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Targeting the aryl hydrocarbon receptor (AhR) with BAY 2416964: a selective small molecule inhibitor for cancer immunotherapy

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ABSTRACT

Background The metabolism of tryptophan to kynurenines (KYN) by indoleamine-2,3-dioxygenase or tryptophan-2,3-dioxygenase is a key pathway of constitutive and adaptive tumor immune resistance. The immunosuppressive effects of KYN in the tumor microenvironment are predominantly mediated by the aryl hydrocarbon receptor (AhR), a cytosolic transcription factor that broadly suppresses immune cell function. Inhibition of AhR thus offers an antitumor therapy opportunity via restoration of immune system functions.

Methods The expression of AhR was evaluated in tissue microarrays of head and neck squamous cell carcinoma (HNSCC), non-small cell lung cancer (NSCLC) and colorectal cancer (CRC). A structure class of inhibitors that block AhR activation by exogenous and endogenous ligands was identified, and further optimized, using a cellular screening cascade. The antagonistic properties of the selected AhR inhibitor candidate BAY 2416964 were determined using transactivation assays. Nuclear translocation, target engagement and the effect of BAY 2416964 on agonist-induced AhR activation were assessed in human and mouse cancer cells. The immunostimulatory properties on gene and cytokine expression were examined in human immune cell subsets. The in vivo efficacy of BAY 2416964 was tested in the syngeneic ovalbumin-expressing B16F10 melanoma model in mice. Coculture of human H1299 NSCLC cells, primary peripheral blood mononuclear cells and fibroblasts mimicking the human stromal-tumor microenvironment was used to assess the effects of AhR inhibition on human immune cells. Furthermore, tumor spheroids cocultured with tumor antigen-specific MART-1 T cells were used to study the antigen-specific cytotoxic T cell responses. The data were analyzed statistically using linear models.

Results AhR expression was observed in tumor cells and tumor-infiltrating immune cells in HNSCC, NSCLC and CRC. BAY 2416964 potently and selectively inhibited AhR activation induced by either exogenous or endogenous AhR ligands. In vitro, BAY 2416964 restored immune cell function in human and mouse cells, and furthermore enhanced antigen-specific cytotoxic T cell responses and killing of tumor spheroids. In vivo, oral application with BAY 2416964 was well tolerated, induced a proinflammatory tumor microenvironment, and demonstrated antitumor efficacy in a syngeneic cancer model in mice.

Conclusions These findings identify AhR inhibition as a novel therapeutic approach to overcome immune resistance in various types of cancers.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ The aryl hydrocarbon receptor (AhR) is a mediator of tumor immune resistance.

WHAT THIS STUDY ADDS

⇒ We identified BAY 2416964, an AhR antagonist which potently and selectively inhibits ligand-induced AhR activation in human and mouse immune cells. BAY 2416964 has proinflammatory immunomodulatory effects resulting in tumor inhibition in vitro and in vivo.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ BAY 2416964, an AhR antagonist, shows potential for the treatment of tumors with AhR-induced-immunosuppression and is currently being tested in a phase I clinical trial.

INTRODUCTION

The essential amino acid tryptophan (Trp) is an important regulator of cancer progression due to its regulatory role in immune cell activity, and Trp catabolism has emerged as an important metabolic regulator of cancer

progression.[1] The rate-limiting enzymes indoleamine-2,3-dioxygenase (IDO1) and tryptophan-2,3-dioxygenase (TDO2) catalyze the degradation of Trp to kynurenines (KYN), thereby modulating immune responses and promoting cancer progression.[2]

IDO1 and TDO2 are expressed in many cancers[3] and high tumor expression of IDO1 is associated with poor patient outcomes.[4] The expression of IDO1 or TDO2 in experimental tumor models results in resistance to immune-mediated rejection, and small molecule inhibitors of IDO1 and TDO2 have been shown to enhance antitumor immune responses in vivo.[3 5–10] However, IDO1 inhibitors have so far failed to achieve efficacy in randomized clinical trials, possibly because they do not address TDO2-mediated KYN generation or production of metabolites downstream of interleukin 4-induced 1 (IL4I1), a metabolic immune checkpoint that has recently been shown to activate the aryl hydrocarbon receptor (AhR).[11 12]

The immunosuppressive effects of KYN are mediated via activation of AhR in infiltrating immune cells.[7] AhR is a broadly expressed cytosolic, ligand-activated transcription factor that binds to different ligands of exogenous and endogenous origin. Exogenous ligands include, for example, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and benzo[a]pyrene (BaP), and endogenous ligands include Trp catabolic products and byproducts of the KYN pathway, such as kynurenic acid (KA).[7 13–17] Upon ligand binding, AhR translocates to the nucleus where it regulates the transcription of a variety of target genes by binding to the dioxin response elements (DREs) in their promoter region. AhR target genes include several enzymes involved in the metabolic degradation of ligands, such as CYP1A1, as well as the AhR repressor (AhRR) protein, a negative regulator of AhR signaling.[18] On the cellular level, AhR modulates the maturation and function of dendritic cells (DCs), controls the generation and function of regulatory T cells (Tregs), and suppresses tumor-specific $CD8^+$ T cells.[7 14 19] Therefore, AhR is suggested to play a key role as an immune checkpoint. This provides strong rationale for targeting AhR to integrate the inhibition of immunosuppressive KYN derived from different enzymatic sources, as well as other potential AhR ligands, in the tumor microenvironment. As recent studies have linked the presence of KYN and IDO activity to the resistance to anti-PD-1 (programmed cell death protein 1) therapy,[20] AhR inhibition may also provide a strategy for overcoming this detrimental effect.

Here, we report the preclinical characterization of the potent and selective AhR inhibitor BAY 2416964. We demonstrate that BAY 2416964 antagonizes agonist-induced AhR activation and relieves immunosuppression in both in vitro and in vivo models.

MATERIALS AND METHODS

Extended materials and methods are presented in online supplemental information.

Compounds and cell lines

BAY 2416964, epacadostat, and the anti-PD-1 antibody were all produced at Bayer AG. Mouse B16F10-OVA melanoma cells (clone 5) were generated at NMI TT Pharmaservices (Reutlingen, Germany). Human U937 lymphoma, U87 glioblastoma, MDA-MB-231 breast cancer and Hep G2 liver cancer cells, and mouse Hepa1c1c7 liver cancer cells were obtained from ATCC (Manassas, VA, USA). Human COLO-800 melanoma cells were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). The cells were routinely cultured according to the manufacturer's protocols, subjected to DNA fingerprinting and regularly tested to be free from Mycoplasma contamination using MycoAlert (Lonza).

Immunohistochemical staining of human tumor tissue microarrays

Human tumor tissue microarrays (TMA; Provitro, Berlin, Germany) representing head and neck squamous cell carcinoma (HNSCC), non-small cell lung cancer (NSCLC) and colorectal cancer (CRC) were stained for AhR using the primary monoclonal AhR antibody ab190797 (1:100, Abcam, Cambridge, UK), scanned and analyzed visually for AhR localization.

Physicochemical properties, metabolic stability, and pharmacokinetics of BAY 2416964

The physicochemical properties, metabolic stability, and pharmacokinetics of BAY 2416964 were evaluated as described in online supplemental methods.

AhR transactivation assay in human and mouse reporter cell lines

Transactivation of the AhR was assessed in a luciferase reporter assay in endogenously AhR-expressing human U87 and mouse Hepa1c1c7 cancer cells. The cells were stably transfected with an AhR-inducible firefly luciferase reporter gene construct carrying DREs in its promoter. U87 cells were additionally transfected with a *Renilla* reporter gene construct with a constitutively active promoter. The Dual-Glo Luciferase Assay System (Promega) and the Steady-Glo Luciferase Assay System (Promega) were used for the detection of luciferase activity in U87 and Hepa1c1c7 cells, respectively. For assessing antagonism, U87 or Hepa1c1c7 cells were stimulated with 150–200 µM KA (Sigma) and incubated in the absence or presence of BAY 2416964 (72 pM–20 µM) for 20 hours. Staurosporin (5 µM) was used as inhibition control. For assessing agonism, U87 or Hepa1c1c7 cells were incubated in the absence or presence of BAY 2416964 (72 pM–20 µM) for 20 hours. As a stimulation control, the cells were incubated with 300–400 µM KA. Firefly luciferase activity was determined using the Steady-Glo Luciferase Assay System. IC_{50} / EC_{50} values were determined from normalized data with the Genedata Screener software package.

