The stress kinase GCN2 does not mediate suppression of antitumor T cell responses by tryptophan catabolism in experimental melanomas

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ABSTRACT
Tryptophan metabolism is a key process that shapes the immunosuppressive tumor microenvironment. The two rate-limiting enzymes that mediate tryptophan depletion, indoleamine-2,3-dioxygenase (IDO) and tryptophan-2,3-dioxygenase (TDO), have already moved into the focus of research and inhibitors targeting IDO and TDO have entered clinical trials. Local tryptophan depletion is generally viewed as the crucial immunosuppressive mechanism. In T cells, the kinase general control non-derepressible 2 (GCN2) has been identified as a molecular sensor of tryptophan deprivation. GCN2 activation by tryptophan depletion induces apoptosis and mitigates T cell proliferation. Here, we investigated whether GCN2 attenuates tumor rejection in experimental B16 melanoma using T cell-specific Gcn2 knockout mice. Our data demonstrate that GCN2 in T cells did not affect immunity to B16 tumors even when animals were treated with antibodies targeting cytotoxic T lymphocyte antigen-4 (CTLA4). GCN2-deficient gp100 TCR-transgenic T cells were equally effective as wild-type pmel T cells against gp100-expressing B16 melanomas after adoptive transfer and gp100 peptide vaccination. Even augmentation of tumoral tryptophan metabolism in B16 tumors by lentiviral overexpression of Tdo did not differentially affect GCN2-proficient vs. GCN2-deficient T cells in vivo. Importantly, GCN2 target genes were not upregulated in tumor-infiltrating T cells. MALDI-TOF MS imaging of B16 melanomas demonstrated maintenance of intratumoral tryptophan levels despite high tryptophan turnover, which prohibits a drop in tryptophan sufficient to activate GCN2 in tumor-infiltrating T cells. In conclusion, our results do not suggest that suppression of antitumor immune responses by tryptophan metabolism is driven by local tryptophan depletion and subsequent GCN2-mediated T cell anergy.

Abbreviations: ACT, Adoptive cell transfer; AHR, Aryl hydrocarbon receptor; ATF4, Activating transcription factor 4; CHOP, C/EBP-homologous protein; CTL, Cytotoxic T cell; CTLA4, Cytotoxic T lymphocyte antigen-4; DC, Dendritic cell; eIF2α, Eukaryotic translation initiation factor 2α; GCN2, General control non-derepressible 2; IDO, Indolamine-2,3-dioxygenase; MALDI-TOF MS, Matrix-assisted laser desorption/ionization-time of flight mass spectrometry; PD1, Programmed death-1; SKCM, Skin cutaneous melanoma; TCGA, The cancer genome atlas; TDO, Tryptophan-2,3-dioxygenase; TH1, T helper type 1; TIL, Tumor infiltrating lymphocyte; Treg, Regulatory T cell; WT, Wild-type

Introduction
Tryptophan metabolism is a central pathway in regulation of immune responses and is known to shape the immunosuppressive tumor microenvironment. Drugs aiming at blocking the activity of indoleamine-2,3-dioxygenase (IDO), a key and rate-limiting enzyme in this metabolic pathway, have already entered clinical trials with the aim to reverse tumor immune escape. Constitutive IDO expression has been reported for most human tumors and in animal models systemic pharmacological inhibition of IDO has been shown to reverse immune resistance mechanisms. Recently, expression of tryptophan-2,3-dioxygenase (TDO), another enzyme involved in tryptophan catabolism, has been shown to drive immune escape in human tumors and systemic TDO inhibition restored tumor rejection in a preclinical model. Despite evidence that tumoral immune resistance as a result of IDO and TDO expression can be attributed to the accumulation of immunosuppressive kynurenines activating the aryl hydrocarbon receptor (AHR), 3,5 tryptophan depletion in...
the local microenvironment by IDO and TDO is generally regarded as the key immunosuppressive effector mechanism. This view is based on preclinical studies identifying the stress kinase general control non-derepressible 2 (GCN2) in T cells as a molecular tryptophan sensor that mediates downstream effects of the immunoregulatory enzyme IDO.\(^6\) GCN2 initiates the integrated stress response upon accumulation of uncharged tRNAs when intracellular amino acid levels are low. Hence, GCN2 acts as a molecular sensor of amino acid homeostasis and mediates phosphorylation of the eukaryotic translation initiation factor 2α (eIF2α) in response to amino acid depletion, thereby attenuating ribosomal translation.\(^7\) Besides inhibiting global protein synthesis, GCN2 activation induces expression of activating transcription factor 4 (ATF4). ATF4 is a key player in the tight regulation of the integrated stress response and mediates translation of genes involved in amino acid synthesis and nutrient catabolism as well as expression of C/EBP-homologous protein (CHOP), a transcription factor that induces apoptosis.\(^6,8\) Although GCN2 activation by IDO-mediated tryptophan depletion mitigates T cell proliferation due to anergy induction \(\text{in vitro}\), its relevance in \(\text{in vivo}\) tumor models is less clear. Previous studies suggested that genetic ablation of GCN2 does not prevent formation of skin tumors after PMA-induced chronic inflammation,\(^9\) whereas \(\text{Ido}\) knockout mice present with dramatically diminished papilloma incidence.\(^10\) However, T cell-mediated effects of \(\text{Gcn2}\) knockout on \(\text{in vivo}\) skin cancer growth remain poorly understood.

In the current study, we tested the hypothesis that the GCN2 pathway is essential in T cell-mediated control of tumor growth in a B16 melanoma mouse model using conditional ablation of GCN2 in T cells.

