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

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ABSTRACT

Infiltration of human melanomas with cytotoxic immune cells correlates with spontaneous type I IFN activation and a favorable prognosis. Therapeutic blockade of immune-inhibitory receptors in patients with preexisting lymphocytic infiltrates prolongs survival, but new complementary strategies are needed to activate cellular antitumor immunity in immune cell-poor melanomas. Here, we show that primary melanomas in Hgf-Cdk4R24C mice, which imitate human immune cell-poor melanomas with a poor outcome, escape IFN-induced immune surveillance and editing. Peritumoral injections of immunostimulatory RNA initiated a cytotoxic inflammatory response in the tumor microenvironment and significantly impaired tumor growth. This critically required the coordinated induction of type I IFN responses by dendritic, myeloid, natural killer, and T cells. Importantly, antibody-mediated blockade of the IFN-induced immune-inhibitory interaction between PD-L1 and PD-1 receptors further prolonged the survival. These results highlight important interconnections between type I IFNs and immune-inhibitory receptors in melanoma pathogenesis, which serve as targets for combination immunotherapies.

SIGNIFICANCE: Using a genetically engineered mouse melanoma model, we demonstrate that targeted activation of the type I IFN system with immunostimulatory RNA in combination with blockade of immune-inhibitory receptors is a rational strategy to expose immune cell-poor tumors to cellular immune surveillance. Cancer Discov; 4(6); 674–87. ©2014 AACR.

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 transplantable 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-Cdk4R mice 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 $\text{CD3D}$ 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-α-treated human melanoma cells (24). Furthermore, high expression of type I IFN–responsive genes or $\text{CD3D}$ 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).

The $\text{Hgf-Cdk}^{\text{R24C}}$ mouse model

**Figure 1.** Immune cell–poor pigmented primary human melanomas show low expression of type I IFN–regulated genes and are morphologically imitated in the $\text{Hgf-Cdk}^{\text{R24C}}$ mouse melanoma model. **A**, representative histomorphology of immune cell–poor (left) and immune cell–rich (right) pigmented primary cutaneous human melanomas in H&E-stained sections. Red arrows, immune cell infiltrates. **B**, heatmap showing expression levels for type I IFN–regulated and T cell–related genes (CD3) in a clinically annotated cohort of 223 human primary melanomas. Samples are ordered by increasing $\text{CD3}$ transcript levels. **C**, corresponding progression-free survival in the indicated patient subgroups. Unbiased median expression value cutoffs were used for patient subgroup classification. $P$ values were determined by a log-rank test. **D**, histomorphology of primary cutaneous melanomas in $\text{Hgf-Cdk}^{\text{R24C}}$ mice showing the typical immune cell–poor pigmented phenotype in H&E-stained sections (left), and schematic diagram depicting the genetic alterations in $\text{Hgf-Cdk}^{\text{R24C}}$ mice (right).
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**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 Ifnar1-competent and Ifnar1-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 Ifnar1-competent (top) and Ifnar1-deficient (bottom) mice over time. C and F, corresponding Kaplan-Meier survival curves of melanoma-bearing (C) or sarcoma-bearing (F) Ifnar1-competent and Ifnar1-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 Ifnar1-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).

