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 interferon (IFN) activation and a favorable prognosis. Therapeutic blockade of immune inhibitory receptors in patients with pre-existing lymphocytic infiltrates prolongs survival, but new complementary strategies are needed to activate cellular anti-tumor 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 bad 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, NK and T cells. Importantly, antibody-mediated blockade of the IFN-induced immune-inhibitory interaction between PD-L1 and PD-1 receptors further prolonged survival. These results highlight important interconnections between type I IFNs and immune-inhibitory receptors in melanoma pathogenesis which serve as targets for combination immunotherapies.

Statement of 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.
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 between 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 IFN-alpha 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 innate immune recognition of transplanted B16 melanomas through signaling on CD8α+ DCs 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 loose their effector functions in the immunosuppressive microenvironment of tumors where 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 up-regulation 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 reanimate 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 melanoma patients with pre-existing anti-tumor 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-Cdk4R24C mouse model where 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 principle hypotheses: 1) immune cell-poor primary melanomas evade innate type I IFN-dependent immune surveillance and thereby avoid the induction of anti-tumoral cytotoxic T cell immunity; 2) targeted activation of type I IFNs can establish cellular immune surveillance; 3) 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

Based on 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 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 0.6) in melanoma cells (Fig. 1A,B and Suppl. Table S1). The type I IFN response signature was generated from a publicly available data set of IFN alpha treated human melanoma cells (24). Furthermore, high expression of type I IFN responsive genes or CD3D was associated with an increased relapse-free survival, consistent with the idea that preexisting antitumoral immune responses determine a favorable prognosis of melanoma patients (Fig. 1C).

In turn, the absence of IFN-regulated genes in immune cell poor melanomas suggested that these tumors escape the immune surveillance function of the type 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-Cdk4R24C 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 anti-tumor immunity.

We therefore crossed Hgf-Cdk4^{R24C} into the Ifnar1^{-/-} 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^{R24C} 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^{R24C} mice (Fig. 2B). Accordingly, DMBA-exposed Ifnar1-deficient and Ifnar1-competent Hgf-Cdk4^{R24C} 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^{R24C} 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 to Ifnar1-competent Hgf-Cdk4^{R24C} mice (Fig. 2E) resulting in shorter survival (Fig. 2F). This ruled out the possibility that the anti-inflammatory properties of HGF, which can promote a tolerogenic dendritic cell phenotype and the expansion of regulatory T cells (26, 27), precluded immune surveillance by the type I IFN system in Hgf-Cdk4^{R24C} mice.

To understand the divergent role of the type I IFN system in the pathogenesis of primary melanomas and fibrosarcomas we performed further morphological, cellular and molecular investigations. Immunohistopathological analyses revealed increased numbers of tumor-infiltrating CD45^{+} immune cells in primary MCA-induced fibrosarcomas when compared to primary DMBA-induced melanomas in Hgf-Cdk4^{R24C} 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 5 different Ifnar1-competent and 5 different Ifnar1-deficient DMBA-induced primary Hgf-Cdk4R24C melanomas onto groups of 3 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, C), indicating the poor immunogenicity of both Ifnar1-competent and Ifnar1-deficient melanomas. Interestingly, a subset of Ifnar1-competent and -deficient melanoma cells grew more rapidly in Ifnar1-deficient mice when compared to Ifnar1-competent or Rag2-deficient mice, pointing towards the previously described role for the host type I IFN system in transplanted melanoma (9).

**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 two weeks strongly induced the expression of type I IFN-regulated genes and promoted the recruitment of immune cells into the tumor microenvironment (Fig. 4A, B). Prolonged treatment with poly(I:C) considerably delayed the growth of primary melanomas and increased survival (Fig. 4C, 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 vs. 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 two 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, G). Prolonged poly(I:C) treatment delayed the growth of transplanted HCmel3 melanomas and significantly increased survival (Fig. 4H, I). Transplanted HCmel3 cells grow with similar kinetics in Ifnar1-competent and Ifnar1-deficient mice (Fig 4I, J, left panel), 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 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 dendritic cells (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 dendritic cells, 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 anti-tumor 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 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 melanoma with poly(I:C) restraints their growth for several weeks but eventually fails (Fig. 4D, I). Both type I and type II IFNs up-regulated the expression of PD-L1 on the Hgf-Cdk4R24C melanoma cell line HCmel3 in vitro (Fig. 6A). We also observed a significant up-regulation of PD-L1 mRNA expression levels in poly(I:C) treated compared to untreated Hgf-Cdk4R24C mouse melanomas that correlated with the upregulation of type I IFN-regulated genes such as IRF7 (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 mAb together with poly(I:C) was able to cause partial regression in established HCmel3 mouse melanomas and significantly prolonged the survival compared to 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 prolonged immune surveillance. Finally, a detailed toxicological 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 (Suppl. Figs. 1 and 2).

