




## ORIGINAL ARTICLE

# The myeloid cell type I IFN system promotes antitumor immunity over pro-tumoral inflammation in cancer T-cell therapy

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2021; 10: e1276**Abstract**

**Objectives.** Type I interferons are evolutionally conserved cytokines, with broad antimicrobial and immunoregulatory functions. Despite well-characterised role in spontaneous cancer immunosurveillance, the function of type I IFNs in cancer immunotherapy remains incompletely understood. **Methods.** We utilised genetic mouse models to explore the role of the type I IFN system in CD8<sup>+</sup> T-cell immunotherapy targeting the melanocytic lineage antigen gp100. **Results.** The therapeutic efficacy of adoptively transferred T cells was found to depend on a functional type I IFN system in myeloid immune cells. Compromised type I IFN signalling in myeloid immune cells did not prevent expansion, tumor infiltration or effector function of melanoma-specific Pmel-1 CD8<sup>+</sup> T cells. However, melanomas growing in globally (*Ifnar1*<sup>-/-</sup>) or conditionally (*Ifnar1*<sup>ΔLysM</sup>) type I IFN system-deficient mice displayed increased myeloid infiltration, hypoxia and melanoma cell dedifferentiation. Mechanistically, hypoxia was found to induce dedifferentiation and loss of the gp100 target antigen in melanoma cells and type I IFN could directly inhibit the inflammatory activation of myeloid cells. Unexpectedly, the immunotherapy induced significant reduction in tumor blood vessel density and whereas host type I IFN system was not required for the vasculosculting, it promoted vessel permeability. **Conclusion.** Our results substantiate a complex and plastic phenotypic interconnection between melanoma and myeloid cells in the context of T-cell immunotherapy. Type I IFN signalling in myeloid cells was identified as a key regulator of the balance between antitumor immunity and disease-promoting inflammation, thus supporting the development of novel combinatorial immunotherapies targeting this immune cell compartment.

**Keywords:** adaptive resistance, cancer immunotherapy, hypoxia, melanoma plasticity, myeloid cells, type I interferon

## INTRODUCTION

Interferons (IFNs) are evolutionarily conserved vertebrate cytokines and were originally discovered because of their interfering effects on viral infections in the 1950s. IFNs are now recognised as a large family of antimicrobial and immunostimulatory cytokines belonging to three different subclasses.<sup>1,2</sup> The role of type I IFNs (predominantly IFN- $\beta$  and members of IFN- $\alpha$ ) in immunity has been extensively studied and has emerged pleiotropic and context dependant. Indeed, type I IFNs have been used in the treatment of both autoimmune multiple sclerosis (MS)<sup>3</sup> and malignancies such as melanoma, where especially ulcerated tumors have been reported to respond well to the therapy.<sup>4,5</sup>

Stemming from research carried out primarily in infection models, IFN- $\alpha$  is considered to be an important 'third signal' in naïve T-cell activation,<sup>6</sup> to promote T-cell expansion, survival and memory differentiation<sup>7,8</sup> and to inhibit regulatory T-cell activation and proliferation.<sup>9</sup> Type I IFN is also an important mediator of spontaneous cancer immunoediting, and it may function primarily through its effects on host immune cells.<sup>10</sup> Consistent with this notion, type I IFN has been reported to have several immunostimulatory functions, which include the activation of dendritic cells,<sup>11</sup> NK cells<sup>12</sup> and T cells.<sup>13</sup> Interestingly, while type I IFNs are produced by and act on almost all nucleated cell types, the signalling is under tight temporal regulation. Concomitant (or slightly delayed) signalling relative to TCR engagement promotes T-cell proliferation, survival and effector cell differentiation. However, premature type I IFN signal can result in T-cell growth arrest or apoptosis.<sup>14</sup> Indeed, DNA vaccinations were reported to be twice as efficient in expanding CD8<sup>+</sup> T cells specific for class I-restricted epitopes in the absence of a type I IFN stimulus.<sup>15</sup> Moreover, several studies have suggested that prolonged type I IFN signalling *in situ* may promote immunosuppressive PD-L1, IL-10 and NOS2 signalling, thus suppressing T-cell-mediated clearance of chronic viral infections<sup>16,17</sup> and cancer.<sup>18,19</sup>

The sequential melanoma regression, remission and relapse phases frequently observed upon adoptive T-cell transfer (ACT) immunotherapy resemble spontaneous cancer immunoediting process. Herein, the host immune system sculpts

tumors resulting in cell variants with acquired genetic loss of antigens (or their presentation machinery) or higher immunosuppressive or immuno-evasive potential. Previously, we identified an additional, dynamic mechanism of resistance, where melanomas can escape ACT immunotherapy targeting the melanocytic lineage antigen gp100 via inflammation-induced dedifferentiation.<sup>20</sup> Indeed, the plasticity of tumor and immune cell phenotypes likely represents a significant source of non-genomic heterogeneity and adaptive resistance in melanomas and other cancers.<sup>21,22</sup> Melanomas that display primary resistance against immune-checkpoint blockade often lack type I IFN responses in the tumor microenvironment and are thus immune cell poor.<sup>11</sup> Accumulating evidence suggests that some tumors lack endogenous type I IFN activation, because they fail to sense cytosolic nucleic acids via the cGAS-STING pathway.<sup>23</sup> Recently, we demonstrated that local activation of type I IFN system by injecting double-stranded RNA analog poly(I:C) exposed mouse melanomas to cytotoxic T-cell attack and consequently to anti-PD-1 immunotherapy.<sup>24</sup> However, the role of the host type I IFN system in ACT immunotherapy has been poorly delineated.

Here, we report that an ACT protocol involving lymphodepletion and transfer of gp100-specific CD8<sup>+</sup> Pmel-1 T cells coupled with adjuvant IL-2 resulted in eradication of Hgf-Cdk4<sup>R24C</sup> mouse-derived HcMel3 melanomas in type I IFN-competent mice. Using global (Ifnar1<sup>-/-</sup>) and conditional (Ifnar1 <sup>$\Delta$ LysM</sup>) type I IFN receptor-deficient mice, we could demonstrate that the therapeutic efficacy was dependent on functional type I IFN system in myeloid cells. These data provide first evidence, suggesting that myeloid type I IFN system may promote antitumor immunity over pro-tumoral inflammation upon ACT immunotherapy and thus play a crucial role in counteracting development of adaptive resistance.

## RESULTS

### Adoptive T-cell therapy efficacy critically depends on host type I IFN system

Certain tumors have been reported to be type I IFN-unresponsive,<sup>25</sup> which could obscure the interpretation of experimental data from genetic mouse models. Thus, we studied the type I IFN

responsiveness of HcMel3 melanoma cells *in vitro* in a type I IFN antiviral assay. Here, HcMel3 cells were stimulated with two concentrations (10 or 100  $\mu\text{M}$ ) of recombinant mIFN $\beta$  and infected with cytolytic but type I IFN-sensitive Semliki Forest virus expressing EGFP (SFV VA7-EGFP).<sup>26</sup> We measured the infection and viability of HcMel3 cells by fluorescence microscopy and crystal violet staining, respectively. As expected, neither of the tested type I IFN concentrations *per se* had major suppressive effects on melanoma cell viability, but type I IFN stimulation efficiently protected HcMel3 cells from viral infection and cytolysis (Supplementary figure 1a and b).

To understand the role of the host type I IFN system on the efficacy of ACT therapy, we transplanted type I IFN-responsive HcMel3 melanomas intracutaneously to *lfnar1*-competent and *lfnar1*-deficient mice. Next, tumor-bearing mice were treated with ACT protocol involving chemotherapeutic preconditioning, transfer of either *lfnar1*<sup>+/+</sup> or *lfnar1*<sup>-/-</sup> CD8<sup>+</sup>CD90.1<sup>+</sup> Pmel-1 T cells, T cell *in vivo* activation by a single injection of Ad-GP100 vector followed by IL-2 injections (100 000 units twice a day for 3 days) to stimulate T-cell expansion. A fully *lfnar1*-competent system (melanoma, Pmel-1 T cells and host, all *lfnar1*<sup>+/+</sup>) resulted in long-term survival of approximately 60% of the treated mice (Figure 1a and b). In line with the well-established role of type I IFN in T-cell activation,<sup>14</sup> the therapeutic efficacy was poor in mice receiving T cells lacking the *lfnar1* receptor (Figure 1c) and the *lfnar1*<sup>-/-</sup> T cells showed a tendency (although non-significant) to expand poorer than the *lfnar1*<sup>+/+</sup> T cells (Supplementary figure 2). Surprisingly, when HcMel3 tumors were treated in *lfnar1*<sup>-/-</sup> mice with *lfnar1*<sup>+/+</sup> T cells, tumors escaped early, suggesting that host type I IFN system is crucial for ACT efficacy and was thus the focus of the further investigations (Figure 1d).

