Targeting HIF-1α abrogates PD-L1-mediated immune evasion in tumor microenvironment but promotes tolerance in normal tissues

Christopher M. Bailey¹,²#, Yan Liu¹#, Mingyue Liu¹, Xuexiang Du¹,³, Martin Devenport⁴, Pan Zheng¹,⁴, Yang Liu¹,⁴*, and Yin Wang¹*

¹Division of Immunotherapy, Institute of Human Virology, Department of Surgery and Comprehensive Cancer Center, University of Maryland School of Medicine, Baltimore, MD 21201
²Institute of Biomedical Sciences, The George Washington University School of Medicine & Health Sciences, Washington DC, 20052
³Key Laboratory of Infection and Immunity of Shandong Province & Department of Immunology, School of Basic Medical Sciences, Shandong University, Jinan, 250012, China.
⁴OncoC4, Inc, Rockville, MD 20852

#co-first authors

*Address correspondence to: Yin Wang, Division of Immunotherapy, Institute of Human Virology, Department of Surgery and Comprehensive Cancer Center, University of Maryland School of Medicine, Baltimore, MD 21201, USA. Phone: 1.410.706.5852; Email: yin.wang@ihv.umaryland.edu. Or to: Yang Liu, Division of Immunotherapy, Institute of Human Virology, Department of Surgery and Comprehensive Cancer Center, University of Maryland School of Medicine, Baltimore, MD 21201, and OncoC4, Inc, Rockville, MD 20852, USA. Phone: 1.410.706.5913; Email: yangl@oncoc4.com.
Abstract

Anti-CTLA-4 + anti-PD-1/PD-L1 combination is the most effective cancer immunotherapy but causes high incidence of immune-related adverse events (irAE). Here we report that targeting of HIF-1α suppressed PD-L1 expression on tumor cells and tumor-infiltrated myeloid cells, but unexpectedly induced PD-L1 in normal tissues by an IFNγ–dependent mechanism. Targeting the HIF-1α-PD-L1 axis in tumor cells reactivated tumor-infiltrating lymphocytes (TILs) and caused tumor rejection. The HIF-1α inhibitor echinomycin potentiated cancer immunotherapeutic effects of anti-CTLA-4 therapy with efficacy comparable to anti-CTLA-4+anti-PD-1 antibodies. However, while anti-PD-1 exacerbated irAE triggered by Iplimumab, echinomycin protected mice against irAE by increasing PD-L1 levels in normal tissues. Our data suggest that targeting HIF-1α fortifies the immune tolerance function of the PD-1:PD-L1 checkpoint in normal tissues but abrogates its immune evasion function in the tumor microenvironment (TME) to achieve safer and more effective immunotherapy.
**Introduction**

Current strategies of immunotherapy, articulated as immune checkpoint blockade, aim to release physiological immune tolerance checkpoints for the benefit of immunotherapeutic effect. As such, immune-related adverse events (irAE) are considered the necessary price for immunotherapy. The relative risk/benefit ratio depends on the significance of the immune checkpoint in immune tolerance vs tumor evasion of host immunity. The PD-1:PD-L1 interaction is less critical than CTLA-4 for immune tolerance as CTLA-4 inactivation leads to more severe autoimmune diseases than that of PD-1 (1-4). Correspondingly, monoclonal antibodies (mAbs) targeting programmed cell death protein 1 (PD-1) and programmed death-ligand 1 (PD-L1) are less toxic than those targeting cytotoxic T-lymphocyte antigen 4 (CTLA-4) (5). In terms of therapeutic efficacy, anti-CTLA-4 + anti-PD-1 combination therapy is considered the most effective immunotherapy strategy (5). However, the combination substantially increases rates of severe irAEs (5) to 50-90% depending on therapeutic setting (6-9). Thus, a major challenge for cancer immunotherapy is to eliminate irAE without compromising synergistic cancer immunotherapeutic effects of dual-immune checkpoint blockade.

