 See related commentary on pg 1185

Skin-Depigmenting Agent Monobenzone Induces Potent T-Cell Autoimmunity toward Pigmented Cells by Tyrosinase Haptenation and Melanosome Autophagy

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In this study, we report the previously unknown mechanism of inducing robust anti-melanoma immunity by the vitiligo-inducing compound monobenzone. We show monobenzone to increase melanocyte and melanoma cell immunogenicity by forming quinone-haptens to the tyrosinase protein and by inducing the release of tyrosinase- and melanoma antigen recognized by T cells-1 (MART-1)-containing CD63+ exosomes following melanosome oxidative stress induction. Monobenzone further augments the processing and shedding of melanocyte-differentiation antigens by inducing melanosome autophagy and enhanced tyrosinase ubiquitination, ultimately activating dendritic cells, which induced cytotoxic human melanoma-reactive T cells. These T cells effectively eradicate melanoma *in vivo*, as we have reported previously. Monobenzone thereby represents a promising and readily applicable compound for immunotherapy in melanoma patients.

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INTRODUCTION

In 1939, Oliver *et al.* discovered that monobenzone is a powerful inducer of leukoderma (Oliver *et al.*, 1939). Clinical monobenzone studies report skin sensitization (Lyon and Beck, 1998) and spreading of depigmentation beyond the skin application site, suggesting a systemic reaction (Mosher *et al.*, 1977). In healthy people who apply monobenzone to acquire a lightened skin tone, it can induce depigmentation that is clinically and histologically indistinguishable from vitiligo (Grojean *et al.*, 1982). Importantly, vitiligo patients using monobenzone to achieve complete depigmentation often experience an application-related dermatitis exclusively in pigmented areas of skin (Nordlund *et al.*, 1985), indicating its dependence on melanocytes. Nevertheless, the working mechanism of monobenzone was unknown. Studies on the structurally related, less potent skin-depigmenting agent 4-methoxyphenol show that this compound is converted into a reactive benzoquinone product by the enzyme tyrosinase (Nair *et al.*, 1993) present in the melanosomes of skin melanocytes. Benzoquinone forms haptens to cysteine residues in proteins (Naish *et al.*, 1988), and was thereby identified as a skin sensitizer (Nazih *et al.*, 1993). Furthermore, animal studies demonstrated that the depigmenting action of phenols or catechols largely depends on their conversion by tyrosinase, and the subsequent formation of benzoquinone-haptens to proteins (Menter *et al.*, 1993). Indeed, the resulting quinone metabolites induced more profound depigmentation than their parental compounds (Tayama and Takahama, 2002).

Vitiligo is mediated by the progressive autoimmune destruction of skin melanocytes by CD8+ T cells (van den Boorn *et al.*, 2009). Furthermore, we have reported previously that by initiating specific autoimmunity against pigmented cells, monobenzone-based immunotherapy effectively inhibits the growth of murine melanoma *in vivo* (van den Boorn *et al.*, 2010). This discovery has provided pivotal *in vivo* data for us to embark on the present study, in which we investigated the cellular mechanisms by which monobenzone induces the systemic CD8+ T-cell response that specifically kills pigmented cells, following its selective

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Abbreviations: AP, autophagosome; CFSE, carboxyfluorescein succinimidyl ester; DC, dendritic cell; MHC, major histocompatibility complex; ROS, reactive oxygen species

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interaction with the enzyme tyrosinase in exposed pigmented cells. Our present study shows that monobenzone confers potent immunogenicity to exposed pigmented cells via the generation of quinone-haptens, melanosome autophagy, and the secretion of melanocyte antigen-containing exosomes in response to monobenzone-induced reactive oxygen species (ROS). We also found these processes to activate dendritic cells (DCs), which prime robust T-cell immunity against melanoma cells.

RESULTS

Tyrosinase is inactivated by monobenzone through quinone-hapten formation

Being an alternative substrate, monobenzone inactivates the pigment-synthesis enzyme tyrosinase (Naish *et al.*, 1988). To investigate this process, we performed spectrophotometry studies with mushroom tyrosinase. Figure 1a shows the conversion of its natural substrate L-3,4-dihydroxyphenylalanine (L-DOPA) into dopachrome (Mason, 1948), with the autoconversion of dopachrome into 5,6-dihydroxyindole (Aroca *et al.*, 1991). The interaction between tyrosinase and monobenzone revealed the formation of covalent quinone–cysteine bonds (quinone-haptens) (Hay *et al.*, 2004) and the reduction of the tyrosinase enzyme (Martin *et al.*, 1981). This suggests that quinone-haptens formed by the monobenzone metabolite 4-benzoxy-1,2-benzoquinon (Figure 1b) reduce and inactivate the enzyme. Incubation of tyrosinase with a higher concentration of monobenzone completely inactivated the enzyme, as no dopachrome was produced in response to L-3,4-dihydroxyphenylalanine addition at the plateau phase of the reaction. Importantly, this excludes substrate competition between monobenzone and L-DOPA. To test whether the monobenzone metabolite 4-benzoxy-1,2-benzoquinon indeed binds cysteine residues within peptides, we incubated various 9-mer peptides containing 0, 1, or 2 cysteine residues with synthetically prepared 4-benzoxy-1,2-benzoquinone. Peptides containing cysteine were incubated with a 2-fold excess of the metabolite. As a control, a peptide containing no cysteine residues was incubated with an 8-fold quinone excess. The liquid chromatography/mass spectrometry results are summarized in Supplementary Table S1 online. The peptides containing cysteines both formed quinone-haptens, whereas the control peptide did not display any quinone haptenation. Collectively, these data reveal that the enzyme tyrosinase is inactivated by monobenzone, likely by the formation of quinone-haptens formed by the 4-benzoxy-1,2-benzoquinon metabolite.

Cellular pigment synthesis is suppressed by monobenzone

To investigate the effect of monobenzone on melanogenesis, we incubated human cutaneous melanoma cells and normal human melanocytes with various concentrations of monobenzone for 72 hours *in vitro*. Importantly, equal numbers of viable cells were used for each experiment, to exclude effects of divergent cell numbers on melanin determinations. Melanin synthesis, induced in melanoma cells by isobutylmethylxanthine and melanocyte-stimulating hormone (α-MSH, Figure 1c), was significantly decreased by monobenzone exposure in a dose-dependent manner. Similarly, melanogenesis was suppressed by monobenzone in normal human melanocytes, which appeared more sensitive to inhibition.

On the basis of the hypothesis that high concentrations of monobenzone can be selectively toxic to tyrosinase-expressing pigment cells (Hariharan *et al.*, 2009), we tested the selective toxicity of monobenzone to pigmented cells as compared with non-pigmented cells. The sensitivity to monobenzone varied between cell lines but did not correlate to the presence of pigmentation (Figure 1d). Although tyrosinase was reexpressed in the amelanotic tyrosinase-negative melanoma cell line BLM (BLM-tyr), sensitivity to monobenzone was not affected. Control experiments using H_2O_2 showed a pigmentation-dependent resistance to oxidative stress, as expected by the antioxidant effect of melanin (Brenner and Hearing, 2008; Figure 1d). These data show that the cytotoxicity of monobenzone is independent of the pigmentation level or the presence of the tyrosinase enzyme.