The effect of BAY 2416964 on AhR ligand-induced *CYP1A1* expression in human U937 cells and mouse splenocytes

U937 cells or freshly isolated mouse splenocytes were stimulated with 200 µM KA. U937 cells were additionally stimulated with 300 nM BaP, 30 µM indole-3-pyruvate (I3P), or 0.1 nM 5,11-dihydro-indolo[3,2-b]carbazole-6-carboxaldehyde (FICZ) for 4 hours in the absence (positive control) or presence of BAY 2416964. RNA isolation was performed using the NucleoSpin 96 RNA-Kit (Macherey-Nagel). *CYP1A1* expression was determined by qPCR (TaqMan-PCR, Applied Biosystems) using the SuperScript VILO cDNA synthesis kit (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and human *HPRT1* as a reference gene. *CYP1A1* expression was calculated as a percentage of the AhR ligand-induced *CYP1A1* expression (positive control=100%) after the unstimulated background (negative control=0%) was subtracted from all values.

Nuclear translocation and cellular thermal shift assay

Hep G2 cells were treated with 100 nM TCDD in the absence (negative control) or presence of BAY 2416964 for 4 hours. Cytoplasmic and nuclear protein fractions were extracted using the NE-PER Nuclear and Cytoplasmic Extraction Kit (Thermo Fisher Scientific). Agonist-induced translocation of AhR from the cytoplasm to the nucleus was analyzed by western blotting using primary antibodies selective against AhR (Clone EPR7119(N)(2)), alpha tubulin (DM1A), and lamin A/C (EPR4100) (all from Abcam) and quantified via densitometric analysis using the ImageJ software. Target engagement was determined by cellular thermal shift assay (CETSA) using MDA-MB-231 cells treated with increasing concentrations of BAY 2416964.

CYP1A1 expression analysis in peripheral blood mononuclear cell subtypes

Peripheral blood mononuclear cells (PBMCs) were isolated using the Ficoll-density gradient method, followed by isolation of individual cell populations using suitable MACS bead isolation kits (Miltenyi Biotec). Total RNA was isolated using the RNeasy Plus Micro Kit (Qiagen, Hilden, Germany), followed by cDNA synthesis using SuperScript VILOMastermix (Thermo Fisher Scientific). Gene expression analysis was performed using the Taqman Fast Universal PCR Master Mix (Applied Biosystems) and the following TaqMan probes: Hs01054797_g1 (*CYP1A1*) and Hs99999905_m1 (*GAPDH*). *CYP1A1* expression was determined using the $2^{-\Delta\Delta Ct}$ method and *GAPDH* as the reference gene.

Immunostimulatory properties of BAY 2416964 in human primary monocytes

Freshly isolated human monocytes were stimulated with lipopolysaccharide (LPS) from *Escherichia coli* (strain O127:B8) alone or in combination with KA, followed by incubation with BAY 2416964 or dimethyl sulfoxide (DMSO) (0.1%, vehicle control) at 37 °C/5% CO_2 for 24 hours. The samples were then centrifuged, and the supernatants and cell pellets were used for further analyses. Tumor necrosis factor α (TNF-α) was determined from the supernatants using the Human TNF-α Tissue Culture Kit (Meso Scale Discovery). The cell pellets were lysed and total RNA was extracted using RNeasy kits (Qiagen, Hilden, Germany). RNA libraries were prepared using the Illumina TruSeq Stranded mRNA Kit (Illumina, San Diego, California, USA) and sequenced on a HiSeq 2500 HTv4 device (single-end, 50 base-pair reads, single-read, dual-indexing, 50 cycles; Illumina). Expression of immunoregulatory genes and inflammatory cytokines was analyzed by genome wide RNA sequencing (RNAseq). For data analysis, generated RNAseq reads were mapped to the human reference genome HG38 using the STAR aligner software,[21] and gene expression was quantified as transcripts per million using the RSEM software.[22] Differential Gene expression analysis was performed using the R programming language. Expression patterns of *AHRR*, *TIPARP*, *IL24*, *CYP1A1*, *CXCL9*, *CXCL10*, *ACOD1*, *TNF*, and *IDO1* across samples were clustered and visualized using the heatmap.2 algorithm. Differential gene expression fold change values were computed based on geometric group means and together with the corresponding Benjamini-Hochberg corrected t-test p values visualized as a volcano plot generated using ggplot functions. In addition, *CYP1A1* and *AHRR* expression was determined by qPCR (TaqMan-PCR, Applied Biosystems) using human *HPRT1* as a reference gene. The probes used were Hs02800695_m1 (*HPRT1*), Hs01054797_g1 (*CYP1A1*), Hs00907314_m1 (*AHR),* and Hs01005075_m1 (*AHRR*).

Human mixed lymphocyte reaction analysis

For the coculture assay, LPS-treated monocyte-derived DCs (moDCs) (1–2×10^4 cells/well) were seeded with T cells (5×10^4 cells/well) from a healthy donor and treated with 3 nM–10 µM BAY 2416964, 3 nM–1 µM epacadostat, or DMSO control at 37°C/5% CO_2 for 3 days. Interleukin-2 (IL-2) and interferon gamma (IFN-γ) secretion was determined using the Human IL-2 and IFN-γ Tissue Culture Kits, respectively (Meso Scale Discovery).

Generation of mouse bone marrow-derived DCs and coculture with mouse OT-I T cells

Bone marrow cells isolated from the hindlimb bones of C57BL/6J mice were cultured in DC medium for 8 days before harvesting the bone marrow-derived DCs (BMDCs). The cells were incubated with 10 µg/mL SIINFEKL (an ovalbumin (OVA)-derived antigen) at 37°C for 2 hours. Then, the SIINFEKL-pulsed DCs were harvested by centrifugation and resuspended at a density of 3×10^6 cells/mL in DC medium.

$CD8^+$ OT-I T cells were isolated from spleens and cervical lymph nodes of Rag1 KO / transgenic OT-I T Cell Receptor mice (B6.129S7-*Rag*^{1tm1Mom}Tg(TcraTcrb)1100Mjb; OT-I 5.2, Taconic) and purified using the CD8a+T Cell Isolation Kit II (Miltenyi Biotech, Bergisch Gladbach,

Germany). BMDCs and CD8+ T cells were cocultured (1:1) and treated with 3 nM–3 µM BAY 2416964 in the presence of 100 µM KA. After 72 hours, IFN-γ secretion was analyzed using the IFN gamma Mouse Uncoated ELISA Kit (Thermo Fisher Scientific). Coculture with OT-I T cells and SIINFEKL-pulsed DCs with or without KA treatment served as negative and positive controls, respectively.

The effect of BAY 2416964 on human naïve CD4+ T cells

IFN-γ was quantified in CD4+ T cells isolated from PBMCs from healthy donors. The T cells were stimulated with IL-2 (50 ng/mL, Gibco), CD3 antibody (coated overnight at 4C in PBS at 10 µg/mL, eBioscience), CD28 antibody (1 µg/mL, R&D Systems) in the presence or absence of TGF-β (5 ng/mL, R&D Systems), and treated with BAY 2416964 (3 nM–10 µM) or DMSO control at 37°C/5% CO_2 for 5 days. IFN-γ levels were measured U-PLEX Biomarker Group 1 (hu) Assays (Meso Scale Discovery) according to manufacturer's instructions.

In vivo antitumor efficacy of BAY 2416964 in the B16F10-OVA mouse model

Animal experiments were performed under the national animal welfare laws in Germany and approved by the local authorities. The in vivo antitumor efficacy of BAY 2416964 was evaluated in the B16F10-OVA (clone 5) mouse melanoma model in male C57BL/Ly5.1 or NSG mice. The mice were treated orally (p.o.) with vehicle or BAY 2416964 (30 mg/kg, QD). At the end of the study, tumor samples were collected from all groups and the percentages of various immune cell populations were determined by flow cytometry.

