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RESEARCH

Normalization of tumor vasculature by imiquimod: proposal for a new anticancer therapeutic indication for a TLR7 agonist

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Abstract

Imiquimod (IMQ), a drug from aminoquinoline group, is the toll-like receptor 7 (TLR7) agonist. It acts as an immunostimulant and radio-sensitizing agent. IMQ stimulates both innate and adaptive immune response. Despite studies conducted, there are no unambiguous data showing how IMQ affects the condition of tumor blood vessels. Tumor vasculature plays the main role in tumor progression. Formation of abnormal blood vessels increases area of hypoxia which recruits suppressor cells, blocks tumor infiltration by $CD8^+$ T lymphocytes, inhibits efficacy of chemoterapeutic drug and leads to cancer relapse. Normalization is a type of therapy targeted at abnormal tumor blood vessels. Here, we demonstrated that 50 µg of IMQ inhibits the growth of melanoma tumors more efficiently, compared to other tested doses and the control group. Dose escalation did not improve the therapeutic antitumor potential of TLR7 agonist. A dose of 50 µg of IMQ most effectively reduced tumor blood vessel density. Imiquimod normalized tumor vasculature both structurally (by reducing vessel tortuosity and increasing pericyte coverage) and functionally (by improving tumor perfusion) in a dose-dependent manner. Hypoxia regions in tumors of treated mice were significantly reduced after IMQ administration. A dose of 50 µg of IMQ had also the greatest impact on the changes in tumor-infiltrating T lymphocytes levels. TLR7 agonist inhibited angiogenesis in treated mice. Functional vascular normalization by IMQ increases the effectiveness of low dose of doxorubicin. Higher dose of IMQ showed worse effects than lower doses including decreased tumor perfusion, increased tumor hypoxia and immunosuppression. This knowledge may help to optimize the combination of the selected IMQ dose with e.g. chemotherapy or radiotherapy to elicit synergistic effect in cancer treatment. To conclude, we outline IMQ repurposing as a vascular normalizing agent. Our research results may contribute to expanding the therapeutic indications for the use of IMQ in anticancer therapy in the future.

Keywords TLR7 agonist · Imiquimod · Antiangiogenic therapy · Blood vessels normalization · Hypoxia · Drug repurposing

Introduction

Tumor microenvironment (TME) is a pathological niche in which progression of cancer occurs. The microenvironment consists of, among others, tumor blood vessels and cells of the immune system that inhibit the anti-tumor immune response [1]. Tumor vascularization develops very rapidly and is often malformed and dysfunctional [2]. Tumor abnormal vasculature is characterized by: sprouting endothelial cells, pericyte detachment, tortuous vascular architecture, irregular blood flow, decreased perfusion and enhanced vascular permeability [3, 4]. The emerging network of blood vessels is defective and blood flow is stagnant and variable. This irregular vasculature creates underoxygenation regions (hypoxia), low pH and elevated interstitial pressure (IFP) within the tumor microenvironment. Hypoxia induces an inflammatory reaction similar to the one present in damaged tissues [5]. Signals of the inflammatory reaction recruit immune cells, which are reprogrammed in a specific way. An example are monocytes that differentiate into specific tumor-associated macrophages (TAMs) which are involved in angiogenesis, immunosuppression, matrix remodelling,

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invasiveness and metastasis [6]. Additionally, hypoxia inhibits the recruitment of cytotoxic T lymphocytes and promotes the accumulation of immunosuppressive regulatory T cells (Tregs). Lymphocytes infiltration into the tumor is further limited by a selective immune cells barrier created by disorganized tumor blood vessels [7]. Vicious circle is created between abnormal tumor blood vessels and immunosuppressive microenvironment. Tumor vasculature directly affects the infiltration of immune effector cells and indirectly promotes immune suppression through synthesis of proangiogenic factors. Increased immunosuppression can in turn induce more abnormal tumor angiogenesis, which disrupt immune activation [7]. Normalization is one type of therapy targeted at abnormal tumor blood vessels. Normalized blood vessels improve tumor oxygenation, reduce IFP, increase the penetration of the tumor mass by the anti-tumor immune cells and enhance drug delivery [8, 9]. Tumor vascular normalization can overcome the antiangiogenic therapy resistance and enhance the anticancer effect of radiotherapy, chemotherapy and immunotherapy [7, 10].

In our study we used imiquimod (1-(2-methylpropyl)-1H-imidazo{4,5-c}quinolin-4-amine; IMQ) [11], a toll-like receptor 7 (TLR7) agonist [12]. IMQ is used in the form of a cream as an anti-viral and anti-allergic agent [13]. In addition, the results of clinical trials indicate the antitumor effect of IMQ [12, 14]. Imiquimod is approved by the Food and Drug Administration (FDA) in a 5% topical formulation for the treatment of external genital warts, superficial basal cell carcinoma and actinic keratosis [15]. It has also been used off-label in the management of different infectious and neoplastic superficial skin disorders [16]. In combination with local radiotherapy, it was used to treat patients with metastatic breast cancer with promising results [17]. In clinic practice IMQ is used as an immunomodulatory agent [16, 18]. Manipulation of TLR response may increase dendritic cells (DCs) activation, enhancing the efficacy of adaptive immune response after irradiation [17]. Imiquimod stimulates both non-specific (DC, macrophages) and specific (cytotoxic $CD8^+$ T lymphocytes) responses. In addition, it reduces the level of regulatory T lymphocytes and the level of inflammatory CCL22 cytokine [14].

The aim of our study was to verify how various doses of a liquid formulation of the IMQ (10 µg, 50 µg or 100 µg) affect the tumor blood vessels condition in murine melanoma. We observed that 50 µg of IMQ significantly inhibits the tumor's growth and neovascularization in treated mice. For the first time we indicate that imiquimod normalized tumor vasculature both structurally and functionally. We observed significant changes in tumor hypoxia and tumor-infiltrating T lymphocytes levels after 50 µg of IMQ treatment. Interestingly, high dose of IMQ (100 µg) increased tumor underoxygenation and immunosuppression. Our results indicate that an antiangiogenic properties of imiquimod normalizes tumor vasculature in a dose-dependent manner. Adequate combination of vascular normalization by IMQ with other form of anticancer therapy (chemo- or/and radiotherapy) should elicit synergistic effect in tumor regression and control further tumor growth. Our results outline the possible future prospects of the repurposed IMQ in clinical application.

Results

Imiquimod inhibits the growth of murine melanoma tumors in a dose-dependent manner

We evaluated the therapeutic effect of different doses of imiquimod in the treatment of B16-F10 murine melanoma tumors. We used three doses of a liquid formulation of IMQ: 10 µg, 50 µg and 100 µg. Imiquimod was subcutaneously injected to the well-developed tumors with volumes of ~ 60–70 mm^3 (Fig. 1A–D). We observed the dose-dependent inhibition of murine melanoma tumor growth. IMQ in a single dose of 10 µg and 100 µg, injected directly into tumors, similarly inhibited the growth of melanoma tumors, compared to the control group (approx. 50%; 225 mm^3 and 206 mm^3 vs 468 mm^3, respectively). 50 µg of IMQ inhibited the growth of tumors more efficiently, compared to other doses (97 mm^3 vs 225 mm^3 IMQ 10 µg and 206 mm^3 IMQ 100 µg) and the control group (97 mm^3 vs 468 mm^3). This data indicates that imiquimod in a single dose of 50 µg is the most effective to inhibit murine melanoma tumor growth. Dose escalation did not improve the therapeutic antitumor potential of TLR7 agonist.