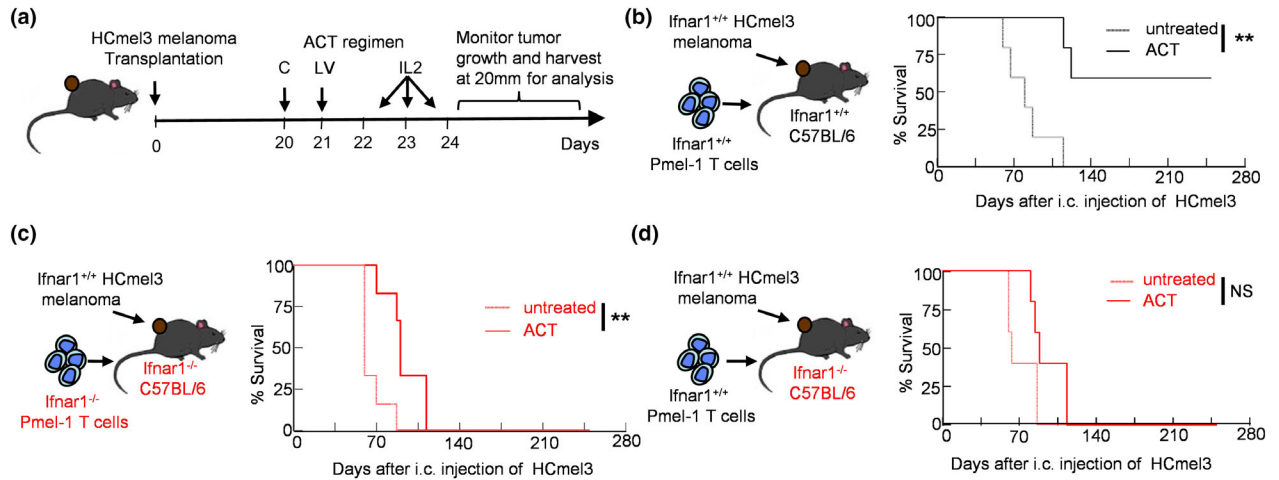
### Host type I IFN system is not required for the T-cell expansion, tumor infiltration and effector functions

Next, we investigated the underlying mechanism for the significantly reduced efficacy of ACT immunotherapy in type I IFN signalling-deficient hosts. Host type I IFN system has been reported to be critical for the expansion and infiltration of antitumor T cells and functions as a 'third signal' in T-cell activation.<sup>27</sup> Thus, we hypothesised that

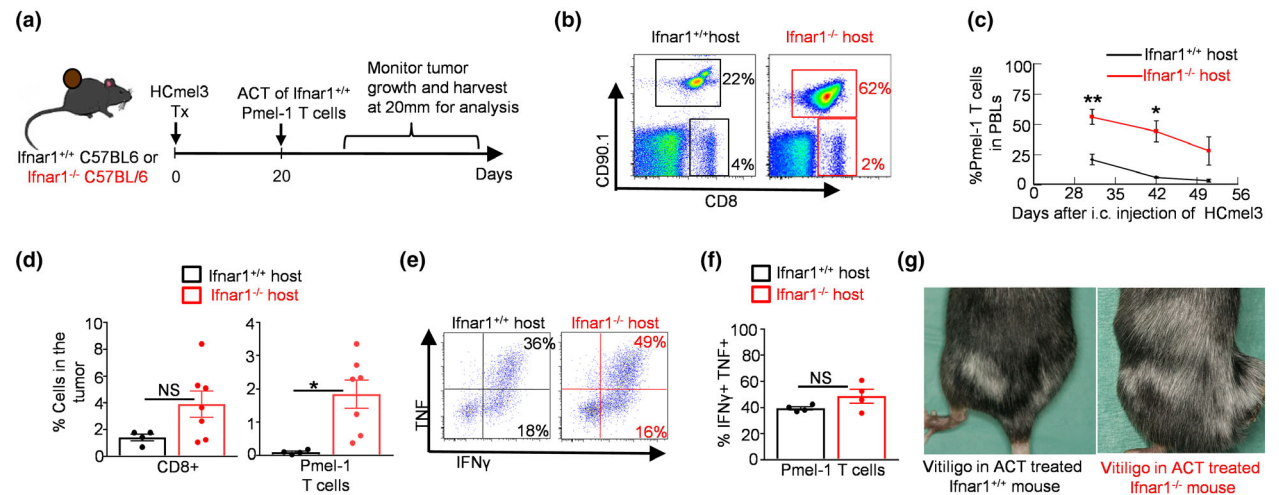
expansion, migration and/or effector function of gp100-specific CD8<sup>+</sup> T cells was impaired in *lfnar1*<sup>-/-</sup> mice. Interestingly, Pmel-1 T cells were observed to expand highly efficiently in *lfnar1*<sup>-/-</sup> mice and were detectable in the blood weeks after transfer (Figure 2a–c). We then analysed the frequency of Pmel-1 T cells infiltrating HcMel3 melanomas in *lfnar1*<sup>-/-</sup> hosts by flow cytometry and observed that the recruitment of Pmel-1 T cells into the HcMel3 tumors was not impaired (Figure 2d). Finally, we assessed the *ex vivo* effector functions of the transferred T cells (isolated 28 days after ACT) by measuring their intracellular synthesis of TNF- $\alpha$  and IFN- $\gamma$  upon gp100 peptide restimulation (Figure 2e and f). T cells from *lfnar1*<sup>-/-</sup> mice had fully retained their effector functions not only *ex vivo*, but also *in vivo* as evidenced by marked vitiligo-like depigmentation of the fur (Figure 2g). Taken together, adoptively transferred Pmel-1 T cells expanded, infiltrated HcMel3 melanomas and acquired effector functions in the *lfnar1*<sup>-/-</sup> mice. These data suggested that the failure of ACT therapy in absence of host type I IFN system was not because of impaired T-cell function.

### Host type I IFN system inhibits myeloid driven inflammation and tumor dedifferentiation

Despite the absence of obvious defects in the transferred Pmel-1 T cells, their ability to control HcMel3 growth in *lfnar1*<sup>-/-</sup> mice was reduced in comparison with *lfnar1*<sup>+/+</sup> mice (Figure 3a and b). Nevertheless, in some mice a short period of tumor control could be observed further, suggesting that the ACT therapy was not failing because of the inactivation of the transferred T cells. We have previously reported that melanomas can resist lineage antigen-targeted T-cell therapy by inflammation-induced dedifferentiation.<sup>20</sup> Thus, we decided to analyse the composition of tumor-infiltrating immune cells in *lfnar1*<sup>+/+</sup> and *lfnar1*<sup>-/-</sup> mice. Surprisingly, ACT resulted in significantly increased frequencies of CD45<sup>+</sup> immune cells and CD11b<sup>+</sup> myeloid cells in *lfnar1*<sup>-/-</sup> mice but not in *lfnar1*<sup>+/+</sup> mice. Further characterisation revealed that the induced myeloid infiltrates were predominantly composed of Ly6C<sup>-</sup>Ly6G<sup>-</sup> cells and to lesser extent Ly6C<sup>+</sup>Ly6G<sup>+</sup> immune cells (Figure 3c). To elucidate why HcMel3 melanomas relapsed early in the *lfnar1*<sup>-/-</sup> mice, we performed histological analysis



**Figure 1.** Adoptive T-cell transfer therapy with gp100 targeted CD8<sup>+</sup> T cells eradicates the majority of type I IFN-responsive HCmel3 melanomas in wild-type mice but tumors escape in Ifnar1-deficient mice. Experimental protocol (a) for adoptive T-cell transfer therapy (ACT) of intracutaneously (i.c.) transplanted Ifnar1<sup>+/+</sup> HCmel3 melanomas in Ifnar1<sup>+/+</sup> vs. Ifnar1<sup>-/-</sup> hosts and corresponding Kaplan–Meier survival curves of Ifnar1<sup>+/+</sup> (b) or Ifnar1<sup>-/-</sup> hosts (c, d) treated as indicated with ACT using either Ifnar1<sup>+/+</sup> (b, d) or Ifnar1<sup>-/-</sup> (c) pmel-1 T cells. 20 d after tumor transplantation, mice were preconditioned with cyclophosphamide (C), received next day Pmel-1 CD8<sup>+</sup> lymphocytes which were activated *in vivo* by Ad5-GP100 viral vector injection (LV) followed by 100 000 units of IL-2 injection twice a day for 3 days. *n* = 5 for all cohorts in b and d, *n* = 6 for cohorts in c. Statistics: log-rank (Mantel–Cox) test, *P*-value < 0.05 (\*) and *P*-value < 0.01 (\*\*). A representative result from two replicate experiments is shown.

