Imiquimod Inhibits Melanoma Development by Promoting pDC Cytotoxic Functions and Impeding Tumor Vascularization

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Imiquimod (IMQ) is a synthetic Toll-like receptor (TLR7/8) ligand that can trigger antiviral and antitumor activities. Despite evidence of potent therapeutic effects, the clinical use of IMQ in melanoma is impeded by incomplete understanding of its mechanisms of action. Mice and humans differ in many aspects of immunity, including TLR7 expression patterns, thus impeding the use of mouse models in translating discoveries into clinical applications. In this article, we investigated the mechanisms behind IMQ effects *in vivo* in a human context of melanoma and immunity using an innovative melanoma-bearing humanized mouse model. In this model, IMQ strongly inhibited melanoma tumor development through prompt mobilization of plasmacytoid dendritic cells and by triggering their cytotoxic functions, and through upregulation of expression of type 1 IFN response genes. IMQ also drastically impeded tumor vascularization by inducing the downregulation of angiogenic factors vascular endothelial growth factor, angiogenin, IL-8, and fibroblast growth factor. Our results revealed the short- and long-term multifactorial effects of IMQ converging toward inhibition of melanoma development. By providing a better understanding of the mechanisms of action of IMQ in melanoma, our study opens the way for its further clinical use in the treatment of metastatic melanoma.

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INTRODUCTION

Imiquimod (IMQ) is a small molecule of the imidazoquinoline family. It displays potent antiviral and antitumor activities through broad direct and indirect effects in the skin and immune system (Smits *et al.*, 2008). A cream formulation of 5% IMQ (ALDARA(TM)) has received Food and Drug Administration approval for topical treatment of basal cell carcinoma, actinic keratoses, and virus-induced skin neoplasms (Stanley, 2002; Gaspari, 2007), and its use has led to high cure rates (Geisse *et al.*, 2004). A few case studies reported the promising potential of IMQ in treating melanoma lesions (Steinmann *et al.*, 2000; Bong *et al.*, 2002). However, because of incomplete understanding of the mechanisms of action and ongoing investigations into its antitumor effects, it is not currently used to treat metastatic melanoma.

IMQ is a synthetic Toll-like receptor (TLR) agonist modulating innate immune responses through TLR7/8 triggering. It has also been shown to have a proapoptotic activity, directly inducing tumor cell apoptosis by triggering the caspase pathway (Schon *et al.*, 2004). Topical application of IMQ in mice and humans induces a strong inflammatory response, characterized by skin inflammation, infiltration, and activation of immune cells, subsequently leading to T-cell activation and antitumor adaptive immune responses (Hemmi *et al.*, 2002; Stanley, 2002). In humans, plasmacytoid dendritic cells (pDCs) are the predominant cell type responding to TLR7-L stimulation (Gibson *et al.*, 2002). These cells have the unique ability to sense single-stranded RNA and unmethylated DNA motifs through expression of TLRs TLR7 and TLR9 (Cao and Liu, 2007; Lande and Gilliet, 2010). Following TLR triggering, pDCs produce large amounts of type 1 IFN and proinflammatory cytokines, which modulate the innate and adaptive immune responses. Interestingly, in melanoma, pDCs are recruited to the tumor site and sentinel lymph nodes (Salio *et al.*, 2003; Vermi *et al.*, 2003; Charles *et al.*, 2010) but they remain in a nonfunctional immature state. However, activation by TLR-L primes pDCs for potent functional responses *in vitro* (Salio *et al.*, 2003; Rothenfusser *et al.*, 2004) and *in vivo* (Liu *et al.*, 2008). Thus, CpG (TLR9-L) can

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Abbreviations: DCs, dendritic cells; HPCs, hematopoietic progenitor cells; IMQ, imiquimod; pDC, plasmacytoid dendritic cell; SCID, severe combined immunodeficient; TLR, Toll-like receptor; VEGF, vascular endothelial growth factor

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promote tumor regression in mice (Furumoto *et al.*, 2004) and can improve melanoma patients' response to vaccination (Speiser *et al.*, 2005) through pDC recruitment and activation (Pashenkov *et al.*, 2006; Molenkamp *et al.*, 2007). It has also been shown that topical application of IMQ (TLR7-L) leads to potent antitumor effects involving pDCs in mice (Palamara *et al.*, 2004). Upon activation, pDCs may also display direct cytotoxic activity toward tumor cells through TRAIL expression, granzyme B, and lysozyme secretion (Chaperot *et al.*, 2006; Stary *et al.*, 2007; Matsui *et al.*, 2009; Jahrsdorfer *et al.*, 2010). Therefore, mobilization of pDCs through TLR agonists could help control melanoma tumors.

Until now, IMQ has been mostly studied in mouse models of melanoma. This has led to key advances, but the relevance of these findings in humans has yet to be assessed. This is essential as the immune systems in mice and humans are different in many aspects (Mestas and Hughes, 2004). Thus, results from animal research do not necessarily reflect human outcomes (Seok *et al.*, 2013). Specifically, mice and humans differ in their TLR expression pattern, especially for TLR7 (Iwasaki and Medzhitov, 2004). In humans, within dendritic cells (DCs), TLR7 is restricted to pDCs, whereas it is expressed by several DC subsets in mice (pDCs, CD4+ DCs, and dominant-negative DCs). Therefore, topical application of IMQ in mice directly activates dermal DC and Langerhans cells, and leads to maturation and migration of cutaneous DCs, whereas in humans it mostly drives pDC mobilization and activation. To date, *in vivo* IMQ studies are limited to murine models of melanoma, and their results cannot be directly translated into clinical applications. Studies in melanoma patients are restricted to case reports or short-term trials. This prevents a detailed dynamic analysis of IMQ effects. A possible tool to circumvent these restrictions is the use of humanized mouse models, based on immunodeficient mice reconstituted with a functional human immune system by injection of human hematopoietic CD34+ stem cells. These animals can then be further xenotransplanted with human tumors (Aspord *et al.*, 2007a,b). Humanized mice have proved to be powerful tools in elucidating the underlying mechanisms of human diseases and testing novel therapies, and have allowed many advances in translational biomedical research, especially in the field of cancer immunotherapy (Shultz *et al.*, 2012).

Many aspects of the mechanisms of IMQ resulting in tumor regression in a human context of melanoma and immunity are still unknown. Moreover, available studies mostly focused on pDCs; yet, other potential effects of IMQ in the context of melanoma have not been investigated. In this article, we investigated the effects of IMQ on melanoma *in vivo* using a melanoma-bearing humanized mouse model. Our results demonstrate that IMQ inhibited melanoma tumor development by promptly mobilizing pDCs, triggering pDC cytotoxic functions, and drastically impeding tumor vascularization. Thus, IMQ triggered a host of actions that converge to inhibit melanoma development. These results contribute to a better understanding of the mechanisms of action of IMQ in the context of human melanoma, underlining its strong therapeutic potential.