**Results**

**T cell-specific Gcn2 knockout does not alter the antitumor immune response to experimental melanoma**

To address the role of \(\text{Gcn2}\) expression in T cell-mediated antitumor immunity in an experimental melanoma model, we employed T cell-specific \(\text{Gcn2}\) knockout mice, where \(\text{Gcn2}\) was conditionally ablated in cells expressing the T cell tyrosine kinase Lck.\(^11\) Loss of GCN2 in T cells neither promoted T cell responses against B16 melanomas (Fig. 1A and B) nor was it involved in attraction of total T cells (Fig. 1C), CD4\(^+\) or CD8\(^+\) T cells (Fig. 1D) or recruitment of T\(_{\text{reg}}\), T helper type 1 (T\(_{\text{H1}}\)) cells, or IFNγ-secretting cytotoxic T cells (CTLs) (Fig. 1E) into B16 melanomas. These data suggest that GCN2 in T cells does not affect their accumulation in syngeneic tumors and is dispensable for T cell-mediated tumor rejection.

**T cell GCN2 is not critical for immune resistance to immune checkpoint blockade**

We next tested the relevance of GCN2 in T cells in an established immunotherapeutic setting, which results in T cell activation and may thus provoke resistance mechanisms involving tryptophan metabolism. IDO-mediated tryptophan catabolism is a critical resistance mechanism during immune checkpoint blockade in experimental melanomas and gliomas.\(^12,13\) Hence, clinical trials combining antibodies targeting cytotoxic T lymphocyte antigen-4 (CTLA4) with IDO inhibitors are underway.\(^14\) We thus conducted a series of experiments employing blockade of CTLA4 in tumor-bearing \(\text{Gcn2}^{\text{ΔLck}}\) mice and control littermates. Checkpoint blockade significantly increased survival; however, loss of GCN2 in T cells did not further prolong survival (Fig. 2A and B). Importantly, accumulation of T cells (Fig. 3A), CD4\(^+\) or CD8\(^+\) T cells (Fig. 3B) as well as T\(_{\text{reg}}\), T\(_{\text{H1}}\) cells, or CTLs (Fig. 3C) remained unchanged within the tumor tissue. Although loss of IDO has been reported to decrease the ratios of T\(_{\text{reg}}\) to effector cells,\(^12\) T cell-specific \(\text{Gcn2}\) knockout did not phenocopy this effect (Fig. 3D). Furthermore, neither proliferation nor programmed death-1 (PD1) expression were altered in response to CTLA4 blockade (Fig. 3E and F). These findings discount the notion that the stress kinase GCN2 in T cells is a key mediator of immune resistance during immune checkpoint blockade.

**GCN2 does not alter phenotype and function of gp100-specific CD8\(^+\) T cells**

The pmel adoptive transfer model\(^15\) allowed us to specifically examine the impact of \(\text{Gcn2}\) deletion on tumor antigen-specific CD8\(^+\) T cell responses. As shown in Fig. 4A and B, transfer of \(\text{in vitro}\)-stimulated gp100-specific CD8\(^+\) pmel T cells in combination with gp100 peptide vaccination significantly delayed tumor growth regardless of the \(\text{Gcn2}\) expression state in pmel T cells. In line with this, accumulation of gp100-specific CD8\(^+\) T cells in the tumor as measured by tetramer staining (Fig. 4C) and expression of the exhaustion marker PD1 (Fig. 4D) were not altered by GCN2 loss in pmel T cells. Detailed \(\text{in vitro}\) analyses of transferred CD8\(^+\) T cells strengthen the finding that \(\text{Gcn2}\) deletion does not reinforce antitumor T cell responses as expression of CD107a on the surface, secretion of both granzyme B and IFNγ as well as proliferation after co-culture with B16 melanoma cells remained unchanged (Fig. 4E).

**T cell-specific GCN2 knockout does not alter growth of constitutively tryptophan-metabolizing B16 melanomas**

We next sought to investigate whether reinforced tryptophan depletion by B16 cells would sensitize tumor-infiltrating T cells to GCN2-mediated impairment of antitumor immunity. To this end, B16 melanoma cells were transduced with a lentiviral construct for murine Tdo expression. \(\text{In vitro}\), cells expressed high Tdo levels and efficiently depleted tryptophan in cell culture supernatants (Fig. S1A and B). Cell biotyping assays\(^16\) also demonstrated a drop of tryptophan within the tumor cells (Fig. S1C). However, implantation of those cells into \(\text{Gcn2}^{\text{ΔLck}}\) mice and control littermates did not reveal any differences in tumor growth (Fig. 5A) despite the fact that B16 melanoma cells maintained Tdo expression \(\text{in vivo}\) (Fig. 5B).

**GCN2 is not activated in tumor-infiltrating T cells**

As our data did not support a crucial role of GCN2 in T cells in mediating immune escape, we asked whether this pathway is at all activated in tumor-infiltrating T cells, which would result in the transcription of GCN2 target genes. The GCN2 target transcription factor CHOP\(^17\) was not transcriptionally induced in
Tumor-infiltrating T cells both upon immune checkpoint blockade and constitutive tryptophan depletion (Fig. 6A and B), whereas Chop expression was efficiently induced by tryptophan deprivation in vitro with a 10-fold increase (Fig. S2A). These data demonstrate that, despite high tryptophan turnover rates, GCN2 is not activated in T cells that infiltrate B16 melanoma tissue. Gene expression analysis of T cells derived from B16 melanomas and spleens as a control showed that neither Gcn2 nor Chop and Atf4 were upregulated in tumor-infiltrating CD4+ T cells, CD8+ T cells, and Treggs, whereas expression of selected chemokines was highly increased in tumor-infiltrating lymphocytes (TILs) (Fig. 6C, Table 1). In addition, Ido expression in bulk B16 melanoma tissue did not correlate with Chop/Atf4 expression in tumor-infiltrating T cells (Fig. 6D). Finally, the analysis of RNA sequencing data retrieved from TCGA datasets16 did not reveal a positive correlation between Chop or Atf4 and Tdo or Ido expression in human skin cutaneous melanoma (SKCM) (Fig. 6E). In contrast to this, we found a highly significant positive correlation between Chop and Atf4 expression with a correlation coefficient similar to that of the two housekeeping genes Gapdh and Actin-β (Fig. S2B). In summary, these results do not suggest that tryptophan metabolism drives GCN2 activation in experimental melanomas and SKCM.