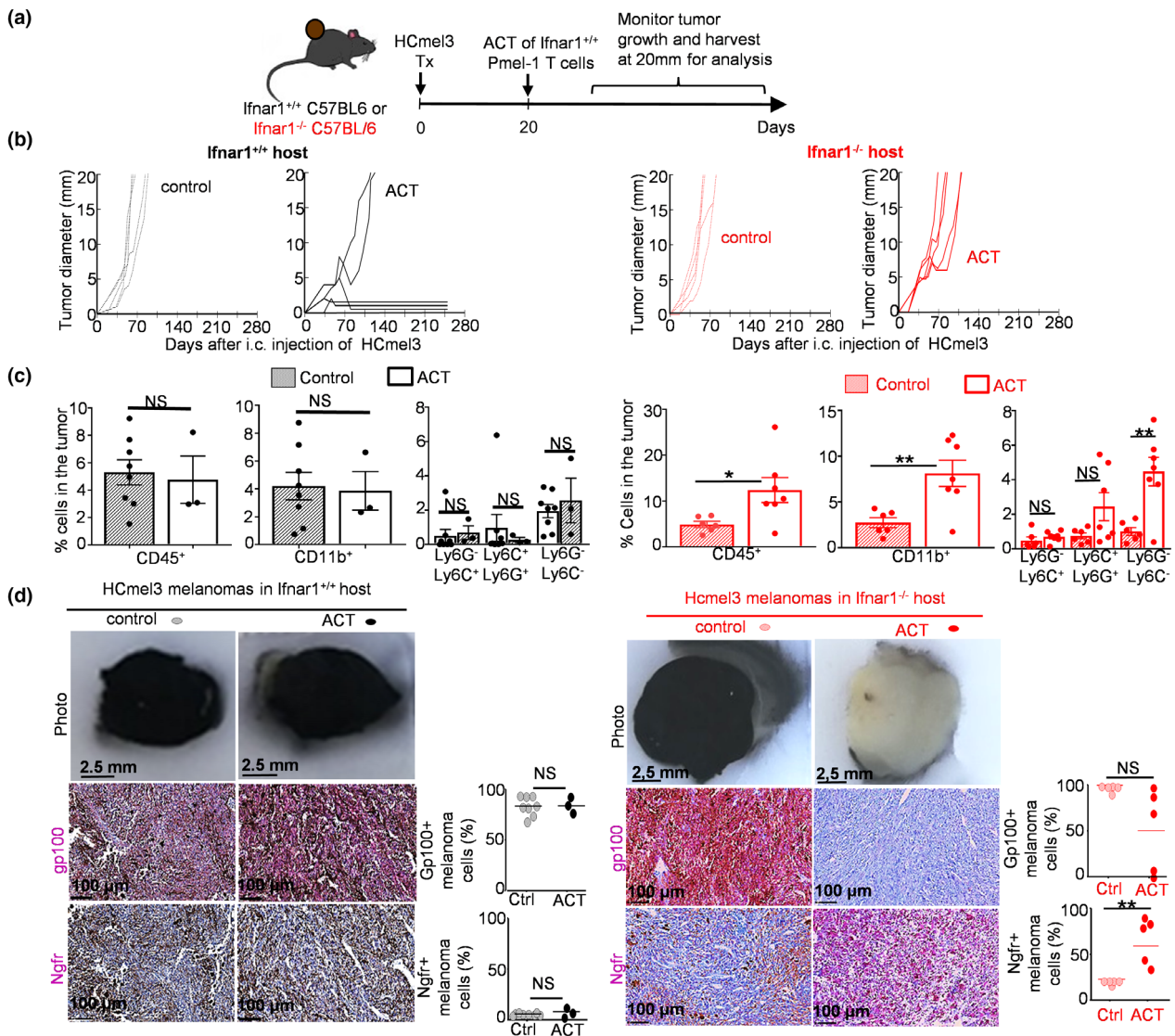


**Figure 2.** The transferred Pmel-1 CD8<sup>+</sup> T cells expand, display effector functions and infiltrate melanomas in Ifnar1-deficient mice. (a) Sample collection timeline (relates to experiments in Figure 1b and d). (b) Gating strategy for c. (c) Quantification of circulating CD8<sup>+</sup> CD90.1<sup>+</sup> Pmel-1 T cells in the blood of Ifnar1<sup>+/+</sup> or Ifnar1<sup>-/-</sup> mice in indicated time points. PBLs = CD45<sup>+</sup> peripheral blood leucocytes. *n* = 4 for Ifnar1<sup>+/+</sup> and *n* = 7 for Ifnar1<sup>-/-</sup> mice. (d) Flow cytometric quantification of CD8<sup>+</sup> and adoptively transferred Pmel-1 T cells infiltrating HCmel3 melanomas growing in Ifnar1<sup>+/+</sup> or Ifnar1<sup>-/-</sup> C57Bl/6 mice. (e) Gating strategy for f. (f) Intracellular IFN-γ and TNF-α cytokine staining after *ex vivo* restimulation of blood-derived (day 28 post-ACT) pmel-1 T cells with GP100 target peptide. (g) Representative photograph showing vitiligo-like depigmentation of the fur in Ifnar1<sup>+/+</sup> and Ifnar1<sup>-/-</sup> mice. Statistics: unpaired *t*-test, error bar = SEM, *P*-value < 0.05 (\*) and *P*-value < 0.01 (\*\*). The results from two replicate experiments are shown.

of the control and ACT-treated melanomas. We noticed marked dedifferentiation and loss of the target antigen gp100 in a proportion of ACT-

treated tumors growing in Ifnar1<sup>-/-</sup> mice, but not in Ifnar1<sup>+/+</sup> mice. Interestingly, the gp100 expression varied significantly in tumors derived

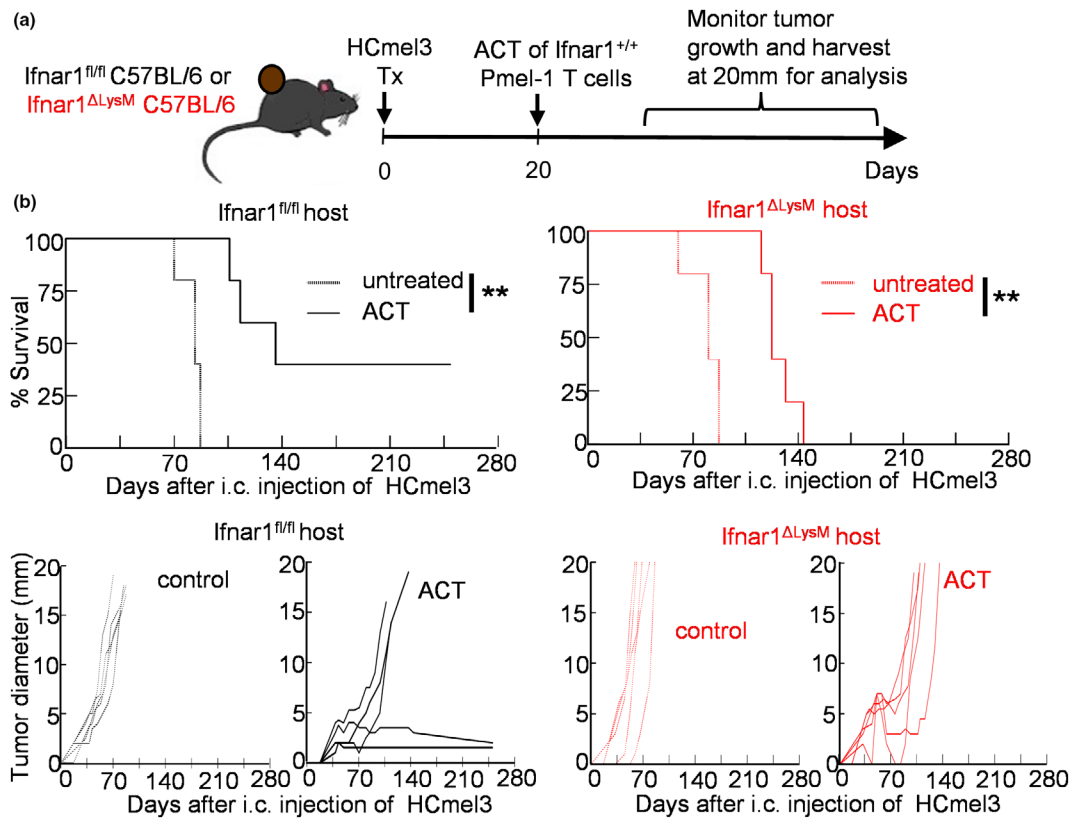




**Figure 3.** The adoptive CD8<sup>+</sup> T-cell transfer therapy results in rapid melanoma dedifferentiation and myeloid cell infiltration in *Ifnar1*<sup>-/-</sup> mice. **(a)** Sample collection timeline, and **(b)** Growth curves of untreated and Pmel-1 CD8<sup>+</sup> T-cell (*Ifnar1*<sup>+/+</sup>) ACT-treated HCmel3 melanomas growing in *Ifnar1*<sup>+/+</sup> or *Ifnar1*<sup>-/-</sup> mice (related to experiments in Figure 1d). **(c)** Flow cytometric analysis of myeloid immune cells infiltrating HCmel3 melanomas growing in *Ifnar1*<sup>+/+</sup> or *Ifnar1*<sup>-/-</sup> hosts treated as indicated. **(d)** Macroscopic pictures and IHC stainings for gp100 and Ngr1 in HCmel3 melanomas growing in *Ifnar1*<sup>+/+</sup> or *Ifnar1*<sup>-/-</sup> mice treated as indicated and the corresponding staining quantifications. Statistics: unpaired *t*-test, error bar = SEM, *P*-value < 0.05 (\*) and *P*-value < 0.01 (\*\*). The results from two replicate experiments are shown in **c** and **d**.

from *Ifnar1*<sup>-/-</sup> mice (*P*-value = 0.0002 in an *F*-test to compare variances) and some ACT-treated tumors displayed almost complete loss of visible pigmentation and gp100 expression (Figure 3d). Moreover, ACT resulted in significant increase (*P*-value = 0.0059 in an unpaired *t*-test) in Ngr1-positive tumor cells only in *Ifnar1*<sup>-/-</sup> mice, further suggesting that ACT-induced tumor dedifferentiation in the absence of host type I IFN system.