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 melanoma dataset shown in Figure 1B. Therefore we interrogated two additional publicly available datasets of either primary melanomas (GSE15605) or melanoma metastasis (TCGA skin cutaneous melanoma) that were generated using a different microarray platform (37) or RNA seq, respectively. Indeed, this analysis confirmed our hypothesis, as we found a strong correlation between CD3D, IFN-responsive genes and PD-L1 expression in both datasets irrespectively of the genomics platform (Fig. 7A, B). Clinical follow-up data was available for the TCGA melanoma metastasis cohort and we classified samples by unbiased median expression value cutoffs as described for the primary melanoma cohort shown in Figure 1B. Consistently, high expression levels of CD3D, IFN-responsive gene and importantly also PD-L1 were associated with a favorable disease course (Fig. 7C).

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^{R24C} mouse strain onto the Ifnar1-deficient background, we show that immune cell-poor primary melanomas do not spontaneously activate the immune surveillance and editing functions of the endogenous type I IFN system. Because Hgf-Cdk4^{R24C} 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^{R24C} mice, confirming a previously published report (25). Since 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 immunological properties of the cells of origin, e.g. melanocytes vs. 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 proinflammatory chemokines and cytokines when compared to malignant melanoma cells. Our observations are in line with recent reports in genetically engineered mouse models where malignant transformation in lung epithelial cells (38) or muscle cells (39) was...
driven by the same genetic events (e.g. introduction of oncogenic K-ras and simultaneous p53 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(I:C) is a prototypic immunostimulatory RNA that potently stimulates innate pattern recognition receptors for viral RNA in macrophages and dendritic cells 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(I:C) 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, dendritic cells, NK and T cells we demonstrate that poly(I:C) 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 where tumor cells spontaneously activated type I IFN responses after transplantation (25). Here, type I IFN-dependent activation of dendritic cell subsets that are specialized for antigen cross-presentation was required for effective induction of anti-tumor immunity (9, 32).

Targeted activation of the type I IFN system in the microenvironment of immune cell poor Hgf-Cdk4R24C mouse melanomas with poly(I:C) was associated with cytotoxic immune cell recruitment, subsequent up-regulation 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 up-regulate PD-L1 expression on melanoma cells in vitro, and because poly(I:C) induces cellular anti-tumor 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(I:C) 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 not only expressed on melanoma cells but also on accessory cells in the tumor stroma (such as fibroblasts and dendritic cells), 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 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 appeared to be particularly effective in melanoma patients 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 predict 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 indicates 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 (48). 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 comparatively poor prognosis (23, 45-47). Hence, there is an obvious need for new therapeutic strategies in this patient subgroup. Our observation that treatment with a combination of poly(I:C) and a blocking anti-PD-1 mAb prolonged survival of mice with immune...
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cell poor melanoma not only highlights the critical importance of immune-inhibitory PD-L1/PD-1 interactions in vivo but also provides a pre-clinical proof of concept that targeted type I IFN activation is a rational strategy to increase the therapeutic benefit of PD-1/PD-L1 blockade also for patients with immune cell-poor melanomas. 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 which 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−/− x HGF-CDK4R24C mice. Ifnar1fl/fl, LysM-Cre x Ifnar1fl/fl (Ifnar1glym) CD11c-Cre x Ifnar1fl/fl (Ifnar1CD11c), CD4-Cre x Ifnar1fl/fl (Ifnar1CD4) and Nc1r-Cre x Ifnar1fl/fl (Ifnar1Nc1r) mice on the C57BL/6 background were bred as described (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 7,12-dimethylbenz(a)anthracene (DMBA) as described previously (13). Alternatively, mice received a single subcutaneous injection of 100 µg 3-methylcholanthrene (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. 2x10^5 cells were injected intracutaneously into the flank and tumor development 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-Cdk4R24C melanoma as described previously (13). Groups of syngeneic C57BL/6 mice were injected intracutaneously with 4x10^5 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 five 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 IFN-γ was performed by twice weekly intraperitoneal injections of 200μg rat anti-mouse CD8 IgG2a (Clone 2.43, BioXcell), 200μg 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-PD1 antibodies intraperitoneally groups of C57BL/6 mice were treated twice weekly for four weeks. A control group of mice was injected with identical volumes of PBS intracutaneously and intraperitoneally. The body weight and general health was observed and documented for 28 days. At necropsy 4 days after the last therapeutic dose blood was collected for clinical chemistry and
hematological 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. Additionally, vital internal organs were isolated, weighed, fixed in formalin and embedded in paraffin for subsequent histopathological analyses. Relative organ weights were calculated as percent of total body weight. H&E-stained sections of several organs were scored for pathological 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 stained with H&E according to 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). Heavily pigmented mouse melanomas were bleached before staining (20 min at 37°C in 30% H2O2 and 0.5% KOH, 20 sec in 1% acetic acid and 5 min 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 Pharmingen) according to standard procedures. Surface expression of PD-L1 and MHC I on HCmel3 melanoma cells was analysed with fluorochrome-conjugated mAbs specific for PD-L1 and MHC I (both BD Pharmingen) according to standard procedures. Data were acquired with a FACSCanto flow cytometer (BD Biosciences) and analysed with FlowJo software (TreeStar, V7.6.5 for Windows).

