Immune Cell–Poor Melanomas Benefit from PD-1 Blockade after Targeted Type I IFN Activation

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INTRODUCTION

Primary and metastatic human melanomas show considerable variability in the composition, density, and distribution of tumor-infiltrating immune cells in different patients. In agreement with the tumor immune-surveillance theory, several studies found a correlation among the presence of T cells in primary melanomas, the expression of MHC class I molecules on tumor cells, and a favorable prognosis (1, 2). Conversely, the absence of tumor-infiltrating T cells in primary melanomas was associated with an increased risk for metastatic spread into the sentinel lymph nodes and decreased survival (3, 4). The underlying mechanisms that recruit immune cells and regulate their function in the tumor microenvironment are poorly understood. Previously, we described an association between the presence of granzyme B–expressing T lymphocytes and a locally activated type I IFN system, indicated by expression of the antiviral protein MxA, in primary melanomas of patients showing signs of spontaneous regression (5). The rationale for our analyses was derived from our observations in experimental mouse models with transplantaible tumors, including the B16 melanoma, where local transgenic expression of IFNα could augment CTL responses in the tumor microenvironment (6–8). More recently, it was shown that activation of the host type I IFN system is indeed a critical requirement for the innate immune recognition of transplanted B16 melanomas through signaling on CD80+ dendritic cells (DC), which then initiate adaptive cellular immunity (9, 10).

However, melanomas also frequently progress despite T-cell infiltration. This was originally thought to be due to the Darwinian selection of tumor-cell variants that escape immune destruction (11). As an alternative explanation, it was found that T cells lose their effector functions in the immunosuppressive microenvironment of tumors in which regulatory immune cell subpopulations accumulate (12). More recently, dynamic adaptive changes of both tumor and immune cells caused by inflammatory cytokines have been described that contribute to the immune escape of melanoma (13–15). Prominent among these is the upregulation of the immune-inhibitory receptor PD-L1 on melanoma cells in response to T cell–derived IFNγ, which in turn engages PD-1 on T cells and attenuates their effector functions (16, 17). Blockade of the interaction between PD-L1 and PD-1 can reactivate effector functions of melanoma-specific T cells both in mouse and man, demonstrating the critical importance of this immunoregulatory mechanism in tumor tissue (18–21).

Given the success of new immunotherapies that abrogate the immune-inhibitory PD-L1–PD-1 interactions in patients with melanoma with preexisting antitumor immunity (16, 20), the treatment of patients with melanomas lacking T-cell infiltrates (“immune cell–poor melanomas”) has emerged as a major clinical challenge. We experimentally addressed this issue in the genetically engineered Hgf-Cdk4<sup>R24C</sup> mouse model.
in which primary melanomas histomorphologically imitate human pigmented melanomas with little immune cell infiltration and metastasize early in lymph nodes and lungs (22).

In our work, we investigated three principal hypotheses: (i) immune cell–poor primary melanomas evade innate type I IFN–dependent immune surveillance and thereby avoid the induction of antitumoral CTL immunity; (ii) targeted activation of type I IFNs can establish cellular immune surveillance; (iii) type I IFNs simultaneously activate immune-inhibitory PD-L1–PD-1 receptor interactions, and therapeutic blockade of this pathway further augments tumor immune surveillance.

RESULTS

Immune Cell–Poor Melanomas Evade Type I IFN–Dependent Immune Surveillance and Editing

On the basis of our immunohistochemical observation that regressive primary melanomas with extensive T-cell infiltration stain positive for markers of an activated type I IFN system (5), we expected that immune cell–poor melanomas lacking T cells would show only low expression levels of type I IFN–regulated genes. A bioinformatic analysis of genome-wide transcriptomic data for 223 primary melanomas (23) indeed showed that the expression of \textit{CD3D} and other T-cell transcripts directly correlated with the expression of a set of genes that are regulated by type I IFNs (Pearson correlation coefficient \(r = 0.6\)) in melanoma cells (Fig. 1A and B and Supplementary Table S1). The type I IFN response signature was generated from a publicly available dataset of IFN\(\alpha\)-treated human melanoma cells (24). Furthermore, high expression of type I IFN–responsive genes or \textit{CD3D} was associated with an increased relapse-free survival, consistent with the idea that preexisting antitumoral immune responses determine a favorable prognosis in patients with melanoma (Fig. 1C). In turn, the absence of IFN-regulated genes in immune cell–poor melanomas suggested that these tumors escape
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the immune-surveillance function of the type I IFN system and thereby avoid the recruitment of immune cells and the subsequent induction of protective tumor-specific cytotoxic immune responses. Primary cutaneous melanomas in Hgf-Cdk4<sup>R24C</sup> mice morphologically imitate immune cell–poor pigmented primary human melanomas (Fig. 1D) and show very low expression of type I IFN–regulated genes. This experimental system therefore allowed us to investigate whether malignant transformation indeed takes place without the induction of type I IFN–dependent activation of cellular antitumor immunity.