TLR7 agonist improves intratumoral vessel function

We checked how different doses of IMQ (10 µg, 50 µg or 100 µg) affect tumor blood vessels of treated mice, compared to the control group. First, we focused on vessels density and the structure after IMQ treatment. Tumor progression depends on its own vascular network. Tumor vessels are structurally and functionally distinct from normal vessels [10]. Here, we have observed that doses of 10 µg and 100 µg do not induce any changes on tumor vessels density. However, we have observed differences in vessels shape and pericytes coverage. The blood vessels in the tumor sections of mice treated with 10 µg of IMQ were mostly large and twisted with an irregular shape and a thin wall, similarly to the control group. While in tumors treated with IMQ at a dose of 100 µg, vessels showed a more regular shape with a wide lumen and a well visible layer of pericytes adhering to their surface (pericyte coverage was approx. 40% higher compared to the control group). Tumor vessels appeared significantly more mature,

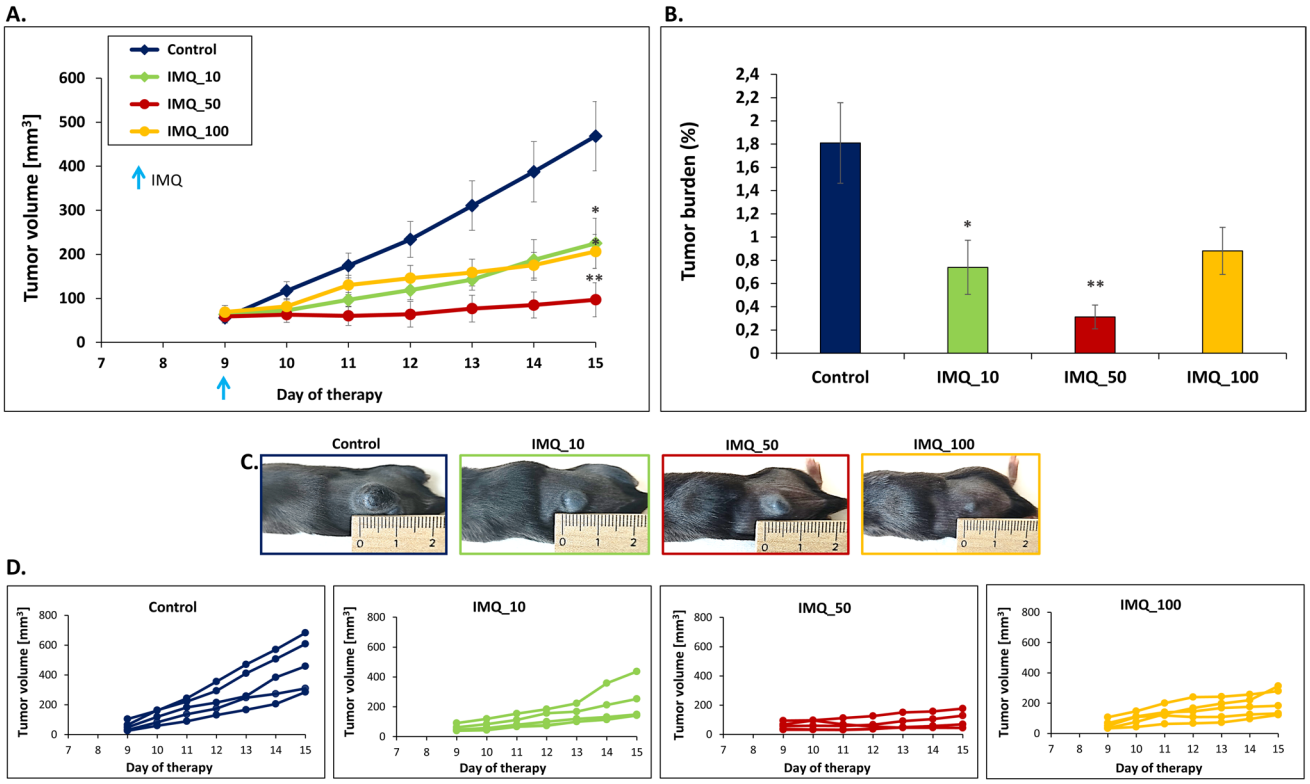

Fig. 1 Inhibition of B16-F10 tumor growth in response to imiquimod. Mice (n = 5; **A**–**D**) with well-developed tumors (~ 60–70 mm^3) were treated with different doses (10–100 µg/100 µl) of imiquimod. 50 µg of IMQ inhibits the growth of tumors the most efficiently (approx. 75%) compared to the control group. Tumor volume was measured with a caliper everyday (mean ± SEM) (**A**, **D**). Data are representative of three independent experiments, n = 5 in each group. **B** Diagram showing tumor burden on the last day of experiment. Each column in the graph represents a mean ± SEM. **C** Photographs were taken on the 15th day of therapy. **D** Individual tumor follow-up. Statistical analysis was performed on the last day of experiment (**A**) $*p < 0.05$ and $**p < 0.001$ compared to the control group; (**C**) $*p < 0.05$, $**p < 0.001$; evaluated by Tukey HSD test

defined by means of the percentage of vessels covered by perivascular cells (PVCs) an increase in the (called as "vascular maturation index" VMI; [8]). Following the injection of 50 µg, the vascular density was reduced by more than 40% and pericyte-covered blood vessels were increased, compared to the control group. The vessels were mostly small, with a thick wall and a regular shape (Fig. 2A–D).

Next, we evaluated how the changes in tumor vessels structure affected tumor perfusion. Functional vessels are detected after perfusion with lectin [8]. Following administration of FITC-labeled isolectin, we observed more than 2.5-times increase of perfused tumor blood vessels in mice treated with 50 µg of IMQ compared to mice treated with 10 µg or 100 µg of IMQ (Fig. 2B, E). This data indicates that imiquimod in a single dose of 50 µg normalized tumor vasculature both structurally (by reducing vessel tortuosity and increasing pericyte coverage) and functionally (by improving tumor perfusion).

Imiquimod reduces hypoxia and immunosuppression in murine melanoma tumors

Hypoxia is a key regulatory factor for tumor growth [5, 17]. Underoxygenation activates the formation of new blood vessels (angiogenesis), recruits immunosuppressive cells of the immune system and impairs T cell functions [5]. Increasing hypoxia and immunosuppression lead to the recurrence of cancer after therapy. In our study, we have evaluated underoxygenation regions and tumor immune infiltration in IMQ treated mice compared to the control group. Pimonidazole staining was conducted to visualize hypoxic regions in tumors. Tumor tissues were collected from mice after Hypoxyprobe™-1 injection. We observed that doses of 10 µg and 100 µg of IMQ slightly reduced hypoxic areas in tumors of treated mice (approx. 20% and 30% respectively). While, in tumors after 50 µg of IMQ treatment, hypoxia areas was approx. 10-times reduced compared to the control group (Fig. 3). Next, we examined the effect of IMQ doses on immunosuppression in tumors of treated mice. We