**Tryptophan is not depleted in B16 melanomas treated with immune checkpoint inhibitors or constitutively tryptophan-metabolizing B16 melanomas**

As GCN2 is activated only at tryptophan levels below 1 μM (Fig. S2A),20 we determined tryptophan concentrations in B16 melanoma tissue. Measurement of tryptophan levels in tumor tissue lysates as well as on tumor sections revealed that tryptophan concentrations are indeed robust in B16 melanoma and do not drop to levels sufficient to activate GCN2 in a highly immunogenic setting (Fig. 7A). Matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) imaging21 was used to assess tryptophan levels with spatial resolution ex vivo. Even in B16 melanomas that exert constitutive tryptophan catabolism, we did not observe a drop in intratumoral tryptophan when compared to control tumors (Fig. 7B). We did not observe heterogeneity of tryptophan distribution within the tumor resectates by MALDI-TOF MS imaging. These data indicate that tryptophan concentrations in B16 melanoma tissue do not drop below a threshold that is sufficient to activate GCN2 in T cells.

**Discussion**

In this study, we demonstrate that the molecular tryptophan sensor GCN2 is dispensable for suppression of T cell-mediated tumor rejection. The concept of tumor immune escape by active tryptophan metabolism is widely accepted and antagonists targeting the two tryptophan-catabolizing enzymes IDO and TDO have already entered clinical trials.2 Our findings are of clinical relevance since prevention of local tryptophan deprivation by IDO and TDO inhibitors is considered to be the key effector mechanism that releases the brakes from an antitumor immune response. First evidence for proliferative arrest in T cells as a consequence of IDO activity in dendritic cells (DCs) came from several independent in vitro studies.22-24 Examination of human tumor cell lines and human tumor tissue...
revealed that IDO expression is not limited to immune cells but is also constitutively expressed in tumor cells. Consequently, treatment of mice bearing IDO-expressing tumors with a common IDO inhibitor, 1-methyl-L-tryptophan, reversed tumor immune resistance in vivo. Here, we challenge the concept that the downstream mechanism of IDO and TDO activity is primarily conferred by local depletion of the essential amino acid tryptophan, which in turn activates the stress kinase GCN2 as a result of uncharged tRNAs. It has previously been shown that Gcn2 knockout does not affect formation of inflammation-induced skin cancer, though we aimed at extending the observation that the GCN2 pathway does not limit T cell-mediated rejection of skin cancers.

To assess the role of the GCN2 pathway in T cell-mediated tumor rejection, we used conditional Gcn2 deletion specifically in T cells in a B16 melanoma mouse model. However, GCN2 deficiency in T cells did not reinforce tumor rejection (Fig. 1). As exhaustion of tumor-specific T cells by engagement of inhibitory receptors may mask a possible role GCN2, we allowed for T cell reinvigoration by immune checkpoint inhibition. Although IDO-mediated tryptophan catabolism has recently been shown to be a key resistance mechanism to checkpoint blockade, we did not observe altered tumor growth or immune cell infiltration in tumors treated with checkpoint inhibitors upon T cell-specific Gcn2 deletion (Figs. 2 and 3). To ensure high tryptophan turnover rates, we induced Tdo expression in B16 melanoma cells, which likewise did not delay tumor outgrowth in mice lacking GCN2 in T cells (Fig. 5). Importantly, these efforts did not even result in GCN2 activation in T cells as measured by Chop expression (Fig. 6). Consistent with this result, HPLC-based quantification in crude tumor lysates indicated that tryptophan concentrations were not affected by GCN2 activation in T cells. Spatially resolved relative quantification of tryptophan by MALDI-TOF MS imaging ruled out the possibility that tumor heterogeneity may have masked local tryptophan depletion. We made use of the novel technique MALDI-TOF MS imaging to analyze tryptophan depletion directly in tumor tissue sections with spatial resolution rather than using crude tissue lysates. This allowed us to uncover that tryptophan is not depleted to concentrations required to activate GCN2 (Fig. 7).

As our data suggest that GCN2 expression in T cells does not mediate tumor immune escape in response to tryptophan catabolism, the question, how IDO and TDO inhibitors exert their effect on tumor rejection, remains unclear. Accumulation of immunosuppressive kynurenines rather than tryptophan depletion may be the crucial mechanism instead. Tryptophan...
metabolites have been shown to efficiently suppress NK and T cell proliferation and induce T cell apoptosis. Furthermore, kynurenines were shown to promote tumor cell survival and motility. These tumor-promoting effects may—at least in part—be attributed to activation of the AHR by tryptophan metabolites as IDO-expressing DCs favor differentiation of naive T cells into Tregs, thereby suppressing antitumor immune responses. Different types of tumors may utilize different pathways of suppressing immune responses through tryptophan metabolism; thus, we cannot exclude that GCN2 may serve as an immunological sensor of tumor-associated tryptophan metabolism in other types of tumors.