We therefore crossed Hgf-Cdk4<sup>R24C</sup> into the I<sup>nfnar1</sup>−/− background to obtain melanoma-prone mice that lack a functional type I IFN system. Cohorts of 8-week-old I<sup>nfnar1</sup>-competent and I<sup>nfnar1</sup>-deficient Hgf-Cdk4<sup>R24C</sup> mice then received a single epicutaneous application of the carcinogen 7,12-dimethylbenz(a)anthracene (DMBA) on the back skin (Fig. 2A) to accelerate and synchronize the development of primary melanomas (13). We found that DMBA-initiated primary melanomas appeared with the same growth kinetics and multiplicity in the skin of I<sup>nfnar1</sup>-deficient and I<sup>nfnar1</sup>-competent Hgf-Cdk4<sup>R24C</sup> mice (Fig. 2B). Accordingly, DMBA-exposed I<sup>nfnar1</sup>-deficient and I<sup>nfnar1</sup>-competent Hgf-Cdk4<sup>R24C</sup> mice showed largely identical survival curves (Fig. 2C). These results indicated that the development of primary melanomas in this experimental system was not affected by type I IFN–dependent immune surveillance.

In subsequent experiments, we injected cohorts of I<sup>nfnar1</sup>-competent and I<sup>nfnar1</sup>-deficient Hgf-Cdk4<sup>R24C</sup> mice subcutaneously with the carcinogen methylcholanthrene (MCA) to induce primary fibrosarcomas (Fig. 2D). In line with previously reported experimental work in this well-established model for IFN-dependent cancer immune surveillance (25), we found that fibrosarcomas developed with decreased latency and increased penetrance in I<sup>nfnar1</sup>-deficient compared with I<sup>nfnar1</sup>-competent Hgf-Cdk4<sup>R24C</sup> mice (Fig. 2E), resulting in shorter survival (Fig. 2F). This ruled out the possibility that the anti-inflammatory properties of hepatocyte growth factor (HGF), which can promote a tolerogenic DC phenotype and the expansion of regulatory T cells (26, 27), precluded immune surveillance by the type I IFN system in Hgf-Cdk4<sup>R24C</sup> mice.

Figure 2. Primary melanomas in Hgf-Cdk4<sup>R24C</sup> mice escape type I IFN-mediated immune surveillance. A and D, experimental protocol for the induction of primary cutaneous melanomas (A) or sarcomas (D) in cohorts of I<sup>nfnar1</sup>-competent and I<sup>nfnar1</sup>-deficient Hgf-Cdk4<sup>R24C</sup> mice with a single epicutaneous application of DMBA or a single subcutaneous injection of MCA, respectively. B and E, tumor growth kinetics of the largest DMBA-induced melanoma (B) and of MCA-induced sarcomas (E) in representative cohorts of 5 individual I<sup>nfnar1</sup>-competent (top) and I<sup>nfnar1</sup>-deficient (bottom) mice over time. C and F, corresponding Kaplan–Meier survival curves of melanoma-bearing (C) or sarcoma-bearing (F) I<sup>nfnar1</sup>-competent and I<sup>nfnar1</sup>-deficient Hgf-Cdk4<sup>R24C</sup> mice. G, representative CD45-stained sections of a primary DMBA-induced melanoma (left) and a primary MCA-induced sarcoma (right) in I<sup>nfnar1</sup>-competent Hgf-Cdk4<sup>R24C</sup> mice. H, flow cytometric quantification of tumor-infiltrating immune cells in primary Hgf-Cdk4<sup>R24C</sup> melanomas and sarcomas (left and middle; mean ± SEM; n = 12) and corresponding real-time PCR analysis of IFN-induced genes (right; mean ± SEM; n = 12; *, P < 0.05).
To understand the divergent role of the type I IFN system in the pathogenesis of primary melanomas and fibrosarcomas, we performed further morphologic, cellular, and molecular investigations. Immunohistopathologic analyses revealed increased numbers of tumor-infiltrating CD45+ immune cells in primary MCA-induced fibrosarcomas when compared with primary DMBA-induced melanomas from 5 individual Ifnar1-competent and Ifnar1-deficient mice onto groups of 3 syngeneic Ifnar1-competent, Ifnar1-deficient, and Rag2-deficient C57BL/6 mice. Tumor development was monitored over time. Figure 3. Both Ifnar1-competent (CT) and Ifnar1-deficient (IFT) Hgf-Cdk4R24C melanomas grow progressively when transplanted in Ifnar1-competent and Ifnar1-deficient hosts. A, experimental protocol for transplantation of primary DMBA-induced melanomas from 5 individual Ifnar1-competent and Ifnar1-deficient Hgf-Cdk4R24C mice onto groups of 3 syngeneic Ifnar1-competent, Ifnar1-deficient, and Rag2-deficient C57BL/6 mice. Tumor development was monitored over time. B, time to tumor onset of Ifnar1-competent Hgf-Cdk4R24C melanomas in the indicated genotypes grouped according to similar (left) or faster (right) growth in Ifnar1-deficient recipient mice. C, corresponding data for Ifnar1-deficient Hgf-Cdk4R24C melanomas. WT, wild-type.