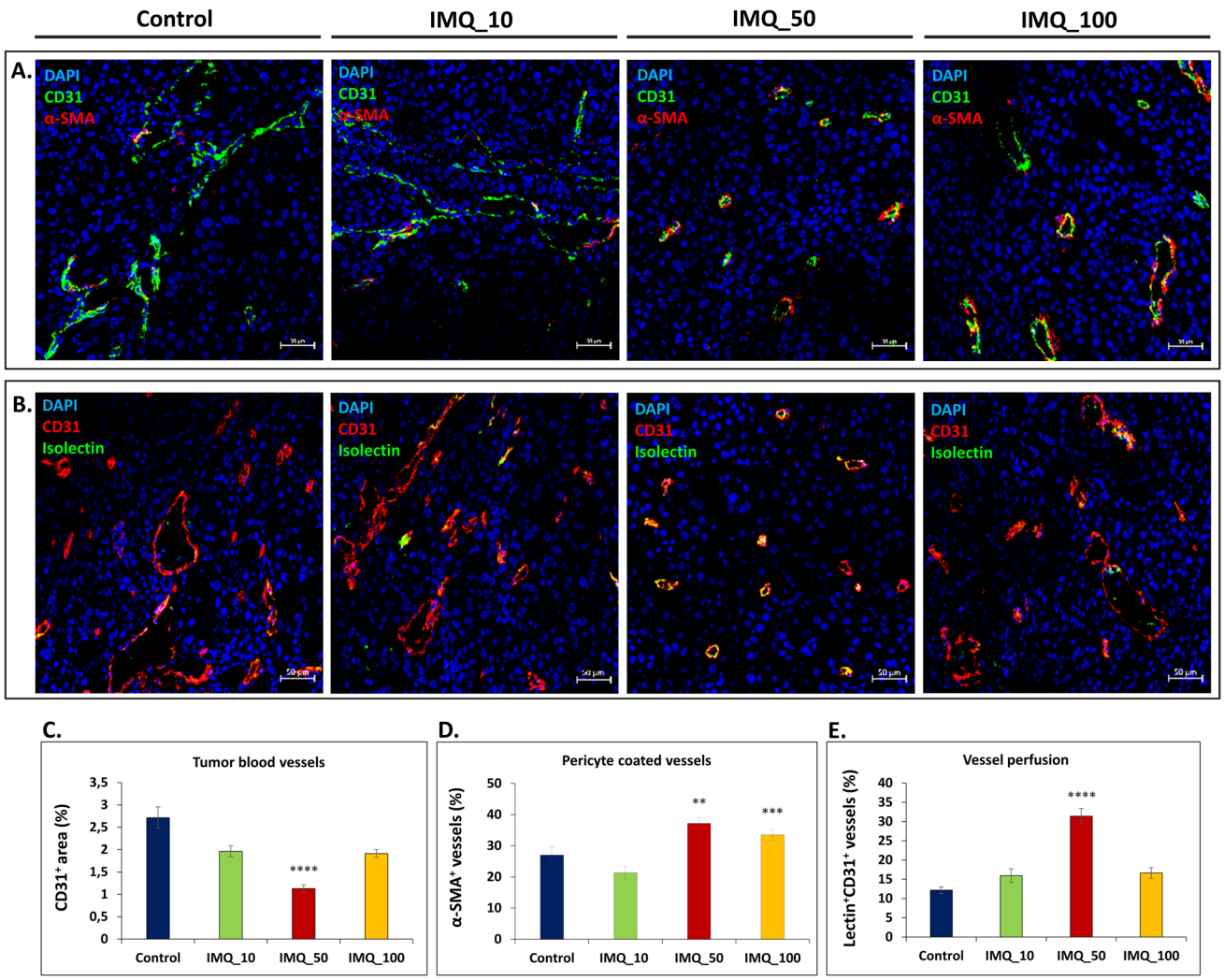

Fig. 2 The effect of different doses of imiquimod on tumor blood vessels. On day 15 of the therapy, mice were sacrificed and tumors were excised for immunohistochemical (IHC) staining. (**A**, **C**) CD31 staining was used to identify tumor blood vessels (10 optical fields were taken from each tumor section; 10 tumors of each group were visualized). **A**, **C**, **D** α-SMA and CD31 staining was used to identify pericyte-covered tumor vessels (α-SMA$^+$CD31$^+$ vessels, % of CD31$^+$ vessels; 10 optical fields were taken from each tumor section; 5 tumors of each group were visualized). **B**, **E** Lectin perfusion test was used to assess vessel permeability in tumors (lectin$^+$CD31$^+$ vessels (% of CD31$^+$ vessels); 10 optical fields were taken from each tumor section; 5 tumors of each group were visualized). Reduced tumor blood vessel density (**A**, **C**), increased the pericyte-covered blood vessels (**A**, **D**) and increased number of perfused blood vessels (**B**, **E**) were observed in tumor sections from mice treated with imiquimod in a single dose of 50 µg. **A**, **B** Representative photographs are shown. Nuclei stained with DAPI (blue), magnification 20×, scale bar represents 50 µm. Percentage of tumor area covered by blood vessels, pericyte coated vessels and lectin$^+$ vessels were calculated using ImageJ software. **C**–**E** Each column in the graph represents a mean ± SEM. **C** ****$p < 0.00001$ compared to other groups; **D** **$p < 0.01$ compared to IMQ_10 and control groups; ***$p < 0.001$ compared to IMQ_10 group; **E** ****$p < 0.00001$ compared to other groups; evaluated by Kruskal–Wallis with multiple comparison of mean ranks test

investigated the presence of cytotoxic CD8$^+$ T lymphocytes and regulatory T cells in the IHC sections obtained from tumors of treated and control mice. These lymphocytes are essential components of the anti-tumor immunity [1]. We observed the doubled number of tumor-infiltrating CD8$^+$ T lymphocytes in mice treated with 10 µg or 100 µg of IMQ compared to the control group. In contrast, 50 µg of IMQ increased the accumulation of CD8$^+$ T lymphocytes more than 3.5 times in tumors of treated mice (Fig. 4A, C). Additionally, the percentage of Ki67$^+$ CD8$^+$ T cells was twice as high compared to other groups (Fig. 4A, D). Moreover, following the injection of 50 µg dose, the number of Tregs in tumors of treated mice was decreased more than 3 times (Fig. 4B, E). In summary, a dose of 50 µg of IMQ had the greatest impact on the hypoxia area and level of tumor-infiltrating T lymphocytes.