Tumor-associated myeloid cells are known to drive a response that resembles physiological wound healing hallmarked by dedifferentiation and vascular remodelling.<sup>28</sup> We hypothesised that differences in the vasculature of tumors growing in wild-type and type I IFN system-deficient hosts might have explained the drastically different therapeutic responses to ACT. To test the hypothesis, we injected Evans blue to cohorts of HCmel3 tumor-bearing *Ifnar1*<sup>+/+</sup> and *Ifnar1*<sup>-/-</sup>

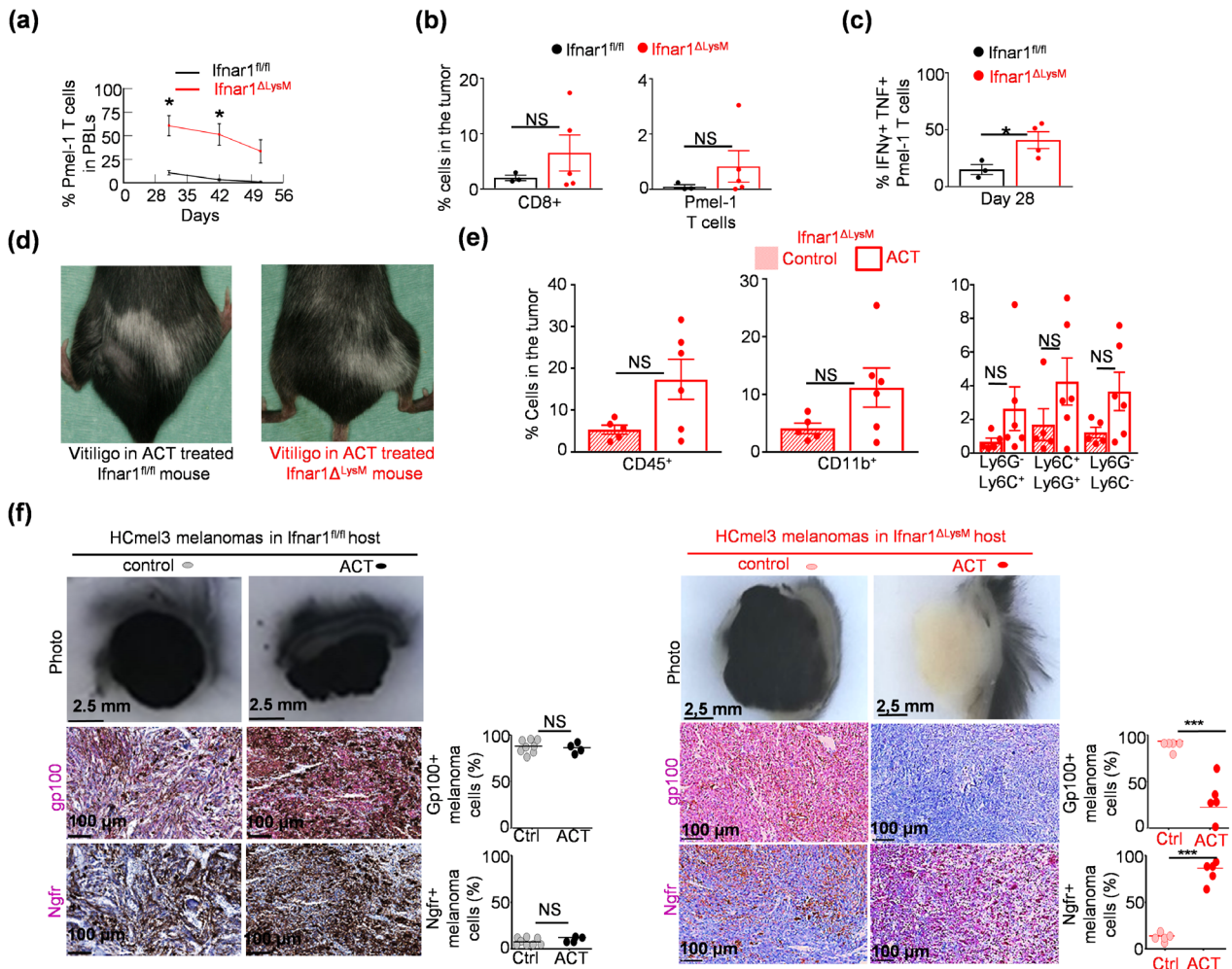


**Figure 4.** Pmel-1 CD8<sup>+</sup> T cells expand, display effector functions and infiltrate melanomas in Ifnar1<sup>ΔLysM</sup> mice but have only limited therapeutic efficacy. **(a)** Sample collection timeline. **(b)** Kaplan–Meier survival and tumor growth curves of Ifnar1<sup>fl/fl</sup> and Ifnar1<sup>ΔLysM</sup> mice. Statistics: log-rank (Mantel–Cox) test, *P*-value < 0.05 (\*) and *P*-value < 0.01 (\*\*), *n* = 5. A representative result from two replicate experiments is shown.

C57Bl/6 mice and assessed the ACT-induced effects on tumor vasculature by quantifying Evans blue extravasation spectrophotometrically. Remarkably, while no significant differences were observed between the control tumors and ACT-treated tumors growing in Ifnar1<sup>+/+</sup> mice, ACT clearly suppressed Evans blue tumor accumulation in Ifnar1<sup>-/-</sup> mice (Supplementary figure 4a and b). To exclude the possibility that the result was derived from differences in the number of tumor blood vessels rather than differences in vascular function, we analysed the tumors for the expression of pan-endothelial marker MECA32 by IHC. Indeed, the blood vessel density was found to be similar in tumors harvested from Ifnar1<sup>+/+</sup> and Ifnar1<sup>-/-</sup> mice. However, ACT unexpectedly provoked a profound vasculoculpting in tumors growing in both type I IFN-proficient and type I IFN-deficient hosts resulting in significant reduction in vascular density (Supplementary figure 4c and d).

### Myeloid cell-intrinsic type I IFN signalling controls adaptive resistance to ACT therapy

Previously, we have reported that myeloid cells contribute to therapeutic inflammation-driven tumor resistance programme upon ACT.<sup>20,29</sup> The observed pronounced intratumoral myeloid cell infiltration in IFN-deficient setting spurred us to hypothesise that myeloid compartment may be a crucial target of host type I IFN system upon ACT. To experimentally test the hypothesis, we transplanted HCmel3 melanomas into mice specifically lacking type I IFN signalling in LysM<sup>+</sup> cells (LysM<sup>Cre/wt</sup>Ifnar1<sup>fl/fl</sup> referred to as Ifnar1<sup>ΔLysM</sup>) or corresponding littermate controls (LysM<sup>wt/wt</sup>Ifnar1<sup>fl/fl</sup> referred to as Ifnar1<sup>fl/fl</sup>) mice<sup>30</sup> and treated them with the ACT protocol. Supporting our hypothesis, ACT resulted in efficient tumor control only in Ifnar1<sup>fl/fl</sup> littermates, whereas the therapeutic efficacy was severely diminished in the Ifnar1<sup>ΔLysM</sup> mice (Figure 4a and b).



**Figure 5.** The adoptive CD8<sup>+</sup> T-cell transfer therapy results in rapid melanoma dedifferentiation and myeloid cell infiltration in *Ifnar1<sup>ΔLysM</sup>* mice. **(a)** Flow cytometric quantification of circulating pmel-1 T cells (CD8<sup>+</sup>CD90.1<sup>+</sup>) in the blood of ACT-treated *Ifnar1<sup>fl/fl</sup>* or *Ifnar1<sup>ΔLysM</sup>* mice at indicated time points after ACT. **(b)** Flow cytometric quantification of CD8<sup>+</sup> and adoptively transferred pmel-1 T cells infiltrating HCmel3 melanomas growing in *Ifnar1<sup>fl/fl</sup>* or *Ifnar1<sup>ΔLysM</sup>* mice. **(c)** Intracellular IFN- $\gamma$  and TNF- $\alpha$  cytokine staining after *ex vivo* restimulation of blood derived Pmel-1 T cells (day 28 post-ACT) with GP100 target peptide. A representative result from two replicate experiments is shown in **a-c**. **(d)** Representative photographs showing vitiligo-like depigmentation of the fur in *Ifnar1<sup>fl/fl</sup>* and *Ifnar1<sup>ΔLysM</sup>* mice. **(e)** Flow cytometric analysis of myeloid immune cells infiltrating HCmel3 melanomas in *Ifnar1<sup>ΔLysM</sup>* mice treated as indicated. **(f)** Macroscopic pictures and IHC stainings of gp100 and Ngr1 in control and ACT-treated HCmel3 melanomas growing either in *Ifnar1<sup>fl/fl</sup>* or *Ifnar1<sup>ΔLysM</sup>* mice. The results from two replicate experiments are shown in **e** and **f**. Statistics: unpaired t-test, error bar = SEM, *P*-value < 0.05 (\*).