Despite being a molecular sensor of tryptophan depletion in T cells, GCN2 has recently been identified as a key molecule in macrophages and DCs to limit inflammation as a result of tolerance induction by apoptotic cell death in an autoimmune diseases and intestinal inflammation. In contrast, GCN2 activation by a yellow fever vaccine boosted both CD4+ and CD8+ T cell responses upon increased antigen presentation by DCs. As myeloid cells may not require a drop in tryptophan levels by a factor of 50 to initiate GCN2 downstream signaling, these recent studies not only highlight the need to study the role of GCN2 in previously neglected immune cell subsets, namely DCs and macrophages, but also shows that immune regulation by GCN2 is highly dependent on the context.

In summary, our data suggest that both IDO and TDO inhibitors do not act via rescue of T cells from tryptophan starvation but prohibit accumulation of immunosuppressive tryptophan catabolites that induce immune escape by Treg differentiation. As the downstream effector mechanism of immunosuppressive tryptophan catabolism is an attractive target integrating both IDO- and TDO-mediated pathways, future studies ought to focus on these mechanisms, also to allow for the identification of faithful biomarkers of IDO/TDO inhibition. Whether the immunosuppressive effects are solely mediated by AHR activation remains to be proven.

**Materials and methods**

**Cell culture**

B16 melanoma cells were a kind gift of Günther J. Hämmerling (Division of Molecular Immunology, DKFZ Heidelberg). Cells were cultured in DMEM (Sigma-Aldrich; D6429)
supplemented with 10% FBS (Sigma-Aldrich; F0804), 100 U/mL penicillin, and 0.1 mg/mL streptomycin (Sigma-Aldrich; P4333) at 37°C, 5% CO₂. The purity of cell lines was validated using the Multiplex cell Contamination Test by Multiplexion (Heidelberg, Germany) as described recently. No Mycoplasma, squirrel monkey retrovirus (SMRV) or interspecies contamination was detected.

**Lentiviral transduction**

The full length sequence of murine Tdo was cloned into the vector backbone of pCCL.PPT.SFFV.MCS.IRES.eGFP, WPRE using the restriction sites BstB1 and BstZ17I. Lentiviral particles for the transduction of B16 melanoma cells were produced by co-transfecting the murine Tdo-containing vector (LV-Tdo) or empty control vector (LV-ctrl) in HEK293T cells using FuGENE HD (Promega; E2311) and the corresponding packaging plasmids (pMDLg/pRRE, pRSV-Rev, pMD2.VSVG). Viruses were harvested by ultracentrifugation 48 h and 96 h after transfection. Infection of B16 melanoma cells was carried out in the presence of 8 mg/mL polybrene (Merck Millipore; TR-1003-G). Transduced cells were sorted for high GFP expression on FACSAria II (BD Biosciences) using BD FACSDiva software. The expression of Tdo as well as enzyme activity was confirmed by qRT-PCR and HPLC, respectively.
Mice

C57BL/6J WT mice were purchased from Charles River. Gcn2^{fl/fl} were purchased from the Jackson Laboratory (B6.129S6-Eif2ak4tm1.1Dron/J) and mated to LckCre mice to generate a T cell-specific Gcn2 knockout (Gcn2^{D/Lck}) as previously described. Mice harboring a rearranged T cell receptor transgene specific for gp100 and the T lymphocyte specific Thy1a (Thy1.1) allele (pmel WT; B6.Cg-Thy1a/Cy Tg(TcraTcrb)8Rest/J) and Foxp3-DTReGFP mice (B6.129(Cg)-Foxp3^{DTR/eGFP}J) were purchased from the Jackson Laboratory and bred at the animal facility of the DKFZ Heidelberg. In order to generate GCN2-deficient pmel mice (pmel Gcn2^{D}), pmel WT mice were bred to Gcn2^{D} mice (B6.129S6-Eif2ak4tm1.1Dron/J; Jackson Laboratory) and offsprings homozygous for both alleles were used for adoptive cell transfer (ACT) experiments.

Sex- and age-matched mice were used for further experiments and all mice were 7–15 weeks of age at use. Experiments were performed according to the rules of the German Animal Welfare Act and were licensed by the Regierungspräsidium Karlsruhe.

Tumor challenge and treatment

Tumors were established by subcutaneous inoculation of 5 × 10^4 cells and were measured in two dimensions (area; width × length) using a caliper. For ACT experiments, pmel splenocytes were cultured in vitro in the presence of 2 μg/mL hgp100(25–33) (Research group GMP & T cell therapy, DKFZ Heidelberg) and 30 IU/mL IL-2 (Novartis) for 3 d. 2.5–5 × 10^6 MACS-purified CD8+ T cells were transferred i.v. 7 d after tumor challenge. On the day of transfer, mice were vaccinated s.c. with 100 μg hgp100(25–33) in Montanide ISA51 (Seppic).