RESULTS

IMQ triggers potent immune-dependent inhibition of melanoma tumor growth in a patient and in tumor-bearing humanized mice

IMQ has rarely been used over a long period to treat metastatic melanoma lesions. To assess whether IMQ could impede long-term tumor development in melanoma patients, we treated a 50-year-old patient with 41 in-transit metastases with IMQ 5% cream 5 days a week for 6 months. After an initial inflammatory episode, all lesions disappeared within 6 months of initiating the treatment (Figure 1). To explore the mechanisms underlying IMQ effects *in vivo* in the context of human melanoma tumor and the human immune system, we developed an innovative humanized mouse model in which immunodeficient NOD-SCID (severe combined immunodeficient) $\beta_2m^{-/-}$ or NOD-SCID IL2R$\gamma_C{}^{-/-}$ mice were first xenotransplanted with human CD34+ hematopoietic progenitor cells (HPCs) before injecting them with human melanoma cells (OncoHumice) (Figure 2a and b). These OncoHumice harbored both a human tumor and a human

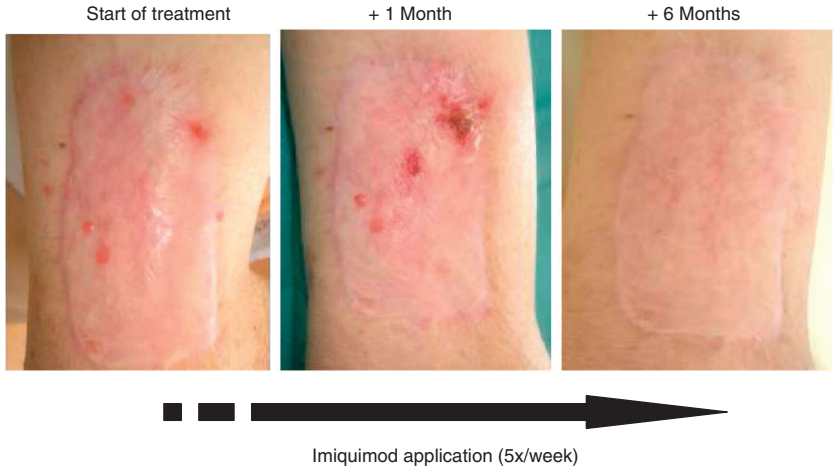

Figure 1. Imiquimod effectively treats cutaneous metastasis in a melanoma patient. A 50-year-old patient with 41 in-transit melanoma metastases was treated with imiquimod 5 days a week (starting in March 2009). After an initial inflammatory episode (April 2009), the lesions had totally disappeared by 6 months (September 2009).

immune system, revealed by the presence of human hematopoietic CD45+ cells including monocytes, B cells, myeloid dendritic cells, and pDCs in NOD-SCID $\beta_2m^{-/-}$ mice 4 weeks post engraftment (Supplementary Figure S1a online) together with NK and T cells in NOD-SCID IL2R$\gamma_C{}^{-/-}$ mice starting 8 weeks post engraftment (Supplementary Figure S1b online). To investigate the overall antitumor effect of IMQ, we treated OncoHumice daily with a topical application of ALDARA(TM) 5% IMQ or control cream to the skin covering the subcutaneous tumor. Melanoma tumor growth was significantly reduced in animals treated with IMQ (Figure 2c and d, Supplementary Figure S2a online). To determine whether the immune system was involved in this antitumor effect, the treatment was also applied in OncoMice, which are immunodeficient NOD-SCID $\beta_2m^{-/-}$ mice engrafted only with human melanoma cells and therefore deprived of human immune cells. In these mice, no difference in the evolution of tumor growth was observed between animals treated with IMQ or the controls (Figure 2e and f). Thus, IMQ triggered potent immune-dependent inhibition of melanoma tumor growth in OncoHumice.

IMQ promptly mobilizes and activates pDCs within tumors in melanoma-bearing humanized mice

We next assessed how IMQ affected mobilization of human pDCs in melanoma-bearing humanized mice. pDCs are the

