtained through ingenuity pathway analysis (IPA) of eight lists of genes corresponding to those differentially expressed (1) in one out of the four subsets, (2) between MHCII+ versus MHCII+ cells, and (3) between melanophages and macrophages (Fig. 7 B). When compared with their MHCII+ counterparts, MHCII+ macrophages and melanophages were significantly
enriched in annotations related to the adaptive immune response such as antigen presentation pathway, dendritic cell maturation, and cytokine signaling (IL-4, IL-6, IL-10, and IL-17). MHCI expression was also associated with a pro-inflammatory signature with higher expression of Ccr7, Il1b, Il1r2, and Il6 and to immune cell chemotaxis with higher expression of Ccl17, Ccl22, Ccl5, and Cxcl13 (Fig. 7 C). When compared with SSC-A\textsuperscript{low} macrophages, melanophages were significantly enriched in annotations related to aryl hydrocarbon receptor signaling and glutathione-mediated detoxification (Fig. 7 B) because of their expression of two genes of the glutathione S transferase family (Gstm1 and 3; Table S1). High and selective expression by melanophages of genes coding for a lysosomal ATPase and a cystein protease (Atp6v0d2 and Ctsk, respectively) led to their enrichment for the annotation phagosome maturation (Fig. 7 B and Table S1), a feature consistent with the fact that they are laden with melanosomes.

**Melanophages are found in melanocytic melanoma**

Tumors depend on the supportive functions of macrophages. Clusters of melanophages have been observed in the periphery of the nevi found in the dermis of mice expressing oncogenic mutated form (BrafV600E) of the serine-threonine-specific protein kinase BRAF (Dhomen et al., 2009). To determine whether such melanoma-associated melanophages correspond to CD64\textsuperscript{+} SSC-A\textsuperscript{high} cells, B6-albino mice, which lack melanophages, were injected with melanocytic (B16) or amelanocytic (BrafV600E) melanoma cells. Analysis of CD45\textsuperscript{+}Lin\textsuperscript{−} cells isolated from tumor masses 14 to 18 d after inoculation showed that SSC-A\textsuperscript{high} cells were present only in the melanocytic melanoma (Fig. 8 A). Their morphology was similar to that of the melanophages identified in the B6 dermis (Fig. 8 B) and they had a CD11b\textsuperscript{+}CD64\textsuperscript{+}F4/80\textsuperscript{+} phenotype (Fig. 8 C).

The melanophages that are identifiable in melanocytic melanoma constitute a unique thread permitting revisiting the phenotype of tumoral macrophages. For instance, the presence of CD64 and absence of Ly-6C permit readily distinguishing macrophages in steady-state tissues (Tamoutounour et al., 2013), and it has been assumed that such a feature holds true within the tumor microenvironment (Salmon et al., 2016). Our analysis of the melanophages found in melanocytic tumors showed, however, ...
that most of them expressed Ly-6C (Fig. 8 C). Therefore, analysis of the melanophages found in melanoma constitute a unique decision support tool permitting to determine whether markers defined as macrophage-specific under steady-state conditions keep or lose their discriminatory potential within the tumor microenvironment.

**Skin macrophages capture and retain tattoo pigment particles**

Using the CD64Δ mouse model and benefiting of our knowledge on melanophages, we determined next the identity, origin, and dynamics of the skin myeloid cells that are capable of capturing and retaining tattoo pigment particles. The frequency of melanophages in the tail skin of B6 mice is fourfold lower than that of melanophages in ear dermis and reflects its scarcity in melanocytes. Moreover, because of their reduced melanin content tail skin melanophages barely merged in the SSC-Ahigh gate, the lower limit of which that was defined by using B6 albino ear (Fig. 4, A and B). Accordingly, we used tail skin of B6 mice to monitor the appearance in the almost-empty SSC-Ahigh gate of those cells that have captured the bright green pigment particles that are contained in the tattooing ink paste used in the present study. Analysis of the percentages of SSC-Alow and SSC-Ahigh cells found among the CD45+Lin– cells isolated from the skin of tails that were tattooed 3 wk before analysis showed that they contained a unique population of SSC-Ahigh cells compared with untreated mice (Fig. 9 A). Further characterization of those SSC-Ahigh cells showed that they had a CD24lowCD11b+CD64+CCR2– to low phenotype (Fig. 9 A), which was identical to that observed for SSC-Ahigh
melanophages and SSC-A\textsuperscript{low} macrophages (Fig. 3 B). Cytospin analysis of SSC-A\textsuperscript{high} cells sorted from tattooed tail skin showed that they were large cells containing prominent cytoplasmic vacuoles loaded with green material, whereas SSC-A\textsuperscript{low} dermal macrophages and cDCs contained no green tattoo pigment (Fig. 9 B). None of the CD45\textsuperscript{+} skin cells, which mostly comprise keratinocytes, contained green material (not depicted). Therefore, 3 wk after tail tattooing, the green pigment particles present at the site of tattooing were primarily found within dermal macrophages.