BioMAP Oncology Panel

The BioMAP Oncology Panel (Eurofins DiscoverX, St Charles, Missouri, USA) was used to assess the effect of BAY 2416964 or epacadostat in a complex human tumor–host microenvironment. The experiments were performed by Eurofins Discovery Services. Briefly, H1299 NSCLC cells were mixed with primary human fibroblasts and PBMCs from healthy donors. T cells were activated via superantigen in the presence or absence of BAY 2416964 or epacadostat for 48 hours. Production of IFN-γ, IL-17, IL-2, IL-6, and TNF-α was quantified by ELISA.

COLO-800 spheroid: MART-1 T cell cocultures

MART-1 (melanoma antigen recognized by T cells 1) T cells were generated by retroviral transduction of PBMCs using a T-cell receptor (TCR) specifically recognizing the MART-1 antigen. The expression of MART-1-specific TCRs was confirmed by flow cytometry. COLO-800 tumor spheroids were generated using the liquid-overlay technique.[23] When the COLO-800 spheroids reached a diameter of 600 µm, MART-1 T cells (1.8×10^4 cells/sample) and 0.0001–10 µM of BAY 2416964 or vehicle (DMSO) were added. Cocultures were cultured for 4 days followed by centrifugation of the sample plates. Supernatants were stored at –20°C for subsequent cytokine analysis. For flow cytometric analysis, single cell suspensions were generated from the cell pellets and stained with human TruStain FcX (BioLegend), Live/Dead Fixable Yellow Dead Cell Stain (Life Technologies), anti-CD45 PE (BioLegend), and anti-CD8 BV650 (BioLegend). Samples were analyzed by flow cytometry using the IntelliCyt iQue Screener PLUS instrument and the iQue ForeCyte software (Sartorius, Göttingen, Germany). IL-2 and Granzyme B secretion was analyzed using the human IL-2 BD OptEIA Set (BD Biosciences) or LEGEND MAX Human Granzyme B ELISA Kit (BioLegend). Total RNA from spheroid-T cell cocultures was isolated using the RNeasyMicro Kit (QIAGEN) followed by cDNA transcription with the RevertAid First Strand cDNA Synthesis Kit (ThermoFisher). Gene expression analysis was performed using PowerUp SYBR Green Master Mix (ThermoFisher) and the following primer sequences: TIPARP F: 5'-CACCCTCTAGCA ATGTCAACTC-3' R: 5'-CAGACTCGGGATACTCTCTCC-3'; CYP1A1 F: 5'-CTTGGACCTCATTGGAGCT-3' R: 5'-GACCTGCCAATCACTGTG-3'; B2M F: 5'- AGATGAG-TATGCCTGCCGTG-3' R: 5'-CTGCTTACATGTCTCG ATCCCA-3'. Gene expression was determined using the 2 ΔΔCt method and B2M as the reference gene.

Statistical analyses

All statistical analyses were performed using linear models estimated with generalized least squares that included separate variance parameters for each study group or a common variance parameter for all study groups. Mean comparisons between the treatment and control groups were performed using either the estimated linear model and corrected for family-wise error rate using Sidak's method; one-way analysis of variance (ANOVA) and Dunnett's multiple comparison test; two-way ANOVA and Tukey multiple comparison test; Mann-Whitney test, or one sample t-test as indicated in the figure legends.

RESULTS

AhR is expressed in tumor cells and tumor-infiltrating immune cells

We first assessed the expression of AhR in TMAs of HNSCC (figure 1A), NSCLC (figure 1B) and CRC (figure 1C). Medium to high AhR protein expression was observed in more than 80% of the tumor samples (figure 1D), and the expression was localized in both tumor cells and tumor-infiltrating immune cells (figure 1E). In tumor cells, AhR expression was weaker and mainly cytoplasmic compared with immune cells, where AhR staining was strong and mainly localized to the nucleus (figure 1E). This suggests that the AhR is particularly active in tumor-infiltrating immune cells and that inhibiting this pathway with an AhR antagonist could restore antitumor immunity.

BAY 2416964 is a highly selective AhR inhibitor

We established a cellular screening cascade to identify AhR inhibitors of the KYN pathway (online supplemental figure S1A). From the initial library of 3.2 million

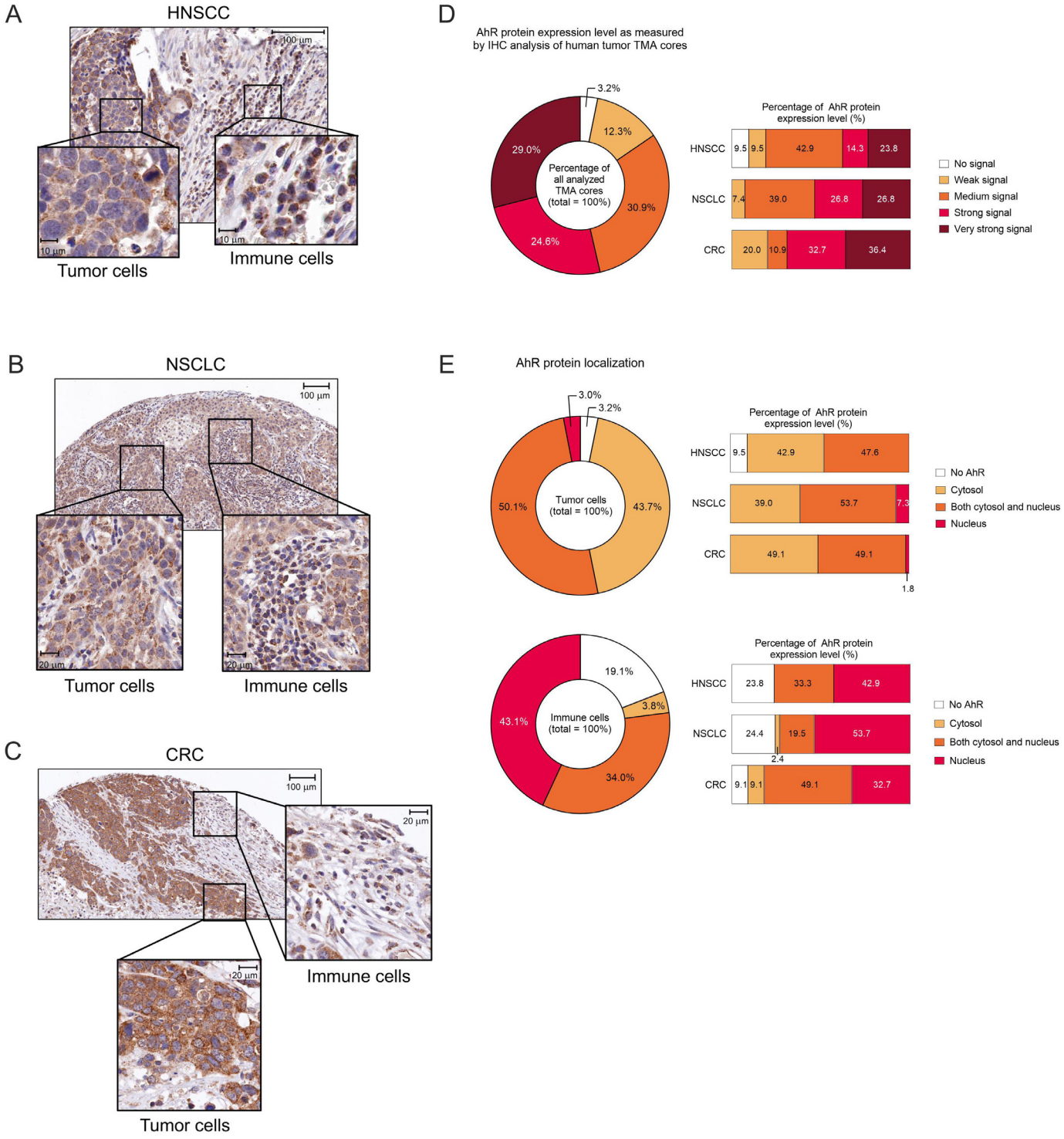

Figure 1 AhR is expressed in tumor cells and tumor-infiltrating immune cells in various cancers. (A–C) Representative IHC images of AhR expression in tissue samples of (A) HNSCC (n=16), (B) NSCLC (n=29) and (C) CRC (n=55). Brown color indicates AhR staining. Scale bars are included in the figures. (D) AhR expression level and (E) localization in tumor samples shown in panels A-C. AhR, aryl hydrocarbon receptor; CRC, colorectal cancer; HNSCC, head and neck squamous cell carcinoma; IHC, immunohistochemistry; NSCLC, head and neck squamous cell carcinoma.