Figure 3. Both Ifnar1-competent (CT) and Ifnar1-deficient (IFT) Hgf-Cdk4R24C melanomas grow progressively when transplanted in Ifnar1-competent and Ifnar1-deficient hosts. A, experimental protocol for transplantation of primary DMBA-induced melanomas from 5 individual Ifnar1-competent and Ifnar1-deficient mice onto groups of 3 syngeneic Ifnar1-competent, Ifnar1-deficient, and Rag2-deficient C57BL/6 mice. Tumor development was monitored over time. B, time to tumor onset of Ifnar1-competent Hgf-Cdk4R24C melanomas in the indicated genotypes grouped according to similar (left) or faster (right) growth in Ifnar1-deficient recipient mice. C, corresponding data for Ifnar1-deficient Hgf-Cdk4R24C melanomas. WT, wild-type.

To understand the divergent role of the type I IFN system in the pathogenesis of primary melanomas and fibrosarcomas, we performed further morphologic, cellular, and molecular investigations. Immunohistopathologic analyses revealed increased numbers of tumor-infiltrating CD45+ immune cells in primary MCA-induced fibrosarcomas when compared with primary DMBA-induced melanomas in Hgf-Cdk4R24C mice (Fig. 2G). This difference was further quantified and confirmed to be significant by flow cytometric analyses of tumor-cell suspensions (Fig. 2H, left). Fibrosarcomas showed increased numbers of tumor-infiltrating CD8+ T cells (Fig. 2H, middle) and significantly higher expression levels of type I IFN-regulated genes (Fig. 2H, right). Taken together, these results demonstrated that melanomas and fibrosarcomas interact with the host immune system in fundamentally different ways, although they were both induced by chemically closely related potent carcinogens.

It was previously described that MCA-induced fibrosarcomas derived from Ifnar1-deficient mice show a highly immunogenic, “unedited” phenotype and therefore do not grow progressively when transplanted onto Ifnar1-competent mice (25). Because primary Hgf-Cdk4R24C melanomas did not activate type I IFNs, we hypothesized that they would also escape the type I IFN-dependent immune-editing process. To test this hypothesis, we transplanted five different Ifnar1-competent and five different Ifnar1-deficient DMBA-induced primary Hgf-Cdk4R24C melanomas onto groups of three Ifnar1-competent, Ifnar1-deficient, and Rag2-deficient syngeneic C57BL/6 mice (Fig. 3A). Each transplanted melanoma grew progressively in all three strains of mice (Fig. 3B and C), indicating the poor immunogenicity of both Ifnar1-competent and Ifnar1-deficient melanomas. Interestingly, a subset of Ifnar1-competent and Ifnar1-deficient melanoma cells grew more rapidly in Ifnar1-deficient mice when compared with Ifnar1-competent or Rag2-deficient mice, pointing toward the previously described role for the host type I IFN system in transplanted melanoma (9).
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Type I IFN system (28, 29). Peritumoral injections of established DMBA-induced primary Hgf-Cdk4R24C melanomas with poly(I:C) for 2 weeks strongly induced the expression of type I IFN–regulated genes and promoted the recruitment of immune cells into the tumor microenvironment (Fig. 4A and B). Prolonged treatment with poly(I:C) considerably delayed the growth of primary melanomas and increased survival (Fig. 4C and D). This effect was completely abrogated in Ifnar1-deficient Hgf-Cdk4R24C mice (Fig. 4E). Thus, targeted type I IFN activation with poly(I:C) exposed immune cell–poor Hgf-Cdk4R24C melanomas to the surveillance functions of cellular immunity.

To investigate the role of the type I IFN system in tumor versus host cells, we used the slowly growing transplantable Hgf-Cdk4R24C melanoma cell line HCmel3, which morphologically imitates primary Hgf-Cdk4R24C melanomas (13) and shows similar low expression levels of type I IFN–regulated genes. Short-term treatment of established HCmel3 melanomas with poly(I:C) for 2 weeks (Fig. 4F) also stimulated type I IFN activation with poly(I:C) exposed immune cell–poor HCmel3 melanomas compared with controls (mean ± SEM, n = 6; **, P < 0.01). Similar results were obtained in two independent treatment cohorts. E and F, corresponding tumor growth kinetics and mean survival in Ifnar1-deficient cohorts of mice treated as indicated (n = 8). Similar results were obtained in two independent treatment cohorts.

**Targeted Type I IFN Activation Establishes Immune Surveillance and Impairs Melanoma Growth**

We hypothesized that therapeutic activation of type I IFNs in the microenvironment of primary Hgf-Cdk4R24C melanomas could alert innate immune surveillance and thereby delay tumor growth. As an experimental strategy, we used the prototypic immunostimulatory RNA polyinosinic:polycytidylic acid [poly(I:C)], which triggers the innate viral recognition receptors TLR3 and MDA5 and efficiently stimulates the type I IFN system (28, 29). Peritumoral injections of established DMBA-induced primary Hgf-Cdk4R24C melanomas with poly(I:C) for 2 weeks strongly induced the expression of type I IFN–regulated genes and promoted the recruitment of immune cells into the tumor microenvironment (Fig. 4A and B).
Prolonged poly(I:C) treatment delayed the growth of transplanted HCmel3 melanomas and significantly increased survival (Fig. 4H and I). Transplanted HCmel3 cells grow with similar kinetics in Ifnar1-competent and Ifnar1-deficient mice (Fig. 4I and J, left), demonstrating that this melanoma cell line does not spontaneously engage the host type I IFN system in the transplantation setting. Importantly, the therapeutic efficacy of poly(I:C) was completely abrogated in Ifnar1-deficient C57BL/6 mice (Fig. 4J), demonstrating the critical requirement for a functional type I IFN system in host cells.