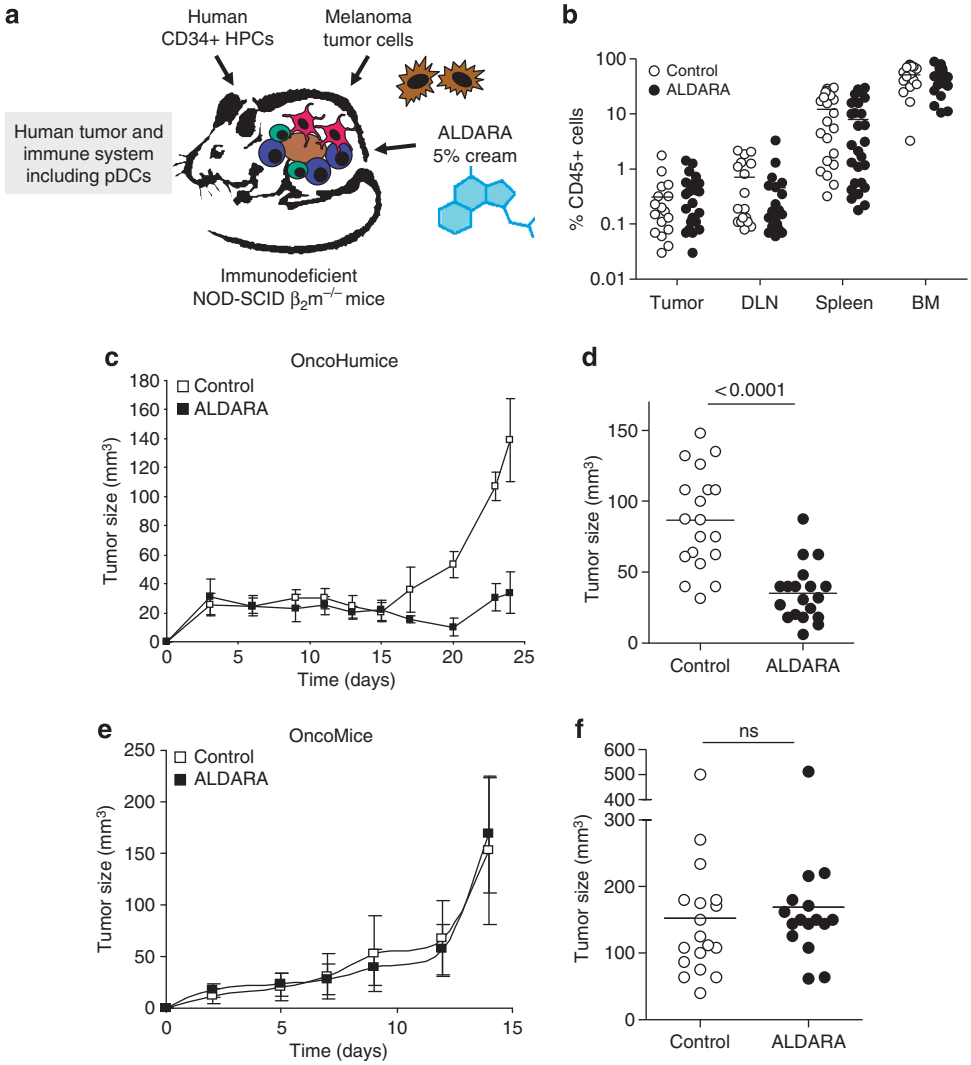

Figure 2. Imiquimod triggers potent immune-dependent inhibition of melanoma tumor growth in tumor-bearing humanized mice. OncoHumice were constructed by intravenous xenotransplantion of human CD34+ hematopoietic progenitor cells (HPCs) into sublethally irradiated NOD-SCID (severe combined immunodeficient) $\beta_2m^{-/-}$ mice, followed by subcutaneous implantation of melanoma tumor cells. OncoHumice thus have both a human immune system, including plasmacytoid dendritic cells (pDCs), and human tumors. OncoMice, in contrast, received only the melanoma cells. All mice received daily topical applications of ALDARA(TM) 5% cream or control cream until sacrifice, and tumor growth was monitored. (**a**) OncoHumouse model. (**b**) Human CD45+ hematopoietic cell percentages measured in different organs on day 3 of treatment ($n=27$ OncoHumice per group). (**c**) Representative tumor growth curves for OncoHumice ($n=5$ OncoHumice per group). (**d**) Tumor size on days 23–26 in OncoHumice ($n=19$ OncoHumice per group). (**e**) Representative tumor growth curves for OncoMice (4–5 OncoMice per group). (**f**) Tumor size on day 14 in OncoMice ($n=16$–18 OncoMice per group). *P*-values: Mann–Whitney test. BM, bone marrow; DLN, draining lymph nodes.

main human cell type expressing TLR7 and are therefore likely to respond to IMQ. Strikingly, following IMQ application, prompt mobilization of pDCs to the melanoma tumor site was observed (Figure 3a and b, Supplementary Figure S2b online); no such mobilization was seen with the control treatment. This recruitment was associated with a concomitant depletion of pDCs from the bone marrow (Figure 3c, Supplementary Figure S2c and S2d online). IMQ did not modulate other human cell populations at the tumor site (Supplementary Figure S3 online). Notably, a signature of pDC activation was detected in the plasma and tumor microenvironment of OncoHumice treated with IMQ, as human IFN-α and IP10 were observed in the plasma and tumor supernatants of OncoHumice treated with IMQ (Figure 3d and e). Furthermore, IMQ rapidly triggered the upregulation of expression of human type 1 IFN genes and type 1 IFN response genes within the tumor microenvironment (Figure 4). Thus, IMQ promptly mobilized and activated pDCs within the melanoma tumor site.

IMQ potently triggers the cytotoxic functions of pDCs

To further investigate how IMQ modulates pDCs, we analyzed the phenotypic and functional changes triggered by IMQ on purified human pDCs *in vitro*, focusing particularly on their cytotoxic potential. Within 24 hours, IMQ elicited a dramatic upregulation of the death-inducing ligand TRAIL on the surface of pDCs (Figure 5a) combined with potent granzyme B degranulation (Figure 5b) as attested by the loss of

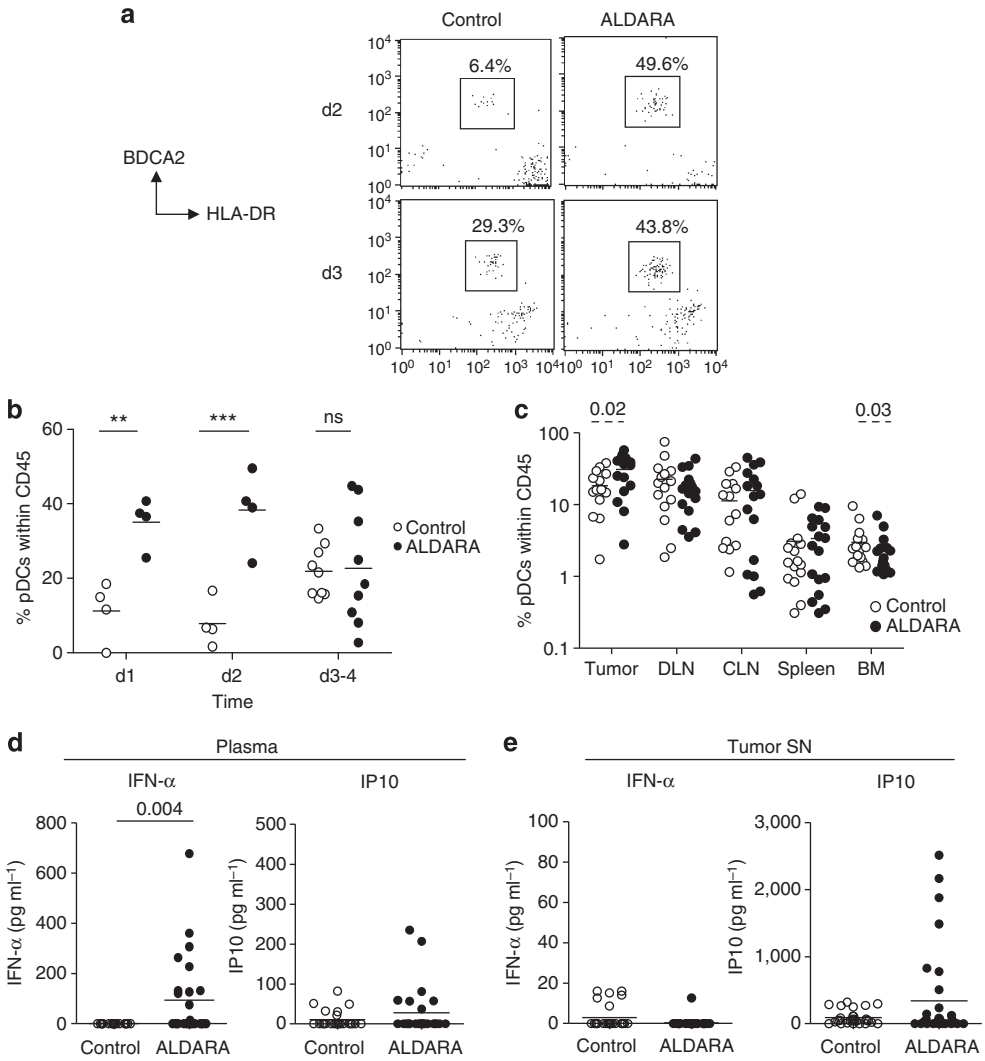

Figure 3. Imiquimod promptly mobilizes plasmacytoid dendritic cells (pDCs) within the tumor site *in vivo* in melanoma-bearing humanized mice. NOD-SCID (severe combined immunodeficient) $\beta_2m^{-/-}$ immunodeficient mice received human CD34+ hematopoietic progenitor cells (HPCs) followed by human melanoma tumor cells. ALDARA(TM) 5% cream or control cream was applied to the skin daily from the day of tumor implantation. (**a**–**c**) Human pDCs were determined within the total human CD45+ hematopoietic cells based on HLA-DR and BDCA2 expression. (**a**) Representative dotplots of pDCs at the tumor site (gated on CD45+ cells). (**b**) Percentage of pDCs at the tumor site at different times ($n=4$–9 OncoHumice/group). *P*-values: two-way analysis of variance test. (**c**) Percentage of pDCs within CD45+ cells in organs within 3 days of treatment ($n=14$ OncoHumice per group) (data from d1, d2, and d3 are pooled). (**d**) Plasma IFN-α and IP10 levels 3 days after initiating imiquimod treatment ($n=14$ OncoHumice per group). (**e**) IFN-α and IP10 levels in tumor supernatants 3 days after the start of imiquimod treatment ($n=14$ OncoHumice per group). *P*-values: Mann–Whitney test.