### Cycles of pigment capture–release–recapture account for long-term tattoo persistence

We used CD64\textsuperscript{dtr} mice to ablate dermal CD64\textsuperscript{+} macrophages laden with green pigment particles (“green” macrophages in short) and to determine whether their death results in tattoo vanishing. Accordingly, CD64\textsuperscript{dtr} mice that had the skin of their tail tattooed with green tattoo ink paste 3 wk before were treated with DT twice and 24 h apart. Analysis of tattooed skin of those mice for the presence of SSC-A\textsuperscript{high} green macrophages showed that they were as expected all ablated within 2 d after DT treatment and that, akin to melanophages, their pool was slowly reconstituted after DT injection (Fig. 9 C). Their granularity, reflecting green-particle content per cell, increased with time and 90 d after DT injection reached levels identical to those present just before DT treatment (Fig. 9, A and C). The pool of SSC-A\textsuperscript{high} cells that was replenished 90 d after DT treatment was exclusively composed of green macrophages with a morphology and CD24\textsuperscript{low}CD11b\textsuperscript{+}CD64\textsuperscript{+}CCR2\textsuperscript{low} phenotype resembling those of green macrophages observed pre-DT treatment (Fig. 9 D and not depicted).

Considering that no green tattoo ink paste was reapplied after DT treatment, it is likely that most of the green pigment particles released after DT-induced green macrophage death remained in an extracellular form at the site of tattooing where they were progressively recaptured by incoming dermal macrophages originating from circulating Ly-6\textsuperscript{C}\textsuperscript{high} blood monocytes (see model A in Fig. 10). Consistent with that model, cytospin preparation of the total cell suspension from tail skin showed that before DT treatment green pigment particles were all associated with cells that had typical features of tissue macrophages, whereas 2 d after the last DT injection no green macrophages were left (Fig. 9 C) and the green pigment particles that were spun down with the cells were all extracellular (Fig. 9 E). Importantly, macroscopic examination of tattooed tails showed that even at time points when green macrophages were absent and green pigment particles were extracellular (for instance, day 2 after DT), the tattoo kept the appearance it had just before DT treatment (Fig. 9 F). Therefore, despite the fact that the green tattoo pigments went, over 90 d, through a full cycle of capture–release–recapture, the tattoo kept its macroscopic appearance.

To further support our pigment capture–release–recapture model without relying on DT ablation, CD45.1\textsuperscript{+} B6 mice were tattooed on the tail. The tattooed tail skin was explanted and grafted on the skin of the back of CD45.2\textsuperscript{+} B6 albino mice. Analysis of the myeloid cells present in the graft 6 wk after transplantation showed that most of the SSC-A\textsuperscript{high} cells found among CD11b\textsuperscript{+}CD64\textsuperscript{+}CCR2\textsuperscript{low} cells were of host origin, suggesting that green tattoo pigments were released from the CD45.1\textsuperscript{+}CD11b\textsuperscript{+}CD64\textsuperscript{+}CCR2\textsuperscript{low} SSC-A\textsuperscript{high} cells originally present in the donor graft and captured by incoming CD45.2\textsuperscript{+}CD11b\textsuperscript{+}CD64\textsuperscript{+}CCR2\textsuperscript{low} macrophages of host origin (Fig. 9 G). As discussed below, those findings provide a cellular basis for long-term tattoo persistence and have implications for improving strategies aimed at removing tattoos.