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 Ifnar1<sup>−/−</sup> background to obtain melanoma-prone mice that lack a functional type I IFN system. Cohorts of 8-week-old Ifnar1-competent and Ifnar1-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 Ifnar1-deficient and Ifnar1-competent Hgf-Cdk4<sup>R24C</sup> mice (Fig. 2B). Accordingly, DMBA-exposed Ifnar1-deficient and Ifnar1-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 Ifnar1-competent and Ifnar1-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 Ifnar1-deficient compared with Ifnar1-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.
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 Hgf-Cdk4R24C mice onto groups of 3 syngeneic Ifnar1-competent, Ifnar1-deficient, and Rag2-deficient C57BL/6 mice. Tumor development was monitored over time. Figure 3, 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 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.
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Figure 4. Peritumoral injections of poly(I:C) induce type I IFN-dependent cytotoxic immunity and delay the growth of primary and transplanted Hgf-Cdk R24C melanomas. Primary DMBA-induced (A–F) and intracutaneously (i.c.) transplanted (F–J) Hgf-Cdk R24C melanoma model. A and F, experimental protocols for short-term treatment of melanomas with poly(I:C). B and G, fold increase of mRNA expression levels for the indicated genes (left) and of the percentage of tumor-infiltrating immune cells (right) in primary and transplanted Hgf-Cdk R24C melanomas compared with controls (mean ± SEM, n = 6). C and H, experimental protocols for long-term treatment of melanomas with poly(I:C). D and I, tumor growth kinetics of melanomas in individual mice treated as indicated (left, middle), and mean survival in each cohort (right; mean ± SEM; n = 6; **, P < 0.01). Similar results were obtained in two independent treatment cohorts. E and J, 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-Cdk R24C 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-Cdk R24C 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-Cdk R24C mice (Fig. 4E). Thus, targeted type I IFN activation with poly(I:C) exposed immune cell–poor Hgf-Cdk R24C 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 transplatable Hgf-Cdk R24C melanoma cell line HCmel3, which morphologically imitates primary Hgf-Cdk R24C 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–regulated genes and increased the number of immune cells in the tumor microenvironment to a similar extent when compared with primary melanomas (Fig. 4B and G).
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 check.
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**Figure 6.** Antibody-mediated blockade of the immune-inhibitory PD-1–PD-L1 signaling pathway prolongs survival in poly(I:C)-treated mice. A, upregulation of MHC class I (MHCI) and PD-L1 on the surface of HCmel3 melanoma cells following exposure to IFNα or IFNγ in vitro. B, correlation of relative mRNA expression levels for PD-L1 and IRF7 in untreated and poly(I:C)-treated HCmel3 melanoma samples determined by quantitative reverse transcriptase PCR (qRT-PCR). C, representative histograms showing the percentage of PD-1–expressing CD8+ T cells (left) and cumulative data for a correlation of PD-1 expression on CD8+ T cells in the blood of HCmel3-bearing mice treated as indicated (right). D, experimental protocol for combination therapy for transplanted HCmel3 melanomas with poly(I:C) and anti–PD-1 antibody. E, representative tumor growth kinetics of HCmel3 melanomas in individual mice treated as indicated (n = 5). Similar results were obtained in at least two independent treatment cohorts. F, corresponding Kaplan-Meier survival curves (left) and mean survival of mice treated as indicated (right); mean ± SEM; n = 10; *, P < 0.05; n.s., nonsignificant; IgG, immunoglobulin G; i.c., intracutaneous.

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 PdilmRNA 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-1–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-Cdk4R24C mouse strain onto the Ifnar1-deficient background, we showed 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-Cdk4R24C 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-Cdk4R24C 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 the IFN response signature, and PD-L1. P values were determined by a log-rank test.

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 gene expression. The color code represents log2-transformed and mean-centered expression values generated with the Affymetrix Hgu133plus microarray platform (bottom). Corresponding barplot of PD-L1 levels and trend line of the IFN response signature matched to the samples shown in the heatmap below. Pearson correlation coefficient (r) is indicated (top). B, PD-L1, IFN response signature, and CD3D expression in human melanoma metastasis (n = 248) from the TCGA melanoma dataset (skin cutaneous melanoma; SKCM). Pearson correlation coefficients (r) are indicated. Expression values represent log2-transformed normalized RNA-seq reads generated with the Illumina platform. 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.

Overall survival (%) Overall survival (%) Overall survival (%)

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proinflammatory chemokines and cytokines when compared with malignant melanoma cells. Our observations are in line with recent reports in genetically engineered mouse models in which malignant transformation in lung epithelial cells (38) or muscle cells (39) was driven by the same genetic events (e.g., introduction of oncogenic Kras and simultaneous Trp53 deletion), but primary lung carcinomas and muscle fibrosarcomas interacted with the immune system in fundamentally different ways (e.g., tolerance induction vs. immune surveillance and editing). Together, these experimental findings emphasize that the spontaneous immune response to cancer is highly diverse and depends on contextual elements, including the cell of origin, the nature of the local immune system, and the type of genetic changes that drive malignant transformation (40).