Figure 6. GCN2 is not activated in tumor-infiltrating T cells. (A) B16 melanoma cells were implanted into Gcn2^{fl/fl} mice and animals were treated with anti-CTLA4 or isotype control. T cells were purified from B16 tumor tissue when the tumor area exceed 150 mm^2 and GCN2 activation was assessed by Chop expression (n = 5). (B) Gcn2^{fl/fl} mice and control littermates were inoculated with B16 Tdo melanoma cells. Chop expression in purified tumor-infiltrating T cells was assessed by qRT-PCR 16 d post-inoculation (n = 3). For (A) and (B), a two-tailed student’s t test was used to assess significance and data are represented as mean ± SEM. (C) Heatmap of the GCN2-ATF4-CHOP pathway in B16 melanoma TILs and spleen T cells generated from microarray gene expression data (n = 3). (D) Ido expression in B16 tumor tissue and Chop/Atf4 expression in TILs from matched samples were correlated and p-values were adjusted for multiple testing according to Benjamini–Hochberg (n = 5). (E) SKCM RNA sequencing data were retrieved from TCGA and correlation between Chop/Atf4 and Tdo and Chop/Atf4 and Ido expression was analyzed.
and treated with 5% Imiquimod (Meda) and 300 ng GM-CSF (Peprotech; 315-03) s.c. For immune checkpoint blockade, mice received i.p. injections of anti-CTLA4 (BioLegend; 9D9; 121613), Pacific Blue anti-mouse CD4+ (BioLegend; RM4-5; 100516), CD8a (BioLegend; 53-6.7; 100725), CD11b (BioLegend; M1/70; 101216), CD45 (BioLegend; 30-F11; 103116), and CD11b (BioLegend; PC61; 102008).

For adoptive transfer experiments murine, CD8+ T cells were purified from in vitro cultured splenocytes by MACS. Single cell suspension was incubated with biotinylated antibodies targeting mouse CD4+ (BioLegend; RM4-5; 100508), CD19 (BioLegend; 6D5; 115504), CD49b (BioLegend; DX5; 108904), CD11c (BioLegend; N418; 117304), and CD11b (BioLegend; M1/70; 101216) prior to labeling with MagniSortTM Streptavidin Negative Selection Beads (eBioscience; MSNB-6002-74) according to the manufacturer’s instructions.

Flow cytometry

Single cell suspensions were stained with the following fluorescent antibodies to surface antigens: APC-Cy7 anti-mouse CD3 (BioLegend; 17A2; 100221), Pacific Blue anti-mouse CD4+ (BioLegend; RM4-5; 100221), PerCP Cy5.5 anti-mouse CD8a (BioLegend; 53-6.7; 100734), BV521 anti-mouse CD45 (BioLegend; 30-F11; 103138), Pacific Orange anti-mouse CD45, APC CD107a (BioLegend; 1D4B; 121613), APC anti-mouse PD-1 (BioLegend; RMP1-30; 109111), and PE anti-mouse PD-1 (eBioscience; J43; 12-9985). For tetramer staining cells were stained with PE H-2Db gp100 Tetramer-EGSRNQDWL (MBL; TS-M546-1) prior to surface marker staining.

Figure 7. Tryptophan is not depleted in B16 melanomas treated with immune checkpoint inhibitors or constitutively tryptophan-metabolizing B16 melanomas. (A) B16 melanoma cells were implanted into Gcn2fl/fl mice and animals were treated with anti-CTLA4 or isotype control. Intratumoral tryptophan levels were determined by HPLC and MALDI-TOF MS imaging. Average tryptophan intensities treated with anti-CTLA4 or isotype control. (B) Gcn2fl/fl mice were inoculated with B16 melanoma cells. Tryptophan levels in B16 melanomas treated with immune checkpoint inhibitors or constitutively tryptophan-metabolizing B16 melanomas. Tryptophan levels in B16 melanoma cells. Tryptophan levels were obtained as described above (n = 4). All data are represented as mean ± SEM and a two-tailed student’s t test was used to assess significance.
For intracellular staining, cells were stained using a FoxP3/ transcription factor staining buffer set (eBioscience; 00-5523) and the following fluorescently labeled antibodies against intracellular molecules: APC anti-mouse FoxP3 (eBioscience; FJK-16s; 17-5773), PerCP-eFluor710 anti-mouse granzyme B (eBioscience; NGZB; 46-8898), FITC anti-mouse IFNγ (eBioscience; XM12.1; 11-7311), PE anti-mouse IFNγ (eBioscience; XM12.1; 12-7311), and PE-Cy7 anti-mouse Ki67 (eBioscience; SolA15; 25-5698).

For quantification of T cell counts, 123count eBeads (eBioscience; 01-1234) were added at a defined volume prior to sample acquisition.

Flow cytometry acquisition was performed on FACS Canto II (BD Biosciences; Heidelberg, Germany) using the BD FACS Diva software. Data were analyzed using FlowJo software version X.

**Quantitative (q)RT-PCR**

Total RNA was isolated using the RNeasy Mini or Micro Kit (Qiagen; 74104 or 74004) and cDNA was synthesized with the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems; 4368814) according to the manufacturer’s protocol. qRT-PCR was performed on a QuantStudio 3 Real-Time PCR System (Thermo Fisher Scientific, Waltham, Massachusetts) using the SYBR Green PCR Master Mix (Applied Biosystems; 4312704). All Ct values were normalized to Gapdh. Murine primer sequences were Atf4-Fwd 5’-CAG GGC GGC CTC CTC GGA ATG-3’, Atf4-Rev 5’-AGG CAT CCT CTC TGG-3’, Chop-Fwd 5’-CTG GAA GCC TGG TAT GAG-3’, Chop-Rev 5’-CAG GGT CAA GAG TAG TGA AGG-3’, Gapdh-Fwd 5’-GCC TTC GTT GTT ACC C-3’, Gapdh-Rev 5’-CAG TGG GCC CTC AGA TGC-3’, Ido-Fwd 5’-GGC TAG AAA TCT GCC GTT GC-3’, Ido-Rev 5’-AGACCTGCGATAGGAACA-3’, Tdo-Fwd 5’-GAA TGC GCA AGA ACT TCA G-3’, Tdo-Rev 5’-CTT CAG AAC CGA GAA CTG CT-3’.