In the tumor microenvironment (TME), tumor cells and tumor-infiltrated myeloid cells express PD-L1 in response to environmental cues including cytokines, hypoxia, or growth factors (10-12). PD-L1/B7-H1 causes T cell apoptosis (13) and/or exhaustion upon binding PD-1 (14). Consequently, PD-1:PD-L1 interaction suppresses T cell-mediated anticancer immunity in the TME, and blocking this interaction reinvigorates immune rejection of tumor cells (15). Though irAEs resulting from anti-PD-1/PD-L1 mAbs are generally less severe than those from anti-CTLA-4 mAbs (16, 17), PD-1/PD-L1 blockade does lead to significant irAE and
administering anti-PD-1 mAbs concurrently with anti-CTLA-4 mAbs substantially worsens irAE incidence and severity (5, 18-21).

A major limitation of anti-PD-1/PD-L1 mAbs is their inability to distinguish PD-1:PD-L1 interactions in the TME, which prevent effective cancer immunity, from PD-1:PD-L1 interactions in normal tissues, which protect against autoimmune diseases. Tumor-specific PD-L1 targeting would be more desirable as it may achieve cancer immunotherapy without causing irAE. This may be possible since the molecular mechanisms governing PD-L1 expression in normal tissues and cancer differ. For example, hypoxia, which is one of the major hallmarks distinguishing solid tumors from normal tissues (22), was reportedly responsible for inducing PD-L1 in tumor (23) and myeloid cells (12) via HIF-1α. These findings raised the intriguing possibility that Hif-1α inhibition may selectively repress PD-L1 expression in cancer. Here, we show pharmaceutical or genetic targeting of HIF-1α suppresses PD-L1 expression in the TME, but paradoxically induces PD-L1 in normal tissues by enhancing T cell production of IFNγ. Our data demonstrate a new approach to differential regulation of PD-L1 for safer and more effective immunotherapy.

**Results**

**Targeting Hif-1α suppresses PD-L1 expression in TME**

Previous studies have shown that hypoxia induces PD-L1 through transcriptional activation of PD-L1 transcription by HIF-1α. Since tumor cells also express HIF-1α under normoxia, we tested if the Hif-1α-PD-L1 axis is also active in tumor cells expressing stable Hif-1α under normoxic conditions. We first examined levels of Hif-1α and PD-L1 in murine breast cancer cell
lines 4T1 and E0771 cultured under normoxia. Both cell lines expressed Hif-1α and PD-L1 protein (Figures 1A and B), and reduction in PD-L1 protein was observed in cells treated by the Hif-1α inhibitor echinomycin (Figure 1B). Consistent with previous reports of hypoxia-induced PD-L1 expression (23), treatment of E0771 cells with hypoxia mimetic CoCl₂ further upregulated PD-L1 from the basal levels seen at normoxia (Figure 1C). To demonstrate the relationship between Hif-1α activity and PD-L1 protein expression in tumor cells in vivo, we transduced 4T1 cells with a lentiviral transcription factor reporter construct containing the core HRE motif upstream of an EGFP reporter. In response to CoCl₂ stimulation, the resultant 4T1-HRE-EGFP cells exhibited a marked increase in EGFP reporter fluorescence activity (Figure 1D). After engrafting the 4T1-HRE-EGFP cells into immunocompetent BALB/c mice and allowing solid tumors to form, we analyzed PD-L1 expression on the isolated tumor cells by flow cytometry. PD-L1 expression was associated with EGFP reporter activity in the tumor cells (Figure 1E). The results suggest that Hif-1α also regulates PD-L1 expression on tumor cells in vivo. To further test this, we evaluated the effect of echinomycin on intratumoral PD-L1 expression by immunofluorescence staining of PD-L1 in the fixed tumor specimens from engrafted tumor cell lines. As shown in Figure 1F, there was a marked reduction in PD-L1 expression in the tumors of echinomycin-treated mice.

To test if Hif-1α inhibition is the mechanism responsible for reduction of PD-L1 protein by echinomycin, we used siRNA to knockdown Hif1α in E0771 cells and quantified PD-L1 expression by flow cytometry after 24-hour incubation with vehicle or echinomycin (Figures 1G-J). Under basal conditions, we found that knockdown of Hif1α reduced PD-L1 protein expression (Figures 1G, 1I, 1J). Moreover, while the inhibitory effect of echinomycin on PD-L1 expression was preserved in E0771 cells transduced with scrambled shRNA (Figures 1H-J)
knockdown of Hif1α abrogated the ability for echinomycin to decrease PD-L1 protein (Figures 1H-J). These results demonstrate that Hif-1α controls PD-L1 expression in E0771 cells and that echinomycin reduced PD-L1 by inhibiting the Hif-1α-PD-L1 axis.