compounds, we first identified a lead series with promising inhibitory potential and evidence for direct competitive binding (online supplemental table S1), and subsequent optimization resulted in the nomination of BAY 2416964 as a selective AhR inhibitor for further characterization (figure 2A). BAY 2416964 potently inhibited agonist-induced AhR activation by KA, as demonstrated by inhibition of DRE-luciferase expression in human U87 (IC_{50} 22 nM; figure 2B) and mouse Hepa-1c1c7 cells (IC_{50} 15 nM; figure 2C) without causing cellular toxicity (online supplemental figure S1B). BAY 2416964 in the absence of KA did not induce luciferase expression,

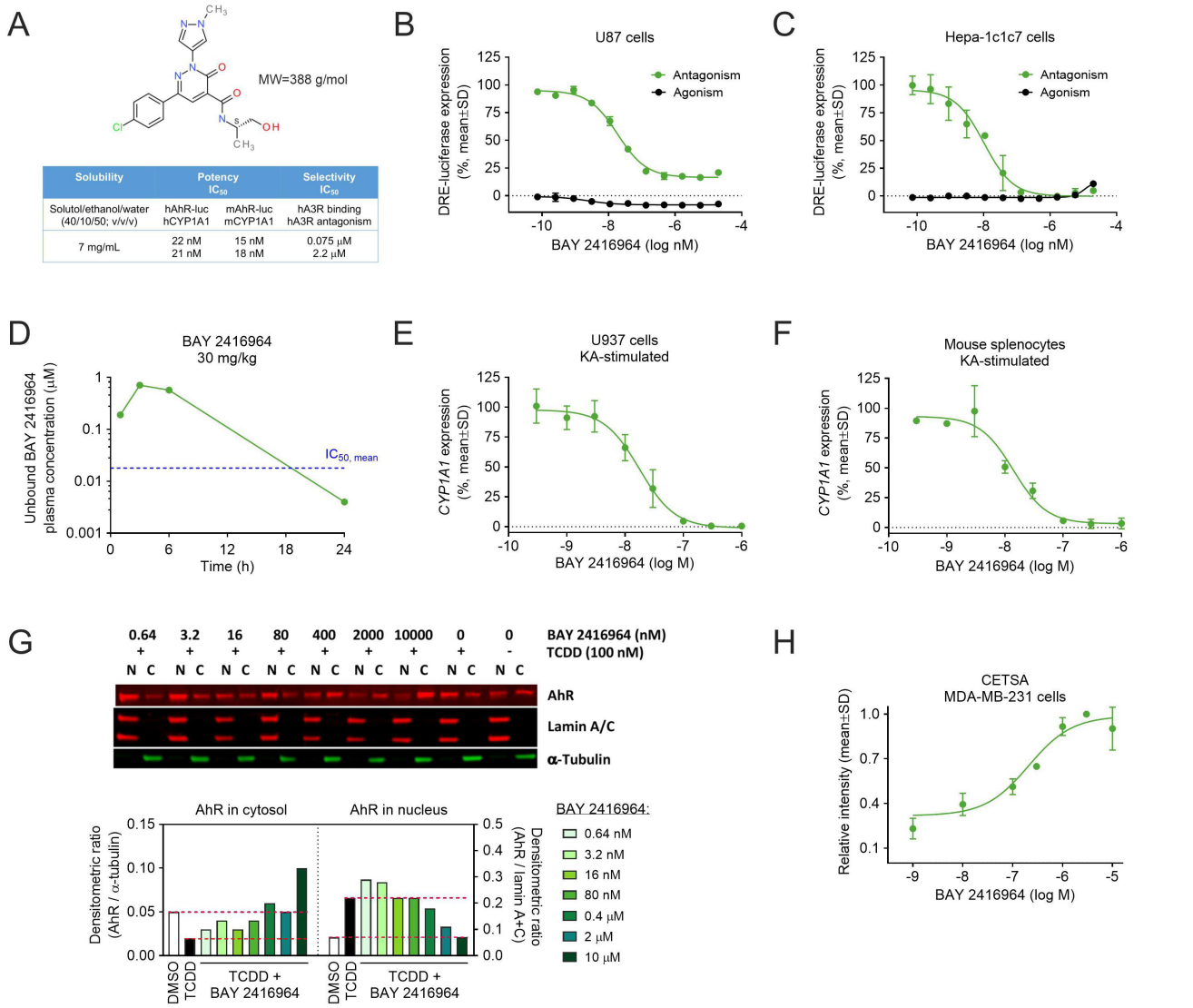

Figure 2 BAY 2416964 inhibits AhR ligand-induced *CYP1A1* transcription and AhR translocation into the nucleus. (A) Molecular structure and characteristics of BAY 2416964 identified in the optimized cellular screening cascade described in online supplemental figure S1. (B–C) Effect of BAY 2416964 on luciferase expression in (B) human U87 cells and (C) mouse Hepa1c1c7 cells in the presence (antagonism) or absence (agonism) of 150 µM or 200 µM KA, respectively, after 20 hours (representative experiments of (B) n=33 and (C) n=34). (D) Unbound BAY 2416964 plasma level in relation to the determined mean cellular unbound IC_{50} value (as determined by *CYP1A1* inhibition in KA-stimulated mouse splenocytes, indicated with a blue dotted line) after repeated dosing with BAY 2416964 at 30 mg/kg (QD) in B16F10-OVA tumor-bearing mice (n=2–3 mice/time point). (E, F) Inhibition of AhR-induced *CYP1A1* expression in BAY 2416964-treated (E) human U937 cells and (F) freshly isolated mouse splenocytes on stimulation with 200 µM KA after 4 hours (representative experiments of n=5). (G) AhR translocation from the cytosol to the nucleus induced with 100 nM TCDD after 4 hours in human Hep G2 cells treated with BAY 2416964 (n=4). N, nucleus; C, cytosol. (H) Direct target engagement of BAY 2416964 with AhR, as determined using CETSA. AhR, aryl hydrocarbon receptor; CETSA, cellular thermal shift assay; DMSO, dimethyl sulfoxide; KA, kynurenic acid; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin.

demonstrating that BAY 2416964 alone did not have any agonistic activity in these cell lines (figure 2B,C). Furthermore, BAY 2416964 demonstrated a favorable pharmacokinetic profile as evidenced by favorable physicochemical properties (topological polar surface area TPSA=100 Å^2, lipophilicity logD=2.4) and a good in vivo PK profile that qualifies the compound for once daily dosing (online supplemental tables S2,3; figure 2D).

We next assessed the potential of BAY 2416964 to inhibit AhR-induced gene expression upon stimulation with various AhR agonists. BAY 2416964 potently inhibited AhR-induced *CYP1A1* expression in human monocytic U937 cells (IC_{50} 21 nM; figure 2E) and in freshly isolated mouse splenocytes (IC_{50} 18 nM; figure 2F) on stimulation with the endogenous agonist KA. BAY 2416964 showed a similar inhibitory effect on AhR-induced *CYP1A1* expression in U937 cells stimulated with the endogenous AhR agonists 6-formylindolo[3,2-b] carbazole (FICZ; IC_{50} 11 nM, online supplemental figure S1C) or indole-3-pyruvate (I3P; IC_{50} 290 nM, online

supplemental figure S1D), or the exogenous BaP (IC_{50} 45 nM; Online supplemental figure S1E). To investigate the mechanism by which BAY 2416964 inhibits AhR activation, we performed nuclear translocation assays. In human Hep G2 liver cancer cells, BAY 2416964 inhibited TCDD-induced translocation of AhR from the cytoplasm to the nucleus, which is required for its transcriptional activity (figure 2G). Finally, using CETSA, we were able to demonstrate that the inhibitory effects of BAY 2416964 resulted from direct target engagement with AhR (EC_{50} 200 nM; figure 2H).