In primary and transplanted MCA-induced sarcomas, Ifnar1 signaling in host hematopoietic cells was shown to be critical for the induction of antitumor immune responses (25). The activation of natural killer (NK) cell responses with poly(I:C) requires signaling through Ifnar1 not only in NK cells but also in other immune cell types, most importantly in DCs (30). To experimentally dissect the contribution of Ifnar1 signaling in various immune cell subsets to the observed antitumor effects, we treated HCmel3 cells with poly(I:C) in mice with cell type-specific conditional deletion of the Ifnar1 gene (31–33). We found that the therapeutic activity of poly(I:C) was largely abrogated in mice lacking the Ifnar1 gene specifically in CD11c+, LysM+, CD4+, or Nc1r+ cells, which are primarily expressed in DCs, macrophages/neutrophils, T cells, and NK cells, respectively (Fig. 5A). These results demonstrate a requirement for the coordinated activation of type I IFN responses in all of these different immune cell subsets to obtain the full antitumor efficacy of poly(I:C).

Next, we explored the antitumor effector mechanisms induced by targeted activation of the type I IFN system in our experimental model. Antibody-mediated depletion of NK cells largely abrogated and depletion of CD8+ T cells severely compromised the efficacy of poly(I:C) treatment (Fig. 5B). This result is consistent with the notion that NK-cell cytotoxicity is required early to keep melanomas in...
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Check and promote the subsequent development of CD8+ T cell–mediated immunity. Both NK cells and CD8+ T cells exert their antitumor activity at least in part through the secretion of IFNγ (34, 35). Antibody-mediated blockade of IFNγ completely abolished the antitumor immune responses induced by poly(I:C) (Fig. 5B). Taken together, our findings indicate that targeted activation of the type I IFN system in immune cell–poor Hgf-Cdk4R24C melanomas induces effective immune cell surveillance through activation of NK and CD8+ T cells and subsequent production of IFNγ.

Combination Immunotherapy with Poly(I:C) and a Blocking Anti–PD-1 mAb Prolongs Survival

Treatment of primary and transplanted Hgf-Cdk4R24C melanomas with poly(I:C) restrains their growth for several weeks but eventually fails (Fig. 4D and I). Both type I and II IFNs upregulated the expression of PD-L1 on the Hgf-Cdk4R24C melanoma cell line HCmel3 in vitro (Fig. 6A). We also observed a significant upregulation of Pdl1 mRNA expression levels in poly(I:C)–treated compared with untreated Hgf-Cdk4R24C mouse melanomas that correlated with the upregulation of type I IFN–regulated genes such as Ifr7 (Fig. 6B). Melanoma–bearing mice treated with poly(I:C) also showed increased numbers of PD-L1–expressing CD8+ T cells in the peripheral blood (Fig. 6C), indicating an activation of the immune-inhibitory PD-L1–PD-1 signaling axis as a counter-regulatory mechanism to attenuate effector functions of both T and NK cells (17, 18, 36).

We therefore reasoned that therapeutic blockade of PD-1 signaling would further augment and sustain the antitumor activity of targeted type I IFN activation with poly(I:C). Indeed, injections of a PD-1–blocking monoclonal antibody (mAb) together with poly(I:C) were able to cause partial regression in established HCmel3 mouse melanomas and significantly prolonged the survival compared with poly(I:C) treatment alone (126 ± 16 vs. 97 ± 13 days; Fig. 6D–F). Injections of anti–PD-1 mAb alone did not show any treatment effect, consistent with our finding that Hgf-Cdk4R24C mouse melanomas escape cellular immune surveillance. Thus, targeted activation of type I IFNs in combination with blockade of the IFN-induced immune-inhibitory PD-L1–PD-1 signaling pathway represents a rational strategy to expose immune cell–poor tumors to prolong immune surveillance. Finally, a detailed toxicologic study revealed that intracutaneous injections of poly(I:C) alone or in combination with intraperitoneal injections of PD-1–blocking mAbs only caused local skin inflammatory responses, without substantial treatment–related acute toxic side effects affecting vital organ structure and function (Supplementary Figs. S1 and S2).

Our experimental findings suggested that the expression of PD-L1 in melanoma tissues correlates with the expression of CD3 and type I IFN–responsive genes. A poor-quality probe precluded the analysis of PD-L1 expression in the primary
DISCUSSION

In our work, we experimentally investigated the role of type I IFN–dependent tumor immune surveillance in a genetically engineered mouse model of melanoma. By crossing the Hgf-Cdk4<sup>R24C</sup> mouse strain onto the Ifnar<sup>-/-</sup>deficient background, we show that immune cell–poor primary melanomas do not spontaneously activate the immune-surveillance and immune-editing functions of the endogenous type I IFN system. Because Hgf-Cdk4<sup>R24C</sup> mouse melanomas imitate immune cell–poor human primary melanomas with a bad prognosis, our results suggest that this subset of tumors also evades type I IFN–dependent immune surveillance.