**Immunotherapeutic effect of Echinomycin**

Given the profound effect of PD-L1 on immune function, it was of interest to test if Hif-1α inhibition results in an immunotherapeutic effect on cancer. To address this, we first compared the effects of pharmacological Hif-1α inhibition with echinomycin on tumor growth rate in mice sufficient or deficient in adaptive immunity (Figure 2A). Echinomycin significantly inhibited 4T1 growth in both immunocompetent (BALB/c) and immunodeficient (NSG) recipients compared to each strain’s respective vehicle control (Figure 2B). However, 4T1 growth was more significantly inhibited in immunocompetent mice than in immunodeficient mice, which suggested an immunotherapeutic effect of echinomycin in addition to potentially tumor-intrinsic therapeutic effects in this model. In a second breast cancer model, E0771, the therapeutic effects of echinomycin were also more pronounced in immune competent mice (Figure 2C). To test if Hif-1α inhibition can confer an immunotherapeutic effect in a non-breast cancer model, we repeated the experiments using MC38 murine colon adenocarcinoma cells. As with E0771, all therapeutic effects required immune competence (Figure 2D).

**Echinomycin inhibits PD-L1 in tumor cells by targeting the Hif-1α-PD-L1 axis**

Next, we used shRNA to compare the effects of Hif1α or Pdl1 targeted knockdown in E0771 cells on the tumor growth kinetics in immunocompetent or immunodeficient recipients. In parallel, we treated both strains with vehicle or echinomycin to measure the impact of tumor cell-
intrinsic Hif1α or Pdl1 on tumor growth therapeutic response to echinomycin (Figure 3A). In C57BL/6, but not NSG recipients, genetic depletion of Hif1α (sh-Hif1α) in E0771 cells significantly inhibited tumor growth compared to E0771 transduced with scrambled shRNA (sh-Scr) in mice of the same respective strains (Figure 3B). Moreover, the tumor growth rates of E0771 with Hif1α knockdown were also significantly reduced in immunocompetent vs immunodeficient recipients (Figure 3B). As in Figure 2C, echinomycin more effectively inhibited sh-Scr E0771 tumor growth in C57BL/6 (Figure 3C), compared to NSG (Figure 3D) recipients; in contrast, echinomycin did not inhibit sh-Hif1α E0771 tumor growth, regardless of the recipient strain (Figures 3C and D). Thus, pharmacologic or genetic targeting of Hif-1α in tumor cells alone can confer an immunotherapeutic effect. Furthermore, the loss of biological activity for echinomycin following knockdown of Hif1α in E0771 provides genetic evidence that echinomycin confers an immunotherapeutic effect in vivo by targeting the Hif-1α in tumor cells.

In the same manner, we analyzed the effects of Pdl1 knockdown to determine whether downregulation of PD-L1 is critical in the immunotherapeutic effect of echinomycin. Much like the knockdown of Hif1α, loss of Pdl1 (sh-Pdl1) in E0771 cells also inhibited tumor growth in C57BL/6 but not NSG recipients (Figure 3B), and echinomycin did not further suppress sh-Pdl1 E0771 tumor growth in C57BL/6 (Figure 3C) or NSG (Figure 3D) recipients. Taken together, the data support the conclusion that echinomycin confers immunotherapeutic effects in vivo by targeting the Hif-1α-PD-L1 axis in tumor cells.