Immunomodulatory effect of BAY 2416964 on human immune cells in vitro

As AhR is expressed in multiple immune cell types and is broadly immunosuppressive, we next assessed AhR activation in KA-treated bead-sorted immune cell subsets isolated from human blood. DCs, $CD3^+$ T cells, $CD14^+$ monocytes, and to a lesser extent $CD19^+$ B cells, were particularly responsive to AhR activation, as assessed by *CYP1A1* upregulation (figure 3A). To test the effects of BAY 2416964 on gene regulation, we assessed its inhibitory potential in KA-treated, LPS-stimulated human primary monocytes by RNAseq analysis. BAY 2416964 suppressed the expression of AhR target genes, such as *AHRR*, *CYP1A1,* and *TIPARP*, and strongly enhanced the expression of genes of proinflammatory cytokines and chemokines, such as *TNF* and *Cxcl9*, respectively, above the level observed with LPS stimulation alone (figure 3B,C, online supplemental table S4). The downregulation of *AHRR* and *CYP1A1* was confirmed by RT-PCR analyses where BAY 2416964 dose-dependently decreased their expression in KA-treated, LPS-stimulated monocytes (figure 3D,E). To examine the immunostimulatory properties of BAY 2416964, we also assessed the production of the proinflammatory cytokine TNF-α in LPS-stimulated monocytes. KA-induced AhR activation suppressed TNF-α production in LPS-stimulated monocytes, and BAY 2416964 dose-dependently rescued this AhR-mediated suppression (figure 3F). These results suggest that BAY 2416964 rescues the proinflammatory phenotype of human monocytes exposed to the immunosuppressive AhR agonist KA.

To study the effect of BAY 2416964 on mature DC-induced T cell responses, we performed a human mixed lymphocyte reaction (MLR) analysis. Since maturation of moDCs with IFN-γ and LPS upregulates *IDO1* expression (online supplemental figure S2), it allows simultaneous assessment of the activity of the IDO1 inhibitor epacadostat. BAY 2416964 enhanced T cell activity as observed by enhanced IL-2 production. BAY 2416964 dose-dependently increased IL-2 production from T cells to a similar extent as anti-PD-1, while no effect was seen with the IDO1 inhibitor epacadostat (figure 3G). Combination treatment with BAY 2416964 and anti-PD-1 resulted in even further increased IL-2 levels compared with the respective monotherapies (figure 3H).

We then investigated if BAY 2416964-induced AhR inhibition directly affected T cells or if T cell cytokine production was altered indirectly via DCs. BAY 2416964 dose-dependently increased IFN-γ production in human naïve $CD4^+$ and $CD8^+$ T cells stimulated with CD3, CD28, and IL-2 (figure 3I, online supplemental figure S3). BAY 2416964 also increased IFN-γ production in $CD4^+$ and $CD8^+$ T cells in the presence of TGF-β, a cytokine that strongly suppresses T cell responses in the tumor microenvironment.[24] Overall the data points toward additional direct effects of BAY 2416964 on T cells.

BAY 2416964 shows antitumor efficacy in vivo

To determine the species-specificity of the immunostimulatory properties of BAY 2416964, we cultured OVA peptide-pulsed mouse BMDCs with transgenic $CD8^+$OT I T cells recognizing the OVA peptide of the BMDCs. Comparable to the human MLR analysis, BAY 2416964 dose-dependently enhanced IFN-γ production demonstrating that this AhR inhibitor can rescue the activation of KA-suppressed antigen-specific T cells also in mice (figure 4A). In a similar assay, we tested the stimulatory potential of BAY 2416964 using BMDCs from Wildtype or AhR-deficient mice. BAY 2416964 failed to further stimulate OT-I T cells in the presence of AhR-deficient BMDCs demonstrating the AhR-dependent effects of the inhibitor in this assay (online supplemental figure S4A).

We assessed the in vivo efficacy of BAY 2416964 in the syngeneic ovalbumin-expressing B16F10 (B16F10-OVA) melanoma mouse model. BAY 2416964 (30 mg/kg, QD, p.o.) suppressed tumor growth compared with vehicle-treated mice in several experiments (representative experiment shown in figure 4B). Furthermore, BAY 2416964 increased the frequency of immunostimulatory tumor-infiltrated $CD8^+$ T cells and natural killer (NK) cells and decreased the frequency of immunosuppressive GR1-positive myeloid cells and $CD206^+$ M2 macrophages (figure 4C). In contrast, BAY 2416964 did not show efficacy in the absence of innate and adaptive immune cells, as evidenced by using immunodeficient NSG mice that were additionally depleted of myeloid cells (figure 4D), while no direct cytotoxic effects on tumor cells were seen in vitro (online supplemental figure S4B). These data suggested that the antitumor effects of BAY 2416964 were immune-mediated. The treatment was well tolerated as demonstrated by stable bodyweights during the course of the treatment (figure 4E) as well as in rodent and non-rodent toxicology studies (data not shown).

Immunostimulatory effects of BAY 2416964 results in antitumor activity in vitro

Finally, we investigated if the observed immune effects of BAY 2416964 would be maintained in a human tumor-like setting and if this would result in antitumor efficacy in humans. To assess the effects of AhR inhibition on human immune cells, we used a coculture of H1299 NSCLC cells, primary human PBMCs, and primary human fibroblasts mimicking a stromal tumor microenvironment.