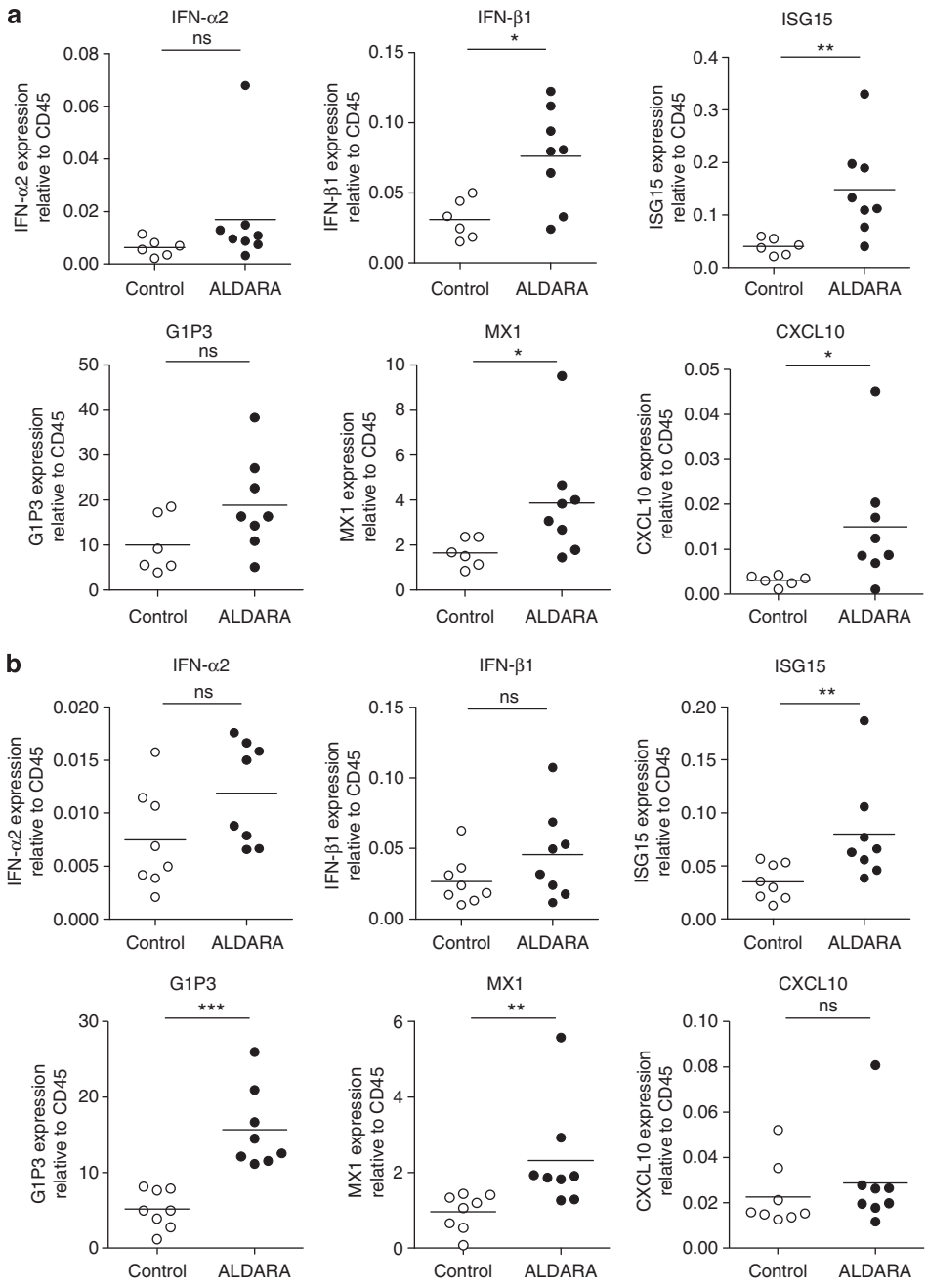

Figure 4. Imiquimod triggers the upregulation of type I IFN-inducible genes at the tumor site of melanoma-bearing humanized mice. NOD-SCID (severe combined immunodeficient) IL2R$\gamma_C^{-/-}$ immunodeficient mice received human CD34+ hematopoietic progenitor cells followed 4 weeks later by human melanoma tumor cells. ALDARA(TM) 5% cream or control cream was applied to the skin daily. The expression of type I IFN (IFN-α2, IFN-β1) as well as human type I IFN-inducible genes (ISG15, G1P3, MX1, CXCL10) was analyzed at the tumor site by reverse transcription-real time PCR on day 1 (**a**, $n = 6$–8 OncoHumice per group) and day 3 (**b**, $n = 8$ OncoHumice per group) after the start of the treatment. *P*-values: Mann–Whitney test.

intracellular granzyme B stocks in pDCs. Strikingly, pDCs treated with IMQ acquired the ability to kill TRAIL-sensitive melanoma tumor cells (Figure 5c, Supplementary Figure S4 online). Given the strong effect of IMQ on pDCs observed *in vitro,* we next investigated the *in vivo* consequences on pDC functions following IMQ administration in melanoma-bearing humanized mice. TRAIL expression was potently upregulated by pDCs within the tumor microenvironment (Figure 5d) and granzyme B degranulation occurred (Figure 5e). IMQ was also found to trigger monocyte chemotactic protein1 and IL-21 production at the tumor site (Supplementary Figure S5a online) and elevated plasma levels of fractalkine, I-transit amplifying cell, and monokine induced by gamma interferon were measured in IMQ-treated OncoHumice (Supplementary Figure S5b online). Thus, IMQ creates a cytokine and chemokine network eliciting pDC and T-cell mobilization