Real-time RT-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:

- **Irf7** (F: CCAGTTGATCCGCATAAGGT; R: AGCATTGCTGAGGCTCACTT);
- **Cxcl10** (F: GCCGTCATTTCCTGCTCAT; R: GCTTCCTATGGGCCCTCATT);
- **Ccl5** (F: TGCTCACCATATGGCTCG; R: GCACCTTGCTGCTGTGA);
- **CD3** (F: GAACCAGTGTAGAGTGACGTG; R: CCAGGTGCTTATCATGCTTCTG);
- **klrb** (F: TTGTTCAGTTAATTAGAGTGCCC; AGCAAAGTGGCTCCTTTTCTAC; R:);
- **Grzb** (F: CTCCAATGACATCATGCTGC; R: TGGCTTCACATCATGCTTCTG);
- **Perf** (F: TGAGAAGACCTATCAGGACC; R: AAGTCAAGGTGGAGTGAGG);
- **PD-L1** (F: AGTATGGGAGCAACGTCACG; R: TCCTTTTCCCAGTACACACTA)
- **Ubc** (F: AGGCAAGACCATCACCTTGACG; R: CCATCACACCCAAGAACAGCACA).

Relative expression to the reference gene **Ubc** was calculated with the delta-Ct method using the following equations: ΔCt(Sample) = Ct(Target) - Ct(Reference); relative quantity = 2^−ΔCt.

**Cell culture and treatment of HCmel3 melanoma cells.**

HCmel3 melanoma cells were generated from primary Hgf-Cdk4R24C mouse melanomas in our laboratory and cultured in complete RPMI 1640 medium containing 10% FCS (Biochrome), 2 mM L-glutamine (Gibco), 10 mM non-essential amino acids (Gibco), 1 mM HEPES (Gibco), 20 μM 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 Cdk4R24C knockin alleles. Melanoma cells were seeded in 6-well plates and treated with 1,000 U/ml recombinant mouse IFN-α (PBL) or IFN-γ (Peprotech). After 24 h 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 preformed 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 interferon (Interferon alpha) induced genes across five human melanoma cell lines (Referred as type I interferon response signature). The gene expression data (GSE19428_series_matrix.txt) was downloaded as normalized data using global scaling with a trimmed mean target intensity of each array set to 100 (24). Expression data was log2-transformed and the top 50 differentially expressed genes were identified by comparing mean expression values of interferon alpha treated cells versus untreated control cells. This type I interferon responsive gene set from melanoma cell lines was used in the further analyses of the human melanoma tissue samples.