**Gene expression analysis**

RNA was extracted from purified murine Tregos, conventional CD4+ T cells, and CD8+ T cells using the RNeasy Plus Micro kit (Qiagen). The DFKZ Genomics and Proteomics Core Facility amplified and hybridized material to the Illumina MouseWG-6 v2.0 Expression BeadChip. Microarray gene expression raw data were imported into Chipster 3.4.0. After built-in quantile normalization, statistical differences between spleen and tumor infiltrating T cell subsets were calculated using empirical Bayes in combination with p-value adjustment according to Benjamini–Hochberg. Differences in log2 expression values were visualized as a heatmap.

SKCM RNA sequencing data were retrieved from data generated by the TCGA Research Network: http://cancer.gov/ctc. Data type was RNASeqV2 data, level 3, and was downloaded on March 4th, 2016. Normalized RSEM expression values for relevant genes were extracted for individual patient sample, log2-transformed, and plotted using R 3.2.3. Correlation was assessed by calculation of Spearman’s rank correlation coefficient using the cor() function and the pairwise significance levels were determined using the cor.test() function. Correction for multiple testing was performed according to Benjamini–Hochberg using the function p.adjust().

**MALDI-TOF MS imaging**

For MALDI-TOF MS imaging fresh-frozen tumor tissue samples were cut into 10 μm sections, thaw-mounted onto indium tin oxide (ITO) slides (Bruker Daltonics; 8237001), and desiccated at room temperature for 2 h. 2.5-Dihydroxybenzoic acid (DHB; Sigma-Aldrich; 8201346) matrix was prepared at a concentration of 60 mg/mL in acetonitrile (Merck; 1000302500)/H2O/trifluoroacetic acid (Merck; 8082600100) (50:49.5:0.5; v/v/v) and deposited onto the tissue sections using a SunCollect sprayer (SunChrom; Friedrichsdorf, Germany). Briefly, matrix was applied in five layers in ascending flow rates (10, 15, 20, 20, 20 μL/min). Data acquisition was performed on an UltraflexXtreme MALDI-TOF/TOF mass spectrometer (Bruker Daltonics; Bremen, Germany) equipped with a 2,000 Hz Smartbeam-II laser. Spectra and images were acquired in the positive reflectron mode within the mass range of m/z 100–1,000 Da using the FlexControl 3.4 software (Bruker Daltonics). Prior to analysis, the acquisition method was calibrated externally on the ITO slide using choline chloride (m/z 104.1070; Sigma-Aldrich; C7017), acetylcholine chloride (m/z 146.1176; Sigma-Aldrich; A6625), the DHB matrix peak (m/z 155.0344; Bruker Daltonics), olanzapine (m/z 313.1481; Selleckchem; S2493), dasatinib (m/z 488.1630; Selleckchem; S1021), and bradykinin 1–7 (m/z 757.3992; Bruker Daltonics; peptide calibration standard II 222570). Tryptophan imaging was performed using the FlexImaging 4.0 software by accumulating 500 laser shots per measurement spot at a raster width of 100 μm. Images were normalized to the DHB matrix peak and average tryptophan intensities were extracted for every tissue section using mMass. Statistical data analysis was performed using a student’s t test.

**MALDI MS cell biotyping**

For MALDI MS cell biotyping Super-DHB (sDHB; Bruker Daltonics; 8209813) matrix was prepared at a concentration of 10 mg/mL in 15 mM ammoniumdihydrogencitrate (Merck; 101154)/ethanol (VWR; 20821.330)/phosphoric acid (Fluka; 64957) (79:20:1 v/v/v). The frozen cell pellets were resuspended in ddH2O to obtain a cell concentration of 5,000 cells/μL. Cells were mixed and disrupted by pipetting. An equal volume of cell suspension and SDHB was mixed. Thereafter, 1 μL of the sample suspensions (2,500 cells) was pipetted onto a 384-well ground steel target plate in octuplets and air dried at room temperature.

Method calibration on the steel target and data acquisition on an UltraflexXtreme MALDI-TOF/TOF mass spectrometer were performed as described above. Sample spots were measured automatically using the AutoXecute function of the flexControl acquisition software by accumulating 2,000 laser shots per measurement spot. Tryptophan peak intensities were obtained using flexAnalysis. Mean values eight technical replicates were displayed using GraphPad Prism.
HPLC

For intratumoral tryptophan measurements, tumor tissue was supplemented with dH2O (2 μL/1 mg tissue) and disrupted using a TissueLyser II (Qiagen; Hilden, Germany) and stainless steel beads (Qiagen). Proteins were precipitated using perchloric acid (Sigma-Aldrich; 244252). Tumor samples were neutralized with potassium hydroxide (Sigma-Aldrich; 221473) prior to HPLC analysis. Tryptophan concentrations were determined by Dionex UltiMate 3000-uHPLC-machine (Thermo Fisher Scientific) with photodiode array detection and an AccucoreTMaQ-column (100 nm, 2.6 μm; Thermo Scientific) as described before.41

Graphical representation and statistics

All data are presented as means (±SEM, SD) as indicated in figure legend. Data were analyzed by two-tailed Student’s t test or one-way ANOVA for comparison of three or more groups combined with correction for multiple testing. Evaluation of survival patterns was performed by the Kaplan–Meier method. p < 0.05 was considered statistically significant (’p < 0.05; ‘’p < 0.01; ‘’’p < 0.001).