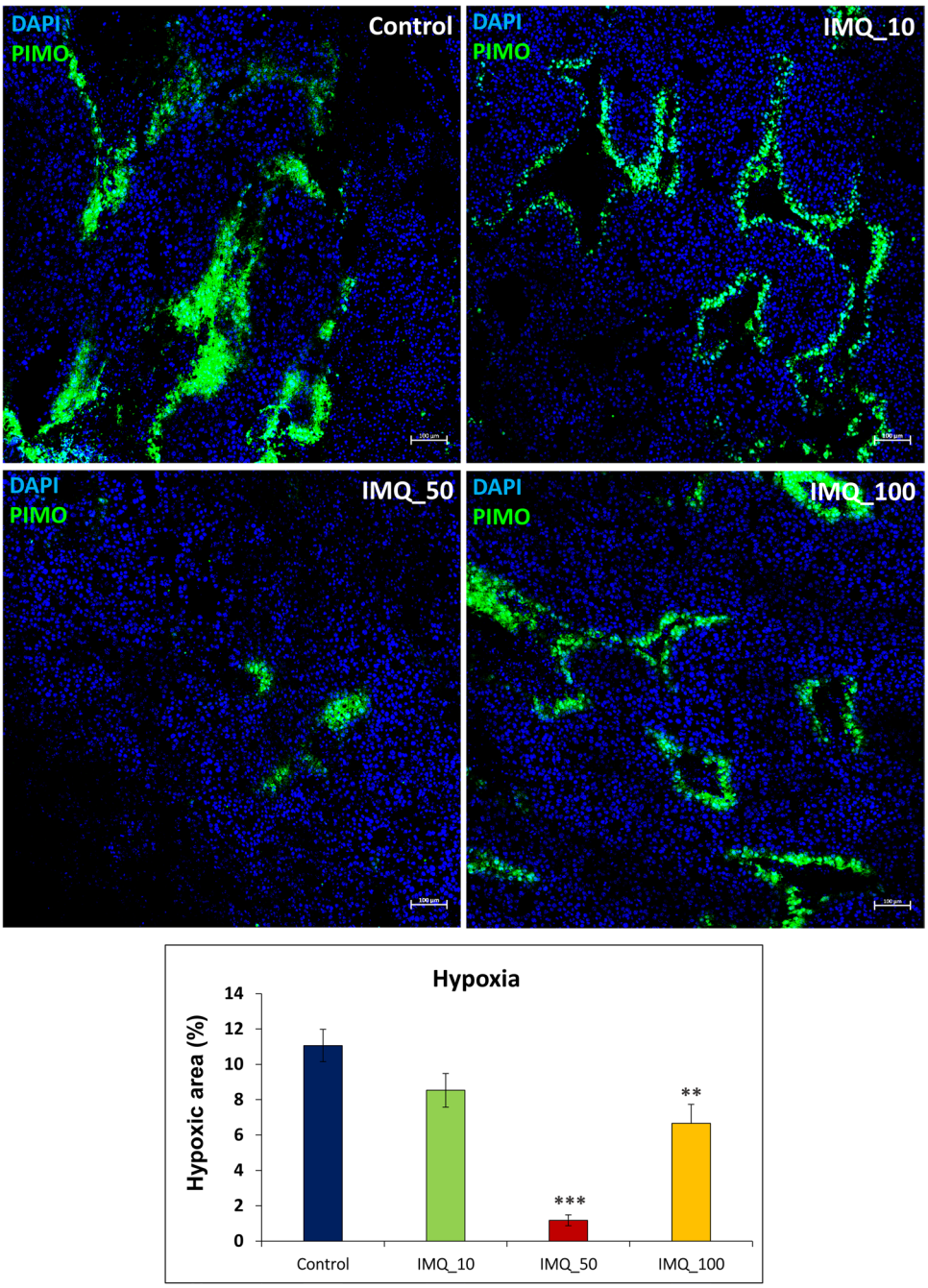

Fig. 3 Effect of different doses of imiquimod on tumor hypoxia in murine melanoma. Mice with well-developed tumors (60–70 mm^3) were treated with different doses (10–100 µg/100 µl) of IMQ. On the 15th day of therapy mice were sacrificed and tumors were excised for IHC staining. Staining with pimonidazole (PIMO) was conducted to visualize hypoxic regions in tumors (n = 5–6 in each group; 2 optical fields per every tumor section which one consist 9 adjacent optical fields of view joined together (summary 18 optical fields/tumor section); magnification 20×). The smallest area of hypoxia were found in tumors of mice treated with IMQ at a dose of 50 µg. Representative photographs which were taken as a scan of 9 adjacent fields at 20× magnification are shown. Nuclei stained with DAPI (blue), scale bar represents 100 µm. Percentage of tumor area covered by hypoxia regions was calculated using ImageJ software. Each column in the graph represents a mean ± SEM. ***$p < 0.001$ compared to other groups; **$p < 0.01$ compared to IMQ_50 and control groups; evaluated by Tukey HSD test

Imiquimod improves tumor penetration of intraperitoneally administered chemotherapy

Hypoxia increases chemotherapeutic drugs efflux and reduces drugs concentration in cancer cells [5]. Functional normalization of tumor blood vessels includes vascularity structural changes with an improvement in perfusion. It improves the delivery of chemotherapeutics to the tumor and as a result inhibits its growth [8]. Here we studied whether functional normalization of tumor blood vessels in murine melanoma using IMQ increases the effectiveness of chemotherapy. For this purpose, mice were treated with 50 µg IMQ and additionally with suboptimal dose of doxorubicin (2.5 mg/kg body mass, 3 times/week; [19]). There were no differences in body weights between the DOX-treatment and other groups of mice (data not shown). The combination of IMQ with low doses of doxorubicin (DOX) effectively inhibits the growth of melanoma tumors. We observed that the tumors of mice treated with the drug combination were more than three times and twice as small (172 mm^3 on the 23th day of therapy) as those treated

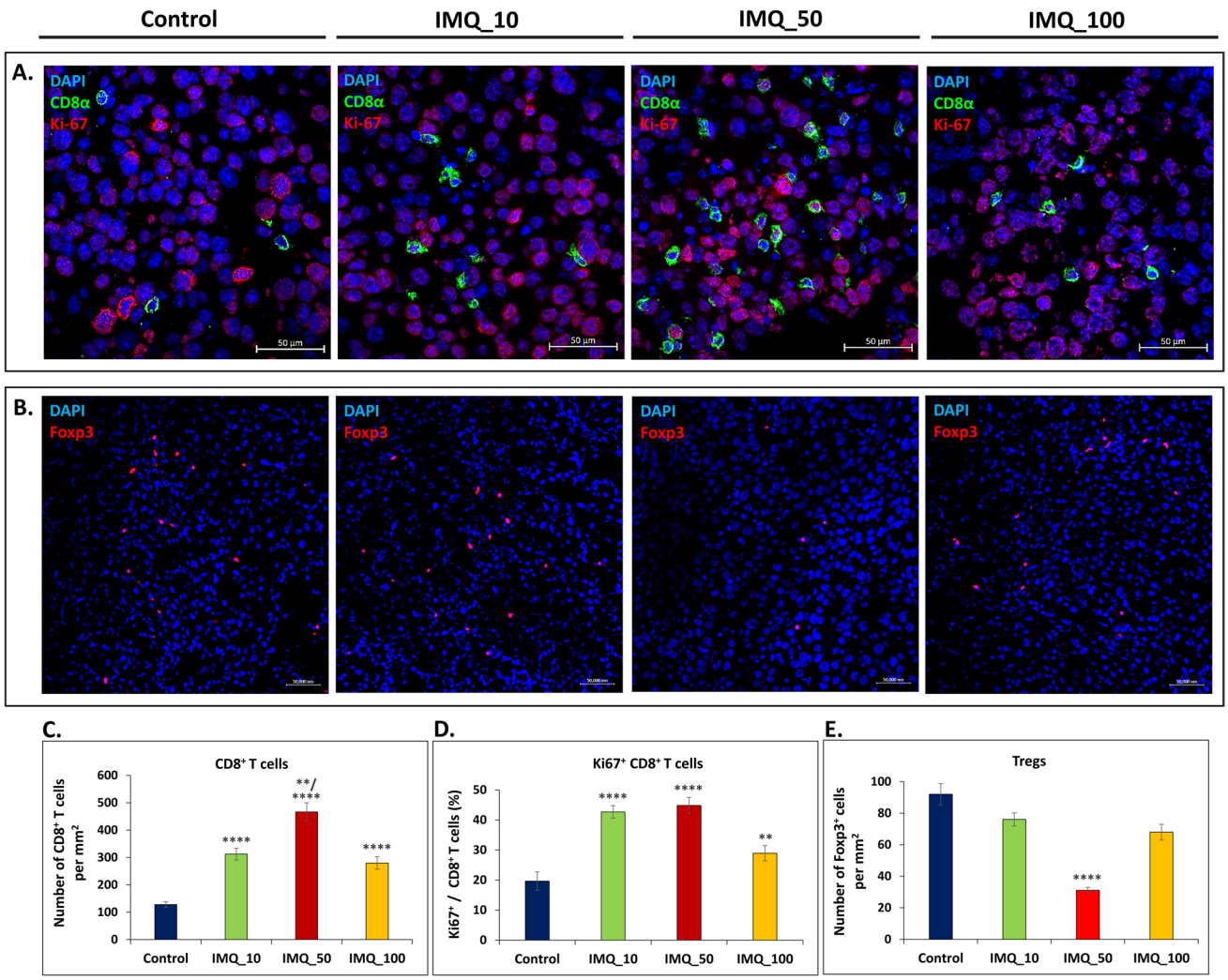

Fig. 4 The effect of imiquimod on the tumor-immune cells infiltration. Six days after IMQ treatment, mice were sacrificed and tumor were collected for IHC. **A**, **C**, **D** Tumor cross-sections were stained with an antibody against CD8a and Ki67 (7–11 optical fields were taken from each tumor section; 5–6 tumors of each group were visualized; magnification 20×) and **B**, **E** also Foxp3 (10–11 optical fields were taken from each tumor section; 5 tumors of each group were visualized; magnification 40×). IMQ at a 50 µg dose has the greatest impact on the level of T lymphocytes in B16-F10 tumors: the number of proliferating ($Ki67^+$) $CD8^+$ T cells increased more than two times (**A**, **D**), while the number of Tregs ($Foxp3^+$) was more than three times lower (B, E) compared to the control group. (A, B) Representative photographs are shown. Nuclei stained with DAPI (blue), scale bar represents 50 µm. The number of immune cells was counted in high power fields (HPF) and calculated per 1 mm^2. (C-E) Each column in the graph represents a mean ± SEM. **C** ****$p < 0.00001$ compared to control group; **$p < 0.01$ compared to IMQ_10 and IMQ_100 groups/ ****$p < 0.00001$ compared to control group; **D** ****$p < 0.00001$ compared to control group; **$p < 0.01$ compared to IMQ_10 and IMQ_50 groups; **E** ****$p < 0.00001$ compared to other groups; evaluated by Kruskal–Wallis with multiple comparison of mean ranks test