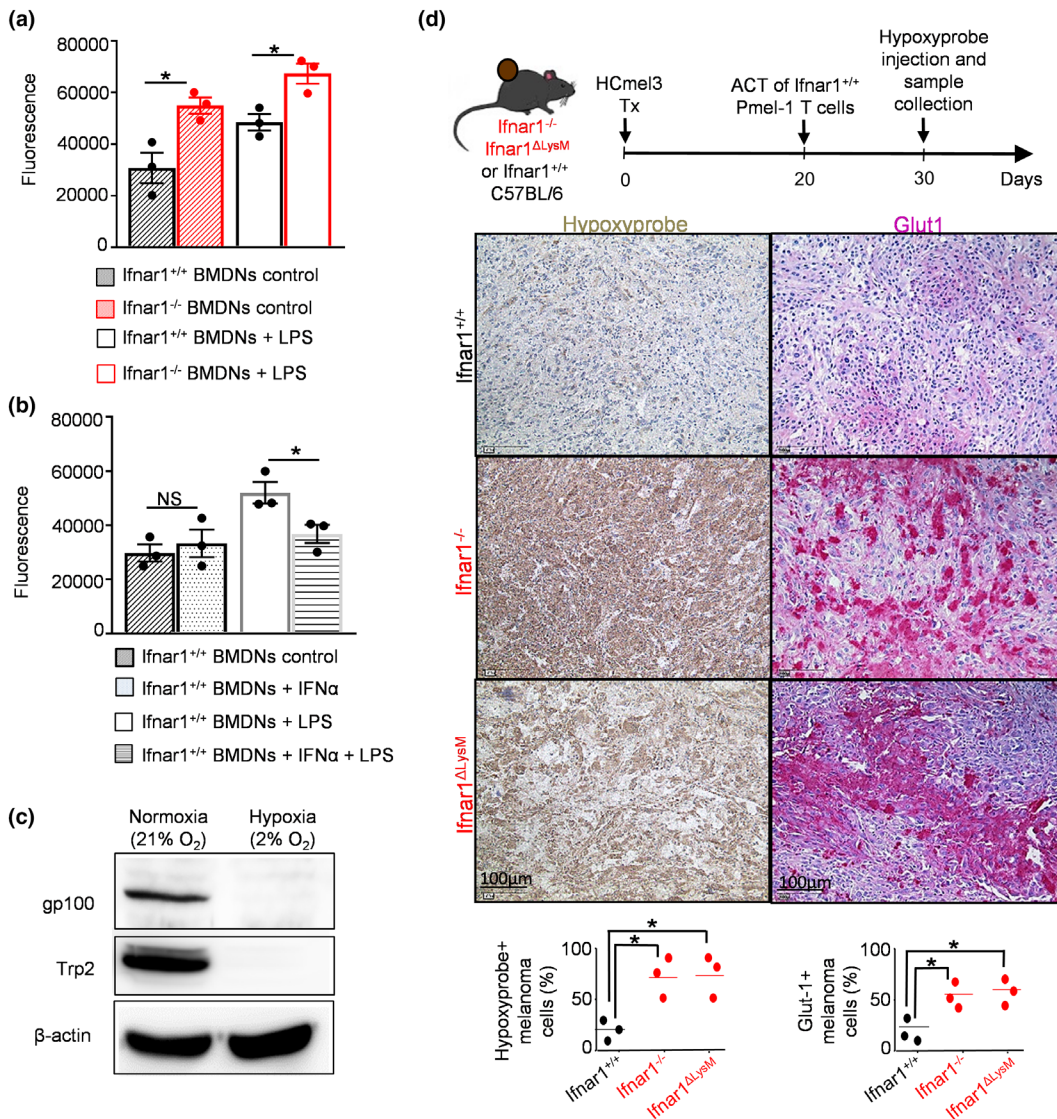
Furthermore, Pmel-1 T cells expanded again well in the *Ifnar1<sup>ΔLysM</sup>* mice, were able to infiltrate the HCmel3 tumors and had retained their effector functions as indicated by effector cytokine production and vitiligo-like depigmentation of the fur (Figure 5a-d). Of note, the lack of a functional myeloid type I IFN system resulted in tendentious increase in myeloid inflammation (Figure 5e) and in frequent (and occasionally nearly complete) dedifferentiation of the tumors growing in *Ifnar1<sup>ΔLysM</sup>* mice but not in wild-type

*Ifnar1<sup>fl/fl</sup>* littermates (Figure 5f). Collectively, these data indicated that the myeloid compartment was a key component of the host type I IFN system in counteracting adaptive resistance upon ACT.

### Myeloid type I IFN system restricts inflammation and hypoxia in the tumor microenvironment upon ACT therapy

It remained unclear as to why the lack of a functional myeloid type I IFN system resulted in





**Figure 6.** Myeloid cell type IFN system controls myeloid inflammation and hypoxia-mediated melanoma cell dedifferentiation. **(a, b)** MPO activity of bone marrow-derived neutrophils (BMDNs) isolated from either *Ifnar1*<sup>+/+</sup> **(a, b)** or *Ifnar1*<sup>-/-</sup> **(a)** mice and treated as indicated. The y-axis represents fluorescence intensity (arbitrary units). **(c)** Western analysis of gp100 and Trp2 of HCmel3 cells subjected to normoxia (21% O<sub>2</sub>) or hypoxia (2% O<sub>2</sub>) for 24 h. **(d)** Sample collection timeline and representative pictures of Hypoxyprobe<sup>TM</sup>-1 and Glut1-immunohistochemical analysis of HCmel3 melanomas transplanted in *Ifnar1*<sup>+/+</sup>, *Ifnar1*<sup>-/-</sup> and *Ifnar1*<sup>ΔLysM</sup> mice and treated as indicated. Statistics: unpaired t-test, error bar = SEM, *P*-value < 0.05 (\*). A representative result from two replicate experiments is shown.

rapid myeloid cell infiltration and escape of the melanomas after ACT therapy. To shed more light on the underlying biology, we next studied the effects of type I IFN on neutrophils, as their activation status can be readily monitored in a myeloperoxidase (MPO) assay. MPO is a haem enzyme that is primarily expressed by activated polymorphonuclear neutrophils. We isolated bone marrow-derived neutrophils (BMDNs) from *Ifnar1*-deficient and *Ifnar1*-competent mice and analysed

changes in MPO activity as a marker for neutrophil activation upon LPS stimulation for 24 h. Interestingly, a significantly higher MPO activity was observed in both untreated and LPS-treated *Ifnar1*-deficient neutrophils than in the respective wild-type controls (Figure 6a). Next, we isolated BMDNs from wild-type mice and left them untreated or treated them with LPS, IFN-α or their combination. As expected, BMDNs treated with the strong pattern recognition receptor

agonist LPS were found to have a higher MPO activity than untreated or IFN- $\alpha$ -treated BMDNs, but the LPS-induced MPO activation was significantly inhibited by the addition of IFN- $\alpha$  (Figure 6b).

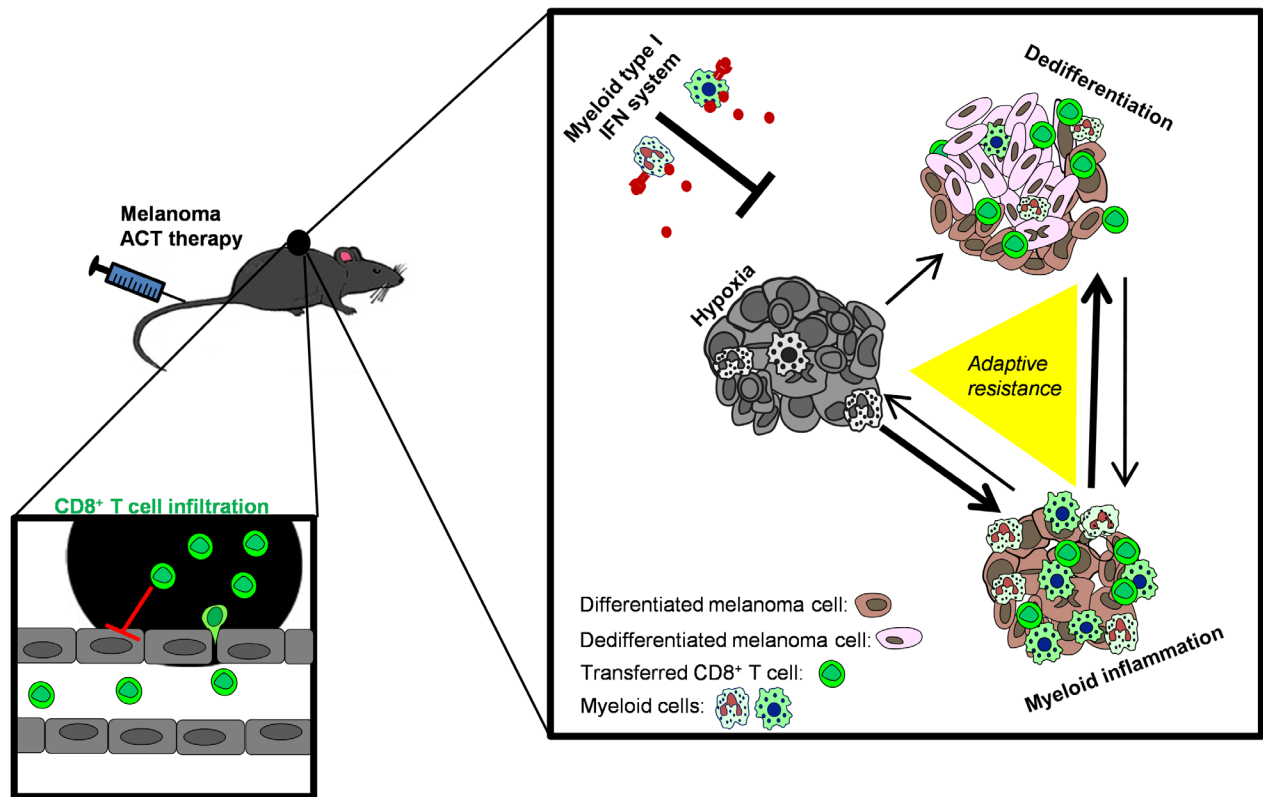
Tumor hypoxia is highly immunosuppressive for T cells and hypoxia-induced tumor dedifferentiation has been well documented, especially in carcinomas,<sup>31–33</sup> while tumor-infiltrating myeloid cells have been shown to induce local hypoxia.<sup>34</sup> The pronounced infiltration of myeloid cells in conjunction with changes in vascular function in the absence of type I IFN signalling prompted us to hypothesise that HcMel3 dedifferentiation may be a result of tumor hypoxia. Indeed, when HcMel3 melanoma cells were cultured *in vitro* under hypoxia for 24 h, the cells completely lost the expression of gp100 and Trp2 differentiation antigens as evidenced by Western analysis (Figure 6c). Next, we treated cohorts of HcMel3-bearing wild-type, *Ifnar1*<sup>-/-</sup> and *Ifnar1* <sup>$\Delta$ LysM</sup> mice with ACT and 10 days after the T-cell transfer injected the mice with Hypoxyprobe<sup>TM</sup>-1. Hypoxyprobe staining of the harvested tumors confirmed that the melanomas growing both in *Ifnar1*<sup>-/-</sup> and in *Ifnar1* <sup>$\Delta$ LysM</sup> mice were more hypoxic than tumors derived from *Ifnar1*-competent mice, and the results could be corroborated by staining for a hypoxia marker Glut1 (Figure 6d).