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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References

1. Sheridan C. IDO inhibitors move center stage in immuno-oncology. Nat Biotechnol 2015; 33:321-2; PMID:25850038; http://dx.doi.org/10.1038/nbt0415-321
2. Uyttenhove C, Pilote L, Theate I, Stroobant V, Colau D, Parmentier N, Boon T, Van den Eynde BJ. Evidence for a tumoral immune resistance mechanism based on tryptophan degradation by indoleamine 2,3-dioxygenase. Nat Med 2003; 9:1269-74; PMID:14502282; http://dx.doi.org/10.1038/nm934
3. Opitz CA, Litzenburger UM, Sahm F, Ott M, Tritscher I, Trump S, Schumacher T, Jestaedt L, Schrenk D, Weller M et al. An endogenous tumour-promoting ligand of the human aryl hydrocarbon receptor. Nature 2011; 478:197-203; PMID:21976023; http://dx.doi.org/10.1038/nature10491
4. Piloite L, Larrieu P, Stroobant V, Colau D, Dolusic E, Frederick R, De Plaen E, Uyttenhove C, Wouters J, Masereel B et al. Reversal of tumoral immune resistance by inhibition of tryptophan 2,3-dioxygenase. Proc Natl Acad Sci USA 2012; 109:2497-502; PMID:22308364; http://dx.doi.org/10.1073/pnas.1113873109
5. Mezrich JD, Fechner NH, Zhang X, Johnson BP, Burlingham WJ, Bradfield CA. An interaction between kynurenine and the aryl hydrocarbon receptor can generate regulatory T cells. J Immunol 2010; 185:3190-8; PMID:20720200; http://dx.doi.org/10.4049/jimmunol.0903670
6. Munn DH, Sharma MD, Baban B, Harding HP, Zhang Y, Ron D, Mellor AL. GCN2 kinase in T cells mediates proliferative arrest and energy induction in response to indoleamine 2,3-dioxygenase. Immunity 2005; 22:633-42; PMID:15894280; http://dx.doi.org/10.1016/j.immuni.2005.03.013
7. Krishnamoorthy T, Pavitt GD, Zhang F, Dever TE, Hinnebusch AG. Tight binding of the phosphorylated alpha subunit of initiation factor 2 (eIF2alpha) to the regulatory subunits of guanine nucleotide exchange factor eIF2B is required for inhibition of translation initiation. Mol Cell Biol 2001; 21:5018-30; PMID:11438658; http://dx.doi.org/10.1128/MCB.21.15.5018-5030.2001
8. Dey S, Baird TD, Zhou D, Palam LR, Spandau DF, Wek RC. Both transcriptional regulation and translational control of ATR4 are central to the integrated stress response. J Biol Chem 2010; 285:33165-74; PMID:20732869; http://dx.doi.org/10.1074/jbc.M110.167213
9. Metz R, Rust S, Duhadaway JB, Maurtino MR, Munn DH, Vahanian NN, Link CJ, Prendergast GC. IDO inhibits a tryptophan sufficiency signal that stimulates mTOR. A novel IDO effector pathway targeted by D-1-methyl-tryptophan. Oncoimmunology 2012; 1(1):1460-8; PMID:23264892; http://dx.doi.org/10.4161/oni.21716
10. Muller AI, Sharma MD, Chandler PR, Duhadaway JB, Everhart ME, Johnson BA, 3rd, Kahler DJ, Pikhala J, Soler AP, Munn DH et al. Chronic inflammation that facilitates tumor progression creates local immune suppression by inducing indoleamine 2,3 dioxygenase. Proc Natl Acad Sci USA 2008; 105:17073-8; PMID:18952840; http://dx.doi.org/10.1073/pnas.0806173105
11. Keil M, Sonner JK, Lanz TV, Oezen I, Bunse T, Bittner S, Meyer HV, Meuth SG, Wick W, Platten M. General control non-derepressible 2 (GCN2) in T cells controls disease progression of autoimmune neuro-inflammation. J Neuroinmunol 2016; 297:117-26; PMID:27397084; http://dx.doi.org/10.1016/j.jneuroim.2016.05.014
12. Holmgaard RB, Zamarin D, Munn DH, Wolchok JD, Allison JP. Indoleamine 2,3-dioxygenase is a critical resistance mechanism in antitumor T cell immunotherapy targeting CTLA-4. J Exp Med 2013; 210:1389-402; PMID:23572227; http://dx.doi.org/10.1084/jem.20130066
13. Wainwright DA, Chang AI, Dey M, Balyasnikova IV, Kim CK, Tobias A, Cheng Y, Kim JW, Qiao J, Zhang L et al. Durable therapeutic efficacy utilizing combinatorial blockade against IDO, CTLA-4, and PD-L1 in mice with brain tumors. Clin Cancer Res 2014; 20:5290-301; PMID:24691018; http://dx.doi.org/10.1158/1078-0432.CCR-14-0514
14. Vaccelli E, Aranda F, Eggermont A, Sautes-Fridman C, Tartour E, Mellor AL. An interaction between kynurenine and the aryl hydrocarbon receptor can generate regulatory T cells. J Immunol 20130066; 185:3190-8; PMID:20720200; http://dx.doi.org/10.4049/jimmunol.0903670
15. Keil M, Sonner JK, Lanz TV, Oezen I, Bunse T, Bittner S, Meyer HV, Meuth SG, Wick W, Platten M. General control non-derepressible 2 (GCN2) in T cells controls disease progression of autoimmune neuro-inflammation. J Neuroinmunol 2016; 297:117-26; PMID:27397084; http://dx.doi.org/10.1016/j.jneuroim.2016.05.014
16. Holmgaard RB, Zamarin D, Munn DH, Wolchok JD, Allison JP. Indoleamine 2,3-dioxygenase is a critical resistance mechanism in antitumor T cell immunotherapy targeting CTLA-4. J Exp Med 2013; 210:1389-402; PMID:23572227; http://dx.doi.org/10.1084/jem.20130066
17. Schumacher T, Jestaedt L, Schrenk D, Weller M et al. An endogenous tumour-promoting ligand of the human aryl hydrocarbon receptor. Nature 2011; 478:197-203; PMID:21976023; http://dx.doi.org/10.1038/nature10491
18. Overwijk WW, Tsung A, Irving KR, Parkhurst MR, Goletz TJ, Tsung K, Carroll MW, Liu C, Moss B, Rosenberg SA et al. gp100/pmel 17 is a murine tumor rejection antigen: induction of ”self”-reactive, tumoricidal T cells using high-affinity, altered peptide ligand. J Exp Med 1998; 188:277-86; PMID:9670040; http://dx.doi.org/10.1084/jem.188.2.277
16. Munteanu B, Meyer B, von Reitzenstein C, Burgermeister E, Bog S, Pahl A, Ebert MP, Hopf C. Label-free in situ monitoring of histone deacetylase drug target engagement by matrix-assisted laser desorption ionization-mass spectrometry biotyping and imaging. Anal Chem 2014; 86:4642-7; PMID:24559101; http://dx.doi.org/10.1021/ac500338