### Discussion

We used a novel model of DT-induced depletion of macrophages to generate niche availability without triggering inflammation and showed that the pool of adult dermal macrophages can be fully reconstituted from circulating Ly-6\textsuperscript{C}\textsuperscript{high} monocytes. By using a model of DT-mediated depletion of liver Kupffer cells, circulating Ly-6\textsuperscript{C}\textsuperscript{high} monocytes were also found capable of colonizing the emptied liver niches and of adopting a Kupffer cell identity (Scott et al., 2016). Likewise, transferred BM-derived monocytes were able to colonize the empty alveolar macrophage...
Figure 7. Gene expression profiling of MHCII⁺ and MHCII⁻ melanophages and macrophages from steady-state dermis. MHCII⁺ and MHCII⁻ CD64⁺CCR2⁻ SSC-Ahi melanophages and MHCII⁺ and MHCII⁻ CD64⁺SSC-Alow macrophages were sorted in triplicates from ear skin and subjected to gene-expression profiling (Affymetrix 1.0ST). (A) PCA of gene expression by MHCII⁺ and MHCII⁻ dermal macrophages and MHCII⁺ and MHCII⁻ melanophages, based on all the 1,054 unique genes differentially expressed between at least one pair of experimental conditions. The percentage of overall variability of the dataset is indicated along each PC axis. (B) Heat map representation of selected functions and pathways found enriched by IPA for the lists of genes differentially expressed in one of the three cell types, between MHCII⁺ versus MHCII⁻ cells, or between melanophages and macrophages. (C) Heat map showing the expression pattern across...
niches of neonatal CSF2RB-deficient mice and generate alveolar macrophages (van de Laar et al., 2016). Therefore, our data showed that the capacity for Ly-6C<sup>hi</sup> blood monocytes to replenish macrophages after DT-induced ablation also extends to the skin dermis. Moreover, we characterized the melanophages that are present in large numbers in the normal ear dermis of mice with a black coat, demonstrating that they correspond to dermal macrophages that have ingested melanosomes originating from neighboring melanocytes.

Limited information has been provided on the identity of the cells that are found in tattooed skin biopsies and capable of retaining tattoo pigment particles. By capitalizing on the knowledge gained on melanophages and on the functionalities offered by CD64<sup>dr</sup> mice, we conducted the first systematic analysis of the identity, origin, and dynamics of the skin myeloid cells that are capable of capturing and retaining tattoo pigment particles. The tattooing process consists of inserting water-insoluble pigment of the desired color into the dermis layer of the skin. Tattoo pigments are primarily metal salts in the submicrometer range. Immediately after tattooing, a fraction of pigments is shed with the epidermis or transported away from the dermis via lymphatic vessels. We demonstrated that the pigment particles that remain at the site of injection and cause the long-term tattoo color were exclusively found within dermal macrophages. Zaba et al. (2007) previously identified a population of cells located in the human dermis that was positive for factor XIIIa and the macrophage scavenger receptor CD163 that lacked CD11c and BDCa-1 and weakly activated alloendic T cells. Those cells have structural and ultrastructural features of macrophages and in biopsies of tattoos they were selectively laden with granular pigments. The fate of tattoo pigment injected into dermal tissues has been studied in the past, and fibroblasts were considered the primary long-term reservoir of the pigment granules (Ferguson et al., 1997; Elsaie et al., 2009). However, the morphological study of Zaba et al. (2007) and our present data combining multiparameter cytometry and the use of CD64<sup>dr</sup> “depleter” mice demonstrate that dermal macrophages constitute the primary store of dermal tattoo pigment in both mice and humans.