with each drug alone (631 mm^3 in DOX group and 427 mm^3 in IMQ_50 group, respectively) and more than six times smaller compared to the control group (1111 mm^3 on the 23th day of therapy; Fig. 5A, C, D). Significantly delayed tumor growth prolonged survival of treated mice (Fig. 5B). These data indicate that vascular normalization improves the effectiveness of used suboptimal dose of chemotherapeutic.

Imiquimod inhibits angiogenesis in vivo

In previous analyses, we have shown that imiquimod reduces the number and condition of tumor blood vessels, in a dose-dependent manner. In the final stage of the study, we assessed whether the reduced number is due to the antiangiogenic properties of imiquimod. A drug that affects tumor vasculature can act in two ways: by inhibiting the formation of new vessels or by destroying existing ones [20]. For

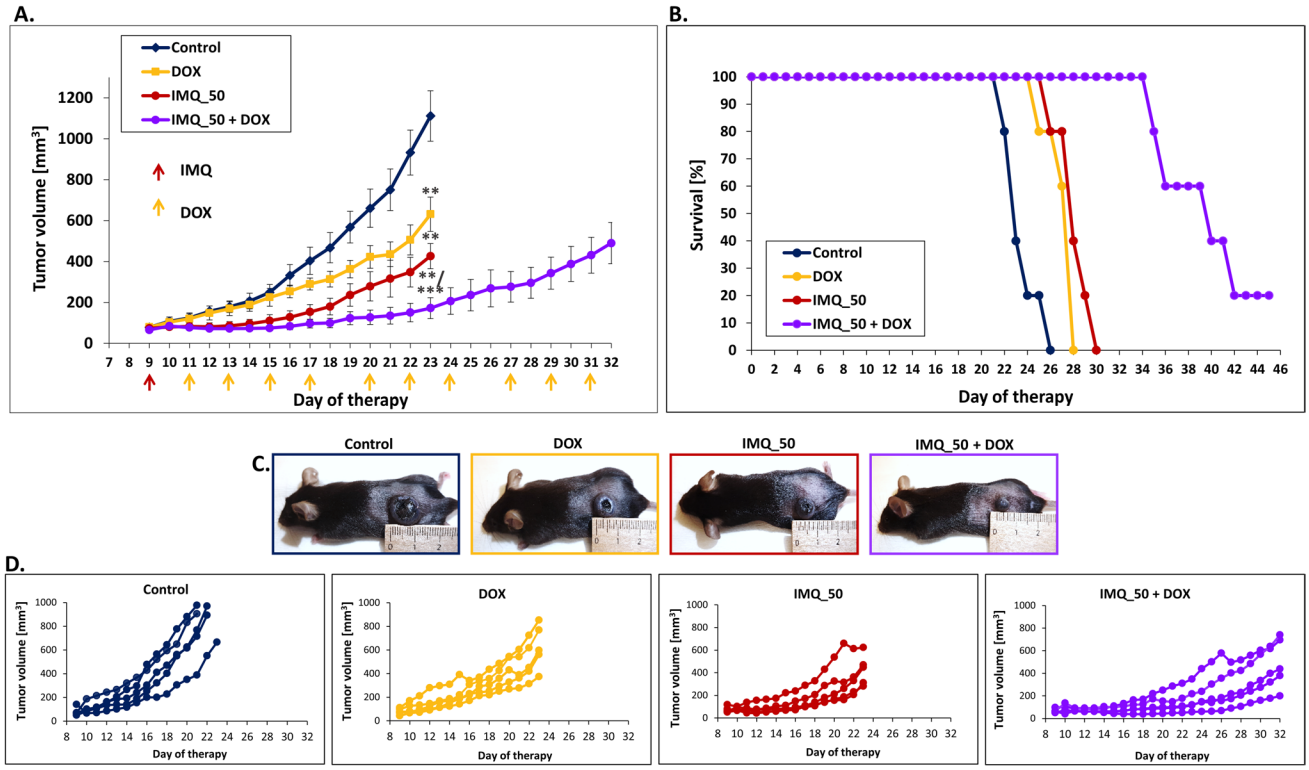

Fig. 5 Inhibition of B16-F10 tumor growth in response to combination imiquimod with chemotherapy. Mice with well-developed tumors (~70–80 mm^3) were treated with combined therapy. Nine days after inoculating mice with B16-F10 cells, IMQ at a dose of 50 µg was once injected directly into tumors. DOX was delivered intraperitoneally at a dose of 2.5 mg/kg, 3 times/week. We observed inhibited growth of tumors treated with the combination of drugs, compared to monotherapy. Tumor volume was measured with a caliper everyday (mean ± SEM) **A** Data are representative of two independent experiments, n = 5 in each group. **B** Animal survival curves of tumor-bearing mice. **C** Photographs of mice were taken on day 22 of the therapy. **D** Individual tumor follow-up. Statistical analysis was performed on the 23th day of experiment. $^{**}p < 0.01$ compared to control group; $^{***}p < 0.001$ compared to DOX group/ $^{**}p < 0.01$ compared to control group; evaluated by Tukey HSD test

this purpose, we performed an in vivo angiogenesis assay using Matrigel™ plugs to confirm antiangiogenic properties of IMQ. After injecting FITC-conjugated isolectin, we visualized the formed vascular network in plugs using a confocal microscope [21]. Vessel density was significantly reduced after dose of 50 µg of IMQ, as compared with Matrigel™ plugs from other tested doses and the control group (0.13% area vs 0.28% area in IMQ_10 group, 0.36% in area IMQ_100 group and 0.68% area in control). These data indicate that imiquimod inhibits neovascularization by suppress angiogenesis process in treated mice (Fig. 6).

Materials and methods

Cell line

The murine melanoma IVISbrite™ B16-F10 Red F-luc tumor cell line (B16-F10; PerkinElmer, MA, USA) was maintained using RPMI (Biowest, Nuaillé, France) supplemented with 10% heat-inactivated fetal bovine serum (Eurx, Gdańsk, Poland) and 1% penicillin–streptomycin (Biowest). The cell cultures were passaged twice a week and cultured under standard conditions (37°C, 5% CO_2, 95% humidity).