Monobenzone induces melanosome degradation and autophagy

We studied the effects of monobenzone on melanosome formation by electron microscopy, in analogy to the reported disruption of melanosomes by the depigmenting compound hydroquinone (Jimbow *et al.*, 1974). Untreated melanoma cells contained all four melanosome developmental stages (Fitzpatrick *et al.*, 1969) (Figure 2a), with cigar-like morphology and the typical striations of stage II and III melanosomes. Exposing the melanoma cells to monobenzone for 60 minutes reduced the number of stage IV melanosomes in the cytoplasm (Figure 2b). Closer examination revealed that the melanosomes, especially stages II and III, were smaller and appeared disorganized or ruptured (arrows). Prolonging monobenzone exposure to 3 hours induced distinct cytoplasmic changes (Figure 2c). The cells no longer contained stage IV melanosomes, and the stage III melanosomes were unmelanized, indicating that monobenzone impeded melanogenesis. Although overall cell morphology appeared normal, structural changes characteristic of melanosome macroautophagy were seen evidenced by electron-dense matter enclosed in a double-membrane structure of $\sim$500 nm diameter, the hallmark of the autophagosome (Mizushima *et al.*, 2010; AP; Figure 2c, arrowheads for double membrane). Moreover, APs fused with electron-lucent lysosome-like vesicles (L; arrows for fusion sites), forming characteristic single-membrane walled autolysosomes (Mizushima, 2007). These results show that monobenzone disturbs melanogenesis, and mediates the disruption and subsequent autophagy of melanosomes, allowing enhanced degradation of melanosomal proteins for antigen processing (Dengjel *et al.*, 2005).

Melanosome autophagy targets tyrosinase to major histocompatibility complex class II compartments via lysosomal degradation involving tyrosinase ubiquitination

Autophagy typically involves fusion of APs with lysosomes, excludes endosome participation (Mizushima, 2007), and

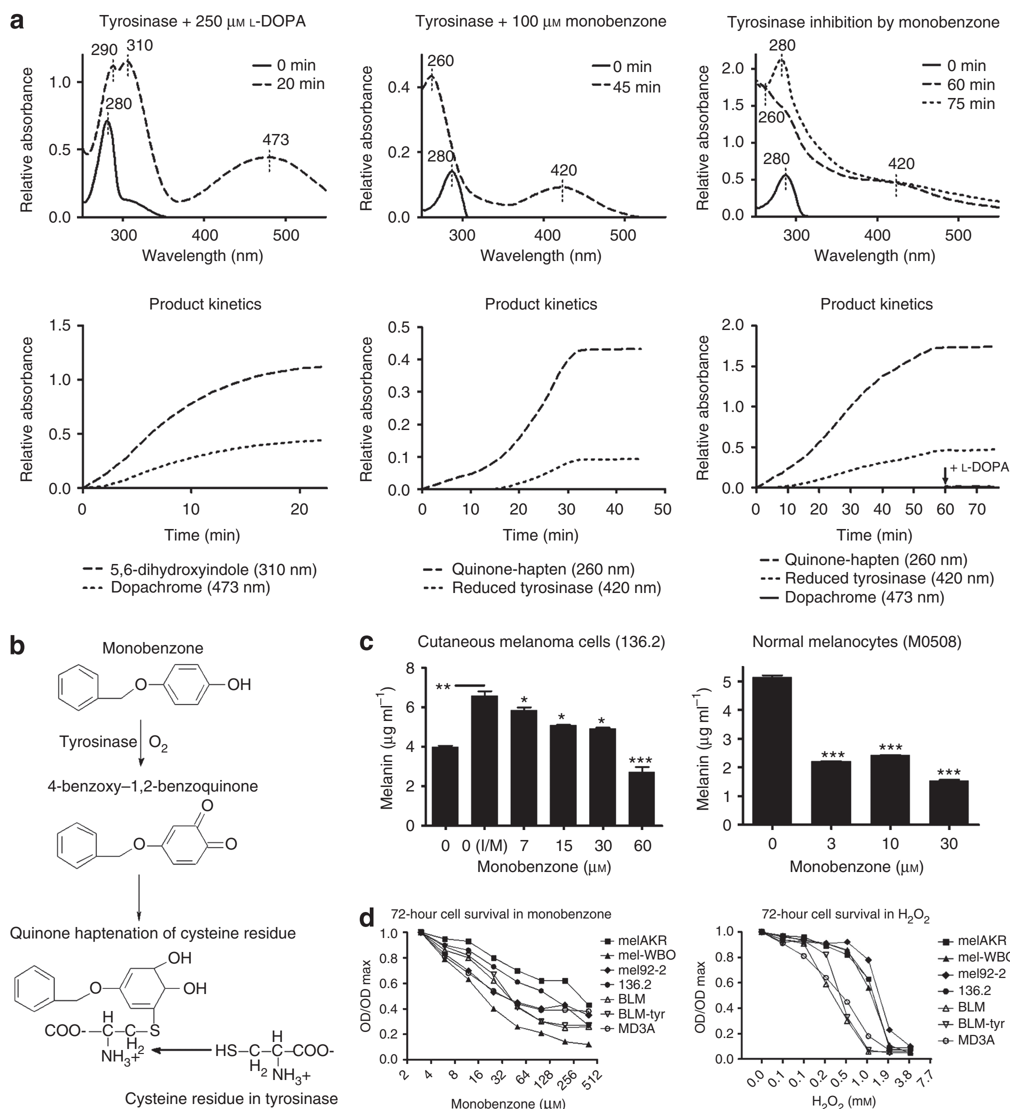


Figure 1. Quinone haptenation reduced melanogenesis, but not selective toxicity to pigmented cells by monobenzone. (**a**) Spectrophotometry showing the reaction of L-3,4-dihydroxyphenylalanine (L-DOPA) or monobenzone with mushroom tyrosinase. Reaction kinetics are depicted for each experiment. Left panels: tyrosinase converted L-DOPA (250 μM) into dopachrome and 5,6-dihydroxyindole (DHI): 473- and 310/290 nm absorption peaks, respectively. Middle panels: covalent quinone–cysteine bonds were formed (260 nm peak) and tyrosinase was reduced (420 nm) on reacting with monobenzone. Right panels: tyrosinase was inactivated by 300 μM monobenzone. No dopachrome or DHI formation was observed when 250 μM L-DOPA was added at $t=60$ minutes. Data represent the results of five independent experiments. (**b**) Schematic overview of monobenzone conversion into 4-benzoxy-1,2-benzoquinon by tyrosinase. The 4-benzoxy-1,2-benzoquinon can bind covalently to cysteine residues by nucleophilic attack of sulfhydryl groups. (**c**) Melanoma cells (136.2; left), pigment synthesis stimulated conditions by isobutylmethylxanthine and melanocyte-stimulating hormone (I/M), or melanocytes (M0508; right) were incubated with increasing concentrations of monobenzone. Graphs show a dose-dependent decrease of cellular melanin content. Both panels are representative of two independent experiments. $^{*}P<0.05$, $^{**}P<0.01$, and $^{***}P<0.0001$ using paired *t*-test (95% confidence interval). Graphs depict mean with SEM. (**d**) Left panel: pigmented (black symbols) or amelanotic (open symbols) human melanoma cells were incubated for 72 hours with monobenzone. Pigmented and amelanotic melanoma cells showed variable survival in the presence of monobenzone, not correlating to cellular pigmentation. Expression of tyrosinase enzyme in the amelanotic melanoma cell line BLM (BLM-tyr) did not affect the survival of this cell line. Right panel: the same cell panel was incubated for 72 hours in the presence of H_2O_2, showing the protective effect of pigmentation on cell survival under oxidative stress.

delivers antigen to major histocompatibility complex (MHC) class II compartments (Schmid and Munz, 2007). We identified the organelles with which the monobenzone-induced AP fuses by confocal laser scanning microscopy. In this study, we used the tyrosinase-specific T311-clone monoclonal antibody (Chen *et al.*, 1995) to detect melanosomes, which specifically recognizes an immunodominant linear tyrosinase determinant (tyrosinase$_{233-247}$) that can be tracked beyond the protein degradation stage (Willers *et al.*, 2005).