Although the dermis lacked macrophages for approximately 1 wk after DT treatment and it took several more weeks for the incoming macrophages to scavenge the released cell-free tattoo pigments, no macroscopic modification of the tattoo occurred. Before our study, the longevity of the undefined tattoo-pigment-laden cells found in the dermis was thought to be in the same order of that of a human adult, thereby accounting for long-term tattoo persistence (Fig. 10 B). Based on the present results, an alternative model for long-term tattoo persistence can be proposed. It takes into consideration the facts that the macrophages found in the dermis of adult mice are continuously replenished from circulating monocytes to compensate for the loss of dying macrophages and that most of the tattoo particles that are released from macrophages after DT treatment of CD64<sup>dr</sup> mice remain in situ and are recaptured by incoming macrophages. According to the pigment capture-release-recapture model (Fig. 10 A), in physiological conditions, when tattoo-pigment-laden macrophages die during the course of adult life, neighboring macrophages recapture the released pigments and ensure in a dynamic manner the macroscopic stability and long-term persistence of tattoos. Note that, in contrast to dermal cDCs, dermal macrophages do not have the capacity to migrate to draining LNs under normal and inflammatory conditions (Tamoutounour et al., 2013), an essential property for insuring tattoo persistence. Whether the pigment capture-release-recapture model of tattoo persistence applies to humans remains to be determined. In the case of the melanophages found in the human dermis, it has been suggested that they persist for years through longevity rather than continual renewal (Bigley et al., 2011).

Professional tattoos last for years or decades, and when no longer desired, they can be removed through the use of quality-switched lasers. Laser pulses lead to lysis of the tattoo-pigment-laden cells, fragmentation of the tattoo pigments, and their lymphatic transportation. Several cycles of laser treatment are required to achieve tattoo removal, and some tattoos remain immune to full removal. These difficulties are generally accounted for by the fact that a fraction of the fragmented pigment particles remains at the tattooing site and are recaptured by neighboring cells, a possibility formally demonstrated here by using the CD64<sup>dr</sup> model. Considering the long duration needed for the reconstitution of the pool of dermal macrophages and the recapture of the whole released pigments, tattoo removal can be likely improved by combining laser surgery with the transient ablation of the macrophages present in the tattoo area. 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.

In conclusion, we have validated a new model of DT-mediated depletion of tissue-resident macrophages, monocytes, and monocyte-derived cells in which DCs are spared. By permitting the emptying of the niche occupied in the skin dermis and intestinal lamina propria by CD64<sup>+</sup> myeloid cells, the CD64<sup>dr</sup> mouse model showed that all the mo-DC and macrophage subsets that were present in adult skin and intestine before DT treatment can be readily reconstituted from circulating Ly-6C<sup>hi</sup> monocytes. Furthermore, we showed that the myeloid cells of the ear skin dermis are dominated by melanophages, and we determined their origin and dynamics. Benefiting from our knowledge on melanophages, we determined the identity, origin, and dynamics of the skin myeloid cells that are capable of capturing tattoo-pigment particles and showed that skin monocytes (P1), mo-DCs (P2, P3), macrophages (P4, P5, Mac), melanophages (Mel), and cDC2s of selected genes contributing to IPA enrichments for the SSC-A<sup>hi</sup> macrophage, SSC-A<sup>lo</sup> melanophage, and MHC<sup>I+</sup> versus MHC<sup>I−</sup> transcriptomic signatures. The cell samples which names are in black font on the top of the heat map originate from Tamoutounour et al. (2013), where the P4 and P5 cells correspond to the MHC<sup>I+</sup> and MHC<sup>I−</sup> macrophages from the present study. The exhaustive lists of genes responsible for the IPA enrichments shown in B are provided in Table S1.
can be improved by combining laser surgery with the transient and specific ablation of the macrophages present in the tattooed area. Finally, we showed that melanocytic melanoma host readily identifiable melanophages that permit determining whether markers defined as macrophage-specific under steady-state conditions keep or lose their discriminatory potential within the tumor microenvironment.

Materials and methods

Mice

Ccr2−/− mice have been described (Jung et al., 2000). Cx3cr1cre/+ (MW126Gsat) mice were generated by N. Heintz (The Rockefeller University, GENSAT, New York) and purchased from MMRRC. Rosa26tdTomato (Ai14[RCL-tdT]-D) have been described (Madisen et al., 2010) and purchased from The Jackson Laboratory. Generation of Fcgr1-IRES-EGFP-hDTR gene-targeted mice is described below. C57BL/6 (B6) and BALB/c mice were purchased from Janvier and B6 albino (B6[Cg]-Tyrc-2J/J) and CD45.1 C57BL/6 mice from Charles River. All mice, except BALB/c, were maintained on a B6 background. Mice were housed under specific pathogen–free conditions, and in vivo procedures were performed following protocols approved by the Ethics Committee of Marseille in accordance with institutional, national, and European directives for animal care (approvals APAFIS 779–2015 0605 10534083 and APAFIS 781–2015 0605 14006182).