Mice and ethic statement

Mice (six-to-eight-week old) C57Bl/6NCrl females (Charles River Breeding Laboratories, Wilmington, MA, USA) were bred in Maria Sklodowska-Curie National Research Institute of Oncology, Gliwice Branch (Poland) in a HEPA-filtered Allentown's IVC System (Allentown Caging Equipment Co, NJ, USA). All efforts were made to minimize animal suffering by qualified personal [22]. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocols were approved by the Committee on the Ethics of Animal Experiments of the Local Ethics Commission (Medical University of Silesia, Katowice, Poland) (Permit Number: 21/ 2020, 50A/ 2023, 50B/

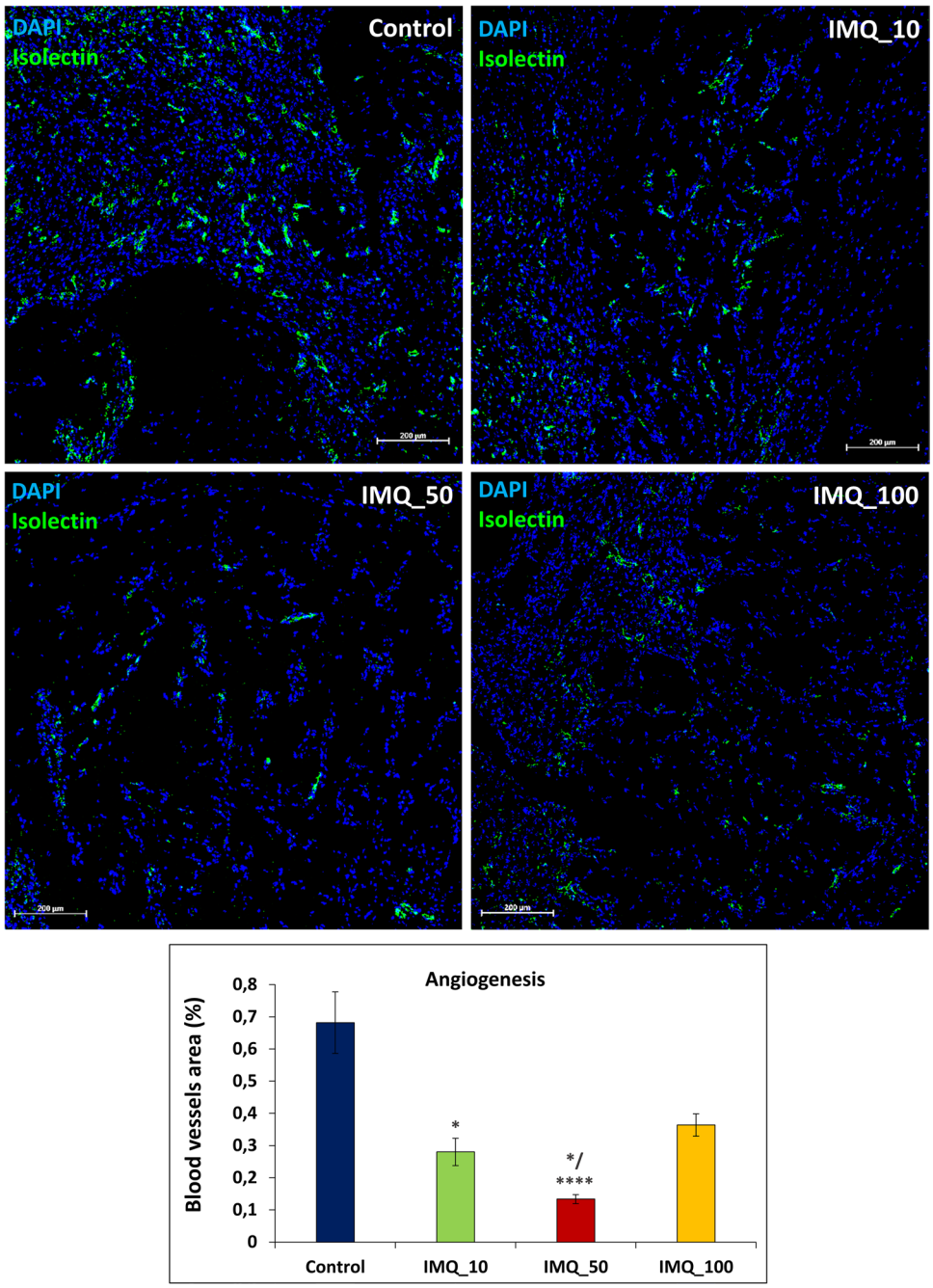

Fig. 6 Suppression of angiogenesis by imiquimod. Growth factor reduced Matrigel™ with recombinant mouse fibroblast growth factor and different doses of IMQ was implanted into mice. Seven days later, intravenous perfusion with FITC-labeled isolectin B4 was used to label endothelium in Matrigel™ plugs (lectin$^+$ vessels, 2–3 optical fields per tumor section which one consist 9 adjacent optical fields of view joined together; 5–6 plugs of each group were visualized, magnification 20×). Neovascularization was significantly reduced in Matrigel™ plugs with imiquimod, compared to control group (approx. twice when compared 10 µg and 100 µg of IMQ to control group and five times when compared 50 µg of IMQ to control group). A single dose of 50 µg of IMQ has the most antiangiogenic effect, as compared to the group of control mice. Representative photographs which were taken as a scan of 9 adjacent fields at 20× magnification are shown. Nuclei stained with DAPI (blue), scale bar represents 200 µm. Each column in the graph represents a mean ± SEM. *$p < 0.05$ compared to IMQ_50 and control groups; *$p < 0.05$ compared to IMQ_10 and IMQ_100 groups/****$p < 0.00001$ compared to control group; evaluated by Kruskal–Wallis with multiple comparison of mean rank test

2023, 50D/ 2023). Mouse were inoculated with B16-F10 tumor cells and were treated as described above. Mice whose tumor size exceeded 1 cm in any dimension (1.2 cm in individual cases) were sacrificed by cervical dislocation [22]. During the experiments we observed no side effects of conducted therapy (the BCS was ≥ 4). Procedures were terminated by cervical dislocation and tumor collection for immunofluorescence analysis.

Inoculation of animals and therapeutic agents

C57Bl/6NCrl mice were injected subcutaneously with 2×10^5 B16-F10 cells in 100 µl PBS. Growing tumors were measured with calipers and tumor volumes were determined using the formula: volume = width^2 × length × 0.52 [22]. After 9–10 days appropriate therapy was performed. A liquid formulation of the toll-like receptor 7 agonist

imiquimod (IMQ; Imiquimod VacciGrade™ (R837), InvivoGen, Toulouse, France), dissolved in sterile water (acc. the manufacturer's specifications), was injected directly into tumors at a dose of 10 µg, 50 µg or 100 µg in 100 µl. Control mice received sterile water directly into tumors. Doxorubicin-Ebewe (2 mg/ml, EBEWE Pharma Ges.m.b.H. Nfg. KG Austria) was delivered intraperitoneally at a dose of 2.5 mg/kg, 3 times/week [19].

Immunohistochemical analysis of tumor vasculature and areas of hypoxia

For immunohistochemical analyses of tumor blood vessels excised tumors were embedded in Leica OTC Tissue Freezing Medium (Leica Biosystems, Wetzlar, Germany) and frozen in liquid nitrogen. Tumors were sectioned into 5 µm slices. Sections were incubated with anti-α-Smooth Muscle Actin (αSMA, Cell Signaling Technology, MA, USA) and anti-CD31 antibodies (Abcam, Cambridge, UK) and subsequently with Texas Red and FITC conjugated secondary antibodies (Vector Laboratories, CA, USA) respectively for pericytes coating vessels assessment [19]; hypoxic regions in tumors were analyzed by Hypoxyprobe™-1 kit (Hypoxyprobe, Inc., MA, USA) following the manufacturer's instructions. For assessment of blood vessels functionality in tumors, FITC-conjugated lectin (100 µg/100 µl, *Lycopersicion esculentum*, Vector Laboratories) was injected into mouse tail vein and allowed to circulate for 15 min prior 4% PFA perfusion. Subsequently tumors were excited, embedded in OCT and frozen in liquid nitrogen. Sections were incubated with anti-CD31 antibody and subsequently with Alexa Fluor 594 conjugated secondary antibody (Vector Laboratories). Sections were mounted in VECTASHIELD Mounting Medium with DAPI (Vector Laboratories). The results were expressed as percentage of area [%] calculated by ImageJ 1.48v where applicable (NIH, Bethesda, MD, USA).