17. Harding HP, Novoa I, Zhang Y, Zeng H, Wek R, Schapira M, Ron D. Regulated translation initiation controls stress-induced gene expression in mammalian cells. Mol Cell 2000; 6:1099-108; PMID:11106749; http://dx.doi.org/10.1016/S1097-2765(00)00108-8

18. Tomczak K, Czerwinska P, Wiznerowicz M. The Cancer Genome Atlas (TCGA): an immeasurable source of knowledge. Contemp Oncol 2015; 19:A68-77.

19. Hinsenkamp I, Schulz S, Suhr AM, Meyer B, Munteanu B, Fuchser J, Schoenberg SO, Ebert MPA, Wissmann A, Voss C. Non-DNA methylation-based in situ multiplexed whole-genome analysis. Nature 2016; 533:639-44; PMID:26837936; http://dx.doi.org/10.1038/nature17186

20. Munn DH, Shafran AL, Shafer AL. Inhibition of T cell proliferation by macrophage tryptophan catabolism. J Exp Med 1999; 189:1363-72; PMID:10224276; http://dx.doi.org/10.1084/jem.189.9.1363

21. Marsching C, Eckhardt M, Grone HJ, Sandhoff R, Hopf C. Imaging of complex sulfatides SM3 and SB1a in mouse kidney using MALDI-TOF/TOF mass spectrometry. Anal Bioanal Chem 2011; 401:53-64; PMID:21359825; http://dx.doi.org/10.1007/s00216-011-4802-0

22. Munn DH, Shafran AL, Ebert MP, Hopf C. Label-free in situ monitoring of histone acetyltransferase drug target engagement by MALDI-TOF/TOF mass spectrometry. Anal Bioanal Chem 2011; 401:53-64; PMID:21359825; http://dx.doi.org/10.1007/s00216-011-4802-0

23. Munn DH, Shafran AL, Ebert MP, Hopf C. Label-free in situ monitoring of histone acetyltransferase drug target engagement by MALDI-TOF/TOF mass spectrometry. Anal Bioanal Chem 2011; 401:53-64; PMID:21359825; http://dx.doi.org/10.1007/s00216-011-4802-0

24. Munn DH, Sharma MD, Lee JR, Jhaver KG, Johnson TS, Keskin DB, Mezrich JD, Fechner JH, Tonetti M, Ferrara GB. T cell proliferation is blocked by indoleamine 2,3-dioxygenase. Transplant Proc 2001; 33:428-30; PMID:11266894; http://dx.doi.org/10.1016/S0041-0045(00)02078-9

25. Hwu P, Du MX, Lapointe R, Do M, Taylor MW, Young HA. Indoleamine 2,3-dioxygenase production by human dendritic cells results in the inhibition of T cell proliferation. J Immunol 2000; 164:3596-9; PMID:10725715; http://dx.doi.org/10.4049/jimmunol.164.7.3596

26. Munn DH, Sharma MD, Lee JR, Jhaver KG, Johnson TS, Keskin DB, Marshall B, Chandler P, Antonia SJ, Burgess R et al. Potential regulatory function of human dendritic cells expressing indoleamine 2,3-dioxygenase. Science 2002; 297:1867-70; PMID:12228717; http://dx.doi.org/10.1126/science.1073514

27. Munn DH, Shafran AL, Ebert MP, Hopf C. Label-free in situ monitoring of histone acetyltransferase drug target engagement by MALDI-TOF/TOF mass spectrometry. Anal Bioanal Chem 2011; 401:53-64; PMID:21359825; http://dx.doi.org/10.1007/s00216-011-4802-0

28. Muller AI, DuHadaway JB, Donover PS, Sutanto-Ward E, Prendergast GC. Inhibition of indoleamine 2,3-dioxygenase, an immunoregulatory target of the cancer suppression gene Bin1, potentiates cancer chemotherapy. Nat Med 2005; 11:312-9; PMID:15711557; http://dx.doi.org/10.1038/nm1196

29. Schmitt M, Pawlita M. High-throughput detection and multiplex identification of cell contaminations. Nucleic Acids Res 2009; 37:e119; PMID:19589807; http://dx.doi.org/10.1093/nar/gkp581

30. Koritzinsky M, Madaio MP, McGaha TL. The amino acid sensor GCN2 inhibits in vivo cell proliferation and natural killer cell proliferation induced by indoleamine 2,3-dioxygenase. J Exp Med 2002; 196:459-68; PMID:12186838; http://dx.doi.org/10.1084/jem.20020121