Construction Fcgr1-IRES-EGFP-hDTR mice

A 6.2-kb genomic fragment encompassing exons 4 to 6 of the Fcgr1 gene (ENS MUSG0000001594 7) was isolated from a BAC clone of B6 origin (clone RP23-427P23; http://www.lifesciences.sourcebioscience.com). By using ET recombination, an IRES-EGFP-2A-hDTR-loxP-Cre-neoR-loxP cassette was introduced in the 3′ untranslated region of the Fcgr1 gene, 33 bp downstream of the stop codon. The targeting construct was abutted to a cassette coding for the DT fragment A expression cassette and linearized with Pme1. JM8.F6 C57BL/6N embryonic stem (ES) cells (Pettitt et al., 2009) were electroporated with the targeting vector. After selection in G418, ES cell clones were screened for proper homologous recombination by Southern blot. When tested on Sca1-digested genomic DNA, the 3′ single-copy probe used to identify proper recombination events hybridized to a 7.0-kb WT fragment and to a 10.3-kb recombinant fragment. When tested on HindIII-digested genomic DNA, the 5′ single-copy probe used to identify proper recombination events hybridized to a 10.7-kb WT fragment and to a 6.3-kb recombinant fragment. A neomycin-specific probe was used to ensure that adventitious nonhomologous recombination events had not occurred in the selected ES clones. Properly recombined ES cells were injected into FVB blastocysts. Germline transmission led to the self-excision of the loxP-Cre-NeoR-loxP cassette in male germinal cells. Fcgr1-IRES-EGFP-hDTR mice were identified by PCR of tail DNA. The pair of primers, sense 5′-CCC CTC TCT CCC AGT GAC AGT ACT TG-3′ and antisense 5′-TGA ACC ACA TCC CTT GTG AG-3′, amplifies a 402-bp band in case of the WT Fcgr1 allele, whereas the pairs of primers, sense 5′-CCC TCT CTC CCA AGT GAC AGT ACT TG-3′ and antisense 5′-CCT GCA AAG GTG CCT AGA C-3′, amplified a 349-bp

![Image](https://example.com/image.jpg)
Figure 9. Dermal macrophages are responsible for uptake and storage of tattoo pigment particles. (A) CD45⁺Lin⁻ cells from the tail skin of B6 mice that were left untreated or tattooed with green tattoo ink paste 3 wk before isolation were analyzed for the percentages of SSC-A<sup>hi</sup> and SSC-A<sup>lo</sup> cells. SSC-A<sup>hi</sup> cells were further analyzed by using a gating strategy resolving cDC2s, monocytes, mo-DCs, and macrophages (Fig. S1 A). (B) Morphological characteristics of representative SSC-A<sup>hi</sup> cells (green macrophages), SSC-A<sup>lo</sup> cells (macrophages), and cDC2s sorted from tail skin of B6 mice tattooed with green tattoo paste 3 wk before isolation and analyzed by hematoxylin and eosin staining after cytospin onto glass slides. Bar, 10 µm. (C) Tails of CD64<sup>dtr</sup> mice were tattooed with green tattoo paste. 3 wk after, mice were treated twice and 24 h apart with 1 µg DT, and tail skin was analyzed 2 or 90 d after the last DT injection for the percentages of SSC-A<sup>lo</sup> and SSC-A<sup>hi</sup> cells found among CD45⁺Lin⁻ cells. (D) SSC-A<sup>hi</sup> cells were sorted from the tail skin of CD64<sup>dtr</sup> mice that were tattooed with green tattoo paste and either left untreated (-DT) or treated with DT and analyzed 90 d (DT + d90) after DT treatment. Sorted cells were analyzed by hematoxylin and eosin staining after cytospin onto glass slides. One representative cell is shown for each population. Bar, 10 µm. (E) Tail skin of CD64<sup>dtr</sup> mice that were tattooed with green tattoo paste and either left untreated (-DT) or treated with DT and analyzed 2 d (DT + d2) after DT treatment was digested, and the resulting cell suspension was directly analyzed by hematoxylin and eosin staining after cytospin onto glass slides. Insets show magnification of selected areas. Bar, 10 µm. (F) Macroscopic view of the tails of CD64<sup>dtr</sup> mice that were tattooed with green tattoo ink paste and either left untreated (-DT) or treated
were analyzed for the percentages of SSC-Alow and SSC-Ahigh cells. The SSC-Ahigh CD64+ cells found in the back of the grafted CD45.2 albino mice were further from the back of untreated CD45.2 albino mice or from the back of CD45.2 albino mice previously grafted with green-tattooed tail skin from CD45.1 B6 animals.