Immunohistochemical analysis of immune cells

Tumors were harvested and frozen in OCT and liquid nitrogen. Immune cells were stained with anti-CD8α (BD Biosciences, NJ, USA) and anti-Ki67 (Cell Signaling Technology, MA, USA) or anti-Foxp3 (Invitrogen, MA, USA) antibodies and subsequently with appropriate secondary antibodies (Vector Laboratories). Sections were mounted in VECTASHIELD Mounting Medium with DAPI. The results were expressed as the number of immune cells which were counted in HPF and calculated per 1 mm^2. Microscopic observations were performed using a LSM710 confocal microscope (Carl Zeiss Microscopy GmbH, Jena, Germany).

Angiogenesis assay

Mice were injected subcutaneously, near the abdominal midline, with 500 µl of growth factor reduced Matrigel™ (BD Pharmingen, NJ, USA) containing 0.4 µg/ml of recombinant mouse fibroblast growth factor-basic (R&D Systems, Inc, MN, USA) [23] and imiquimod at a dose of 10 µg, 50 µg or 100 µg in 100 µl. After 7 days, fluorescein labeled *Griffonia simplicifolia* lectin I, Isolectin B4 (50 µg/100 µl; Vector Laboratories) was injected into mouse tail vein and allowed to circulate for 25 min prior 4% PFA perfusion. Then, Matrigel™ plugs were excised and frozen in OCT and liquid nitrogen. Plugs were sectioned into 5 µm slices and counterstained with DAPI (Sigma-Aldrich) [21]. The results were expressed as percentage of area [%] calculated by ImageJ 1.48v where applicable.

Statistical analysis

The normality of distribution was verified by the Shapiro–Wilk test. The homogeneity of variance was verified by the Levene test or the Brown-Forsythe test. For variables meeting the conditions of parametric tests, analysis of variance (ANOVA) with post-hoc Tukey HSD or LSD tests was performed. For variables not meeting the conditions of parametric tests, the Kruskal–Wallis with multiple comparison of mean rank test was performed Variables are shown as mean ± SEM. All statistical comparisons were performed using Statistica 13 software.

Discussion

Imiquimod (IMQ), a TLR7 agonist [11], is used as an antiviral and anti-allergic agent [13]. The FDA approved IMQ in a 5% topical formulation (Aldara) for the treatment of external genital warts, superficial basal cell carcinoma and actinic keratosis [15]. It has also been used off-label to treat patients with skin cancers [16]. We focused on the anticancer properties for IMQ repurposing. Drug repurposing is a promising strategy that has become attention among researchers. This approach includes the identification of new applications for clinically approved drugs. Advantages of this strategy among others include: cost-effectiveness, confirmed safety and accelerated clinical trial process in a new therapeutic indication [24, 25]. Clinical trials indicate the antitumor effect of TLR7 [12, 14]. The results of the literature data show the anticancer effects of IMQ in the form of cream [11]. Commercially, IMQ is available as an oil-in-water-based 3.75–5% varnishing cream in sachets [16]. The molecular weight of imiquimod is 240.31 kDa, therefore it is an appropriate drug for transdermal administration (the

molecular weight of the drug cannot exceed 350 kDa). When given cutaneously, it induces tumor-specific $CD8^+$ T cytotoxic lymphocytes response [13]. Hirobe et al. [26] have shown that the administration of IMQ enhanced B cell differentiation into plasma cells and germinal center B cells in the draining lymph nodes and spleen. There are also attempts to encapsulate IMQ into micelles which exhibit precise TME targeting [27] and IMQ-loaded nanoparticles to evaluate antitumor activity [28, 29]. Dias et al. [28] have noticed that despite the advantages of IMQ topical use (easy application, less pain, reduced cost) there are some limitations (low skin permeation, adverse effects encompassing skin reactions). A liquid form of IMQ is tested in a phase 2 of clinical trials. This novel formulation of IMQ called TMX-101 (Vesimune) is tested in the treatment of bladder cancer. TMX-101 is optimized for intravesical delivery and the results are promising [30]. We also used a liquid form of IMQ. We observed tumor growth inhibition of murine melanoma in a dose depended manner. A dose of 50 µg of IMQ showed the therapeutic antitumor efficiency but dose escalation did not improve the antitumor potential of TLR7 agonist. Cytotoxic effect of IMQ on B16-F10 melanoma cells and also on normal endothelial cell lines was observed in vitro (data not shown). Chang et al. [31] showed, that IMQ induces mitochondrial-mediated apoptosis and autophagic cell death in murine and human basal cell carcinoma and melanoma cell lines. Authors demonstrated that IMQ-induced reactive oxygen species (ROS) accumulation causes lysosomal membrane permeabilization and caspase-8 activation which promotes lysosomal cell death [31].

In our study, we focused on effect of different IMQ doses on the blood vessels condition in tumors. Tumor vasculature plays main role in tumor progression. Tumor blood vessels differ structurally and functionally from normal vessels. Immature and unstable vessels, irregular vascular branching, heterogeneous flow, collapsed vessels lumens, leakiness and low perfusion cause hypoxia high IFP and an acidic microenvironment. These processes promote escape of tumor cells from the attack by the immune system, tumor progression and chemo- and radiotherapy resistance [3, 10]. We assessed structurally and functionally tumor blood vessels of IMQ-treated mice. We observed that after a single dose of IMQ treatment, tumor vascular density was reduced and pericyte-covered blood vessels were increased. The vessels were mostly small, with a thick wall and a regular shape. We observed a higher number of perfused blood vessels. Our data indicate that the dose of 50 µg of IMQ caused the most visible changes in condition of tumor blood vessels. Tumor vasculature was structurally and functionally normalized: vessel shape was more regular, tortuosity was reduced, pericyte coverage was increased and tumor perfusion was improved. The influence of IMQ on tumor blood vessel condition was demonstrated for the first time by these results.

Improving supportive functions of blood vessels leads to better oxygenation of the tumor. Underoxygenation is a key regulatory factor for tumor growth. It reduces oxygen-dependent DNA damage and induces HIF-1α-mediated cell survival. Hypoxia activates the formation of tumor angiogenesis and leads to tumor progression [5, 17]. In tumors of IMQ-treated mice we observed reduced areas of hypoxia. After treatment with 50 µg of IMQ, where tumor vessels were normalized, hypoxia areas was approx. ten times reduced compared to control group. There is a correlation between the presence of hypoxia in the tumor and immunosuppression. Hypoxia induces an inflammatory reaction similar to the one present in damaged tissues. It recruits cells of the immune system, which are reprogrammed in a specific way toward pro-tumor phenotypes. Hypoxia also impairs T cell functions. Chen et al. [5] showed that tumor $CD8^+$ and $CD4^+$ T cells infiltration is significantly diminished in hypoxic areas. Underoxygenation stimulates cancer cells to express chemokines that recruit regulatory T cells. Induced HIF-1α promotes Treg polarization and significantly contributes to cancer development and progression [5]. Moreover, Luo et al. [10] pointed out a strong correlation between an increase $CD8^+$ T lymphocytes infiltration and vascular normalization in tumors, which may be a potential biomarker to choose "vascular normalizing" therapy. We observed the highest number of cytotoxic $CD8^+$ T lymphocytes in more oxygenated tumors with more regular normalized structure of vasculature. The number of tumor-infiltrating immunosuppressive regulatory T cells was decreased. There is a lot of data confirming the immunostimulatory properties of IMQ. The effect of TLR7 is pleiotropic, stimulating both innate (monocytes/macrophages; dendritic cells, NK/NKT cells) and adaptive (T cells, B cells) immune responses [11, 16, 32]. IMQ stimulates macrophages survival, induces DC maturation and migration to lymph nodes, upregulates costimulatory receptors in an antigen-presenting cell (APC) and enhances antigen presentation to T cells [16]. IMQ also promotes Th1 skewing and increased T lymphocytes infiltrating to the tumor, in part by the indirect effect of type I IFN derived from plasmacytoid DC (pDC) activated by IMQ [33]. In addition, it reduces the level of regulatory T lymphocytes and the level of CCL22 cytokine [14]. IMQ has an impact on synthesis of pro-inflammatory molecules: IFN-α, IFN-γ, TNF-α, IL-1a, IL-2, IL-6, IL-8, IL-10, IL-12, G-CSF and GM-CSF via macrophages and DC. IMQ also upregulates CCL5, CXCL9 and CXCL10 for homing T lymphocytes [16]. Unlike Maulodin et al. [34] we noticed decreased CXCL10 levels. In the serum of mice treated with IMQ, we observed statistically significant three-fold