**Hif-1α inhibition potentiates anti-CTLA-4 immunotherapy**

Co-targeting targeting CTLA-4 and PD-1/PD-L1 immune checkpoints simultaneously with their respective blocking mAbs is the most efficacious strategy currently available for cancer
immunotherapy. Having established that echinomycin can target PD-L1 in tumor cells and promote an immunotherapeutic effect in vivo, we next asked whether this strategy may potentiate immunotherapeutic effects in the context of anti-CTLA-4 therapy. We examined the therapeutic effects of CTLA-4 blocking mAbs, with or without echinomycin, using 4T1, E0771, or MC38 syngeneic mouse models of cancer (Figure 4A). As shown in Figure 4B, anti-mouse CTLA-4 mAb (9D9) in combination with echinomycin significantly inhibited 4T1 tumor growth more effectively than either monotherapy. To further investigate the combination efficacy of targeting Hif-1α during anti-CTLA-4 therapy, we performed similar drug treatment experiments using immunocompetent C57BL/6 recipients and the E0771 breast cancer (Figure 4C), or MC38 colon adenocarcinoma (Figure 4D) models and observed synergistic effect in all models. We also compared the effects of echinomycin or anti-PD-1 (RMP1-14) in conjunction with 9D9. Again, we observed significant inhibition of tumor growth by echinomycin or 9D9 monotherapies compared to vehicle, while the greatest inhibition was achieved by 9D9 + echinomycin or RMP1-14 (Figures 4C and D). These data demonstrated therapeutic effect of blocking PD-1:PD-L1 interaction can be similarly achieved by either anti-PD-1 or echinomycin.

**Echinomycin inhibits PD-L1 on tumor cells and tumor-infiltrated myeloid cells**

We have shown systemic Hif-1α inhibition suppressed PD-L1 expression in multiple tumors (Figure 1F). To gain insight as to the cellular landscape and cell-specific expression patterns of PD-L1 in the TME following echinomycin and/or 9D9 treatment, we analyzed E0771 tumors from C57BL/6 mice treated with vehicle, echinomycin, 9D9, or 9D9 + echinomycin for the composition of immune cells. While echinomycin did not significantly impact the frequencies of tumor-infiltrated lymphocytes or myeloid subsets, 9D9 reduced the frequencies of
polymorphonuclear MDSCs (PMN-MDSCs) (Supplemental Figure S1). However, echinomycin significantly reduced PD-L1 expression on tumor cells (Figure 5A), and tumor-infiltrated monocytic MDSCs (M-MDSC) (Figure 5B), PMN-MDSCs (Figure 5C), and CD11b⁺CD11c⁺ double-positive cells (Figure 5D), with or without anti-CTLA-4 therapy. Further analysis revealed that most of the CD11b⁺CD11c⁺ cells were TAMs, as roughly 90% expressed F4/80, consistent with the earlier report (24) (Supplemental Figure S2). The results show that, in addition to tumor cells, in vivo HIF-1α inhibition can also suppress PD-L1 on tumor-infiltrated myeloid cells, and these effects persist in the context of anti-CTLA-4 therapy. More importantly, the results provide evidence that Hif-1α is involved in coordinating PD-L1 expression on tumor-infiltrated myeloid cells in the TME.

To test whether Hif-1α inhibition can rescue TIL function in the TME, we used flow cytometry to measure frequencies of IFNγ-expressing T cells in the E0771 tumors. Compared to vehicle, all treatments increased the frequencies of both IFNγ⁺CD8⁺ (Tc1) and IFNγ⁺CD4⁺ (Th1) subsets, although the highest frequencies of Tc1 were observed in mice receiving 9D9 + echinomycin (Figures 5E and F). The absolute numbers of Tc1 and Th1 cells were also highest in 9D9 + echinomycin treated mice as shown in Supplemental Figure S3.

Apart from boosting CD8 TIL responses, anti-PD-L1 promoted an inflammatory TAM phenotype in the TME, which may contribute to its CITE (25). In this regard, we also noted increased MHCII expression 9D9 + LEM vs vehicle treated mice (Supplemental Figure S4).

To better understand the impact of pharmacologic Hif-1α targeting in the context of immunotherapy, we performed more detailed analysis of TILs. E0771 mice treated with 9D9 had higher expression of exhaustion marker PD-1 on CD8 TILs compared to vehicle, which was reversed by echinomycin (Figure 6A). The same was seen for PD-1 expression on CD4 TILs.
(Figure 6B), and to a less extent, for CTLA-4 expression on CD8 TILs (Supplemental Figure S5A). The drug treatments had minimal impact on CD4 expression of CTLA-4 (Supplemental Figure S5B). In addition to TIL exhaustion (14), PD-L1 can also induce TIL apoptosis (13). Annexin V staining revealed more apoptotic CD8 and CD4 TILs in tumors of 9D9-treated mice compared to vehicle and adding echinomycin appeared to repress this effect in CD8 TILs (Figure 6C). The same trend was seen for CD4 TILs, although the difference between 9D9 and 9D9 + LEM groups was not significant (Figure 6D). Higher expression of cytolytic effector molecules granzyme B and perforin were noted in CD8 TILs of 9D9 + LEM treated mice vs vehicle (Figure 6E and F). Roughly one fifth of CD4 TILs were granzyme B\(^+\), which was not significantly affected by drug treatments (Figure 6G). On the other hand, in all treated groups, the mean frequencies of CD4 TILs expressing perforin roughly doubled that of the control group (Figure 6H).