Melanoma cells, but not MCF7 control cells (not shown), selectively initiated a lysosomal degradation response on 1 hour of monobenzone exposure (Figure 3). Lysosome-associated membrane protein-1 (LAMP1) expression was

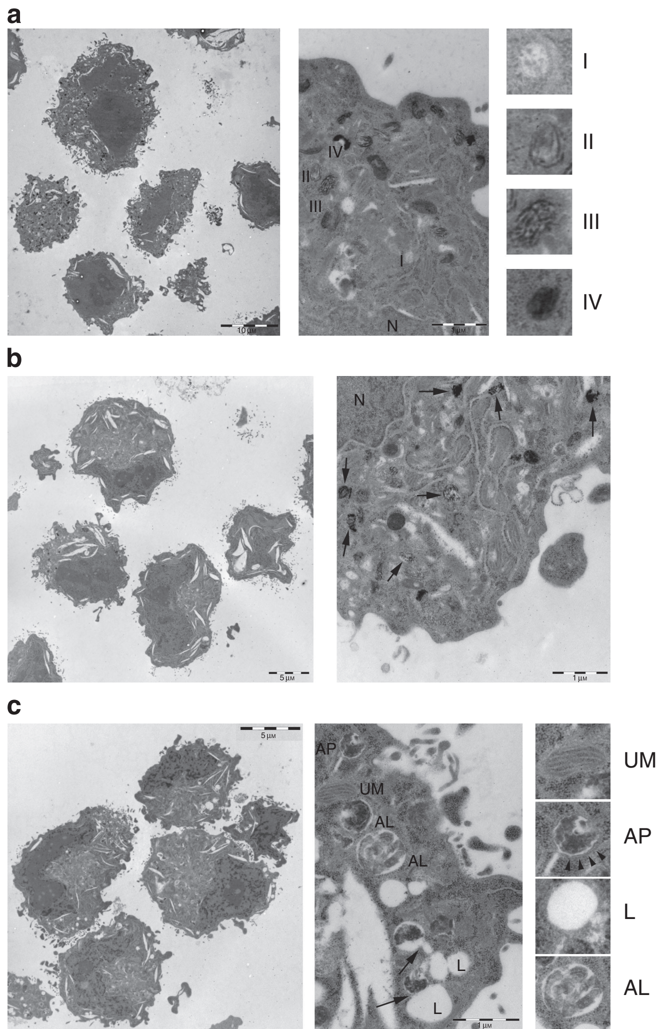


Figure 2. Monobenzone induces melanosome autophagy. Electron microscopy analysis of pigmented human melanoma cells (92.2) exposed to 20 μM monobenzone for 3 hours. (**a**) Unexposed melanoma cells contain all normal melanosome developmental stages, with typical cigar-like morphology and the striations of stage II and III melanosomes. Scale bars indicate 10 and 1 μm. (**b**) On 60 minutes of monobenzone exposure, melanoma cells contained fewer stage IV melanosomes. Stage II and III melanosomes appeared disorganized (arrows). Scale bars indicate 5 and 1 μm. (**c**) Melanoma cells no longer contained stage IV melanosomes on 3 hours of monobenzone exposure. Unmelanized stage III melanosomes (UM) were visible. Double-membrane walled structures containing electron-dense material were found, typical for melanin-containing autophagosomes (APs; arrowheads for double membrane). APs fused with electron-lucent lysosome-like structures (L; arrows for fusion), forming single-membrane-walled autolysosomes (ALs). Data represent the results of two independent experiments on two human pigmented melanoma cell lines (92.2 and 136.2). Scale bars indicate 5 and 1 μm. N, nucleus.

upregulated (Figure 3b), as well as the number of HLA–DR-positive vesicles (Figure 3c), whereas early endosome antigen-1 expression was unaffected (Figure 3a). Moreover, tyrosinase colocalized with LAMP1-positive vesicles (arrowheads in magnification Figure 3b and c). Furthermore, HLA–DR expression reached a maximum at 3 hours of exposure, revealing enhanced colocalization between tyrosinase and HLA–DR-positive vesicles (arrowheads in magnification Figure 3c). These HLA–DR/tyrosinase-positive vesicles were slightly larger organelles, suggestive of multivesicular bodies characteristic of late-stage APs processing antigen for MHC class II presentation (Schmid *et al.*, 2007).

As the vitiligo-inducing effect of monobenzone is mediated by topical exposure of the skin, we verified the effects of monobenzone in human skin melanocytes. As shown in Supplementary Figure S1A and S1B online, melanocytes also initiated a lysosomal response on 1 hour of monobenzone exposure, consisting of upregulation of LAMP1 and HLA–DR expression, colocalizing with cytoplasmic tyrosinase (arrows in magnification), whereas early endosome antigen-1 expression was unaffected.

As ubiquitination of protein aggregates can enhance autophagy (Kirkin *et al.*, 2009), besides providing antigen for the MHC class I presentation route, we investigated whether monobenzone induced ubiquitination of tyrosinase, in particular during prolonged monobenzone exposure. Unexposed melanoma cells analyzed by confocal laser scanning microscopy displayed low ubiquitin expression (Figure 4a). After 1 hour of monobenzone exposure, cellular ubiquitin upregulation and foci of tyrosinase-associated ubiquitination were observed (Figure 4a, arrows). Following 3 hours of monobenzone incubation, numerous small foci and larger complexes were identified showing colocalization of tyrosinase and ubiquitin (Figure 4a, arrowheads). Western blot analysis of these exposed melanoma cells indicated that monobenzone induced increased protein ubiquitination (Figure 4b). The predominance of heavy ubiquitinated proteins suggests polyubiquitination. Immunoprecipitation of tyrosinase confirmed that tyrosinase was subjected to (poly)ubiquitination following monobenzone exposure. Increased ubiquitination was absent in monobenzone-treated tyrosinase-negative MCF7 control cells (Figure 4b). Together, these results demonstrate that exposure of pigmented cells to monobenzone induces tyrosinase ubiquitination and the autophagocytic degradation of melanosomes by means of lysosomal uptake and subsequent fusion with HLA–DR-containing vesicles.

Monobenzone augments the release of tyrosinase- and melanoma antigen recognized by T cells-1 (MART-1)-containing exosomes by pigmented cells

As monobenzone may induce oxidative stress in pigmented cells (Boissy and Manga, 2004), which can promote the release of exosomes (Soderberg *et al.*, 2007), we tested the generation of ROS in a panel of monobenzone-exposed pigmented cells and the tyrosinase-negative control MCF7 cell line. Monobenzone induced ROS in exposed melanoma cells and melanocytes, whereas MCF7 cells displayed background levels of ROS during exposure (Figure 5a). Importantly, the induced ROS were not cytotoxic as monobenzone was not selectively toxic to pigmented cells (Figure 1d).

Thereafter, we analyzed exosome release from pigmented cells exposed to monobenzone. Exosomes were isolated from the culture supernatant by ultracentrifugation