## DISCUSSION

While ACT effectively controlled HcMel3 tumors in wild-type mice, we observed only a transient tumor control in mice lacking a functional type I IFN system in all host cells (*Ifnar1*<sup>-/-</sup>) or specifically in myeloid immune cells (*Ifnar1* <sup>$\Delta$ LysM</sup>). Interestingly, some HcMel3 tumors escaped the therapy also in type I IFN-proficient hosts despite remaining pigmented (Figures 3 and 5) potentially because of relatively low circulating levels of transferred T cells (Figures 2c and 5a). In agreement with previous studies,<sup>13,35</sup> we also observed that *Ifnar1*<sup>-/-</sup> Pmel-1 T cells had a tendency (although statistically non-significant) to expand poorer than *Ifnar1*<sup>+/+</sup> T cells in wild-type mice (Supplementary figure 2). Because of the well-known positive feedback signalling of type I IFN induction,<sup>14</sup> we reasoned that the *Ifnar1*<sup>-/-</sup> hosts might not have provided enough type I IFN for efficient T-cell expansion. However, *Ifnar1*<sup>+/+</sup> Pmel-1 T cells were found to expand very well

both in the *Ifnar1*<sup>-/-</sup> and *Ifnar1* <sup>$\Delta$ LysM</sup> mice and Pmel-1 T cells could be detected in the therapy-resistant tumors (Figures 2 and 5). These data suggest that despite the important function as a danger signal in attraction of endogenous T cells<sup>36</sup> the host type I IFN system may not be crucial for the T-cell infiltration into tumors at least in the setting of adoptive T-cell transfer. Indeed, stimulation of type I IFN system in the tumor cells themselves rather than host cells either endogenously via cGAS/STING<sup>23</sup> or therapeutically<sup>36</sup> may be sufficient to stimulate T-cell infiltration. Interestingly, modest tumor growth control was seen in globally and especially in conditionally *Ifnar1*-deficient mice upon ACT therapy, Pmel-1 T cells could be restimulated *ex vivo* and the mice frequently experienced vitiligo-like depigmentation of the fur. The *in vivo* effector functions of transferred T cells *per se* did not thus seem to require the myeloid type I IFN system.

Here, we have presented evidence that type I IFN system in myeloid cells not only inhibits their own infiltration into melanomas upon T-cell therapy, but also restricts their inflammatory activation. Indeed, the absence of a functional type I IFN system in myeloid cells was found to result in their hyper-activation, as evidenced by increased tumor infiltration *in vivo* (Figures 3 and 5) and MPO activity *in vitro* (Figure 6). This likely contributed to the observed tumor dedifferentiation.<sup>37</sup> In accordance with these data, type I IFN has been reported to interfere with Cxcr2-driven neutrophil recruitment in both cancer and infection,<sup>38,39</sup> and adjuvant IFN- $\alpha$  benefits, especially in patients with ulcerated high-risk melanomas, which are typically infiltrated by neutrophils.<sup>5</sup> Indeed, large HcMel3 tumors frequently ulcerate in both the type I IFN-proficient and type I IFN-deficient mice (unpublished observations), which may promote myeloid cell inflammation, especially in the absence of inhibitory type I IFN signal. Moreover, despite the non-redundancy of the type I IFN system in myeloid compartment, our data suggest that host type I IFN signalling beyond the LysM<sup>+</sup> myeloid compartment may also be required for the full ACT efficacy. This observation is in line with our previous results demonstrating that multiple different immune cell subsets require coordinated type I IFN signalling in the context of checkpoint blockade immunotherapy of melanoma.<sup>36</sup>



**Figure 7.** Myeloid type I IFN system promotes antitumor immunity over pro-tumoral inflammation. The myeloid type I IFN system promotes an antitumor (cytotoxic) immunity and limits pro-tumoral (regenerative) inflammation by regulating the function of adaptive resistance network in the tumor microenvironment. The network consists of regenerative myeloid inflammation, hypoxia and tumor dedifferentiation. The self-amplifying adaptive resistance circuit is likely instigated by CD8<sup>+</sup> T-cell-mediated suppression of tumor vasculature resulting in hypoxia. The resistance is further promoted by hyper-activation of myeloid cells and ultimately leads to tumor dedifferentiation.

Here, we have demonstrated that upon ACT immunotherapy the absence of myeloid type I IFN system stimulates myeloid cell tumor infiltration, dedifferentiation and hypoxia. These observations are concurring with previous studies showing that over the course of tumor progression hypoxia can promote phenotype switching in a population of melanoma cells from a highly proliferative to a highly invasive and therapy-resistant state.<sup>40</sup> Highlighting the complexity of type I IFN signalling in malignancy, exogenous type I IFN was previously shown to stabilise B16 melanoma vasculature<sup>41</sup> while endogenous IFN- $\beta$  suppressed angiogenesis by inhibiting expression of proangiogenic and homing factors in neutrophils.<sup>42</sup> Indeed, we observed that functional endogenous type I IFN system promoted higher tumor blood vessel permeability upon ACT resulting in increased accumulation of Evans blue dye (Supplementary figure 4a and b). Unexpectedly, CD8<sup>+</sup> T-cell ACT also resulted in

profound suppression of tumor vasculature in both type I IFN-proficient and type I IFN-deficient hosts (Supplementary figure 4c and d). The underlying mechanism for the vasculosculting remains to be shown in future but might relate to IFN- $\gamma$  secreted by the antitumor CD8<sup>+</sup> T cells.<sup>43</sup>

Myeloid inflammation, dedifferentiation and local hypoxia are all characteristics associated with regenerative wound-healing response.<sup>28</sup> In the context of melanoma, these responses can contribute to the development of adaptive immunotherapy resistance.<sup>20,29,44</sup> In the current work, we have provided direct evidence showing that myeloid type I IFN system can counteract this resistance network. The exact spatiotemporal dynamics of the phenotypic traits will be difficult to address because of their highly complex mechanistic interconnections. On the one hand, type I IFN *per se* is known to enhance the expression of gp100 and other melanocytic antigens along with MHC-I required for antigen

presentation.<sup>45</sup> On the other hand, while hypoxia and regenerative inflammatory reactions are immunosuppressive in their own right, they are also avid inducers of melanoma dedifferentiation. Furthermore, mesenchymal melanomas not only downregulate melanocytic antigen expression but are also more resistant to apoptosis and thus T-cell attack.<sup>22,46,47</sup> Finally, myeloid cells are known to adapt immunosuppressive phenotype under inflammatory and hypoxic conditions.<sup>21</sup> However, a hypoxic microenvironment could be either the result or the cause of myeloid infiltration<sup>34,48</sup> and while myeloid cells can foster melanoma dedifferentiation<sup>37</sup> they also preferentially infiltrate dedifferentiated, mesenchymal tumors.<sup>44</sup>

Our data indicate that ACT-induced suppression of tumor vasculature may have instigated the observed tumor hypoxia in *Ifnar1*-deficient hosts. However, the vasculosculpting occurred irrespective of host type I IFN system and thus did not explain the observed adaptive resistance phenotype in the absence of type I IFN system as did not more dysfunctional tumor vasculature. On the contrary, whereas dramatic vasculosculpting could be seen in all ACT-treated tumors from *Ifnar1*<sup>+/+</sup> mice, half of the tumors displayed high accumulation of Evans blue dye, suggesting that the remaining vessels in these tumors were highly aberrant.

Based on the presented experimental evidence, we postulate a model, where myeloid type I IFN system promotes antitumor (cytotoxic) immunity over pro-tumoral (regenerative) inflammation upon T-cell therapy (Figure 7). In this model, myeloid type I IFN system works in T-cell therapy as a key regulator between microenvironmental phenotypes switch from pro-tumoral inflammation to antitumor immunity through inhibition of therapy-induced hypoxia and myeloid inflammation. Herein, the tumor cells, endothelial cells, transferred T cells and myeloid cells reside in a complex, plastic and reciprocally interconnected network, where the balance between immunity and inflammation is coordinated by the myeloid type I IFN system. The temporal sequence of the phenotypic traits remains to be addressed in future. However, irrespective of the exact dynamics, it seems clear that local, myeloid cell-driven type I IFN responses are a prerequisite for efficient melanoma T-cell therapy, a notion potentially exploitable in the rational design of novel combinatorial T-cell therapies.

## METHODS

### Mice

C57Bl/6 wild-type and *Ifnar1*<sup>-/-</sup> mice were purchased from the Jackson Laboratory. *Ifnar1*<sup>fl/fl</sup>, *LysM-Cre* x *Ifnar1*<sup>fl/fl</sup> (*Ifnar1*<sup>ΔLysM</sup>)<sup>49</sup> and Pmel-1 T-cell receptor transgenic mice<sup>50</sup> were all on the C57Bl/6 background and were bred as described previously. Age- and sex-matched cohorts of mice were randomly distributed to the different experimental groups at the beginning of each experiment. All animal experiments were approved by the local government authorities (LANUV, NRW, Germany) and were performed according to the institutional and national guidelines for the care and use of laboratory animals.