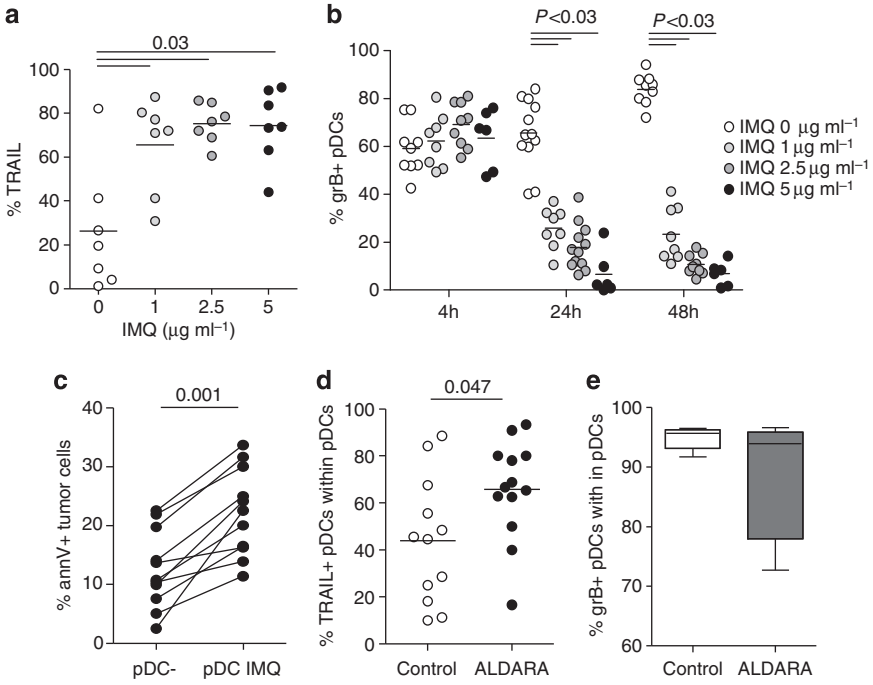

Figure 5. Imiquimod triggers the cytotoxic functions of pDCs *in vitro* and *in vivo* in melanoma-bearing humanized mice. (**a**–**c**) pDCs purified from healthy donor blood were treated with imiquimod. pDC features were assessed after 4, 24, and 48 hours. (**a**) TRAIL expression at 24 hours ($n=7$). (**b**) Intracellular granzyme B (grB) staining ($n=6$–12). (**c**) Cytotoxicity of pDCs toward melanoma tumor cells measured by annexinV (annV) staining of COLO829 and A375 after 18-hour co-cultures with imiquimod (IMQ) (2.5 μg ml^{-1})-stimulated plasmacytoid dendritic cells (pDCs) (E:T ratios: 40:1 or 20:1) ($n=11$). (**d**, **e**) NOD-SCID (severe combined immunodeficient) $\beta_2m^{-/-}$ immunodeficient mice received human CD34+ hematopoietic progenitor cells followed by subcutaneous implantation of human melanoma tumor cells. ALDARA(TM) 5% cream or control cream was applied topically to the tumor daily. (**d**) TRAIL expression on pDCs within the tumor microenvironment on day 3 of imiquimod treatment ($n=12$–13). (**e**) Granzyme B (grB)+ pDCs in different organs on day 3 of imiquimod treatment ($n=5$). *P*-values: Wilcoxon's matched pairs test.

and function, and endowing them with potent cytotoxic activity toward melanoma tumor cells.

IMQ drastically inhibits tumor vascularization *in vivo* in melanoma-bearing humanized mice

Vascularization being important in supporting tumor growth, we investigated the effects of IMQ administration on the levels of proangiogenic factors. Levels of vascular endothelial growth factor (VEGF), angiogenin, IL-8, and fibroblast growth factor were significantly decreased within the melanoma microenvironment of OncoHumice treated with ALDARA(TM) compared with levels in control animals (Figure 6a, upper panels). This difference was not observed in OncoMice deprived of the human immune system (Figure 6a, lower panels). We then assessed whether these features could impact tumor vascularization. Strikingly, the overall density of blood vessels within tumors was significantly lower in IMQ-treated OncoHumice than in animals receiving control treatment at day 16 and day 24 after the start of treatment (Figure 6b and c, left panels). Moreover, by counting the number and measuring the area of blood vessels, the structure of the vasculature within tumors was also found to be significantly different with IMQ-treated tumors displaying significantly more small (50–200 μm^2) and fewer large (>500 μm^2) blood vessels compared with tumors receiving the control treatment (Figure 6d, left panels). In contrast, tumor vascularization was not affected in IMQ-treated OncoMice (Figure 6b–d, right panels). Thus, IMQ drastically remodeled the tumor vasculature and inhibited vascularization in an immune-dependent manner.

DISCUSSION

In view of the clinical interest for TLR7 agonists in metastatic melanoma, it is essential to determine the mechanism of action of IMQ. Our study uses a highly relevant model of humanized mice to confirm the key role of pDCs in translating the effects of IMQ into potent tumor growth restriction. Our results also revealed a previously unexpected role for IMQ inhibiting tumor vascularization. These additional insights will have a major impact on the use of IMQ as treatment against metastatic melanoma.

Humanized mice were used in this study to elucidate the effects of IMQ in the context of melanoma. Studies in humans are hindered by restricted access to tissues and ethical concerns, and do not allow to investigate mechanisms of action or disease pathogenesis. Studies of IMQ effects in humans are limited to case reports (Steinmann *et al.*, 2000; Bong *et al.*, 2002; Zeitouni *et al.*, 2005), being mostly descriptive and not investigational. The few data available regarding the effects of IMQ on immunity in melanoma patients revealed an IFN signature in the serum of patients (Dummer *et al.*, 2008) suggesting an activation of pDCs and an increase in T cells in the treated area (Narayan *et al.*, 2012).