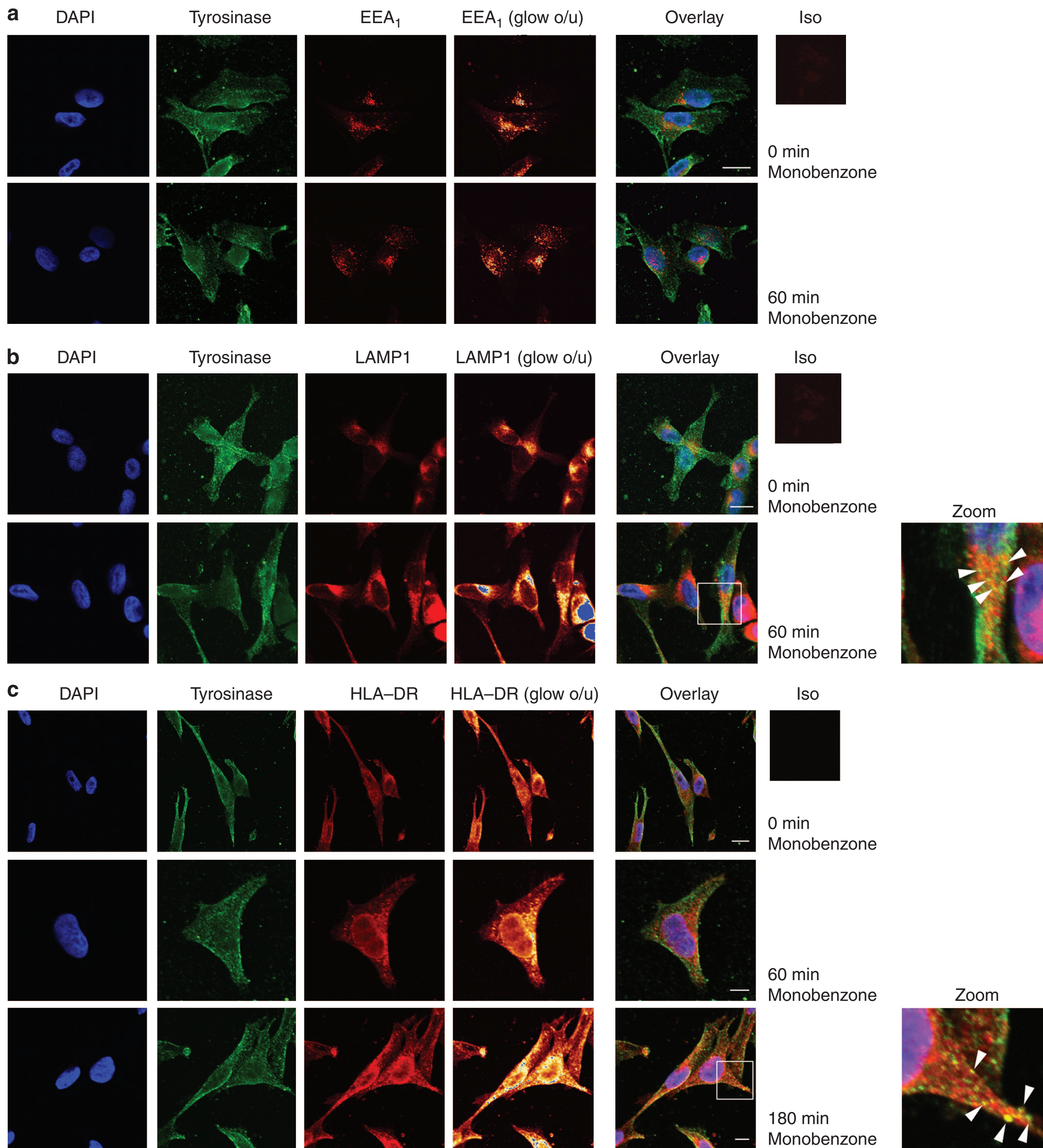


Figure 3. Lysosome-dependent melanosome degradation by monobenzone. Confocal laser scanning microscopy analysis of pigmented human melanoma cells (92.2) exposed to 20 μM monobenzone. Tyrosinase (green) for melanosomes and 4′,6-diamidino-2-phenylindole (DAPI) for nucleus (blue). Glow over/under panels (glow o/u) display fluorescence intensity of designated marker, indicating expression level from low (red/orange) to high (white/blue). Isotype mAb control incubations were negative (iso panels). (**a**) Early endosome antigen-1 (EEA_1; red) expression was not upregulated by 60 minutes of exposure, nor did tyrosinase colocalize with EEA_1. Scale bar = 20 μm for all panels. (**b**) Lysosome-associated membrane protein-1 expression (LAMP1; red) was upregulated after 60 minutes of exposure. Tyrosinase colocalized with lysosomes (arrowheads). Scale bar = 20 μm for all panels. (**c**) Major histocompatibility complex class II (HLA–DR; red) was upregulated after 60 minutes of exposure, reaching maximum expression after 180 minutes of exposure. Thereafter, tyrosinase colocalized to HLA–DR-containing vesicles (arrowheads). Scale bar = 20 μm for 0 minutes, and 8 μm for 60 and 180 minutes. The data represent the results of two independent experiments on two pigmented human melanoma cell lines (92.2 and 136.2).