The gene expression data set (Illumina WG-DASL array platform) of 223 primary melanomas was previously described (23). A median expression cutoff value for CD3D expression as T cell marker was used to analyze relapse free survival of the CD3D\textsuperscript{high} versus the CD3D\textsuperscript{low} subgroup. The mean expression of the interferon induced gene set was used to define IFN signature\textsuperscript{high} and IFN signature\textsuperscript{low} 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 between 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 SKCM, melanoma metastasis, Ilumina RNA Seq 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 RMA. Gene expression values were log2-transformed and mean centered for heatmap visualization. The gene probe for PD-L1 (CD274) was 227458_at. Expression values of the type I interferon response signature genes were averaged (mean) and scaled for the barplot representation. CD3D\textsuperscript{high} and CD3D\textsuperscript{low} were defined by the median cutoff. RNA Seq based gene expression data of the TCGA melanoma samples (SKCM, Skin cutaneous melanoma) for CD3D, PD-L1 (CD274) and the type I interferon response signature was retrieved through the CGDS server of the cBioportal hosted by the Memorial Sloan-Kettering Cancer Center using the R-package cgdsr (50). The TCGA clinical annotation data file was downloaded (01/2014) from the TCGA Data Portal (https://tcga-data.nci.nih.gov/tcga/tcgaHome2.jsp).
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 due to low case numbers and short prospective clinical follow-up. We included only samples 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" was transformed to "years to death" and "years to last follow-up". Within this selected TGCA metastasis cohort, the clinical follow-up of many cases started far 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 non-metastatic e.g. stage I or stage 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 since 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 interferon response signature genes were averaged (mean) prior to 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.

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Figure Legends

Figure 1. Immune cell poor pigmented primary human melanomas show low expression of type I IFN regulated genes and are morphologically imitated in the Hgf-Cdk4<sup>R24C</sup> mouse melanoma model. 

A, representative histomorphology of immune-cell poor (left) and immune cell-rich (right) pigmented primary cutaneous human melanomas in HE-stained sections. Red arrows indicate 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 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 Hgf-Cdk4<sup>R24C</sup> mice showing the typical immune-cell poor pigmented phenotype in HE-stained sections (left) and schematic diagram depicting the genetic alterations in Hgf-Cdk4<sup>R24C</sup> mice (right).

Figure 2. Primary melanomas in Hgf-Cdk4<sup>R24C</sup> mice escape type I IFN-mediated immune surveillance. 

A, 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, 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, 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 ± s.e.m., n=12) and corresponding real-time PCR analysis of IFN-induced genes (right, mean ± s.e.m., n=12, *p<0.05).

Figure 3. Ifnar1-competent and -deficient Hgf-Cdk4<sup>R24C</sup> melanomas both grow progressively when transplanted in Ifnar1-competent and -deficient hosts. A, experimental protocol for transplantation of primary DMBA-induced melanomas from 5 individual Ifnar1-competent and Ifnar1-deficient Hgf-Cdk4<sup>R24C</sup> 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-
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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.

**Figure 4.** Peritumoral injections of poly(I:C) induce type I IFN-dependent cytotoxic immunity and delay the growth of primary and transplanted Hgf-Cdk4R24C melanomas. Primary DMBA-induced (A-F) and intracutaneously transplanted (F-J) Hgf-Cdk4R24C melanoma model. A,F, experimental protocols for short-term treatment of melanomas with poly(I:C). B,G, fold increase of mRNA expression levels for the indicated genes (left panel) and of the percentage tumor infiltrating immune cells (right panel) in primary and transplanted Hgf-Cdk4R24C melanomas compared to controls (mean ± s.e.m., n=6). C,H, experimental protocols for long-term treatment of melanomas with poly(I:C). D,I, tumor growth kinetics of melanomas in individual mice treated as indicated (left, middle) and mean survival in each cohort (right, mean ± s.e.m., n=6, ** p<0.01). Similar results were obtained in two independent treatment cohorts. E,J, corresponding tumor growth kinetics and mean survival in Ifnar1-deficient cohorts of mice treated as indicated (n=6). Similar results were obtained in two independent treatment cohorts.