### Cell isolation and culture

HCmel3 melanoma cells and bone marrow-derived neutrophils (BMDNs) were cultured in complete RPMI 1640 medium containing 10% FCS (Biochrom, Cambourne, UK), 2 mM L-glutamine (Gibco, Dublin, Ireland), 10 mM non-essential amino acids (Gibco, 1 mM HEPES), 20 μM 2-mercaptoethanol (Sigma-Aldrich, St. Louis, MO, USA), 100 IU mL<sup>-1</sup> penicillin and 100 mg mL<sup>-1</sup> streptomycin (Invitrogen, Waltham, Massachusetts, USA). All cells were incubated at 37°C, 5% CO<sub>2</sub> and 21% O<sub>2</sub>, apart from hypoxia experiments when 2% O<sub>2</sub> was used. For isolation of BMDNs, the bone marrow from *Ifnar1*-competent and *Ifnar1*-deficient mice was harvested from femurs and tibias under sterile conditions and suspended in PBS. Erythrocytes were removed by hypotonic lysis. BMDNs were sorted using magnetic microbead selection following the manufacturer's instructions (Miltenyi Biotec, Bergisch Gladbach, Germany). A negative selection against CD11c<sup>+</sup>, DX5<sup>+</sup>, CD4<sup>+</sup>, CD8<sup>+</sup> and CD19<sup>+</sup> cells was first performed, and the remaining cells were positively selected for Ly6G<sup>+</sup> cell population. Cells were cultured in RPMI medium (1640; Gibco). The purity of MACS-sorted cells was confirmed by flow cytometry.

### Type I IFN antiviral assay

The type I IFN antiviral assay was carried out by treating HCmel3 melanoma cells with either 10 or 100 u mL<sup>-1</sup> mIFNβ (Sigma-Aldrich) for 12 h followed by MOI = 1 infection with type I IFN-sensitive virus SFV VA7-EGFP.<sup>26</sup> 24 h post-infection, the infection was monitored in fluorescence microscopy, and at 72 h, the cell viability was quantified by measuring crystal violet (diluted in methanol) staining intensity as described.<sup>44</sup>

### Myeloperoxidase activity assay

BMDNs (1 × 10<sup>6</sup>) derived from *Ifnar1*-competent and *Ifnar1*-deficient mice were left untreated or treated with LPS (100 ng mL<sup>-1</sup>; Invivogen, San Diego, CA, USA) or IFN-α (1000 u mL<sup>-1</sup>; PBL Assay Science, Piscataway, NJ, USA) overnight and lysed by freeze/thaw in 200 μL of PBS. The samples were further diluted 1:50 in PBS for the myeloperoxidase assay, which was performed according to



the manufacturer's instructions (EnzChek MPO Kit; Thermo Fisher Scientific, Waltham, MA, USA). The fluorescence of each sample was measured using a BioTek (Winooski, VT, USA) plate reader with 530 and 590 nm excitation and emission wavelengths, respectively.

### HCmel3 tumor transplantation and adoptive T-cell transfer

The HCmel3 melanoma cell line was generated from an Hgf-Cdk4<sup>R24C</sup> primary melanoma as described previously.<sup>20</sup> Cohorts of syngeneic Ifnar1-competent and Ifnar1-deficient C57Bl/6 mice received  $5 \times 10^5$  HCmel3 melanoma cells intracutaneously into the right flank. Tumor size was measured weekly and recorded as mean diameter in millimetres. Blood was collected by facial vein puncture, mice were sacrificed and tumors were analysed when they reached 20 mm in diameter. Mice bearing palpable HCmel3 melanomas were preconditioned by an i.p. injection of 2 mg cyclophosphamide (Baxter Oncology GmbH, Halle, Westfalen, Germany) a day before intravenous transfer of  $2 \times 10^6$  naive gp100-specific CTLs harvested from spleens of TCR-transgenic Pmel-1 mice that were then activated *in vivo* by single i.p. injection of  $5 \times 10^8$  plaque-forming units of recombinant adenoviral vector Ad-GP100. After 2 days, 100 000 units of human IL-2 (Proleukin, Novartis, Basel, Switzerland) in 100  $\mu$ L PBS was injected twice a day for 3 days.

### Histological and immunohistochemical analyses

Murine melanoma samples were immersed in a zinc-based fixative (BD Pharmingen, Franklin Lakes, NJ, USA) over night. Samples were then embedded in paraffin, cut in sections and subjected to standard H&E staining. Immunohistochemistry was performed with rabbit anti-mouse gp100 polyclonal antibody (NBP1-69571; Novus Biologicals, Littleton, CO, USA), goat anti-mouse Ngfr polyclonal antibody (BAF1157; R&D Systems, Minneapolis, MN, USA) and rabbit anti-mouse Glut1 polyclonal antibody (ab652; Abcam, Cambridge, UK) followed by suitable enzyme-conjugated secondary antibodies and the LSAB-2 colour development system (Dako, Jena, Germany). Heavily pigmented mouse melanomas were first bleached before staining (20 min at 37°C in 30% H<sub>2</sub>O<sub>2</sub> and 0.5% KOH, 20 s in 1% acetic acid and 5 min in Tris buffer). For tissue hypoxia analysis, Ifnar1-competent, Ifnar1-deficient and Ifnar1<sup>ALysM</sup> mice bearing HCmel3 melanomas were treated with ACT. Ten days after, ACT mice received intravenous injection of 2 mg of Hypoxyprobe<sup>TM</sup>-1 (Hypoxyprobe, Burlington), and 45 min later, melanomas were harvested. Hypoxyprobe<sup>TM</sup>-1 adducts with thiol groups were detected with FITC-MAb1 antibody by immunohistochemistry as described above. Stained sections were examined with a Leica (Wetzlar, Germany) DMLB microscope, images were acquired with a JVC (Yokohama) digital camera KY-75FU and images were processed with Adobe (San Jose) Photoshop. For quantification of antigen-positive melanoma cells, a blinded investigator calculated the average percentage of positive cells from 10 fields of view

(20 $\times$ ) on each IHC slide. Five tissue slides were analysed from each tumor.

### Flow cytometry and intracellular cytokine staining

Tumor-infiltrating and blood immune cells were isolated and stained as described previously<sup>36</sup> with fluorochrome-conjugated mAbs specific for mouse: PerCP-labelled anti-CD45, FITC-labelled anti-CD11b, PE-labelled anti-Ly6G, APC-labelled anti-Ly6C, PE-labelled anti-CD8 and APC-labelled anti-CD90.1 (all from BD Pharmingen). The percentage of Pmel-1 T cells out of peripheral blood leucocytes (PBLs) was analysed utilising APC-labelled anti-CD45, FITC-labelled anti-CD8 and PerCP-labelled anti-CD90.1 antibodies (BD Pharmingen). For intracellular cytokine staining, cells were first restimulated with human GP100 peptide (KVPRNQDWL) for 6 h and golgi-stop (BD Biosciences, San Jose, CA, USA) and golgi-plug (BD Biosciences) were added to the medium according to manufacturer's instructions. Cells were then washed and stained for surface CD90.1 and CD8 markers. Afterwards, cells were fixed with 2% PFA and permeabilised using saponin buffer (0.5%) and subsequently stained with APC-labelled anti-IFN- $\gamma$  and PE-labelled anti-TNF- $\alpha$  (BD Pharmingen). Data were acquired with a FACSCanto Flow Cytometer (BD Biosciences) and analysed with FlowJo Software (Treestar, San Carlos, CA, USA, V7.6.5, for Windows).

### Western analysis

HCmel3 cell-derived protein lysates were prepared using the M-PER mammalian protein reagent (Fermentas, Vilnius, Lithuania) supplemented with protease inhibitors (Roche, Basel, Switzerland). Protein lysates were then separated by SDS-PAGE and transferred to Immobilon-P transfer membranes (Millipore, Burlington, VT, USA). The detection was carried out using goat anti-mouse gp100 (Novus Biologicals), rabbit anti-mouse/human TRP2 (sc.25544; Santa Cruz Biotechnology, Dallas, TX, USA) and mouse anti- $\beta$ -actin (C4) (sc-47778; Santa Cruz Biotechnology) antibodies followed by incubation with suitable HRP-conjugated secondary antibodies and the Super Signal West Dura Extended Duration Chemiluminescence Substrate (Thermo Scientific, Waltham, MA, USA). Blots were analysed using ImageQuant LAS 4000 (GE Healthcare, Chicago, IL, USA).

### Statistical analysis

Unpaired, two-tailed t-tests and log-rank (Mantel-Cox) tests were carried out using GraphPad Prism 4 Software (GraphPad Software, San Diego, CA, USA). Results were considered statistically significant when  $P < 0.05$  (\*),  $P < 0.01$  (\*\*),  $P < 0.001$  (\*\*\*) and  $P < 0.0001$  (\*\*\*\*). Error bars represent SEM.

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## CONFLICT OF INTEREST

The authors declare no conflict of interest.