Alexa Fluor 700–conjugated rat IgG2b κ isotype control (R35-38); anti-CD43 (S7); Alexa Fluor 700–conjugated anti-CD45.1 (A20); anti-CCR2 (475301) were from R&D. BV711-conjugated anti-CD11b (M1/70), FITC-conjugated anti-CD11b (M1/70), FITC-conjugated rat IgG2b κ isotype control (RTK2758), APC-conjugated anti-CD11c (N418), APC-conjugated Armenian hamster IgG isotype control (HTK888), APC-conjugated anti-CD5 (53–73), APC-conjugated anti-CD19 (6D5), APC-Cy7–conjugated anti-CD11b (M1/70), FITC-conjugated anti-CD11b (M1/70), FITC-conjugated rat IgG2b κ isotype control (MRG25-8T), BV711-conjugated anti-CD64 (X54-5/7.1), BV711-conjugated mouse IgG1 κ isotype control (MOPC-21), and BV510- and Alexa-700–conjugated anti-MHC Class II (IA/I-E; M5/I14.15.2) were from Biolegend. Pe-Cy5.5–conjugated anti-CD45 (50–1F1) and Pe-Cy7–conjugated anti-CD11b (AFS98) were from eBioscience. Biotin-conjugated antibodies were detected by using streptavidin conjugated with PE-CFS94 or Pe-Cy7 (BD PharMingen).

Flow cytometry
Cells were stained and acquired on a FACS LSRII system and analyzed with FlowJo software (Tree Star). Neutrophils and B, T, and NK cells were systematically gated out by using a Lin+ gate (Fig. S1 and Fig. 2).

Cell sorting
The different cell types were sorted on a FACS Aria II according to the marker combination specified on Fig. S1 A and Fig. 3. For microarrays (Affymetrix 1.0ST), cells were sorted directly into lysis buffer provided from the Qiagen RNAeasy PLUS extraction kit.

Cell morphology
Sorted cells were spun onto Shandon Cytoslides with a Thermo Shandon Cytospin 4 cytofuge (4 min at 400 g), fixed with methanol and stained with hematoxylin and eosin.

Electron microscopy
Sorted cells were incubated at 37°C on poly-Lysine–coated cover slides. After 30 min, attached cells were fixed by using 2% glutaraldehyde in 0.1 M cacodylate buffer, pH 7 4 for 1 h, postfixed for 1 h with 2% buffered osmium tetroxide, dehydrated in a graded series of ethanol solution, and then embedded in epoxy resin. Images were acquired with a digital camera Quemesa (SIS) mounted on a Tecnai Spirit transmission electron microscope (FEI Company) operated at 80 kV.