Poly(IC) is a prototypic immunostimulatory RNA that potently stimulates innate pattern recognition receptors for viral RNA in macrophages and DCs, leading to the induction of type I IFNs and the activation of innate and adaptive cellular immune responses (29, 41, 42). Our experimental results in a genetically engineered mouse model show that poly(IC) can alert the cellular immune system to nascent primary cutaneous melanomas that evade IFN-dependent immune surveillance. Using the Ifnar1–competent transplantable Hgf-Cdk4R24C melanoma cell line HCmel3 that does not spontaneously activate type I IFNs and mice with conditional deletion of the Ifnar1 gene in different immune cell subsets, including macrophages/neutrophils, DCs, NK, and T cells, we demonstrate that poly(IC) induces the coordinate type I IFN–dependent activation of all these cell types to promote effective tumor immunity. In the effector phase, this depends on the presence of both NK cells and CD8+ T cells and the production of IFNγ in line with a large body of experimental evidence in different tumor models (34, 35, 43, 44). These findings underscore the critical importance of a functional type I IFN system in cells of the host immune system that was also found in experimental models in which tumor cells spontaneously activated type I IFN responses after transplantation (25). Here, type I IFN–dependent activation of DC subsets that are specialized for antigen cross-presentation was required for effective induction of antitumor immunity (9, 32).

Targeted activation of the type I IFN system in the microenvironment 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 peripheral blood CD8+ T cells. Because type I and type II IFNs upregulate 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 antibody-mediated PD-1 blockade prolonged the survival of mice only in combination with poly(IC) but not given as a monotherapy 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 upregulation of the PD-1–PD-L1 signaling axis in tumor tissue, 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 immunohistochemistry are functionally and clinically relevant and may explain discrepancies addressing the prognostic and predictive 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 melanosmas, in which type I IFN–regulated genes, T cell–related genes, and PD-L1 are expressed at low levels, had a comparatively 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 combination 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−/−, Ly4M-Cre × Ifnar1−/− (Ifnar1ΔMΔ), CD11c-Cre × Ifnar1−/− (Ifnar1Δ11cΔ11c), CD4-Cre × Ifnar1−/− (Ifnar1ΔcΔc), and

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planted onto 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-Cdk4 ΔNc1r 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-Cdk4 ΔNc1r 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 × 10^5 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.

**HCmel3 Tumor Transplantation**

The HCmel3 melanoma cell line was generated from a primary Hgf-Cdk4 ΔNc1r melanoma as described previously (13). Groups of syngeneic C57BL/6 mice were injected intracutaneously with 4 × 10^6 HCmel3 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 200 μg rat anti-mouse IFNγ (clone R7B5; BioXcell), respectively. Control groups again received 200 μg/mouse of the irrelevant 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/mouse of anti-mouse CD8 IgG2a (clone 2.43; BioXcell), 200 μg/mouse of anti-mouse NK1.1 IgG2a (clone PK136; BioXcell), or 100 μg/mouse of 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 retro-orbital 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**

Mice 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-7SFU 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 Pharmingen) according to the standard procedures. Surface expression of PD-L1 and CCL5 in tumor infiltrating immune cells was analyzed using flow cytometry (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). Reverse transcription 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:

- **Irp7** (F: CCAGTGTACCCGATAAGGT; R: AGACATTGCTGAGGCT CACCT);
- **Ccl5** (F: GCCGTCTATTTCCTGCTCAT; R: GCTTCCCTATGGCC CTCATT);
- **Cd8** (F: TGCCCTCATAATGCCGTCG; R: GACCTTCTGC TGGTA);
- **Cd4** (F: GAACAGTTGTAGATGTCATGG; R: CCACTGCCTTAT CATGCCTTG);
- **Xbp1** (F: TGTTCAGTATTAGTCAGGCC; R: AGCAATGTCGC TCTTCTTCTAC);
- **Grb** (F: CTTCAATGACATGTCG; R: TGCTTCTCATTGAGG);
- **Per1** (F: TGAAGAGACCTACATGAGC; R: AGACTCAAGTGAGG TCGAGG);
- **Pdi1** (F: AGTATGGGACGAGACCTAC; R: TCCCTTCTCCAGTA CACACTA); and
- **Lif** (F: AGGCAAGACCATCACCTTGGAC; R: GCCATCACACCCA AGAACAAGACA).