## REFERENCES

- Isaacs A, Lindenmann J. Virus interference. I. The interferon. *Proc R Soc London* 1957; **7**: 429–438.
- Parker BS, Rautela J, Hertzog PJ. Antitumour actions of interferons: implications for cancer therapy. *Nat Rev Cancer* 2016; **16**: 131–144.
- Reder AT, Feng X. How type I interferons work in multiple sclerosis and other diseases: some unexpected mechanisms. *J Interferon Cytokine Res* 2014; **34**: 589–599.
- Woo SR, Corrales L, Gajewski TF. The STING pathway and the T cell-inflamed tumor microenvironment. *Trends Immunol* 2015; **36**: 250–256.
- Eggermont AMM, Suci S, Testori A et al. Long-term results of the randomized phase III trial EORTC 18991 of adjuvant therapy with pegylated interferon alfa-2b versus observation in resected stage III melanoma. *J Clin Oncol* 2012; **30**: 3810–3818.
- Curtsinger JM, Valenzuela JO, Agarwal P, Lins D, Mescher MF. Type I IFNs provide a third signal to CD8 T cells to stimulate clonal expansion and differentiation. *J Immunol* 2005; **174**: 4465–4469.
- Marrack P, Kappler J, Mitchell T. Type I interferons keep activated T cells alive. *J Exp Med* 1999; **189**: 521–530.
- Kolumam GA, Thomas S, Thompson LJ, Sprent J, Murali-Krishna K. Type I interferons act directly on CD8 T cells to allow clonal expansion and memory formation in response to viral infection. *J Exp Med* 2005; **202**: 637–650.
- Srivastava S, Koch MA, Pepper M, Campbell DJ. Type I interferons directly inhibit regulatory T cells to allow optimal antiviral T cell responses during acute LCMV infection. *J Exp Med* 2014; **211**: 961–974.
- Dunn GP, Koebel CM, Schreiber RD. Interferons, immunity and cancer immunoediting. *Nat Rev Immunol* 2006; **6**: 836–848.
- Woo S-R, Fuertes M, Corrales L et al. STING-dependent cytosolic DNA sensing mediates innate immune recognition of immunogenic tumors. *Immunity* 2014; **41**: 830–842.
- Nguyen KB, Watford WT, Salomon RA et al. Critical role for STAT4 activation by type 1 interferons in the interferon-gamma response to viral infection. *Science* 2002; **297**: 2063–2066.
- Xu H, Grusdat M, Pandya A et al. Type I interferon protects antiviral CD8<sup>+</sup> T cells from NK cell cytotoxicity. *Immunity* 2014; **40**: 949–960.
- Crouse J, Kalinke U, Oxenius A. Regulation of antiviral T cell responses by type I interferons. *Nat Rev Immunol* 2015; **15**: 231–242.
- Dikopoulos N, Bertolotti A, Kröger A, Hauser H, Schirmbeck R, Reimann J. Type I IFN negatively regulates CD8<sup>+</sup> T cell responses through IL-10-producing CD4<sup>+</sup> T regulatory 1 cells. *J Immunol* 2005; **174**: 99–109.
- Teijaro JR, Ng C, Lee AM et al. Persistent LCMV infection is controlled by blockade of type I interferon signaling. *Science* 2013; **340**: 207–211.
- Wilson EB, Yamada DH, Elsaesser H et al. Blockade of chronic type I interferon signaling to control persistent LCMV infection. *Science* 2013; **340**: 202–207.
- Benci JL, Xu B, Qiu YU et al. Tumor interferon signaling regulates a multigenic resistance program to immune checkpoint blockade. *Cell* 2016; **167**: 1540–1554.
- Jacquelot N, Yamazaki T, Roberti MP et al. Sustained Type I interferon signaling as a mechanism of resistance to PD-1 blockade. *Cell Res* 2019; **29**: 846–861.
- Landsberg J, Kohlmeyer J, Renn M et al. Melanomas resist T-cell therapy through inflammation-induced reversible dedifferentiation. *Nature* 2012; **490**: 412–416.
- Visser KE, Tüting T. How neutrophils promote metastasis. *Science* 2016; **352**: 145–146.
- Hölzel M, Tüting T. Inflammation-induced plasticity in melanoma therapy and metastasis. *Trends Immunol* 2016; **37**: 364–374.
- Mackenzie KJ, Carroll P, Martin C-A et al. cGAS surveillance of micronuclei links genome instability to innate immunity. *Nature* 2017; **548**: 461–465.
- Steitz J, Brück J, Lenz J, Knop J, Tüting T. Depletion of CD25<sup>+</sup> CD4<sup>+</sup> T cells and treatment with tyrosinase-related protein 2-transduced dendritic cells enhance the interferon alpha-induced, CD8<sup>+</sup> T-cell-dependent immune defense of B16 melanoma. *Cancer Res* 2001; **61**: 8643–8646.
- Shin DS, Zaretsky JM, Escuin-Ordinas H et al. Primary resistance to PD-1 blockade mediated by JAK1/2 mutations. *Cancer Discov* 2017; **7**: 188–201.
- Ruotsalainen J, Martikainen M, Niittykoski M et al. Interferon-β sensitivity of tumor cells correlates with poor response to VA7 virotherapy in mouse glioma models. *Mol Ther* 2012; **20**: 1529–1539.
- Von Essen MR, Kongsbak M, Geisler C. Mechanisms behind functional avidity maturation in T cells. *Clin Dev Immunol* 2012; **2012**: e163453.
- Singel KL, Segal BH. Neutrophils in the tumor microenvironment: trying to heal the wound that cannot heal. *Immunol Rev* 2016; **273**: 329–343.
- Glodde N, Bald T, van den Boorn-Konijnenberg D et al. Reactive neutrophil responses dependent on the receptor tyrosine kinase c-MET limit cancer immunotherapy. *Immunity* 2017; **47**: 789–802.
- Clausen BE, Burkhardt C, Reith W, Renkawitz R, Förster I. Conditional gene targeting in macrophages and granulocytes using LysMcre mice. *Transgenic Res* 1999; **8**: 265–277.
- Barsoum IB, Koti M, Siemens DR, Graham CH. Mechanisms of hypoxia-mediated immune escape in cancer. *Cancer Res* 2014; **74**: 7185–7190.
- Ohta A. A metabolic immune checkpoint: adenosine in tumor microenvironment. *Front Immunol* 2016; **7**: 1–11.

33. Ye L-Y, Chen W, Bai X-L et al. Hypoxia-induced epithelial-to-mesenchymal transition in hepatocellular carcinoma induces an immunosuppressive tumor microenvironment to promote metastasis. *Cancer Res* 2016; **76**: 818–830.
34. Campbell E, Bruyninckx W, Kelly C et al. Transmigrating neutrophils shape the mucosal microenvironment through localized oxygen depletion to influence resolution of inflammation. *Immunity* 2014; **40**: 66–77.
35. Sikora AG, Jaffarad N, Hailemichael Y et al. IFN- $\alpha$  enhances peptide vaccine-induced CD8<sup>+</sup> T cell numbers, effector function, and antitumor activity. *J Immunol* 2009; **182**: 7398–7407.
36. Bald T, Landsberg J, Lopez-Ramos D et al. Immune cell-poor melanomas benefit from PD-1 blockade after targeted type I IFN activation. *Cancer Discov* 2014; **4**: 674–687.
37. Bald T, Quast T, Landsberg J et al. Ultraviolet-radiation-induced inflammation promotes angiogenesis and metastasis in melanoma. *Nature* 2014; **507**: 109–113.
38. Stock AT, Smith JM, Carbone FR. Type I IFN suppresses Cxcr2 driven neutrophil recruitment into the sensory ganglia during viral infection. *J Exp Med* 2014; **211**: 751–759.
39. Jablonska J, Wu C-F, Andzinski L, Leschner S, Weiss S. CXCR2-mediated tumor-associated neutrophil recruitment is regulated by IFN- $\beta$ . *Int J Cancer* 2014; **134**: 1346–1358.
40. Widmer DS, Hoek KS, Cheng PF et al. Hypoxia contributes to melanoma heterogeneity by triggering HIF1 $\alpha$ -dependent phenotype switching. *J Invest Dermatol* 2013; **133**: 2436–2443.
41. Liu P, Zhang C, Chen J et al. Combinational therapy of interferon- $\alpha$  and chemotherapy normalizes tumor vasculature by regulating pericytes including the novel marker RGS5 in melanoma. *J Immunother* 2011; **34**: 320–326.
42. Jablonska J, Leschner S, Westphal K, Lienenklaus S, Weiss S. Neutrophils responsive to endogenous IFN- $\beta$  regulate tumor angiogenesis and growth in a mouse tumor model. *J Clin Invest* 2010; **120**: 1151–1164.
43. Johansson A, Hamzah J, Payne CJ, Ganss R. Tumor-targeted TNF $\alpha$  stabilizes tumor vessels and enhances active immunotherapy. *Proc Natl Acad Sci USA* 2012; **109**: 7841–7846.
44. Riesenberger S, Groethen A, Siddaway R et al. MITF and c-Jun antagonism interconnects melanoma dedifferentiation with pro-inflammatory cytokine responsiveness and myeloid cell recruitment. *Nat Commun* 2015; **6**: 8755.
45. Dunn IS, Haggerty TJ, Kono M et al. Enhancement of human melanoma antigen expression by IFN- $\beta$ . *J Immunol* 2007; **179**: 2134–2142.
46. Hölzel M, Bovier A, Tüting T. Plasticity of tumour and immune cells: a source of heterogeneity and a cause for therapy resistance? *Nat Rev Cancer* 2013; **13**: 365–376.
47. Pérez-Guijarro E, Yang HH, Araya RE et al. Multimodal preclinical platform predicts clinical response of melanoma to immunotherapy. *Nat Med* 2020; **26**: 781–791.
48. Murdoch C, Giannoudis A, Lewis CE. Mechanisms regulating the recruitment of macrophages into hypoxic areas of tumors and other ischemic tissues. *Blood* 2004; **104**: 2224–2234.
49. Diamond MS, Kinder M, Matsushita H et al. Type I interferon is selectively required by dendritic cells for immune rejection of tumors. *J Exp Med* 2011; **208**: 1989–2003.
50. Kohlmeyer J, Cron M, Landsberg J et al. Complete regression of advanced primary and metastatic mouse melanomas following combination chemoimmunotherapy. *Cancer Res* 2009; **69**: 6265–6274.

## Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.



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