In contrast to DMBA-induced melanomas, we found that primary MCA-induced sarcomas spontaneously activated the host type I IFN system in Hgf-Cdk4<sup>R24C</sup> mice, confirming a previously published report (25). Because both tumor types were induced by chemically related and highly potent carcinogens, one would expect a similar spectrum of tumor antigens due to genetic mutations. The divergent interaction of primary melanomas and sarcomas with the innate immune system might therefore reflect the different immunologic properties of the cells of origin, for example, melanocytes versus fibroblasts. It is tempting to speculate that malignant fibrosarcoma cells dictate an immunologically much more active microenvironment as they express higher levels of MHC class I molecules and secrete increased amounts of...

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**Figure 7.** PD-L1 expression correlates with T-cell markers and an IFN response signature in human melanomas. A, bottom, heatmap of primary melanoma samples (n = 46; GSE15605) ordered by increasing T-cell marker gene levels (CD3) and visualization of corresponding IFN response signature in the primary melanoma cohort shown in Fig. 1B. Clinical follow-up data were available for the TCGA melanoma metastasis (n = 248) from the TCGA melanoma dataset (skin cutaneous melanoma; SKCM). Pearson correlation coefficients (r) are indicated (top). B, PD-L1, IFN response signature, and CD3D expression in human melanoma metastasis (n = 248) are indicated (top). C, Kaplan–Meier analysis of overall survival (calculated as years to death or years to last follow-up) using the TCGA cohort (melanoma metastasis; n = 248) and median expression value cutoffs for CD3D, the IFN response signature, and PD-L1. P values were determined by a log-rank test.
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Cdk4

late PD-L1 expression on melanoma cells

leads to subsequent functional activation of the PD-L1–PD-1

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in vivo

infections after transplantation (25). Here, type I IFN–dependent activation of DC subsets that are

specialized for antigen cross-presentation was required for effective

injection of antitumor immunity (9, 32).

Targeted activation of the type I IFN system in the microen-

vironment of immune cell–poor Hgf-Cdk4R24C mouse melanomas with poly(IC) was associated with cytotoxic immune cell recruitment, subsequent upregulation of PD-L1 expression in tumor tissue, and an increased expression of PD-1 on periph-

eral blood CD8+ T cells. Because type I and type II IFNs upregu-

late PD-L1 expression on melanoma cells in vitro, and because poly(IC) induces cellular antitumor immunity that critically

depends on type I and type II IFNs in vivo, we hypothesized that the interaction between melanoma and T cells through

PD-L1 and PD-1 receptors represents an adaptive resistance program to IFN-driven cytotoxic immunity that attenuates effector functions of T and NK cells. Our observation that anti-

body-mediated PD-1 blockade prolonged the survival of mice only in combination with poly(IC) but not given as a mono-

therapy demonstrates that activation of the type I IFN system leads to subsequent functional activation of the PD-L1–PD-1

immune-inhibitory signaling axis in immune cell–poor Hgf-

Cdk4R24C melanomas. Because PD-L1 is expressed not only on melanoma cells but also on accessory cells in the tumor stroma

(such as fibroblasts and DCs), the relative contribution of these
cell types for PD-1–mediated interaction with T cells will have to be experimentally resolved in future work. This would have to include studies in other experimental systems to confirm the generality of our findings beyond the Hgf-Cdk4R24C mouse melanoma model used in our work.

Recent clinical trials have demonstrated that blockade of the immune-inhibitory PD-L1–PD-1 pathway can achieve high

response rates in some patients with advanced metastatic melanoma and other types of cancers (19–21). PD-L1–PD-1 blockade seemed to be particularly effective in patients with melanoma with an ongoing cellular immune response (20). Our experimental results confirm the notion that upregula-

tion of the PD-1–PD-L1 signaling axis in tumor tissue, and as a consequence of type I IFN activation and invasion by NK and T cells, predicts therapeutic benefit from therapeutic

PD-L1–PD-1 blockade alone. We therefore propose that the expression of PD-L1 and type I IFN–responsive genes in tumor tissues could serve as a sensitive biomarker for patient stratification in clinical trials investigating PD-1–PD-L1 antibody-containing regimens. RNA-seq data from the TCGA melanoma project indicate a comparatively low abundance of PD-L1 mRNA (lower third) relative to all other detected reference transcripts (data not shown). Taking our survival analysis into account, it is therefore conceivable that PD-L1 levels below the current detection threshold of immunohis-

tochemistry are functionally and clinically relevant and may explain discrepancies addressing the prognostic and predic-
tive value of PD-L1 expression.

Approximately one third of all metastatic melanomas are only poorly infiltrated with immune cells (45). Consistent with recently published work, our bioinformatic analysis revealed that patients with these immune cell–poor melanomas, in which type I IFN–regulated genes, T cell–related genes, and PD-L1 are expressed at low levels, had a com-

paratively poor prognosis (23, 46–48). Hence, there is an obvious need for new therapeutic strategies in this patient subgroup. Our observation that treatment with a combina-
on of poly(IC) and a blocking anti–PD-1 mAb prolonged the survival of mice with immune cell–poor melanoma not only highlights the critical importance of immune-inhibitory

PD-L1–PD-1 interactions in vivo but also provides a preclinical proof-of-concept that targeted type I IFN activation is a rational strategy to increase the therapeutic benefit of PD-1–PD-L1 blockade for patients with immune cell–poor melanomas as well. These insights underscore the clinical relevance of our work and provide a rationale for further experimental investigations to develop similar combination treatment protocols. These may further augment cytotoxic immunity by additionally targeting other IFN-driven counter-regulatory mechanisms that attenuate NK- and T-cell effector functions in the tumor microenvironment (49).