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Relative expression to the reference gene Ubc was calculated with the ΔCt method using the following equations: ΔCt (sample) = Ct (target) - Ct (reference); relative quantity = 2^-ΔΔCt.

Cell Culture and Treatment of HCMel3 Melanoma Cells

HCMel3 melanoma cells were generated from primary Hgf Cdk4 R24C 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 μg/ml streptomycin (Invitrogen). HCMel3 cells were authenticated by genomic PCR for the Hgf transgene and the Cdk4 R24C 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's 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 log2-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 IFN-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 CD3D hi versus the CD3D lo subgroup. The mean expression of the IFN-induced gene set was used to define IFN signature hi and IFN signature lo subgroups using an unbiased median expression cutoff value. Relapse-free survival was determined using the Kaplan–Meier method and significance was assessed by a log-rank test. A gene probe for PD-L1 (C274) on the Illumina WG-DASL array platform failed our quality control and was considered as not reliable. Correlations among PD-L1, IFN 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 RNAseq platform (https://tcga-data.nci.nih.gov/tcga/tcgaHome2.jsp). Primary melanoma samples (n = 46) were selected from the GSE15605 dataset, and raw CEL files were normalized by Robust Multidimensional Array Average (RMA). Gene expression values were log2-transformed and mean centered for heatmap visualization. The gene probe for PD-L1 (C274) was 227458_at. Expression values of the type I IFN response signature genes were averaged (mean) and scaled for the barplot representation. CD3 hi and CD3 lo were defined by the median cutoff: RNA-seq-based gene expression data of the TCGA melanoma samples (SKCM) for CD3D, PD-L1 (C2724), and the type I IFN response signature were retrieved through the CGDS server of the eBioportal hosted by the Memorial Sloan-Kettering Cancer Center (New York, NY) using the R-package cgdsr (50). The TCGA clinical annotation data file was downloaded (January 2014) from the TCGA Data Portal (https://tcga-data.nci.nih.gov/tcga/) using the Data Matrix download option. We used the data columns "vital status," "days to death," "days to last follow-up," and "tumor tissue site" to select samples from melanoma metastasis and to analyze survival in cohorts stratified by median gene expression level cutoffs. Primary melanomas were excluded because of low case numbers and short prospective clinical follow-up. We included samples only from regional lymph node metastasis, regional cutaneous or subcutaneous metastasis (including satellite and in-transit metastasis), and distant metastasis at various anatomic sites such as trunk, extremities, and head/neck region. We obtained a total of 248 samples with clinical annotation and RNA-seq gene expression data. For convenience, the survival data provided as "days to death" and "days to last follow-up" were transformed to "years to death" and "years to last follow-up." Within this selected TCGA metastasis cohort, the clinical follow-up of many cases started well before the sample collection and molecular characterization of the respective metastatic lesion that occurred later in the course of the disease. As exemplification, the cohort contains many samples from metastatic lesions of patients that were initially diagnosed with a nonmetastatic, for example, stage I or II, melanoma several years ago, but have developed a melanoma metastasis later in the course of their disease. Hence, survival ("days to death or last follow-up") reflects a combination of retrospective and prospective survival data as initiation of the clinical follow-up and serves as an assessment of the overall disease course.

RNA-seq read counts were log2, normalized, and unbiased median gene expression value cutoffs were applied for the analysis of high/low gene expression subgroups and their potential associations with overall disease outcome ("days to death or last follow-up"). Expression values of the type I IFN response signature genes were averaged (mean) before calculation of the median expression cutoff value. Overall survival was calculated by the Kaplan–Meier method and significance was determined by the log-rank test.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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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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REFERENCES
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