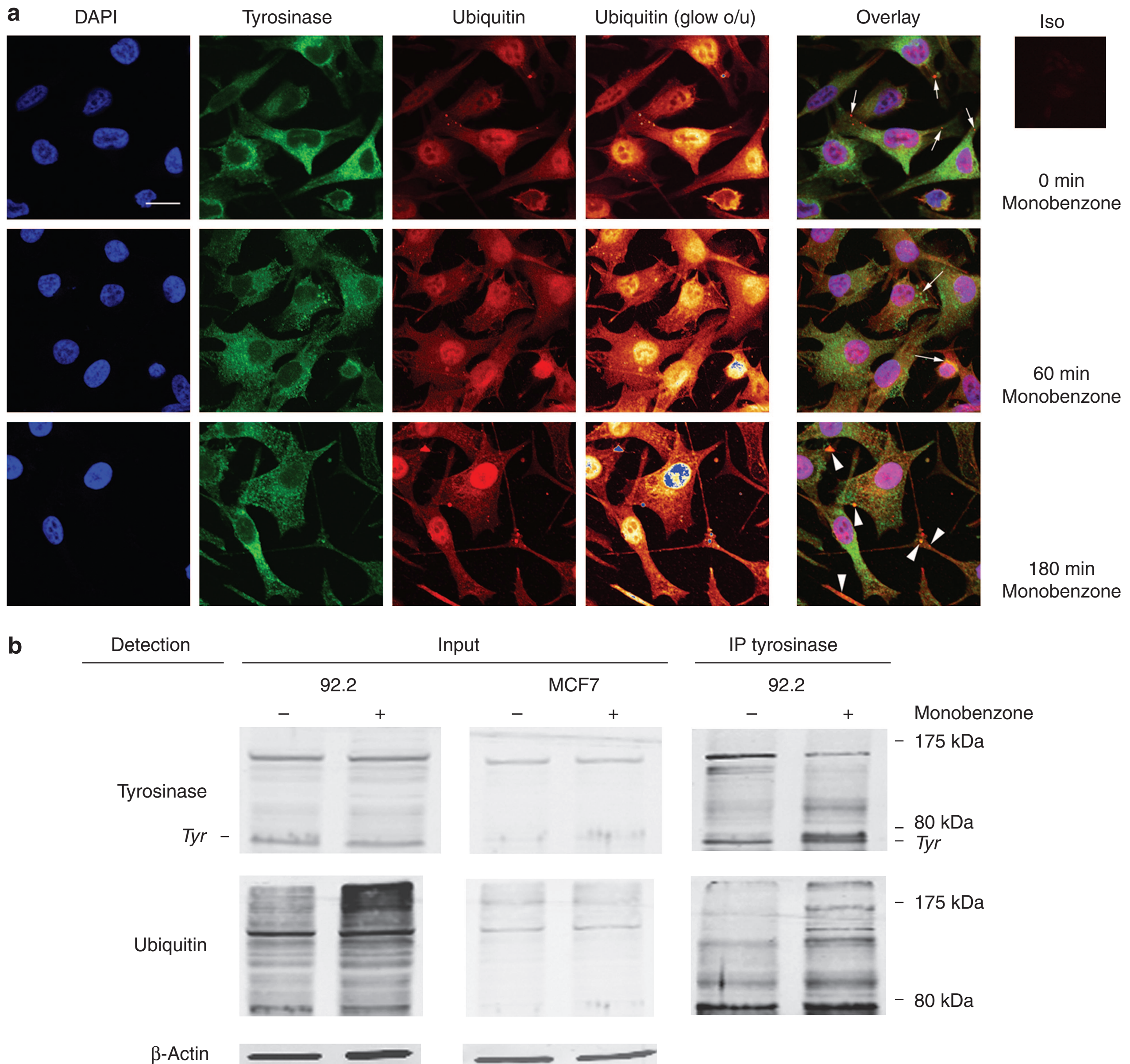


Figure 4. Enhanced tyrosinase ubiquitination following monobenzone exposure. (**a**) Confocal laser scanning microscopy analysis of pigmented human melanoma cells (92.2) exposed to 20 μM monobenzone. Staining with 4′,6-diamidino-2-phenylindole (DAPI) for nucleus (blue). Glow over/under panels (glow o/u) show the fluorescence intensity of ubiquitin, indicating its expression level from low (red/orange) to high (white/blue). Upper row: low ubiquitin (red) expression in untreated melanoma cells, only small ubiquitination complexes were present (arrows). Middle row: ubiquitin expression was upregulated on 60 minutes of incubation, foci of tyrosinase-associated ubiquitination (arrows) were found. Lower row: after 180 minutes of exposure, melanoma cells displayed large complexes and small foci of tyrosinase/ubiquitin colocalization (arrowheads). Scale bar = 16 μm for all panels. Isotype mAb control incubations were negative (iso panel). Data represent the results of two independent experiments in two different melanoma cell lines (92.2 and 136.2). (**b**) Western blot analysis of pigmented human melanoma cells (92.2) and tyrosinase-negative control cells (MCF7) exposed to 20 μM monobenzone for 2 hours. Analysis of whole-cell lysate (input) showed that melanoma cells express the tyrosinase protein (*Tyr*), whereas MCF7 cells do not. Monobenzone treatment did not alter tyrosinase expression level, but enhanced protein ubiquitination in the 92.2 cells. The predominance of heavy ubiquitinated proteins suggests polyubiquitination. Immunoprecipitation of tyrosinase (IP tyrosinase) showed altered tyrosinase weight distribution and (poly)ubiquitination following monobenzone exposure. Data represent the results of two independent experiments.

(Savina *et al.*, 2003) and identified by transmission electron microscopy (Figure 5b). We analyzed the exosomes by their adsorption on aldehyde/sulfate latex beads and flow cytometric detection of specific antigens (Mathivanan and Simpson, 2009). Figure 5c shows that melanocytes or melanoma cells release increasing number of exosomes when exposed to monobenzone over time. These exosomes carried the CD63 exosome marker and tyrosinase and MART-1 antigens. In contrast, MCF7 cells did not enhance exosome release following monobenzone exposure, nor did its exosomes contain

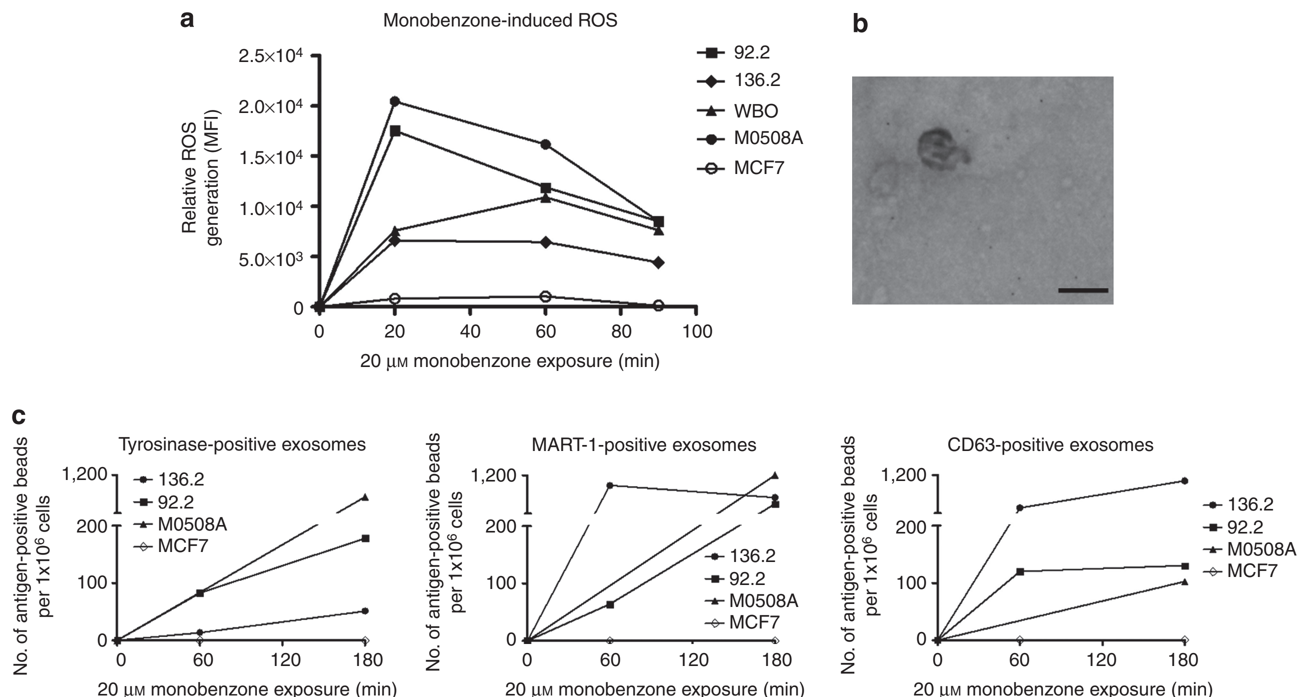


Figure 5. Monobenzone induces reactive oxygen species (ROS) and antigen-containing exosome release in pigmented cells. (**a**) Human melanocytes (M0508A), human melanoma cells (92.2, 136.2, and WBO), and tyrosinase-negative MCF7 control cells were incubated with 20 μM monobenzone for 90 minutes. During exposure, ROS were formed in the pigmented cells. MCF7 cells display background levels of ROS on exposure. Data are normalized to the ROS levels present in each cell line without monobenzone addition. (**b**) Transmission electron microscopy image of an exosome from the prepared culture supernatants of melanoma cells treated with 20 μM monobenzone for 3 hours (136.2 and 92.2). Data represent the results of two independent experiments. Scale bar = 100 nm. (**c**) Human melanocytes (M0508A), human melanoma cells (136.2 and 92.2), or tyrosinase-negative control cells (MCF7) were incubated with 20 μM monobenzone for 180 minutes. During exposure, increasing numbers of tyrosinase- and MART-1 antigen-containing exosomes were released by pigmented cells. In contrast, monobenzone did not induce exosome release from MCF7 cells. Released exosomes were found to express the exosome-specific marker CD63. Data are normalized to the exosome release levels of untreated cells for each cell line. MART-1, melanoma antigen recognized by T cells-1.

tyrosinase or MART-1 antigen (data not shown). These data demonstrate that monobenzone, by inducing cellular oxidative stress, augments the release of melanocyte-differentiation antigen-containing exosomes from exposed pigmented cells.

Monobenzone-exposed melanoma cells activate DCs

Next we investigated whether monobenzone-exposed melanoma cells induced DC activation. Supplementary Figure S2A online shows immature DCs to express low levels of the activation markers CD86 and HLA–DR. Expression of the CD14 monocyte marker was negative (data not shown). DCs were incubated with polyinosinic:polycytidylic acid (poly I:C) to induce optimal DC maturation. Lysate of monobenzone-exposed melanoma cells induced DC activation comparable to untreated melanoma cell lysate. Interestingly, monobenzone-treated intact melanoma cells induced DC activation, whereas monobenzone-treated intact tyrosinase-negative Epstein-Barr virus (EBV)-B cells did not. This demonstrates the dependence of the observed DC activation on the interaction of monobenzone with tyrosinase. Importantly, monobenzone did not disrupt melanoma cells during overnight incubation (data not shown). Control incubations of DCs with monobenzone, melanoma, or EBV-B cells alone did not trigger DC activation (data not shown).