METHODS

Mice

Wild-type, Ifnar1+/− and Rag2+/− C57BL/6 mice were purchased from The Jackson Laboratory. Ifnar1+/− C57BL/6 mice were crossed with melanoma-prone Hgf-Cdk4R24C mice to obtain Ifnar1+/− × Hgf-

Cdk4R24C mice. Ifnar1+/−, Ly5.5-Cre × Ifnar1+/− (Ifnar1+/−Cre) or LysM-Cre × Ifnar1+/− (Ifnar1+/−LysM-Cre), CD11c-Cre × Ifnar1+/− (Ifnar1+/−CD11c-Cre), CD4-Cre × Ifnar1+/− (Ifnar1+/−CD4-Cre), and

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Ncrr-Cre × Ifnar1−/− (Ifnar1−/−/C57BL/6) mice on the C57BL/6 background were bred as described previously (31–33). All animal experiments were approved by the local government authorities (LANUV, NRW, Germany) and performed according to the institutional and national guidelines for the care and use of laboratory animals.

Induction and Analysis of Primary Melanomas and Primary Sarcomas

The development of primary melanomas on the shaved back skins of 8-week-old Ifnar1-competent and Ifnar1-deficient Hgf-Cdk4R24C mice was accelerated and synchronized by a single epicutaneous application of 100 nmol DMBA as described previously (13). Alternatively, mice received a single subcutaneous injection of 100 μg MCA into the flank to induce fibrosarcomas. Tumor development was monitored by inspection, palpation, and digital photography. Tumor sizes were measured weekly using a vernier calliper and recorded as mean diameter. Mice were sacrificed when progressively growing melanomas or sarcomas exceeded 10 mm or when signs of illness were observed.

Serial Tumor Transplantation

Primary DMBA-induced melanomas from Ifnar1-competent (CT) and Ifnar1-deficient (IFT) Hgf-Cdk4R24C mice were serially transplanted onto Ifnar1-competent, Ifnar1-deficient, or Rag2-deficient syngeneic C57BL/6 mice. For this, tumors were excised, dissociated mechanically, filtered through 70-μm cell strainers (BD Biosciences), and washed in PBS. A total of 2 × 10⁶ cells were injected intracutaneously into the flank, and tumor development was monitored by inspection and palpation. Tumor onset was defined as the day when a tumor reached 2 mm in diameter and grew progressively. Tumor sizes were measured weekly and recorded as mean diameter.

HCme3 Tumor Transplantation

The HCme3 melanoma cell line was generated from a primary Hgf-Cdk4R24C melanoma as described previously (13). Groups of syngeneic C57BL/6 mice were injected intracutaneously with 4 × 10⁶ HCme3 melanoma cells into the flank, and tumor size was measured weekly and recorded as mean diameter in millimeters. Mice with tumors exceeding 20 mm were sacrificed. Experiments were performed in groups of 5 or more mice and repeated at least twice.

Tumor Treatment

When primary or transplanted melanomas became palpable, twice weekly peritumoral injections with 50 μg poly(I:C) (Invivogen) were performed. Therapeutic blockade of PD-1 was performed by twice weekly intraperitoneal injections of 250 μg rat anti-mouse PD-1 IgG2a (clone RMP1-14; BioXcell) or control rat IgG2a mAb (clone 2A3; BioXcell). Antibody-mediated depletion of CD8+ T cells or NK cells and neutralization of IFNγ was performed by twice-weekly intraperitoneal injections of 200 μg rat anti-mouse CD8 IgG2a (clone 24-3; BioXcell), 200 μg rat anti-mouse NK1.1 IgG2a (clone PK136; BioXcell), or 100 μg rat anti-mouse IFNγ IgG1 (clone XMG1.2; BioXcell), respectively. Control groups again received 200 μg/mouse of the irrelevant rat IgG2a mAb (clone 2A3; BioXcell).

Repeated-Dose Acute Toxicity Study

To evaluate the potential toxicity of the combination treatment with 50 μg poly(I:C) intracutaneously and 250 μg anti-PD-1 antibodies intraperitoneally, groups of C57BL/6 mice were treated twice weekly for 4 weeks. A control group of mice was injected with identical volumes of PBS intracutaneously and intraperitoneally. The body weight and general health were observed and documented for 28 days. At necropsy 4 days after the last therapeutic dose, blood was collected for clinical chemistry and hematologic analyses by retroorbital puncture. Various biochemical parameters were measured in the sera with the Vitros 250 (Ortho-Clinical Diagnostics) clinical chemistry automated system. Counts of white blood cells, red blood cells, and platelets as well as hemoglobin levels were determined in EDTA-blood using the Celltac α (Nihon Khoden Europe) instrument. In addition, vital internal organs were isolated, weighed, fixed in formalin, and embedded in paraffin for subsequent histopathologic analyses. Relative organ weights were calculated as a percentage of total body weight. Hematoxylin and eosin (H&E)-stained sections of several organs were scored for pathologic alterations, including degenerative changes and immune cell infiltration.