We used depletory antibodies to assess the impact of CD4, CD8, and NK cells in the combinational efficacy of 9D9 + LEM in E0771 mice. These studies revealed that optimal efficacy required all three cell types, with CD8 being the most critical, followed by NK and CD4 cells (Figure 6I). Thus, the immunotherapeutic effects of pharmacological Hif-1\(\alpha\) inhibition in context of anti-CTLA-4 are multi-cell dependent, but primarily depend on CD8 T cells.

Since Hif-1\(\alpha\) regulates Treg and Th differentiation (26), we further examined the impact of echinomycin on lineage-specific transcription factors and cytokines in the TILs. The proportions of CD8 and CD4 TILs expressing T-bet were significantly increased in 9D9 + LEM treated mice vs vehicle (Supplemental Figures S5C and D). TNF\(\alpha\) was unaffected by the therapies in CD4 TILs, though we observed increased TNF\(\alpha^+\)CD8 TILs in groups receiving 9D9 (Supplemental Figures S5E and F). The proportions of CD8 and CD4 TILs expressing ROR\(\gamma\)t
were unchanged among different groups (Supplemental Figures S5G and H). None of the therapies significantly impacted frequencies of tumor-infiltrating Treg or Th17, except for a modest decrease in Treg for groups receiving 9D9 (Supplemental Figures S5I and J). The proportion of tumor-infiltrated NK cells expressing granzyme B and perforin were decreased and increased, respectively, in mice treated with 9D9 alone (Supplemental Figures S5K and L). 9D9 significantly increased and decreased the frequencies of effector and memory CD8 T cells in the tumors, respectively (Supplemental Figure S6).

Echinomycin alone inhibited PD-L1 on tumor cells and tumor-infiltrating myeloid cells but increased the proportion of CD8 TILs expressing IFNγ (Figure 5). An important question arose as to whether echinomycin improves CD8 TIL function directly by a T cell-intrinsic mechanism, or indirectly through reducing PD-L1 on tumor and/or myeloid cells. To test this, we generated mice with conditional knockout of Hif1a in T lineages using the cre lox system. Loss of Hif1a did not significantly impact proportion of CD4 and CD8 TILs expressing IFNγ, T-bet, or RORγt, or the frequencies of Tregs or Th17. In CD4, but not CD8 TILs, we found an increased frequency of PD-1+ cells (Supplemental Figure S7). Granzyme B and perforin in CD8 TILs was slightly reduced in Hif1a KO mice, but not significantly. In contrast, knockdown of Pdl1 in the tumor cells significantly increased the frequencies of Tc1 and Th1 cells, phenocopying the effects of echinomycin (Supplemental Figure S8). Therefore, suppression of PD-L1 on tumor cells can at least partially account for the enhanced CD8 TIL function and therapeutic effects provided by echinomycin in the immune competent mouse. Notably, inhibition of PD-L1 on tumor cells by echinomycin was preserved in mice with conditional knockout of Hif1a in T cells. These data indicate that the decreased PD-L1 expression is not due to an T cell-intrinsic effect echinomycin (Supplemental Figure S9).
Sitkovsky’s group previously reported that TILs tend to avoid hypoxic zones in the TME (27). Using the methods from Hatfield et al (27), we noted an increase in CD3 TIL infiltration into hypoxic areas of the tumors in echinomycin-treated mice (Supplemental Figure S10).