To analyze the nature of this DC activation, we compared the uptake of cell-derived matter from monobenzone-treated and -untreated melanoma cells by DCs, using carboxyfluorescein succinimidyl ester (CFSE)-labeled melanoma cells. CFSE fluorescently labels intracellular proteins covalently (Lyons and Parish, 1994) and does not diffuse out of cells. CFSE-labeled, thoroughly washed, melanoma cells were cocultured with immature DCs for 3 hours. Subsequently, uptake of CFSE-labeled matter by DCs was analyzed by confocal laser scanning microscopy. As Supplementary Figure S2B online shows, control incubations with DCs alone showed CFSE-negative DCs. DCs cocultured with intact CFSE-labeled melanoma cells did not ingest any matter from these cells. In contrast, monobenzone treatment of intact melanoma cells led to the uptake of CFSE-positive matter by DCs. DCs had also taken up CFSE-labeled debris from lysed melanoma cells, either monobenzone treated or unexposed. These results show that monobenzone exposure of melanoma cells triggers the release of intracellular-derived antigenic matter, likely by the monobenzone-induced exosomes, which is readily taken up by DCs.

Monobenzone-exposed pigmented cells induce melanoma-reactive CD8+ T cells within 7 days *in vitro*

On the basis of the observation that monobenzone induces leukoderma, which can spread to non-exposed sites, we

investigated the nature of this systemic reaction. In particular, we studied whether monobenzone can induce CD8+ T-cell reactivity to pigmented cells. Therefore, we performed a series of cross-presentation experiments using monocyte-derived DCs and autologous T cells from nine healthy human donors. The DCs were loaded with either a freeze–thaw melanoma cell lysate or with melanoma cells previously exposed to monobenzone. Importantly, no DC-activating stimuli were added to the cultures. The DCs were confirmed to be immature before coculture (Supplementary Figure S2A online upper row). Melanoma cell lysate is well suited to prime tumor-antigen-specific T cells by DCs (Kotera *et al*., 2001), and thereby functions as a reference stimulation. Furthermore, it provides a representative source of melanoma-specific antigen to the DCs, as procedures such as UV- or γ-irradiation are known to significantly alter autoantigen expression, processing, and surface presentation (Reits *et al*., 2006).

Figure 6a depicts representative healthy donor DC–T-cell stimulations. The columns designated "*ex vivo*" show that CD8+ T cells from healthy donors did not *ex vivo* recognize autologous DCs presenting melanoma cells exposed or unexposed to monobenzone (both either lysed or intact). On 7 days of coculture under these conditions and subsequent restimulation, a population of CD8+ T cells reactive to DCs cross-presenting monobenzone-exposed melanoma cell lysate had emerged, whereas DCs loaded with unexposed melanoma cell lysate (or intact cells) did not induce T-cell activation. Strikingly, profound T-cell activation was induced by DCs cross-presenting intact monobenzone-exposed melanoma cells, illustrating that monobenzone confers functional immunogenicity to intact pigmented cells. Control stimulations neither revealed T-cell reactivity against unloaded DCs, nor direct T-cell recognition of intact melanoma cells (data not shown). Monobenzone-induced T-cell activation was found in eight additional healthy human donors as shown in Figure 6b. Here, significant activation of CD8+ T cells reactive to 20 μM monobenzone-exposed intact melanoma cells was induced by day 7. Moreover, melanoma cells exposed to 40 μM monobenzone induced even more CD8+ T-cell activation. Further increasing monobenzone exposure of melanoma cells did not enhance T-cell reactivity (data not shown). In contrast, no T cells reactive to untreated melanoma cells were found, indicating that monobenzone increased the immunogenicity of the melanoma cells.

To exclude melanoma cell alloreactivity induced by monobenzone, we performed cross-presentation experiments using the melanoma cell line mel-WBO and the autologous EBV-transformed B-cell line EBV-WBO. The CD8+ T cells activated by monobenzone-exposed mel-WBO during the 7-day coculture did not respond to autologous EBV-WBO cells. This shows that monobenzone-exposed melanoma cells did not induce T-cell alloreactivity but selectively activated melanoma-reactive T cells. Figure 6c shows that EBV-WBO lysate did not induce a T-cell response, not even when these cells were exposed to monobenzone before coculture. Thereby monobenzone did not increase the immunogenicity of these unpigmented cells.

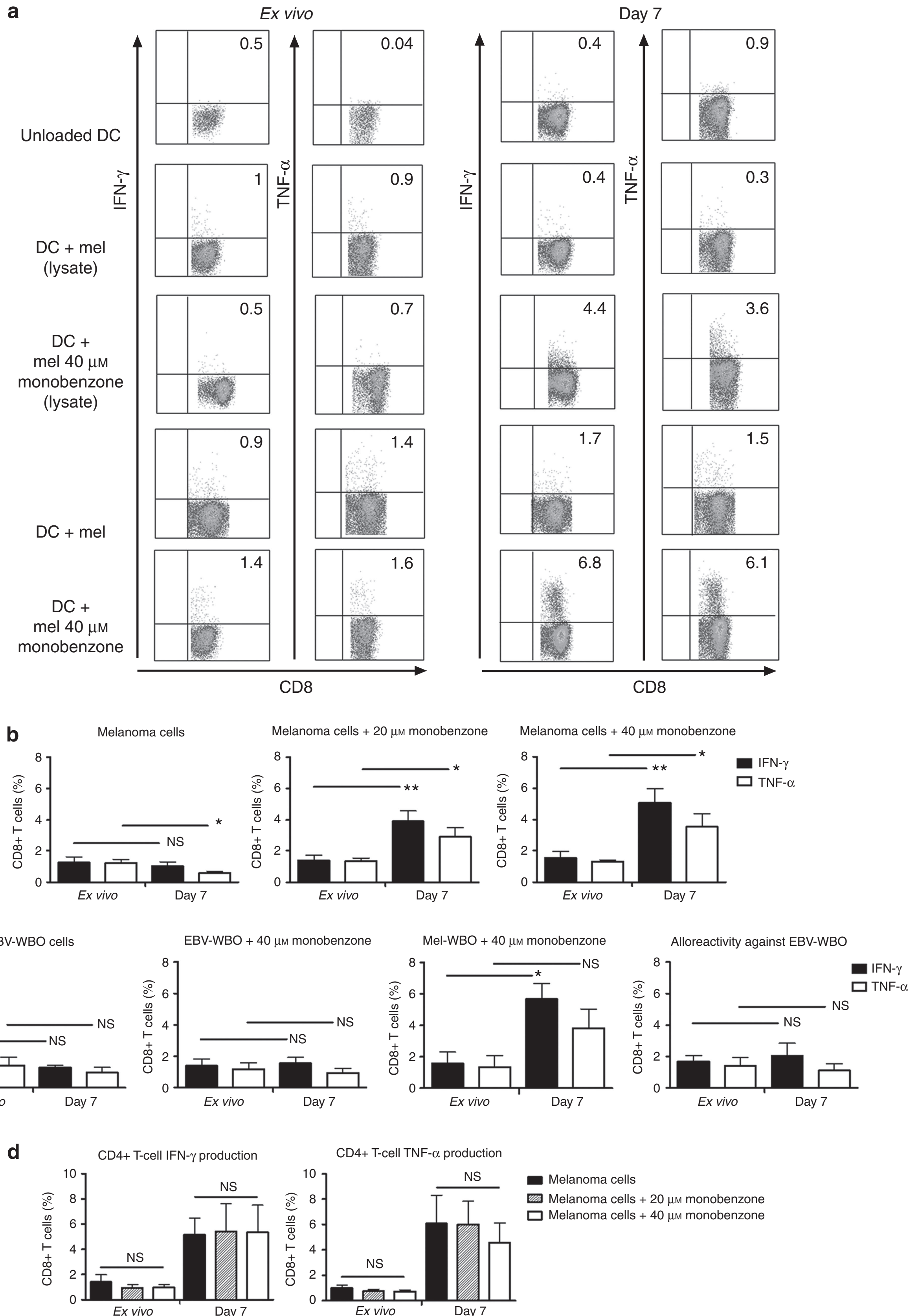
As shown in Figure 6d, CD4+ T-cell activation was found in all stimulation conditions of monobenzone-exposed and -unexposed melanoma cells to equal degrees. These results demonstrate that the monobenzone exposure of the melanoma cells selectively affected the generation of melanoma-reactive CD8+ T cells. Taken together, these results demonstrate that the interaction of monobenzone with tyrosinase elevated the immunogenicity of melanoma cells, resulting in the effective induction of a melanoma cell-reactive CD8+ T-cell response.