with DT and analyzed 2 (DT + d2) and 90 d (DT + d90) after DT treatment. Data are representative of at least three experiments. (C) CD11b–CD64–CCR2– cells from the back of untreated CD45.2 albino mice or from the back of CD45.2 albino mice previously grafted with green-tattooed tail skin from CD45.1 B6 animals were analyzed for the percentages of SSC-Ahigh and SSC-Alow cells. The SSC-Ahigh CD64+ cells found in the back of the grafted CD45.2 albino mice were further analyzed for the percentages of CD45.1 (donor) and CD45.2 (host) expression. Data are representative of two to three independent experiments involving two to four animals per group.
Confocal microscopy
Trunk and ear skin pieces were fixed in Antigen Fix (Microm Microtech) for 2 h, washed in 0.1 M phosphate buffer, and dehydrated overnight in 30% sucrose in 0.1M phosphate buffer. Tissues were snap frozen, and 25-µm sections were stained with DAPI. To obtain an en face section of ear dermis, the dorsal part was split from the ventral one 2 h after intradermal ear injection of CD64-APC (2 µg), fixed in Antigen Fix for 2 h, washed in phosphate buffer, stained with DAPI, and clarified with Rapiclear 1.47 (SunJin Lab). Immunofluorescence confocal microscopy was performed by using a Zeiss LSM 880 confocal microscope. Separate images were collected for each fluorochrome and merged to obtain a multicolor image. Final image processing was performed with Imaris software (Bitplane) and Adobe Photoshop.

RNA isolation and microarray analysis
Transcriptomic and bioinformatics analyses were performed as previously described (Tamoutounour et al., 2013). To perform a PCA focused on all variability across the four biological samples examined (Fig. 7 A), we selected all ProbeSets showing a fold change ≥1.2 between at least one pair of conditions, taking into account only those ProbeSets nonambiguously annotated.
to one gene only and further keeping only the highest ProbeSet for each gene. This led to selection of 1,054 unique genes. To select the genes showing a significant differential expression in one type of condition compared with the other three, between MHCI+ versus MHCI− cells, or between melanophages and macrophages (Fig. 7 B), we used the GeneSign module of the BubbleGUM software using the metrics Minimal pairwise [Mean(test)/Mean(ref)] with a linear fold change threshold of 1.5 and a P value threshold of 0.05. The microarray data have been deposited in the GEO database under accession number GSE103339.

**Generation of parabiotic mice**

Parabiotic mice were generated by using 8–9-wk-old male mice as described previously (Tamoutounour et al., 2013). For WT-WT parabiosis, CD45.1 B6 mice were sutured together with CD45.2 B6 mice, whereas for WT-Ccr2−/− parabiosis, WT CD45.1 B6 mice were sutured with Ccr2−/− CD45.2 B6 mice and subsequently treated with Bactrim for 8 wk before analysis.

**In vivo depletion of CD64+ cells**

CD64dr mice were injected intraperitoneally twice and 24 h apart with 1 µg DT (Calbiochem, EMD Millipore) in PBS. CD64dr mice injected with PBS showed no ablation of monocyte-derived cells and macrophages.

**Tumor model**

B6-albino mice were injected subcutaneously with 105 amelanotic (BrafV600E) or melanocytic (B16) melanoma cells (Brown et al., 2001; Zelenay et al., 2015).

**Tattooing**

Tattooing was performed on the tail skin of mice under general anesthesia. Tail skin was tattooed by using the Animal Tattoo Ink Paste (Ketchum Mfg. Co, Inc.) and a 25-gauge needle. The ink-coated needle permits ink delivery in a topical manner. Before analysis, mice were kept for at least 3 wk until complete healing of the treated skin.

**Skin grafting**

CD45.1 B6 mice were tattooed as described above. After 5 wk, the tattooed skin was explanted and grafted on the back of CD45.2 albino mice under general anesthesia. The back skin of the host was analyzed 6 wk after the graft.

**Statistical analyses**

The unpaired Student t test was used for statistical analyses with GraphPad Prism software.

**Online supplemental material**

Fig. S1 shows the gating strategy used to identify CD11b+ cell subsets within the skin of WT and CD64dr mice. Fig. S2 shows the ablation of CD64+ cells present in the lamina propria of the large intestine upon DT treatment of CD64dr mice. Fig. S3 shows the replenishment kinetics of the monocyte precursors and monocytes found in the BM after DT treatment of CD64dr mice. Fig. S4 shows the lack of major impact of DT treatment on cDCs found in the skin of CD64dr mice. Table S1, a separate Excel file, shows the exhaustive lists of genes responsible for the IPA enrichments shown in Fig. 7 B.

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