reduction in the level of CXCL10 (IP-10) in mice treated with a single dose of 50 µg, compared to the blood of control group mice (data not shown). IP-10 is a pleiotropic cytokine which may recruits immunosuppressive $CXCR3^{+}Foxp3^{+}$ regulatory T cells in tumors [35]. Taken together, IMQ decreased hypoxic regions and immunosuppression in mice.

Abnormal tumor blood vessels cause increase of tumor hypoxic and necrotic regions, high interstitial fluid pressure and substantially decrease anticancer drugs penetration [10]. This significantly reduces the effectiveness of used therapies. Therefore, one of the therapeutic methods aimed at tumor blood vessels is their normalization [8, 9]. It involves the use of antiangiogenic drugs in treatment regimens and doses that not only inhibit the angiogenesis process but also transform the vessels into more similar to normal ones. The vessels become more tight, with a thick wall covered with pericytes and an open lumen. This leads to oxidation of the surrounding tumor tissue, reducing areas of hypoxia and IFP inside the tumor. As a consequence, there is a better tumor penetration by the chemotherapeutic drugs [7, 10]. As part of our research, we used an immunostimulating drug IMQ, which, as our results showed, can also be used as a drug targeting tumor blood vessels. In a properly selected dose, it transforms the vessels into more normal ones, reduces areas of hypoxia and immunosuppression in tumors. This, in turn, leads to better control of tumor growth. We used the acquired knowledge to combine the selected dose of IMQ with a chemotherapeutic agent widely used in the clinic. Doxorubicin (DOX) have been approved by the FDA for the treatment of various cancers and is currently widely used in clinic. DOX is recognized as one of the most efficient chemotherapeutics [36]. We used suboptimal doses of DOX to reduce its toxicity and combine with IMQ for improvement of the effectiveness of low doses DOX. In our previous study, we showed that combination of normalized therapy (endoglin-based DNA vaccine with IL-12) increases antitumor effect of low doses of doxorubicin and significantly inhibits tumor growth [22]. In this study, we observed murine melanoma tumors growth inhibition after combined therapy with IMQ and DOX. Synergistic effect in controlling tumor growth prolonged survival of treated mice. This knowledge can be used in the future to combine selected drugs in the clinic. Wen et al. [27] proposed precise delivery of doxorubicin and imiquimod encapsulated in dual pH-sensitive and TME-active targeting micelles. The authors demonstrated that micelles with drugs were selectively toxic to cancer cells and have changed the tumor microenvironment toward immunostimulatory one, resulting in tumor growth inhibition [27].

Reduction of the number of tumor blood vessels may be the result of the action of two classes drugs: antiangiogenic (inhibiting the formation of new vessels) and antivascular (destroying existing ones) [20]. The tumor may become resistant to the use of any of these drugs, therefore new possibilities of using drugs already known and used in clinical application are being sought. As we previously mentioned, normalization with antiangiogenic drugs is one of these methods. As part of our analyses, we used the in vivo Matrigel™ plug assay and FITC-labeled isolectin B4 for endothelium staining [21]. We observed significantly reduced neovascularization by suppressed angiogenesis process in Matrigel™ plugs with imiquimod, compared to control group. Liotti et al. [37] used in vitro Matrigel™ plug assay with HUVEC cells to formation of capillary-like tube structures to measure angiogenesis process. The authors indicate that TLR7 regulates the angiogenic potential of NSCLC cells. Majewski et al. [38] reported inhibition of tumor cells-induced formation of new blood vessels by IMQ in vivo. The authors showed that using 5% IMQ in the form of a cream, mediated antiangiogenic effect of IMQ due to secretion of IL-18 [38]. Dias et al. [28] used IMQ-loaded nanoparticles to evaluated antiangiogenic activity of IMQ in the chicken embryo chorioallantoic membrane (CAM). In the group treated with IMQ macrovessels are absent and the number of small blood vessels was significantly reduced compared to control group. Our results provide new data about IMQ that by selecting the appropriate dose, we can not only inhibit the formation of new blood vessels (angiogenesis) but also normalize tumor blood vessels leading to reduction of areas of hypoxia and immunosuppression and increase of the effectiveness of antitumor low doses chemotherapy. This knowledge can be translated into the clinic to use IMQ not only as an immunostimulating drug but also as an antiangiogenic, blood vessels normalizing one when used in appropriate doses. Additionally, IMQ can be combined with chemotherapy or radiotherapy to increase the anticancer effect [27, 33, 39]. Cho et al. propose the use of imiquimod as a radiosensitizer and immune booster during radiotherapy in patients with melanoma [40]. Zhao et al. [39] designed a TLR7 agonist-based nanodrug which up-regulated ICAM-1 expression and inhibited the growth of nonirradiated tumors when combined with RT. After systemic delivery IMQ-based nanoparticles enhances the abscopal effects of RT. It should be remember that normalization of tumor blood vessels and improvement of tumor oxygenation may also lead to further tumor growth and recurrence. While selecting IMQ for new therapeutic applications, its effect on tumor blood vessels should be taken into account. High dose of antiangiogenic agent may has negative consequences due to its anti-vascular effects resulting in the promotion of tumor hypoxia which is a key of aggressive phenotype and tumor therapy resistance [2, 8]. We observed that higher dose of IMQ (100 µg) showed worse effects than lower doses including decreased tumor perfusion, increased tumor hypoxia and immunosuppression. Therefore, knowing the mode of

action of the drug, determination of the tumor blood vessels normalization window is necessary to effectively combine the action of an antiangiogenic drug with chemotherapy (better tumor penetration) or radiotherapy (better tumor oxygenation). The repurpose of IMQ in the proposed new therapeutic indication (as an IMQ-based normalization strategy) indicates significant opportunities in anticancer therapy.

In summary, we have shown that the immunostimulatory TLR7 agonist – IMQ, which has also antiangiogenic properties, changes tumor vasculature. A dose of 50 µg normalizes tumor blood vessels both structurally and functionally, leads to inhibition of murine melanoma tumor growth and improves the effectiveness of low doses of doxorubicin. Our data may help to expand the potential of imiquimod clinical application in the treatment of cancer patients. Normalizing of tumor vasculature by IMQ is a new proposal for anti-cancer TLR-7 agonist therapeutic indication. In the future, combining repurposed IMQ with first-line anticancer strategy may create new opportunities in cancer treatment.

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Author contributions M.J.-B. conceptualized and supervised the study. M.J.-B., J.Cz., J.C., R.S., A.D., D.S.-L., E.P., S.M., T.C. performed experiments and analyzed data. T.C. provided assistance with in vivo studies. M.J.-B. drafted the original manuscript. All authors revised and approved the manuscript.

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Data availability No datasets were generated or analysed during the current study.

Declarations

Conflict of interest The authors declare no competing interests.

Ethical approval This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. Studies on mice were performed according to the protocols approved by the Local Ethics Committee for Animal Experiments in Katowice (Permit Number: 21/2020, 50A/2023, 50B/2023, 50D/2023).

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