Histology and Immunohistology

Mouse tumors were immersed in a zinc-based fixative (BD Pharmingen) and human melanoma samples in buffered paraformaldehyde (DAKO). Informed consent to use melanoma biopsy material for scientific purposes was obtained from all patients. Tissues were embedded in paraffin and sections were stained with H&E according to the standard protocols. Immunohistochemistry was performed with rat anti-mouse CD45 mAb (BD Biosciences), followed by enzyme-conjugated secondary antibodies and the LSAB-2 color development system (DAKO). Heavy pigmented mouse melanomas were bleached before staining (20 minutes at 37°C in 30% H₂O₂, and 0.5% KOH, 20 seconds in 1% acetic acid and 5 minutes in TRIS Buffer). Stained sections were examined with a Leica DMLB microscope. Images were acquired with a JVC digital camera KY-75FU and processed with Adobe Photoshop.

Flow Cytometry

Melanoma-infiltrating immune cells were isolated and stained with fluorochrome-conjugated mAbs specific for mouse CD45, CD11b, CD8, NK1.1, and MHC I (all from BD Pharmaningen) according to the standard procedures. Surface expression of PD-L1 and PD-L2 was measured weekly using a vernier calliper and recorded as mean diameter in millimeters. Mice with tumors exceeding 20 mm were sacrificed. Experiments were performed in groups of 5 or more mice and repeated at least twice.

Tumor Treatment

When primary or transplanted melanomas became palpable, twice weekly peritumoral injections with 50 μg poly(I:C) (Invivogen) were performed. Therapeutic blockade of PD-1 was performed by twice weekly intraperitoneal injections of 250 μg rat anti-mouse PD-1 IgG2a (clone RMP1-14; BioXcell) or control rat IgG2a mAb (clone 2A3; BioXcell). Antibody-mediated depletion of CD8+ T cells or NK cells and neutralization of IFNγ was performed by twice-weekly intraperitoneal injections of 200 μg rat anti-mouse CD8 IgG2a (clone 24-3; BioXcell), 200 μg rat anti-mouse NK1.1 IgG2a (clone PK136; BioXcell), or 100 μg rat anti-mouse IFNγ IgG1 (clone XMG1.2; BioXcell), respectively. Control groups again received 200 μg/mouse of the irrelevant rat IgG2a mAb (clone 2A3; BioXcell).

RepeteD-Dose Acute Toxicity Study

To evaluate the potential toxicity of the combination treatment with 50 μg poly(I:C) intracutaneously and 250 μg anti-PD-1 antibodies intraperitoneally, groups of C57BL/6 mice were treated twice weekly for 4 weeks. A control group of mice was injected with identical volumes of PBS intracutaneously and intraperitoneally. The body weight and general health were observed and documented for 28 days. At necropsy 4 days after the last therapeutic dose, blood was collected for clinical chemistry and hematologic analyses by retroorbital puncture. Various biochemical parameters were measured in the sera with the Vitros 250 (Ortho-Clinical Diagnostics) clinical chemistry automated system. Counts of white blood cells, red blood cells, and platelets as well as hemoglobin levels were determined in EDTA-blood using the Celltac α (Nihon Khoden Europe) instrument. In addition, vital internal organs were isolated, weighed, fixed in formalin, and embedded in paraffin for subsequent histopathologic analyses. Relative organ weights were calculated as a percentage of total body weight. Hematoxylin and eosin (H&E)-stained sections of several organs were scored for pathologic alterations, including degenerative changes and immune cell infiltration.

Histology and Immunohistology

Mouse tumors were immersed in a zinc-based fixative (BD Pharmingen) and human melanoma samples in buffered paraformaldehyde (DAKO). Informed consent to use melanoma biopsy material for scientific purposes was obtained from all patients. Tissues were embedded in paraffin and sections were stained with H&E according to the standard procedures. Immunohistochemistry was performed with rat anti-mouse CD45 mAb (BD Biosciences), followed by enzyme-conjugated secondary antibodies and the LSAB-2 color development system (DAKO). Heavy pigmented mouse melanomas were bleached before staining (20 minutes at 37°C in 30% H₂O₂, and 0.5% KOH, 20 seconds in 1% acetic acid and 5 minutes in TRIS Buffer). Stained sections were examined with a Leica DMLB microscope. Images were acquired with a JVC digital camera KY-75FU and processed with Adobe Photoshop.

Flow Cytometry

Melanoma-infiltrating immune cells were isolated and stained with fluorochrome-conjugated mAbs specific for mouse CD45, CD11b, CD8, NK1.1, and MHC I (all from BD Pharmaningen) according to the standard procedures. Surface expression of PD-L1 and MHC I on HCme3 melanoma cells was analyzed with fluorochrome-conjugated mAbs specific for PD-L1 and MHC I (BD Pharmaningen) according to the standard procedures. Data were acquired with a FACScanto Flow Cytometer (BD Biosciences) and analyzed with FlowJo software (TreeStar, V7.6.5 for Windows).