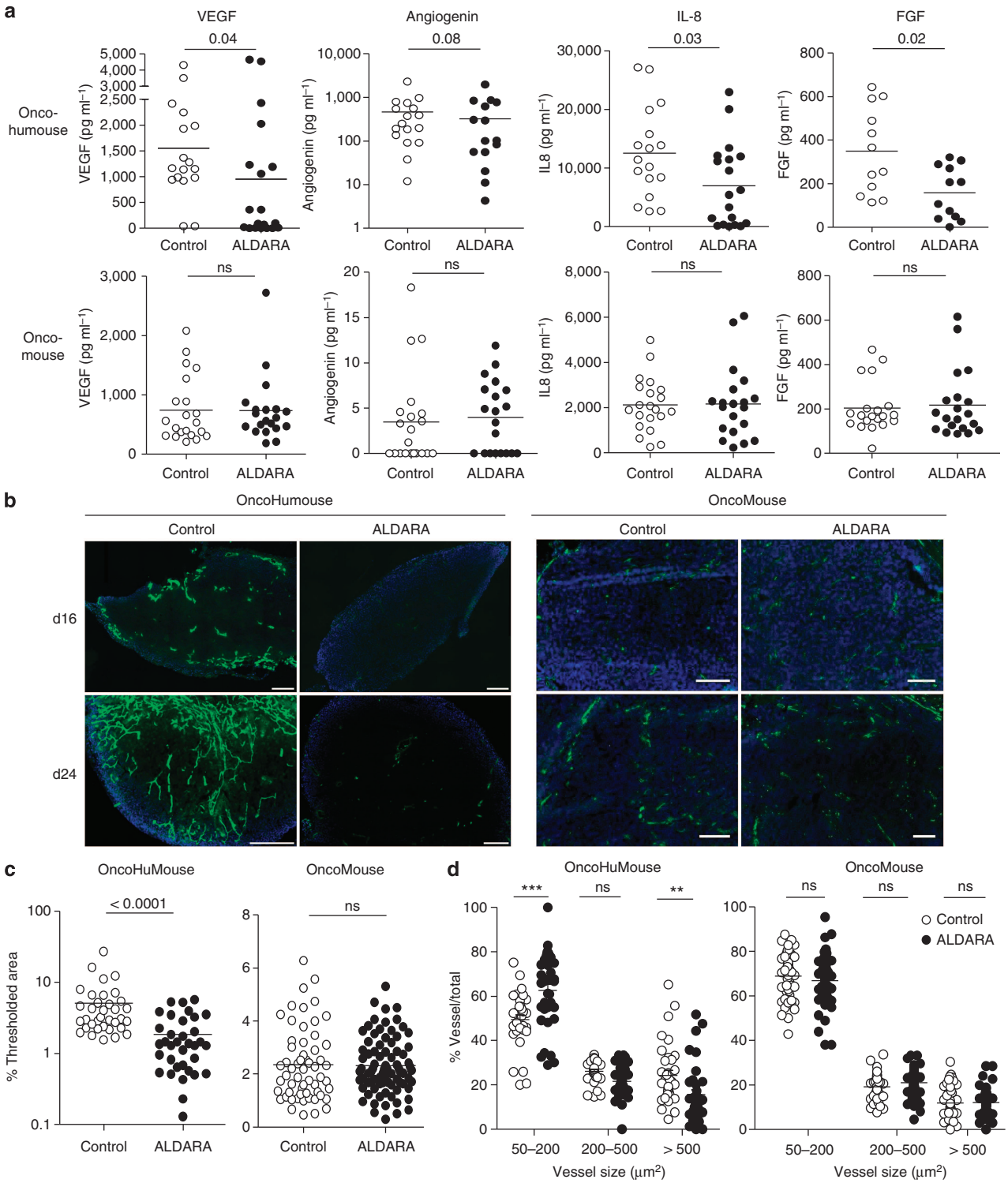

Figure 6. Imiquimod drastically inhibits tumor vascularization *in vivo* in melanoma-bearing humanized mice. NOD-SCID (severe combined immunodeficient) $\beta_2m^{-/-}$ immunodeficient mice were transplanted with human CD34+ hematopoietic progenitor cells (OncoHuMouse) or not transplanted (OncoMouse) followed by subcutaneous injection of human melanoma tumor cells. Tumors were treated daily by topical application of ALDARA(TM) 5% cream or control cream. (**a**) Vascular endothelial growth factor (VEGF), angiogenin, IL-8, and fibroblast growth factor (FGF) levels at the tumor site on days 3–30 of treatment ($n = 17$–19 OncoHumice per group; $n = 20$ OncoMice per group). (**b**–**d**) Tumor vascularization on days 16 and 24 of treatment. Vascularization was visualized after FITC-lectin injection and fluorescence microscopy analysis of tumor sections. (**b**) Representative tumor sections (bar = 200 µm). (**c**) Surface occupied by blood vessels (percentage of thresholded area) on days 16–24 (left panel: $n = 35$ sections from 8 OncoHumice per group; right panel: $n = 58$–81 sections from 8–10 OncoMice per group). (**d**) Size distribution for vessels 16–24 days after starting treatment (left panel: $n = 35$ sections from 8 OncoHumice per group; right panel: $n = 52$ sections from 8–10 OncoMice per group). *P*-values: (**a**, **c**) Mann–Whitney test; (**d**) two-way analysis of variance (ANOVA).

Strikingly, our results not only describe immune effects but also reveal vascular effects. Human CD34+ HPCs, which were used to reconstitute the immune system in these animals, also contain endothelial progenitor cells, which are known to contribute to tumor angiogenesis (Hristov *et al.*, 2003; Ribatti, 2004). Thus, humanized mice represent a relevant model with which to decipher interactions between immune and tumor cells, to perform dynamic *in vivo* analysis, and to further translate discoveries into clinical applications.

IMQ triggered prompt mobilization of pDCs to the tumor site; this was not observed with the control treatment. Notably, the early IFN-α and IP10 signature in the plasma and tumor microenvironment of humanized mice treated with ALDARA(TM) revealed a potent activation of the mobilized pDCs. Interestingly, a short-term trial using the TLR7-L agonist 852A in metastatic melanoma patients demonstrated similar increases in serum levels of type I IFN and IP10 in most treated patients (Dummer *et al.*, 2008). Our results also showed elevated levels of monocyte chemotactic protein1/CCL2 and IL-21 in the tumor microenvironment following IMQ application but not with control treatment. monocyte chemotactic protein1/CCL2 may contribute to pDC recruitment (Drobits *et al.*, 2012), whereas IL-21 has been shown to be implicated in triggering pDC degranulation (Karrich *et al.*, 2013), increasing the potential of cytotoxic effector T and NK cells (Park *et al.*, 2012; Turksma *et al.*, 2013) and hampering Treg expansion (Battaglia *et al.*, 2013). Interestingly, IL-21 was found to be clinically active in a phase II study in melanoma patients (Petrella *et al.*, 2012). Other chemokines were also elevated in the plasma of IMQ-treated humanized mice, including fractalkine/CX3CL1, I-transit amplifying cell/CXCL11, and monokine induced by gamma interferon/CXCL9. In the context of melanoma, these chemokines have been shown to favor CD8 T-cell infiltration and to be potent lymphocyte chemoattractants (Hensbergen *et al.*, 2005; Nukiwa *et al.*, 2006; Harlin *et al.*, 2009). Thus, IMQ triggers events that may potentiate subsequent adaptive antitumor immune responses.

The pDCs recruited by IMQ may contribute to inhibiting tumor development in many ways. They could directly kill tumor cells, as IMQ potently triggered pDC cytotoxic functions through TRAIL upregulation and granzyme B degranulation both *in vitro* and *in vivo* in the humanized mouse model. This mechanism has been described *in vitro* (Kalb *et al.*, 2012) and is supported by data from a mouse model of melanoma showing that IMQ drives pDC recruitment and their subsequent conversion into tumor-killing effector cells through TRAIL and granzyme B secretion (Drobits *et al.*, 2012). Tumor cell killing may generate apoptotic bodies and release antigens that can then be processed by DCs for cross-presentation to T cells to elicit specific antitumor responses. In response to IMQ, pDCs secrete IFN-α, which can also directly trigger tumor cell apoptosis through a caspase-dependent mechanism (Thyrell *et al.*, 2002). This leads to upregulation of cytotoxic molecules and their corresponding ligands on effectors and target tumor cells (Stary *et al.*, 2007), thereby potentiating the destruction of tumor cells. IP10 produced by pDCs may promote a Th1-prone immunity, which will tend to elicit efficient antitumor responses. Thus, IMQ triggers a number of pathways through pDCs, which converge to inhibit tumor development.