**Echinomycin induces PD-L1 expression to limit anti-CTLA-4-induced T cell infiltration in irAE target organs**

To test if PD-L1 is induced on the tissue level in response to anti-CTLA-4 therapy, we performed immunofluorescence staining of PD-L1 and CD3 in the liver and kidney of tumor-bearing mice treated with 9D9 alone or in combination with other therapies (Figure 7A). PD-L1 expression in these tissues was elevated in mice treated with 9D9 (Figure 7A). Interestingly, echinomycin also induced PD-L1 (Figure 7A), but only 9D9 resulted in hepatic and renal infiltration of T cells (Figures 7B-D). T cell infiltration was reduced in 9D9 + LEM treated mice when compared to 9D9 alone (Figures 7B-D). In contrast, the frequency of T cells, as well as the frequencies Tc1 and Th1, expanded in the spleen of mice that received 9D9 + echinomycin treatment (Figures 7E-G). Thus, the reductions of T cells in liver and kidney by echinomycin were not the result of general T cell inactivation. Rather, cleaved-caspase 3 staining suggested that the induced PD-L1 regulates T infiltration by triggering apoptosis (Figure 7H). When 9D9 was combined with anti-PD-1 (RMP1-14), the mice had high intensity of T cell infiltration in liver and kidney (Figures 7B-D). The frequencies of T cells in the spleen, including Tc1 and Th1, were comparable when either anti-PD-1 or echinomycin were used in conjunction with anti-CTLA-4 mAb (Figures 7E-G). Since IFNγ is known to upregulate PD-L1 in normal tissues (28, 29), we hypothesized PD-L1 induction by echinomycin could be due to IFNγ. Echinomycin alone did not stimulate increased infiltration of T or NK cells in the tissues with PD-L1.
expression (Figures 7B-D, and Supplemental Figure S11), but led to modest increase in IFNγ detected in the serum (Supplemental Figure S12).

We further tested the importance of IFNγ by using the anti-IFNγ neutralizing mAb, XMG1.2, which abrogated PD-L1 induction by 9D9 + echinomycin treatment and increased T cell infiltration in the kidneys and liver (Figures 7B-D). XMG1.2 also abrogated PD-L1 expression in the kidney and liver in absence of 9D9, indicating that IFNγ is responsible for PD-L1 induction by LEM in these tissues (Supplemental Figure S13). Conditional knockout of Hif1a in T cells did not phenocopy the effects of echinomycin on PD-L1 induction in the liver, but PD-L1 induction was preserved regardless of the mouse genotype (Supplemental Figure S14). To test whether LEM can reduce irAE in the adult tumor-bearing mouse, we measured serum biomarkers for hepatic, renal, and gastrointestinal irAE. However, the adult mouse tolerated high dose of anti-CTLA-4 antibody without significant irAE (Supplemental Figure S15).

**Echinomycin protected Ipilimumab-induced irAEs in human CTLA4-knockin mice**

To circumvent this caveat, we used human Ctl4 knockin mice, which are susceptible to irAE induction by Ipilimumab at young age (30). Gastrointestinal tract is the most frequent target of irAEs (31). Therefore, we used intestinal permeability to orally administrated FITC-dextran and histology as the readout for irAE (Figure 8A). As what was described for liver and kidney, Ipilimumab treatment result in elevated PD-L1 expression (Figure 8B) and T cell accumulation (Figure 8C) in the intestines. To explore whether PD-L1 could serve a functional role in the protection from GI-irAEs induced by Ipilimumab, we compared the fluorescence intensity of FITC-dextran measured in the sera among those with high or low PD-L1 staining in the intestines. We observed that mice with high levels of intestinal PD-L1 had much lower intestinal
permeability (Figure 8D). The association between intestinal permeability and PD-L1 expression supports the hypothesis that Ipilimumab-induced PD-L1 serves as a limiting factor against Ipilimumab-induced GI-irAEs.