Monobenzone-induced melanoma-reactive CD8+ T cells also recognize unexposed melanoma cells

As monobenzone-induced vitiligo progressively spreads to unexposed skin sites, we tested whether CD8+ T cells induced by the monobenzone-exposed melanoma cells could recognize both monobenzone-exposed and -unexposed melanoma cells. We cloned the activated T cells by single-cell flow cytometry sorting of IFN-γ-producing T cells, following day 7 restimulation. CD8+ T-cell clones were established from five healthy donor cultures. Shown in Supplementary table S2 online, all T-cell clones recognized autologous DCs loaded with intact monobenzone-exposed melanoma cells. None of the T-cell clones recognized unloaded DCs, or DCs loaded with MCF7 control cells, indicating their melanoma-specific activation. Importantly, seven out of nine clones (78%) equally recognized autologous DCs loaded with either monobenzone-exposed or -unexposed melanoma cells. Interestingly, two out of nine T-cell clones were less reactive with monobenzone-untreated melanoma cells (no. 3 and 9), one being unresponsive to unexposed melanoma cells (no. 9). Possibly, these two clones were reactive with an epitope containing a quinone-hapten, an epitope absent from unexposed cells. Importantly, these data provide immunological evidence for the clinical observation that monobenzone-induced vitiligo eventually spreads beyond the monobenzone application site to unexposed skin areas, and illustrates that monobenzone exposure of melanoma cells leads to the activation of a T-cell response against antigens present on both monobenzone-treated and -untreated melanoma cells.

DISCUSSION

The present study establishes how monobenzone induces distinct immunological effects, which each contributes to induce potent immunity specifically against pigmented cells. First, we have shown that monobenzone inactivated the tyrosinase pigment-synthesis enzyme, and that quinone-haptens were formed to its cysteine residues by the monobenzone metabolite 4-benzoxy-1,2-benzoquinone. Monobenzone exposure reduced pigment-cell melanogenesis, without exerting selective toxicity toward these cells. In addition, exclusively in pigmented cells, monobenzone induced ROS, the release of tyrosinase- and MART-1-containing exosomes, and melanosome autophagy. The autophagocytic response was found to target melanosomal tyrosinase protein via lysosomal degradation to MHC class II compartments. Moreover, ubiquitination of tyrosinase protein


a
Ex vivo
Day 7
Unloaded DC
DC + mel
(lysate)
DC +
mel 40 μM
monobenzone
(lysate)
DC + mel
DC +
mel 40 μM
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IFN-γ
TNF-α
CD8
0.5
0.04
0.4
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1
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4.4
3.6
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1.7
1.5
1.4
1.6
6.8
6.1
b
Melanoma cells
Melanoma cells + 20 μM monobenzone
Melanoma cells + 40 μM monobenzone
CD8+ T cells (%)
Ex vivo
Day 7
NS
IFN-γ
TNF-α
c
EBV-WBO cells
EBV-WBO + 40 μM monobenzone
Mel-WBO + 40 μM monobenzone
Alloreactivity against EBV-WBO
d
CD4+ T-cell IFN-γ production
CD4+ T-cell TNF-α production
Melanoma cells
Melanoma cells + 20 μM monobenzone
Melanoma cells + 40 μM monobenzone

was augmented upon monobenzone exposure. Strikingly, intact monobenzone-exposed melanoma cells activated DCs that had taken up matter from these cells. Subsequently, these DCs induced a robust melanoma-reactive CD8+ T-cell response within 7 days *in vitro* in nine healthy human donors. The potency of monobenzone to induce such immune responses was found to be pigment-cell-dependent and did not represent alloreactivity.

Although autophagy can precede imminent cell apoptosis under conditions of enduring stress (Hotchkiss *et al*., 2009), the monobenzone-induced ROS was not toxic to the pigmented cells. Autophagy has a clear relationship to the generation of antigen for presentation in MHC class I and class II molecules. MHC class II antigen-loading compartments regularly receive antigen input from APs (Schmid *et al*., 2007), while it also enhances endogenous antigen presentation in MHC class I (English *et al*., 2009). Importantly, the observed exosomes may also result from the autophagy process. Although macroautophagy degrades whole organelles, microautophagy engulfs small portions of cytoplasm, possibly containing proteins released from damaged organelles such as tyrosinase and MART-1 (Mizushima, 2007). This typically occurs via membrane invaginations of the multivesicular endosome (Thery *et al*., 2009), a late endosomal compartment, which thereby forms many cytosol-containing vesicles that can eventually be released as exosomes (Fevrier and Raposo, 2004). Collectively, monobenzone exposure of pigmented cells leads to the constant engagement of exposed cells in the degradation, processing, and presentation of melanosomal proteins. This enhanced melanosomal antigen presentation can result in the priming of autoreactive T cells, and provides the most likely explanation for the elevated immunogenicity of monobenzone-exposed pigmented cells. Interestingly, melanocytes are quite unique cells in the sense that they express MHC class II, similar to DCs, and display effective antigen-presenting capabilities (Le Poole *et al*., 1993). This implies that the exposed melanocyte by itself can initiate melanocyte antigen-specific T-cell immunity.

We also observed augmented tyrosinase (poly)ubiquitination in monobenzone-exposed cells, which suggests protein ubiquitination in damaged melanosomes. Besides targeting proteins to the MHC class I degradation route, ubiquitination has a distinct role in autophagy. Ubiquitination of peptide aggregates functions as a receptor for the initiation of macroautophagy (Kirkin *et al*., 2009). Furthermore, ubiquitinated transmembrane proteins, such as tyrosinase and MART-1 in the melanosome, are directed to the vesicles of the multivesicular endosome (Hicke, 2001). Thereby, active shuttling of these proteins into the exosomal pathway may be enhanced by monobenzone. Monobenzone-induced exosomes were found to contain tyrosinase and MART-1 antigen. The active release of cell-specific antigen-containing exosomes is a potent inducer of adaptive immunity (Zeelenberg *et al*., 2008). The tyrosinase protein in these exosomes will carry quinone-haptens, whereas other proteins such as MART-1 or gp100 are likely to be present in their native form. The excretion of these antigens explains why monobenzone-exposed melanoma cells induce T cells to be reactive to both monobenzone-exposed and -unexposed melanoma cells.

Increased exosome secretion alone cannot account for the observed DC activation by monobenzone-exposed melanoma cells. The quinone-haptens we observed can partly explain this effect. In a recent study (Palm and Medzhitov, 2009), protein haptenation was found to overcome the need for Toll-like receptor signaling in inducing immunity against an otherwise non-immunogenic protein. Moreover, contact-sensitizing compounds generating haptens such as trinitro-chlorobenzene can activate DCs via triggering of the NALP3 inflammasome (Yazdi *et al*., 2007). Furthermore, ROS are known to induce adenosine triphosphate release from tumor cells (Efferth and Oesch, 2004), which is a potent metabolic danger stimulus activating the NALP3 inflammasome (Schroder *et al*., 2010). Thereby, it is possible that monobenzone-exposed pigmented cells activate DCs in two different ways: first, the generation of ROS may mediate adenosine triphosphate release from exposed cells, stimulating activation of the NALP3 inflammasome in local DCs. Second, DC uptake of quinone-haptenated tyrosinase-containing exosomes may augment DC activation via additional triggering of the NALP3 inflammasome.