Melanoma tumor cells secrete many potent angiogenic factors such as VEGF, fibroblast growth factor, angiogenin, and IL-8, which drive angiogenesis, critical for tumor growth and invasiveness, and also display autocrine and paracrine growth factor properties (Graeven *et al.*, 2001). Tumor cell elimination may thus directly reduce the level of tumor-derived factors, especially proangiogenic factors. This hypothesis is supported by the decreased levels of VEGF, angiogenin, IL-8, and fibroblast growth factor observed in the tumor microenvironment in this study. The large amounts of IFN-α and IP10 produced by pDCs in response to IMQ stimulation are described to be antiangiogenic (Sidky and Borden, 1987; Sgadari *et al.*, 1996), and can downregulate VEGF and fibroblast growth factor production (Riedel *et al.*, 2000). Strikingly, the findings presented here are confirmed in a case report in melanoma patients where IMQ treatment of a skin metastasis caused changes to the expression levels of genes involved in metastasis and angiogenesis (Hesling *et al.*, 2004). Indeed, the analysis of biopsies of skin lesions before and after topical IMQ treatment revealed that IMQ triggered the upregulation of IFN-α and angiogenesis inhibitors while downregulating genes involved in angiogenesis and metastatic invasion. In melanoma, VEGF is considered to be the main proangiogenic factor promoting tumor growth and invasiveness (Graeven *et al.*, 2001). VEGF has mitogenic properties on endothelial cells, promotes vascular permeability, and triggers the release of matrix metalloproteinases by endothelial cells to facilitate invasion into the surrounding tissue. Vascularization is critical for tumor growth and invasiveness. By impeding it, IMQ blocks an essential step of tumor development. Notably, high serum levels of the angiogenic factors VEGF, angiogenin, and IL-8 are associated with poor clinical outcome in metastatic melanoma patients, correlating with tumor progression and reduced survival (Ugurel *et al.*, 2001). These factors are therefore predictive markers of overall and progression-free survival.

pDCs are key regulators of immune responses (Colonna *et al.*, 2004; Lande and Gilliet, 2010). Depending on their maturation state and on the microenvironment, pDCs can induce immunity or tolerance. The extensive functional plasticity of pDCs gives them the capacity to direct immunity toward multiple profiles depending on the surrounding signals. In melanoma, pDCs within the tumor microenvironment are in a nonfunctional state and are associated with a poor clinical outcome (Jensen *et al.*, 2012). However, several studies demonstrated that mobilization and activation of pDCs using TLR7 and TLR9 agonists allowed potent functional antitumor responses, eventually leading to tumor regression in animal models. pDC reactivation seems to be essential for the antitumor response induced by IMQ (Drobits *et al.*, 2012). Thus, IMQ could tip the balance in favor of immunity over tolerance. Our results corroborate the hypothesis that reactivation of pDCs in the context of melanoma using TLR agonists is a way of impeding tumor development.

IMQ may contribute to antitumor responses by affecting tumor cells, tumor-infiltrating immune cells, and the tumor

microenvironment. This broad range of antitumor activities make IMQ a good candidate for its further clinical use as adjuvant combined with others therapies, particularly to enhance immunotherapy (Smits *et al.*, 2008). Its therapeutic potential is currently being investigated in preclinical studies in animals and in patients in whom topical IMQ is used in combination with other strategies. IMQ improved response to DC-based vaccination in mice by promoting tumor-specific T-cell priming and trafficking (Prins *et al.*, 2006) and drove specific responses toward coadministered antigens in patients (Adams *et al.*, 2008). The combination of topical IMQ and photodynamic therapy in patients induces regression of distant metastases and triggers beneficial immune responses (Naylor *et al.*, 2006). IMQ together with intralesional IL-2 induced systemic immunological effects, reversing the Th1/Th2 balance (Green *et al.*, 2008).

Our study highlights the relevance of IMQ in a human context of immune cells and melanoma tumor cells and provides further insights into the antitumor effects of IMQ. Thanks to our innovative model of melanoma-bearing humanized mice, we were able to show that pDCs, promptly mobilized by IMQ, may also contribute to triggering long-term inhibition of angiogenesis. Humanized mice could be used further to design IMQ dosing schedules, optimize timing of administration, or test IMQ in combination with other immunotherapies in preclinical trials, thereby opening opportunities for future changes in clinical practice. Manipulating the immune system using IMQ may be a potent means to trigger several pathways converging into inhibition of tumor development. The potent antitumor effects of IMQ through pleiotropic pathways make it very attractive for further clinical developments.

MATERIALS AND METHODS

Melanoma patient

A 50-year-old patient was diagnosed with a primary melanoma on the arm in 2007 (Breslow 3.5 mm, ulcerated). The melanoma was surgically removed, but 41 in-transit cutaneous melanoma metastases developed in March 2009. IMQ 5% cream (ALDARA(TM)), marketed in France by MEDA Laboratories, was applied to all lesions 5 days a week for 6 months (3 packages of 12.5 mg per day for all lesions). A spectacular disappearance of all metastases was observed in September 2009. IMQ application was then reduced to twice a week. The patient received no other treatment before and during IMQ application. The patient gave written informed consent for participation in the study. The study was approved by the Ethics committee of Grenoble University Hospital and performed with adherence to Helsinki Guidelines.

Melanoma cell lines and human CD34+ HPCs isolation

Human melanoma lines COLO829 and A375 were purchased from ATCC (LGC Standards, Molsheim, France) and cultured in RPMI 1640 Glutamax supplemented with 1% non-essential amino acids, 1 mM sodium pyruvate (Sigma, Lyon, France), 100 $\mu g\,ml^{-1}$ gentamycin, and 10% fetal calf serum (all from Invitrogen, Saint Aubin, France, unless indicated). Human CD34+ HPCs were purified from cord blood using a CD34 HPCs isolation kit (Miltenyi, Paris, France) and stored frozen until use. This procedure was approved by the French Blood Service's Institutional Review Board, and donors gave written informed consent.