To test this hypothesis, we assessed whether blockade of PD-1:PD-L1 checkpoint during Ipilimumab treatment would also worsen GI-irAEs in the CTLA4 knockin model, and how this approach might compare to substitution of anti-PD-1 mAbs with echinomycin. We evaluated % of mice with significantly higher serum FITC-dextran than control mice, using means+2 SD as boundary for intestinal leakage. As shown in Figure 8E, echinomycin protected against Ipilimumab-induced intestinal leakage by a PD-L1-dependent mechanism as this protection is abrogated by anti-PD-1 (Figure 8E). Moreover, in mice that received Ipilimumab + echinomycin treatment, addition of anti-IFNγ antibody increased the frequency of mice with intestinal leakage from 7.7% to 20.0% (Figure 8E). Collectively, the data suggested that through induction of IFNγ, echinomycin confers protection against ipilimumab-induced GI-irAEs by elevating PD-L1 expression to fortify the PD-1:PD-L1 checkpoint.

To further investigate PD-L1 expression in the intestinal tissues in response to Ipilimumab and to validate its role in conferring protection from Ipilimumab-induced GI-irAEs, we performed histological analysis of the intestinal tissue and immunofluorescence staining of PD-L1. Consistent with the FITC-dextran data in Figure 8E, Ipilimumab induced intestinal inflammation (Supplemental Figure S16 for additional information). The inflammation is largely abrogated by echinomycin as mice treated with Ipilimumab + echinomycin for the most part exhibited normal intestinal pathology (Figure 8F). Echinomycin enhanced PD-L1 expression in the intestine compared to Ipilimumab alone (Figure 8G). The elevated PD-L1 expression was confirmed by flow cytometry using digested intestinal tissues (Figure 8H).
To confirm the significance of induced PD-L1 in protection against inflammation in the intestine, we used anti-PD-1 mAb to block PD-1/PDL-1 interaction. These data showed that the protective effect of echinomycin is abrogated by the anti-PD-1 antibodies (Figure 8F). Moreover, IFNγ was elevated in intestine from mice that received treatment of both echinomycin and Ipilimumab (Supplemental Figure S17), and the protective effect of echinomycin depends on IFNγ as the effect was abolished by the anti-IFNγ mAb XMG1.2 (Figure 8F). These data suggested that the IFNγ-PD-L1 axis is responsible for the echinomycin-mediated protection against Ipilimumab-induced GI-irAE.

**Discussion**

HIF-1α inhibition is an area of active investigation in cancer therapy (32, 33). We have reported that echinomycin effectively eliminated leukemia stem cells (34). However, clinical development of echinomycin for solid tumor has met with minimal success. In our studies of breast cancer, we found that reformulating echinomycin with liposomes enabled potent therapeutic effects in orthotopic xenograft mouse models of triple-negative breast cancer (TNBC), including primary tumor growth and metastasis in the MDA-MB-231 and SUM-159 models (35). The current study supports echinomycin’s re-emergence as an immunotherapeutic agent.

Targeting Hif-1α in immunotherapy is a relatively new concept, for which proof of principle has been amply demonstrated by others (12, 23, 27). While our studies expand on these early studies which established the significance of HIF1α pathway in immunosuppression, they propose a new
paradigm: exploiting differential regulatory pathways of PDL1 expression simultaneously can uncouple immunotherapeutic effects and irAE. In this study, we demonstrate that targeting HIF1α can achieve such an effect in preclinical models of cancer. These findings provide a new perspective for immunotherapy drug development.

Whether hypoxia/Hif-1α pathways are pro- or anti-inflammatory is a topic of debate. Sitkovsky’s group originally demonstrated the first in vitro evidence that hypoxia is immunosuppressive for T cells (36), and the first in vivo genetic evidence that hypoxia is immunosuppressive for T and B cells of adaptive immune system (37). Subsequently it was shown that direct elimination of TME hypoxia can improve cancer immunotherapy in mice (27, 38, 39). On the other hand, studies by Johnson and colleagues found Hif-1α to be essential for myeloid cell-mediated inflammation (40). In T cells, Hif-1α is transiently stabilized in T cells following TCR activation (41), and plays an important role in regulating Treg and Th17 balance (26). According to Doedens et al., HIFs enhance the effector responses of CD8+ T cells to persistent antigen (42). Palazon et al showed that selective Hif-1α targeting in T cells inhibited IFNγ and granzyme B production in CD8 TILs (43). Notably, echinomycin treatment had the opposite effect. Therefore, abrogating Hif-1α-mediated immune suppression in tumor cells and tumor-associated myeloid cells may be more important for immunotherapeutic efficacy than preserving Hif-1α function in T cells.