Real-Time Reverse Transcriptase PCR

Tumor samples were harvested and immediately snap-frozen in liquid nitrogen. Total RNA was isolated using TRI Reagent (Sigma-Aldrich) and purified using RNeasy columns (Qiagen). Real-time PCR analysis was performed with the SuperScript II system and oligo-dT18 primers (Invitrogen). Real-time PCR analysis was performed with diluted cDNA and Fast SYBR Green Master Mix (Applied Biosystems) using a 7500 Real-time PCR system (Applied Biosystems). Sequences of primers:

- Ifγ: (F: CAGGTTGACCGCATAGAGT; R:AGACATTGCTGAAGCT CACTT);
- Ccl10: (F:GCCGTACTTTCTGCTCTAT;R:GCTTCCCTATGGCC CTCACT);
- Cd8: (F: TGCCATCATATGAGCTG; R: GACCTTGCTGTCG TTGAAG);
- Cd7: (F: GAACCATGGTGGATGTCGTTG R: CCAAGTCTCATT CATGCTCTCG);
- Kbb: (F: TTGTTCAACATTAGATGGCC; AGCAAGTGGCC TCCCTTCACAG);
- GB: (F: CTCTCAATGACATGTCG; TCAGTTCCACATTGA TGAGGCAG);
- Per: (F: TGGAGAAGACCATAGGGACC; R:AAGCTCAAGGTTGAGGGAG);
- Pdi: (F: AGTATGGCAAGACCTCAG; R:TCCAATTCCATAGA CACTACTA); and
- L6: (F: AGGCAAGACATCCACCTTGGGAGC; R:CCATCACACCCA AGAACAAC).

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- Relative expression to the reference gene Ubc was calculated with the ΔCt method using the following equations: \( \Delta C_t \) (sample) = \( C_t \) (target) − \( C_t \) (reference); relative quantity = 2−ΔCt.

**Cell Culture and Treatment of HCMel3 Melanoma Cells**

HCMel3 melanoma cells were generated from primary Hgf-Cdk4P2c knockout mouse melanomas in our laboratory and cultured in complete RPMI-1640 medium containing 10% fetal calf serum (FCS; Biochrome), 2 mmol/L L-glutamine (Gibco), 10 mmol/L nonessential amino acids (Gibco), 1 mmol/L HEPES (Gibco), 20 μmol/L 2-mercaptoethanol, 100 IU/mL penicillin, and 100 mg/mL streptomycin (Invitrogen).

HCMel3 cells were authenticated by genomic PCR for the Hgf transgene and the Cdk4P2c knockin alleles. Melanoma cells were seeded in 6-well plates and treated with 1,000 U/mL recombinant mouse IFNα (PBL) or IFNγ (Peprotech). After 24 hours of stimulation, surface expression of MHC I and PD-L1 was analyzed.

**Statistical Analyses**

Statistical analyses of experimental results were evaluated with the GraphPad Prism 4 software. Two-tailed Student t test analyses were performed as indicated. Results were considered statistically significant when *, \( P < 0.05; **, P < 0.01; \) and ***, \( P < 0.001. \)

**Bioinformatic Analyses of Gene Expression Array Data for Human Melanomas**

We used the R programming environment and the Bioconductor platform for our bioinformatic analysis. The GSE19428 dataset was used to identify a core signature of type I IFNα-induced genes across five human melanoma cell lines (referred to as type I IFN response signature). The gene expression data (GSE19428_series_matrix.txt) were downloaded as normalized data using global scaling with a trimmed mean target intensity of each array set to 100 (24). Expression data were log-transformed, and the top 50 differentially expressed genes were identified by comparing mean expression values of IFNα-treated cells versus untreated control cells. This type I IFNα-responsive gene set from melanoma cell lines was used in the further analyses of the human melanoma tissue samples.

The gene expression dataset (Illumina WG-DASL array platform) of 223 primary melanomas was previously described (23). A median expression cutoff value for CD3D expression as a T-cell marker was used to analyze relapse-free survival of the CD3Dhigh versus the CD3Dlow subgroup. The mean expression of the IFNα-induced gene set was used to define IFNα signaturehigh and IFNα signaturelow subgroups using an unbiased median expression cutoff value. Relapse-free survival was determined by the Kaplan–Meier analysis and significance was assessed by a log-rank test. A gene probe for PD-L1 (CD274) on the Illumina WG-DASL array platform failed our quality control and was considered as not reliable. Correlations among PD-L1, IFNα signature, and CD3D expression were determined using two independent melanoma datasets and genomics platforms: (i) primary melanomas, GSE15605, Hgu133plus2 Affymetrix microarray platform (37); (ii) TCGA skin cutaneous melanoma (SKCM), melanoma metastasis, Illumina RNA-seq platform (https://tcga-data.nci.nih.gov/tcga/tcgaHome2.jsp).

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Authors’ Contributions**

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Study supervision: T. Tüting

Performance of experimental work and participation in discussions: D. Lopez-Ramos

Design, performance, and analysis of animal study for toxicity evaluation: J. Steitz

Performance of the repeated dose toxicity studies: R. Tolba

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the mouse colony and performing histopathology, immunohistochemistry, and quantitative PCR.

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