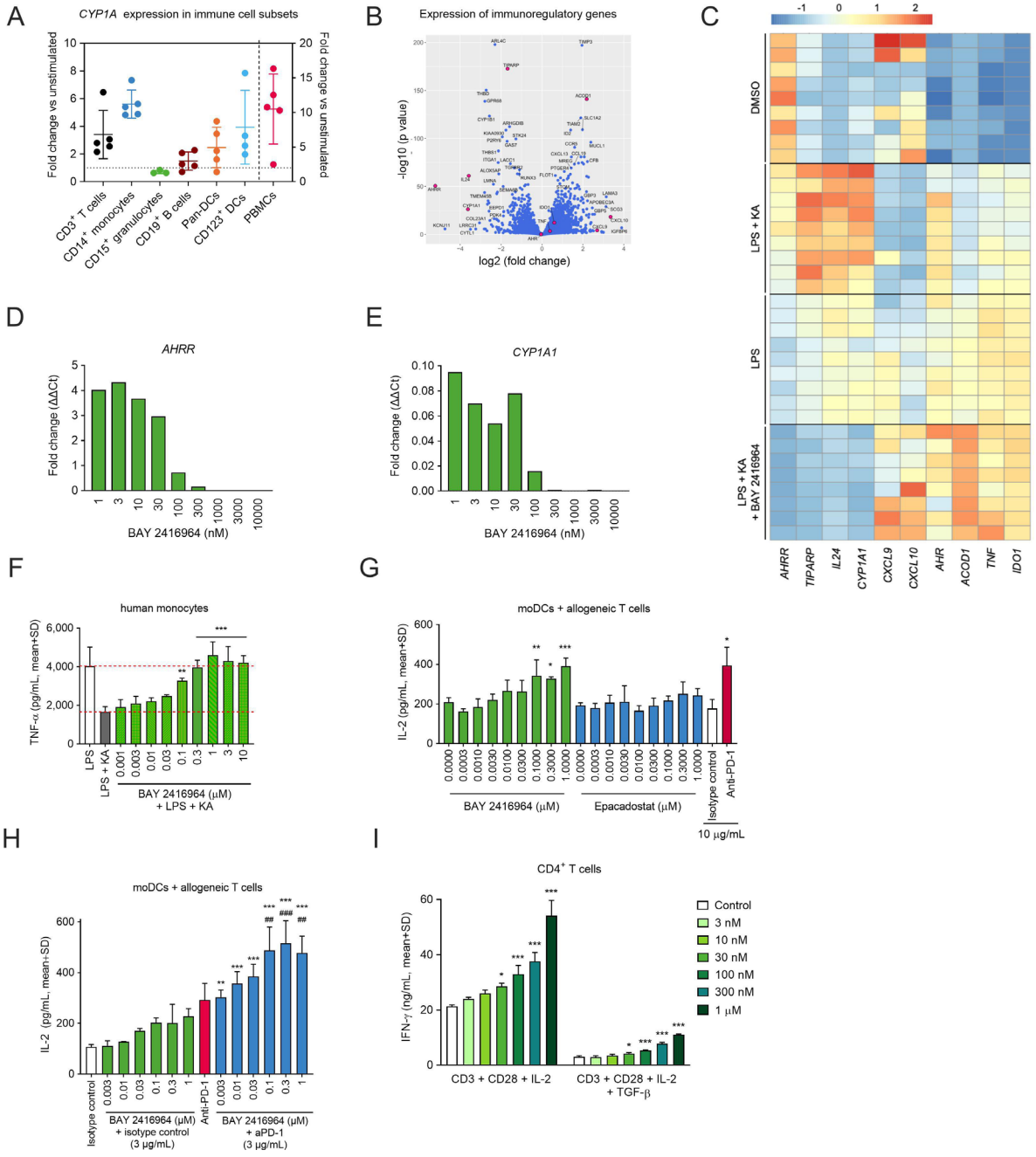

Figure 3 BAY 2416964 induces proinflammatory effects in various human immune cell subsets in vitro. (A) *CYP1A1* expression in 200 µM KA-treated human immune cell subsets from 3 to 5 donors. (B) Effect of BAY 2416964 (300nM) on the expression of immunoregulatory genes in KA-treated, LPS-stimulated human primary monocytes, as determined by RNAseq analysis. (C) Expression of AhR target genes and inflammatory cytokines in human primary monocytes (isolated from six different donors) treated with LPS, LPS and KA, or LPS+KA+BAY 2416964 (300 nM), as determined by RNAseq analysis. (D, E) Effect of BAY 2416964 (1 nM–10 µM) on (D) *AHRR* and (E) *CYP1A1* expression (fold change vs vehicle) in 200 µM KA-treated, 10 ng/mL LPS-stimulated monocytes after treatment with BAY 2416964 (1 nM–10 µM), as determined by qRT-PCR. (F) TNF-α production after 24 hours in 200 µM KA-treated, 10 ng/mL LPS-stimulated monocytes on treatment with BAY 2416964 (1 nM–10 µM) (one representative of 6 donors). Statistical analyses were performed using one-way ANOVA and Dunnett's multiple comparisons test. **p<0.01, ***p<0.001 compared with LPS+KA. (G) IL-2 production by human T cells cocultured with LPS and IFN-γ-matured moDCs treated with 1 nM–1 µM BAY 2416964 or epacadostat for 3 days (representative of n≥3). *p<0.05, **p<0.01, ***p<0.001 compared with control. (H) IL-2 production by T cells cocultured with LPS and IFN-γ-matured moDCs treated with 3 nM–1 µM BAY 2416964 alone or in combination with 3 µg/mL anti-PD-1 (n=4). *p<0.05, **p<0.01, ***p<0.001 compared with the respective concentration of isotype control; ##p<0.01, ###p<0.001 compared with anti-PD-1 alone. (I) Effect of BAY 2416964 (3 nM–1 µM) on IFN-γ production in naïve CD4+ T cells stimulated with CD3, CD28, and IL-2 in the absence or presence of 5 ng/mL TGF-β (n=3). *p<0.05, **p<0.01, ***p<0.001 compared with control. Statistical analyses were performed using the estimated linear model and corrected for family-wise error rate using Sidak's method. DCs, dendritic cells; KA, kynurenic acid; LPS, lipopolysaccharide; moDCs, monocyte-derived DCs; PBMCs, peripheral blood mononuclear cells.

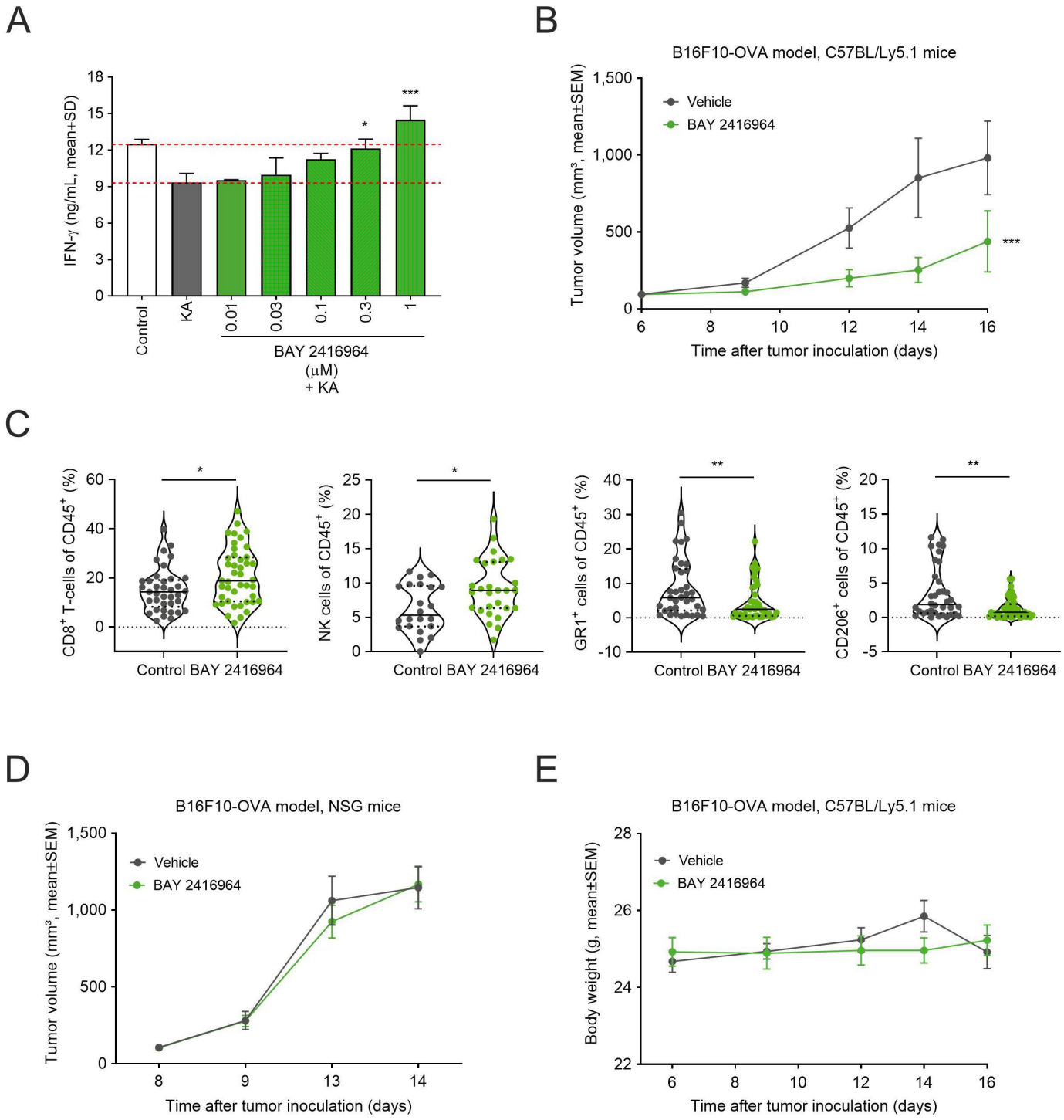

Figure 4 BAY 2416964 decreases tumor growth in an immune cell-dependent manner. (A) IFN-γ production on treatment with different doses of BAY 2416964 in OVA peptide SIINFEKL (H2–Kb)-pulsed mouse bone marrow-derived dendritic cells cultured with transgenic CD8⁺ OT-I T cells in vitro (n=3). (B) Tumor growth in the syngeneic B16F10-OVA melanoma mouse model in one of 5 experiments (n=8 mice/group) treated with vehicle or BAY 2416964 at 30 mg/kg (QD, p.o.). Statistical analyses were performed using two-way ANOVA and Tukey multiple comparison test. ***p<0.001 vs vehicle control. (C) Immune composition of the B16F10-OVA tumor microenvironment on treatment with BAY 2416964 (30 mg/kg, QD). Statistical analyses were performed using the Mann Whitney test. *p<0.05; **p<0.01 vs control. (D) Tumor growth in B16F10-OVA tumor-bearing immunodeficient NSG mice treated with vehicle or BAY 2416964 at 30 mg/kg (QD, p.o.). (E) Body weight of the B16F10-OVA tumor-bearing mice shown in A. ANOVA, analysis of variance; KA, kynurenic acid.