In vitro modulation of pDC phenotype and function by IMQ

Blood samples were obtained from 38 healthy volunteers. This procedure was approved by the French Blood Agency's Institutional Review Board and participants signed informed consent forms. PBMCs were purified from blood by Ficoll-Hypaque density-gradient centrifugation (Eurobio, Courtaboeuf, France) and pDCs were further purified using the EasySep Human pDC enrichment kit (StemCell, Grenoble, France) (purity > 97%). pDCs were stimulated for 4, 24, or 48 hours with the indicated doses of IMQ (R837). Flow cytometric analysis (FACSCalibur, BD, Paris , France) was used to determine levels of costimulatory and death-inducing molecules using anti-CD40, -CD80, -CD86 (Beckman), -TRAIL, and -granzyme B (BD) antibodies. A Cytometric Bead Array assay (BD) was used to quantify IFN-α, IP10, IL-6, IL-8, and TNF-α cytokines in culture supernatants. The sensitivity of COLO829 and A375 melanoma tumor cells to TRAIL-mediated killing was assessed by incubating the tumor cells with soluble TRAIL and labeling with 7 aminoactinomycin D after 4, 18, and 48 hours. Killer TRAIL (100 $ng\,ml^{-1}$) (Axxora, Villeurbanne, France) was used as positive control of TRAIL-induced death. pDC cytotoxicity toward melanoma tumor cells was measured in co-cultures of IMQ-stimulated (2.5 $\mu g\,ml^{-1}$) pDCs with COLO829 and A375 melanoma tumor cells at 20:1 to 1:1 ratios for 18 hours followed by annexinV FITC and 7 aminoactinomycin D labeling of tumor cells.

Humanized mice

NOD-SCID $\beta_2m^{-/-}$ (NOD.Cg-Prkdc$^{SCID}\beta_2m^{Tm1Unc}$/J) and NOD-SCID IL2R$\gamma_C{}^{-/-}$ (NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ) immunodeficient mice were purchased from Jackson ImmunoResearch Laboratories (Bar Harbor, MI) and bred at the Plateforme de Haute Technologie Animale (PHTA, La Tronche, France). Humanized mice were constructed by intravenous xenotransplantion of $1–2 \times 10^5$ human CD34+ HPCs into sublethally irradiated 4-week-old immunodeficient mice (100–120 cGy). The human immune system was reconstituted within 4 weeks in both strains, except for human NK and T cells, which developed from 8 weeks post transplantation only in NOD-SCID IL2R$\gamma_C{}^{-/-}$ recipient mice. Then, 10×10^6 human melanoma tumor cells were implanted subcutaneously into the flank of the humanized mice (OncoHumice). In some experiments, NOD-SCID $\beta_2m^{-/-}$ immunodeficient mice were only implanted with 10×10^6 human melanoma tumor cells without prior immune reconstitution (OncoMice). All *in vivo* studies were carried out in strict accordance with European Union guidelines (86/609/CEE) and French National Charter guidelines; protocols were approved by the local Ethics Committee for Animal Experimentation, Grenoble (ComEth).

ALDARA(TM) treatment of melanoma-bearing humanized mice

Tumors received daily topical treatment with ALDARA(TM) 5% cream (3 mg per mouse) (Meda Pharma, Paris, France) or control cream without IMQ from the day of implantation until sacrifice. Tumor growth was measured every 2–3 days using a caliper, and the tumor volume was calculated using the ellipsoid formula: $(\text{length} \times \text{width})^2/2$.

Analysis of pDC phenotype and function by flow cytometry

At different time points after the start of ALDARA(TM) treatment, tumors, draining lymph nodes, control lymph nodes, spleen, and bone marrow were harvested. Organs were digested with 2 $mg\,ml^{-1}$ collagenase D (Roche, Boulogne, France) and the resultant cell

suspensions were filtered, washed, resuspended in phosphate-buffered saline supplemented with 2% fetal calf serum, and counted before staining with anti-human antibodies. pDCs ($CD45^+$ $HLA\text{-}DR^+$ $BDCA2^+$) were identified using anti-CD45 FITC, anti-HLA-DR PerCP (BD), and anti-BDCA2 APC antibodies (Miltenyi). Their basal activation level was determined using anti-CD40, -CD80, and -CD86 FITC/PE antibodies and their isotype-matched controls (Beckman Coulter, Paris, France). TRAIL expression was measured by surface staining using anti-CD253 Abs (BD), and intracellular granzyme B was quantified using anti-grB Abs (BD). Suspensions were then submitted to flow cytometric analysis on a FACSCalibur using Cell Quest software (BD) for acquisition and anlaysis.

RTqPCR quantification of gene expression

Reverse transcription-real time PCR assays with Universal Probe Library probes have been performed in this study according to the recommendations of the supplier (Roche). The primers and Universal Probe Library probes used are listed in Supplementary Table S1 online. Tumors were taken from mice and put immediately in RNAlater and kept at $-20\,^{\circ}C$. Total RNA was extracted following disruption of the tumor with tissue ruptor apparatus (Qiagen, Courtaboeuf, France) and submitted to an extraction procedure using the Qiagen kit according to the recommendations of the supplier. Following treatment with a Turbo DNAse (Ambion, Saint Aubin, France) to remove genomic DNA, cDNA was obtained from RNA using the expand transcriptase reverse enzyme from Roche and submitted to qPCR.

Cytometric bead array for plasma and tumor factor quantification

Blood samples were collected from humanized mice in heparinized tubes and plasma was separated. Small tumor fragments (about $10\,mm^3$) were incubated in 100 µl complete RPMI 1640 Glutamax supplemented with 10% fetal calf serum. Culture medium was recovered 24 hours later as tumor supernatants. The following human cytokines, chemokines, and angiogenic factors were quantified in plasma and tumor supernatants by Cytometric Bead Array assay (BD): IFN-α, IP10, IL-8, IL-21, fractalkine, I-transit amplifying cell, monokine induced by gamma interferon, monocyte chemotactic protein1, VEGF, and angiogenin.

Measuring tumor vascularization *in vivo*

Tumor vasculature was analyzed after intravenous injection of FITC-labeled Lycopersicon esculentum lectin (100 µg per 100 µl) (Sigma) followed 10 minutes later by perfusion of 0.5% glutaraldehyde 1% paraformaldehyde in phosphate-buffered saline. Tumors were immediately frozen in tissue-tek OCT and stored at $-80\,^{\circ}C$. Cryosections (5 µm) were prepared using a cryostat and mounted with diamidinophenylindol-containing mounting medium (Invitrogen). Sections were analyzed by fluorescence microscopy using a motorized Axio Imager M2 apparatus (Zeiss, Marly le Roi, France) with plan-Apochromat ×20/0.75 NA and Axiovision software equipped with the MosaiX module. The surface occupied by blood vessels was quantified (percentage of thresholded area) and the vessel number and area were determined after thresholding and segmentation using Metamorph software (Molecular Devices, St Gregoire, France). To correct for heterogeneity of vasculature, sections were imaged at 3–4 different depths in each tumor sample, and whole tumor sections were analyzed. For each section, the number and area of each vessel were determined, and the relative proportion of vessels ranked by size was then calculated.

Statistical analysis

All statistical analyses were performed using Prism software (GraphPad software, La Jolla, CA) by using the Mann–Whitney nonparametric *U*-test, the unpaired *t*-test, and one- and two-way analysis of variance.

CONFLICT OF INTEREST

The 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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