In conclusion, we here show that conversion of monobenzone by the tyrosinase enzyme confers potent immunogenicity to pigmented cells by effectively integrating different cellular and immunological processes. This, to our knowledge previously unreported mechanism of monobenzone-induced autoimmune vitiligo, provides the effectual basis for

Figure 6. Melanoma cells exposed to monobenzone prime melanoma-specific cytotoxic T cells. (**a**) Left panels: *in vitro* overnight T-cell stimulation *ex vivo* with autologous dendritic cells (DCs) loaded with melanoma cells previously exposed overnight to 40 μm monobenzone or unexposed melanoma cells (both either lysed or intact). CD8+ T cells were not activated by these conditions. Right panels: after 7 days of incubation, cultures were restimulated. CD8+ T cells were reactive to DCs loaded with monobenzone-exposed melanoma cells (intact or as a lysate), whereas reactivity against DCs loaded with monobenzone-untreated melanoma cells was absent. Intact monobenzone-exposed melanoma cells induced the most profound T-cell activation. (**b**) CD8+ T-cell reactivity against 20 or 40 μm monobenzone-exposed intact melanoma cells was found in nine healthy human donors. Graphs show T-cell activation as IFN-γ and TNF-α production by CD8+ T cells. Monobenzone-exposed melanoma cells increased CD8+ T-cell activation after 7 days of stimulation, whereas monobenzone-unexposed melanoma cells did not. Data show the mean result of nine healthy donors. (**c**) The control Epstein-Barr virus (EBV)-transformed B-cell line EBV-WBO did not induce CD8+ T-cell reactivity within 7 days, similar to monobenzone-treated EBV-WBO. CD8+ T cells induced by the monobenzone-exposed melanoma cell line mel-WBO did not display alloreactivity to autologous EBV-WBO cells. Data show the mean result of four healthy donors. (**d**) CD4+ T-cell activation was comparable to DCs incubated with either monobenzone-exposed or -unexposed melanoma cells. Data represent the results of four independent experiments. NS, not significant ($P > 0.05$); *$P < 0.05$, **$P < 0.01$, using unpaired *t*-test (95% confidence interval) in **b** and **c**, and paired *t*-test in **d**. Graphs depict mean with SEM.

our successful and easily applicable monobenzone-imiquimod-CpG therapy for melanoma, which we previously developed in the B16-B6 model of murine melanoma (van den Boorn *et al.*, 2010). Taken together, monobenzone represents a potent and innovative way of inducing potent immunity against pigmented cells, with great promise to the field of cancer immunotherapy.

MATERIALS AND METHODS

Spectrophotometric studies

Mushroom tyrosinase (Sigma-Aldrich, Steinheim, Germany) was dissolved in 50 mM phosphate buffer (pH 7.1). A volume of $10\,U\,ml^{-1}$ tyrosinase was supplemented with either 250 μM L-DOPA (Fluka, Sigma-Aldrich) or 100 μM monobenzone (Sigma-Aldrich). For complete inactivation of tyrosinase, 300 μM monobenzone was used. Reactions were carried out in suprasil quartz cuvettes (Hellma, Aartselaar, Belgium) at room temperature and oxygenized before incubation by vortexing. At 2-minute intervals, the 250–550 nm absorption spectrum was determined by a spectrophotometer (V-560; Jasco, Great Dunmow, UK).

Peptide haptenation analysis

Synthesis of 4-benzoxy-1,2-benzoquinon: to an ice-cooled solution of KH_2PO_4 (50 mg) in water (25 ml) was added Fremy's salt (potassium peroxylamine disulfonate, 500 mg). The reaction mixture was extracted with chloroform (3×25 ml) and the collected organic layers were dried on $MgSO_4$. The solvent was evaporated *in vacuo* after which the residue was used directly. Reaction of 9-mer peptides with 4-benzoxy-1,2-benzoquinon: 5 mM of peptide solution in a 9:1 mixture of 50 mM phosphate-buffered saline, pH 7.2/acetonitrile, total volume 250 μl, was treated with (0–40 μl of a 0.25 M stock solution in acetonitrile) 4-benzoxy-1,2-benzoquinon. After 1 hour, the reaction was quenched with 30 μl trifluoroacetic acid and the mixture was analyzed by liquid chromatography–mass spectrometry (reverse phase high performance liquid chromatography, 10–90% gradient of acetonitrile). Quinone adduct was detected when MH^+ and MH_2^{2+} of the 4-benzoxy-1,2-benzoquinon-incubated peptides showed an increase of 214.2 or 107.1, respectively, as compared with starting material.

DC–T-cell autologous cocultures and restimulation

Peripheral blood mononuclear cells were obtained from blood donated by healthy human subjects. Blood donation followed approval by the ethical committee of the Academic Medical Center (Amsterdam, the Netherlands). The present study was conducted according to the Declaration of Helsinki Principles. DCs were generated as described previously (de Jong *et al.*, 2002). DCs were cultured in 48-well plates at 10^5 DC per well in 1 ml IMDM (Life Technologies, Carlsbad, CA) supplemented with GM-CSF ($500\,U\,ml^{-1}$, Schering-Plough, Kenilworth, NJ), and were cocultured with 2×10^5 melanoma cells (1:1 mixture of melanoma cell lines mel-WBO and mel-136.2, or mel-WBO alone) or EBV-WBO previously incubated overnight with 0, 20, or 40 μM monobenzone in 100 μl of IMDM per 2×10^5 cells at 37 °C 5% CO_2. When required, melanoma cells were lysed by $3\times$ freeze–thawing using liquid N_2 before DC loading. After 6 hours, autologous peripheral blood mononuclear cells were added to the DCs at 2×10^5 cells per well. peripheral blood mononuclear cells were previously cultured for 7 days in IMDM (PAA GmbH, Pasching, Austria) with 10% FBS (Hyclone, Cramlington, UK), supplemented with $15\,\mu g\,ml^{-1}$ gentamycin (Duchefa, Haarlem, the Netherlands), $20\,U\,ml^{-1}$ IL-2 (Novartis, Arnhem, the Netherlands), and $5\,ng\,ml^{-1}$ IL-15 (CLB/Sanquin, Amsterdam, the Netherlands). IL-2, IL-15, and gentamycin were added to the DC–T coculture. To test T-cell activation, Brefeldin-A was added (Golgiplug, 1:1000; BD Biosciences, San Diego, CA) during overnight incubation to allow determination of cytokine production by flow cytometry the following day. Alternatively, the coculture was cultured for 7 days, followed by an overnight restimulation and T-cell activation analysis. Unloaded DCs and phorbol 12-myristate 13-acetate/ionomycine-stimulated T cells were included as controls in the stimulations (Leukocyte activation cocktail, 1:500, BD Biosciences).

Exosome isolation and analysis

Exosomes were isolated as described before (Mears *et al.*, 2004), with the modification of filtering supernatants at 0.2 μm and centrifugation of filtered supernatants at $100\,000 \times g$. Flow cytometric analysis of exosomes was performed using aldehyde/sulfate latex, 4% (w/v) 4 μm beads (Invitrogen, Breda, The Netherlands), as described before (Logozzi *et al.*, 2009), using mAb specific for tyrosinase (clone T311, 1:200, Invitrogen), melanA (1:50, DAKO cytomation, Glostrup, Denmark), and CD63 (1:100, BD Pharmingen, San Diego, CA). mAb binding was detected by goat anti-mouse Alexa-488 for IgG2a-isotype primary mAb (1:400, Invitrogen), and goat anti-mouse Cy3 for IgG1-isotype primary mAb (1:100, Jackson immunoresearch, West Grove, PA). Exosome electron microscopy was performed as described previously (Merendino *et al.*, 2010).

Other assays

Cell culture, cellular melanin determination, cell survival assay, electron microscopy, confocal microscopy analysis, immunoprecipitation, flow cytometry, and T-cell cloning by single-cell sorting were carried out as described in the Supplementary Materials and Methods section online.

CONFLICT OF INTEREST

CJM is employed for 0.75 fte as CSO of ISA Pharmaceuticals and has stock appreciation rights in ISA. The other authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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