BAY 2416964 increased the production of the proinflammatory cytokines IFN-γ, IL-2 and TNF-α, which is consistent with our previous results in human monocytes (figure 3F) and T cells (figure 3G–I), whereas epacadostat treatment led to predominantly anti-inflammatory responses with decreases in IFN-γ, IL-2, IL-6, and IL-17

(figure 5A). This suggested that the effects of AhR inhibition were independent of IDO1 in this assay. To confirm that the immune-mediated effects of BAY 2416964 result in antitumor responses, COLO-800 melanoma tumor spheroids were cocultured with tumor antigen-specific MART-1 (melanoma antigen recognized by T cells 1) T cells and treated with different doses of BAY 2416964 (online supplemental figure S5A). BAY 2416964 dose-dependently enhanced the activity of MART-1 T cells as evidenced by increased production of IL-2 (figure 5B) and granzyme B (figure 5C) and showed decreased expression of AhR-regulated genes *CYP1A1* (figure 5D) and *TIPARP* (figure 5E). Moreover, increased MART-1 T cell activation by BAY 2416964 resulted in a dose-dependent increase in the cytotoxic capacity of MART-1 T cells as demonstrated by increased COLO-800 tumor cell death (figure 5F), which occurred in a T cell-dependent manner (figure 5F,G, online supplemental figure S5B).

DISCUSSION

The KYN pathway controlled by the rate limiting enzymes IDO1 and TDO2 is a critical intracellular immune checkpoint, and hence, a target of interest for cancer immunotherapy. KYN is produced by many cancer cells[3 25–27] and increased KYN concentrations in tumor tissue and plasma are associated with increased cancer risk, suppression of antitumor immunity, and poor prognosis of patients with progressive tumors.[28] Thus, therapies targeting the Trp-metabolizing enzymes IDO1 and TDO2 or the KYN-degrading enzyme kynureninase to inhibit the production and/or activity of KYN, respectively, are expected to relieve immunosuppression and increase antitumor immune responses.[3 6 29] Recently, the metabolic immune checkpoint IL4I1 has been shown to catalyze Trp to indole-3-pyruvate with the subsequent generation of KA, in addition to other AhR-activating ligands, and it associates more frequently with AhR activity in cancer than IDO1 or TDO2.[12] Therefore, we and others propose that targeting the KYN pathway downstream of IDO1, TDO2 and/or IL4I1, at the convergence of these pathways, may represent a superior approach to targeting the upstream enzymes.[27 30] AhR inhibition, instead of dual IDO1/TDO2 or kynureninase inhibition, has the advantage that in addition to inhibiting the KYN pathway, immunosuppression induced by other potential AhR ligands in the tumor microenvironment is also relieved.

Here, we investigated the mechanism of action, immunostimulatory properties, and efficacy of the AhR inhibitor BAY 2416964, identified using a cellular screening cascade. On ligand-induced activation, AhR translocates from the cytoplasm to the nucleus[31] where it controls the transcription of multiple target genes, for example, by upregulating the expression of *CYP1A1*.[18] BAY 2416964 inhibited KA-induced AhR activation in both human U87 and mouse Hepa-1c1c7 cells. BAY 2416964 also inhibited the upregulation of *CYP1A1* expression by several endogenous AhR ligands and prevented its translocation into the nucleus. The inhibition of AhR activity by BAY 2416964 also translated into a modulation of immune responses, as evidenced by reduced AhR-mediated suppression of TNF-α production. In KA-treated, LPS-stimulated human primary monocytes, BAY 2416964 decreased the expression of AhR target genes and at the same time upregulated genes associated with proinflammatory cytokines and chemokines thus reversing the anti-inflammatory phenotype of human monocytes exposed to immunosuppressive AhR activation.

In the tumor microenvironment, the immunosuppressive effects of AhR activation occur in infiltrating immune cells. This has significant effects in the control of adaptive immunity, modulating T cell differentiation and function both directly and indirectly through its effects on antigen presenting cells such as DCs.[7 18 19] AhR modulates DC function by altering antigen presentation as well as by inducing the expression of IDO1 and IDO2 which catalyze KYN production.[32 33] Our results demonstrated that IDO1 inhibition by epacadostat was not sufficient to restore IL-2 production from T cells in an MLR, whereas direct inhibition of AhR by BAY 2416964 dose-dependently increased IL-2 production to a similar extent as anti-PD-1. This indicates that AhR-induced immunosuppression can occur through multiple pathways and cell types, and thus supports the rationale for inhibiting Ahr ligand binding and subsequent AhR nuclear translocation, the point at which these upstream pathways converge. Furthermore, BAY 2416964 directly increased the activity of $CD4^+$ and $CD8^+$ T cells, even when cultured in the presence of TGF-β, a well-known suppressor of T cell responses in the tumor microenvironment. This could be considered unexpected given data from Veldhoen *et al* showing almost exclusive expression of AhR in Th17 cells and an essential role in their differentiation.[34 35] However, it is in line with previous reports demonstrating that AhR is expressed by and influences the phenotype and function of other immune cell subsets including $CD8^+$ T cells, gamma delta (γδ) T cells and NK cells, while $CD4^+$ or $CD8^+$ T cells have been identified as direct targets of ligand-induced AhR activation.[18 36–39] Our in vivo data support an immune-based mechanism for BAY 2416964 in driving efficacy whereby treatment resulted in increased proinflammatory $CD8^+$ T cells and NK cells in B16-OVA tumors. Nevertheless, further functional dissection is required to elucidate the contribution of specific immune cell subsets to BAY 2416964 activity in vivo.

Several cancer treatments targeting the Trp pathway have already been developed, but challenges still remain. In a previous phase 3 trial, the addition of the IDO1 inhibitor epacadostat to the treatment regimen failed to enhance the efficacy of pembrolizumab monotherapy in patients with melanoma.[40 41] Here, BAY 2416964 monotherapy demonstrated superior efficacy compared with epacadostat in vitro, suggesting that targeting AhR may enhance immune activity independent of IDO1 and that targeting the KYN pathway downstream of both IDO1 and TDO2 may be critical for a therapeutic effect. The