**Figure 5.** The therapeutic efficacy of poly(I:C) requires a functional type I IFN system in dendritic cells, macrophages/neutrophils, NK cells and T cells. A, experimental protocol for treatment of HCmel3 melanomas in Ifnar1^+/+^, Ifnar1^LYM^, Ifnar1^CD11c^, Ifnar1^CD4^ and Ifnar1^Nc1r^ mice (top) and Kaplan Meier survival curves in groups of 5 mice with the indicated genotypes (middle, bottom). Similar results were obtained in 2 independent experiments. B, experimental protocol (top) and Kaplan Meier survival curves in groups of 5 mice treated as indicated (bottom). Similar results were obtained in 2 independent experiments.

**Figure 6.** Antibody-mediated blockade of the immune inhibitory PD-1/PD-L1 signaling pathway prolongs survival in poly(I:C)-treated mice. A, up-regulation of MHC class I 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 qRT-PCR. C, representative histograms showing the percentage of PD1 expressing CD8+ T cells (left) and cumulative data for a correlation of PD1 expression on CD8+ T cells in the blood of HCmel3 bearing mice treated as indicated (right). D, experimental protocol for combination
therapy of transplanted HCmel3 melanomas with poly(I:C) and anti-PD1 antibody. **E,** representative tumor growth kinetics of HCmel3 melanomas in individual mice treated as indicated (n=5). Similar results were obtained in at least 2 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. = non-significant).

**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 panel). 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 is indicated (top panel). **B,** PD-L1, IFN response signature and CD3D expression in human melanoma metastasis (n=248) from the TCGA melanoma dataset (SKCM). Pearson correlation coefficients 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 interferon response signature and PD-L1. P-values were determined by a log-rank test.
Figure 1 - Bald et al.

A

Immune cell poor pigmented primary human melanoma

Immune cell rich pigmented primary human melanoma

B

Primary melanomas, n=223

C

IFN response signature

CD3

D

The Hgf-Cdk4 mouse model

Growth factor signaling

Cell cycle control

18,6 x 7,9 cm / 7 1/3 x 3 1/8 inch
Figure 2 - Bald et al.
Figure 3 - Bald et al.

(A) Schematic representation of the experimental setup. Ifnar1-competent (CT) and Ifnar1-deficient (IFT) mice are used to monitor the development of Hgf-Cdk4R24C melanomas.

(B) Kinetics of tumor onset showing similar growth in Ifnar1-deficient recipients and faster growth in Ifnar1-competent recipients.

(C) Kinetics of tumor onset showing similar growth and faster growth in Ifnar1-deficient recipients.

10.7 x 7.9 cm / 4 1/5 x 3 1/8 inch
Figure 4 - Bald et al.
Figure 5 - Bald et al.

**A**

- HCmel3 Control or poly(I:C)
- % Survival vs Days after i.c. injection of HCmel3
- Lines indicate different genotypes: Ifnar1fl/fl, Ifnar1/g507LysM, Ifnar1/g507CD11c, Ifnar1/g507Nc1r, Ifnar1/g507CD4

**B**

- HCmel3 Control or poly(I:C)
- % Survival vs Days after i.c. injection of HCmel3
- Treatment groups: Ctrl IgG, anti-CD8, anti-NK1.1, anti-IFNγ mAb
- Lines indicate different treatments: control, poly(I:C) + Ctrl IgG, poly(I:C) + anti-CD8, poly(I:C) + anti-NK1.1, poly(I:C) + anti-IFNγ
Figure 6 - Bald et al.

15.4 x 7.9 cm / 6 1/8 x 3 1/8 inch
Figure 7 - Bald et al.

A. IFN response signature

Primary melanomas, n=46 (GSE15605)

PD-L1 expression (log2)

CD3low, n=23

CD3high, n=23

r=0.75

B. PD-L1 expression (RNA Seq log2)

Overall survival (%)

C. TCGA melanoma metastasis

IFN response signature

PD-L1, expression (RNA Seq log2)

Overall survival (%)

CD3D

p= 0.00533

IFN response signature

Low, n=124

High, n=124

p= 0.00387

PD-L1, expression (RNA Seq log2)

Overall survival (%)

CD3D

p= 0.00509

Years to death or last follow-up

11 x 15.5 cm / 4 1/3 x 6 1/8 inch
Immune-cell poor melanomas benefit from PD-1 blockade after targeted type I IFN activation

Tobias Bald, Jennifer Landsberg, Dorys Lopez-Ramos, et al.

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