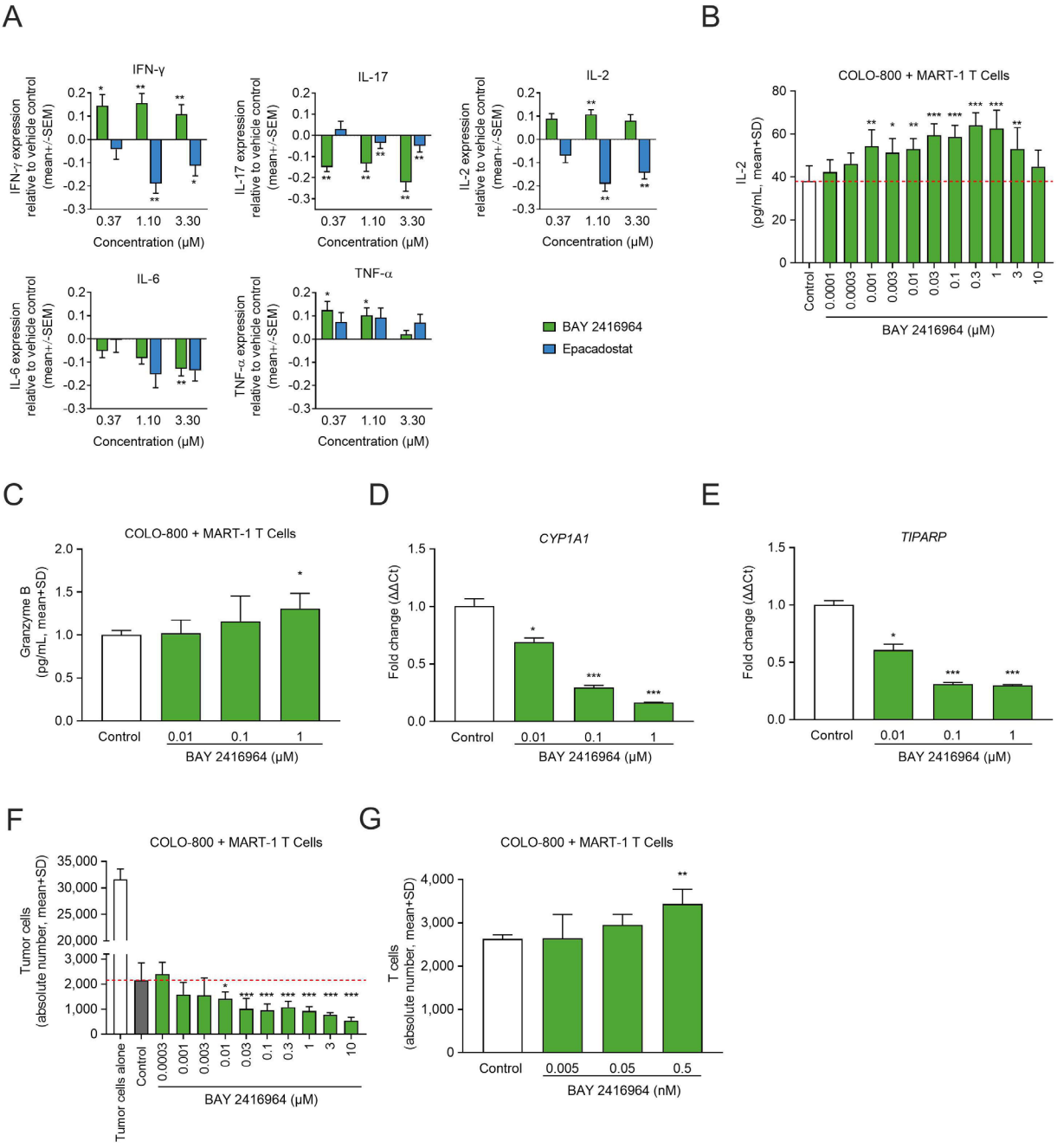

Figure 5 Proinflammatory activity of BAY 2416964 enhances antigen-specific T cell killing of human tumor spheroid in vitro. (A) Expression of IFN-γ, IL-17, IL-2, IL-6, and TNF-α in a coculture of H1299 NSCLC cells, primary human PBMCs, and primary human fibroblasts treated with 0.37, 1.1, or 3.3 µM BAY 2416964 or epacadostat determined using BioMAP Oncology Panel. Statistical analyses were performed using the estimated linear model and corrected for family-wise error rate using Sidak's method. *p<0.05, **p<0.01 vs vehicle control. (B, C) Effect of BAY 2416964 (0.1 nM–10 µM) on (B) IL-2 and (C) granzyme B production by MART-1 T cells cocultured with COLO-800 tumor spheroids for 4 days. Granzyme B concentrations were normalized to vehicle-treated control samples. Statistical analyses were performed using one-way ANOVA and Dunnett's multiple comparisons test. *p<0.05, ***p<0.001 vs vehicle control. (D) *CYP1A1* and (E) *TIPARP* expression (fold change vs vehicle) in COLO-800 spheroid—MART-1 T cell cocultures after 4 days in culture as analyzed by RT-PCR. Statistical analyses were performed using one sample t-test with a hypothetical value of 1 and assumed Gaussian distribution. *p<0.05; ***p<0.001 vs vehicle-treated control. (F) Effect of BAY 2416964 (0.3 nM–10 µM) on the cytotoxic capacity of MART-1 T cells to kill human COLO-800 tumor spheroids measured as absolute tumor cell counts of COLO-800 spheroids after 4 days of coculture with MART-1 T cells determined by flow cytometry. One representative experiment with six technical replicates per condition is shown (n=4). Statistical analyses were performed using one-way ANOVA and Dunnett multiple comparisons test. *p<0.05, ***p<0.001 vs control (COLO-800 spheroid only). (G) Effect of BAY 2416964 (5–500 nM) on the absolute MART-1 T cell count after 4 days of coculture with COLO-800 spheroids determined by flow cytometry. Statistical analyses were performed using one-way ANOVA and Dunnett's multiple comparisons test. **p<0.01 vs vehicle-treated control. ANOVA, analysis of variance; NSCLC, non-small cell lung cancer; PBMCs, peripheral blood mononuclear cells.

advantage of AhR inhibition, instead of dual IDO/TDO or KYN inhibition, is that immunosuppression induced by any other potential AhR ligands in the tumor microenvironment, such as those generated by IL4I1,[12] will also be relieved. On the contrary, AhR inhibition will not affect all activities of Kyn nor will it alter depletion of tryptophan that leads to activation of the general control nonderepressible 2 (GCN2) kinase in T cells to induce their apoptosis.[42 43] However, we have previously published that local tryptophan depletion does not result in anergy of tumor-infiltrating T cells and that intratumoral tryptophan levels are maintained despite high tryptophan turnover.[44] Further studies are needed to elucidate the dependence of BAY 2416964 on upstream IDO/TDO/IL4I1 activity. BAY 2416964 has many advantages compared with other known AhR antagonists such as SR1 and CH-223191. SR1 is not mouse cross-reactive and was developed for ex vivo stimulation of human $CD34^+$ hematopoietic stem cells, while CH-223191 has AhR-independent proproliferative activities.[45 46] In contrast to SR1, BAY 2416964 is orally bioavailable and showed immunomodulation of the TME and tumor efficacy in vivo suggesting it has potential to counter the resistance resulting from KYN and IDO upregulation in the patient setting. Metabolic resistance mechanisms that may arise from targeting the immunosuppressive KYN pathway still require further understsanding. Odunsi *et al* showed that IDO1 blockade in ovarian cancer patients resulted in a metabolic adaptation of the tumor microenvironment whereby increased NAD+ resulted in suppressed antitumor responses.[47] For AhR, activity of the receptor is fine-tuned by positive and negative feedback loops that result from direct AhR-induced upregulation of target genes such as *CYP1A1*, *AhRR* and *IDO*.[48 49] While evidence for emerging resistance mechanisms was not observed in the current study, the impact of inhibiting this regulatory circuit long-term remains to be seen. Increased proinflammatory responses within the TME and consequent resistance via PD-1 pathway activation suggests that an AhR inhibitor, similar to other immuno-oncology approaches, will be more effective in combination with anti-PD(L)1 and even sensitize tumors to such T cell activating strategies, which have been shown to induce resistance via the KYN-AhR axis.[26] Here, we show combination effects of BAY 2416964 and anti-PD-1 on T cell responses in vitro while others have shown the potential to combine an AhR inhibitor with PD-1 Ab in vivo.[27 30] Overall, the development of BAY 2416964 and other AhR inhibitors with further improved pharmacological characteristics is essential and may lead to innovative and effective cancer treatment via targeting of the immunosuppressive Trp pathway. Ph1 investigation of BAY 2416964 as an immunotherapy in patients with advanced cancer (NCT04069026) is currently ongoing.

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