The pioneering work in developing immunotherapy targeting PD-1 and PD-L1 (13, 44-47) has led to the most important breakthrough in cancer therapy, with rapidly expanding indications of anti-PD1/PD-L1 antibodies adopted for treatment of both hematological and non-hematological
malignancies (48). However, the current approach that overcomes tumor evasion of host immunity also disables the immune tolerance checkpoint, leading to significant irAEs, particularly when used in conjunction with anti-CTLA-4 antibodies. Here, we showed that targeting HIF1α not only overcomes immune evasion in the TME, but also fortifies the immune tolerance checkpoint in normal tissues.

HIF-1α is generally inactivated in normal tissues but frequently stabilized in tumor cells regardless of oxygen tension (49, 50). This fundamental difference allows us to selectively inhibit PD-L1 expression in the tumor microenvironment using echinomycin. Surprisingly, echinomycin induced PD-L1 expression in normal tissues of immunocompetent mice, including liver, kidney, and colon. The unexpected induction of PD-L1 was attributable to elevated IFNγ production associated with echinomycin-induced expansion of IFNγ-producing T cells, including Tc1 and Th1 cells. The induced PD-L1 is causatively associated with reduction of inflammation and intestinal leakage induced by anti-CTLA-4 antibodies as it is abrogated by anti-PD-1 antibody.

The ability of anti-PD-1 to abrogate protection by echinomycin also suggests an interesting explanation on how anti-PD-1 exacerbates irAE when used in conjunction with anti-CTLA-4: PD-L1 is induced by anti-CTLA-4-induced IFNγ as a negative feedback mechanism to control irAE. By preventing PD-L1 from interacting with PD-1, anti-PD-1/PD-L1 antibodies exacerbate irAE caused by anti-CTLA-4 antibodies. In contrast to anti-PD-1, echinomycin not only further enhanced anti-CTLA-4 induced PD-L1 in normal tissue, but also allows PD-L1 to signal through PD-1 to supercharge the immune tolerance checkpoint function.
While HIF-1α has been shown to be involved in degradation of Foxp3 and induce Th17 (26), its function in inducing Th1 has also been reported (51). Our data presented herein show a strong effect of echinomycin in inducing IFNγ-producing cells, including Tc1 and Th1 cells. It is unclear whether echinomycin promotes Tc1 expansion in vivo by cell-intrinsic targeting of HIF-1α or indirectly by reduction of regulatory T cells. Regardless of whether the effect is T cell-intrinsic, the induction of PD-L1 normal tissues are tissue cell-extrinsic. In contrast, in cancer cells, targeting HIF-1α resulted in a cell-intrinsic inhibition of PD-L1. Thus, the data presented herein revealed a cancer cell-intrinsic inhibition of PD-L1 and normal tissue cell-extrinsic induction of PD-L1 by echinomycin. Together, these two activities provide what we believe is the first example in cancer immunotherapy of an approach which abrogates the PD-1-PD-L1 checkpoint in the TME to eliminate immune evasion by cancer cells, while fortifying its immune tolerance checkpoint activity in normal tissues. Therefore, HIF-1α inhibitors represent an ideal partner for CTLA-4-targeted immunotherapy.

Methods

Cell Lines 4T1, E0771, and MC38 cells were obtained from American Type Culture Collection (Manassas, VA).

Therapeutic Agents Echinomycin was formulated with liposomes as previously described (35). Recombinant Ipilimumab was provided by Lakepharma Inc. (San Francisco, CA). Remaining therapeutic antibodies were from BioXCell (West Lebanon, NH) as follows: anti-mouse CTLA-
4, clone 9D9 (BE0164); anti-mouse PD-1, clone RMP1-14 (BE0146); anti-mouse IFNγ, clone XMG1.2 (BE0055); anti-mouse CD4, clone GK1.5 (BE0003-1); anti-mouse CD8α, clone YTS 169.4 (BE0117); anti-mouse NK1.1, clone PK136 (BE0036).

**Flow Cytometry** Data was acquired on BD FACSCanto II or Cytek Aurora and analyzed using FlowJo software (v10.7.0). Detailed description of fluorescent antibodies used are provided in supplemental methods.

**Mice** BALB/cAnNCr and C57BL/6NCr were obtained from NCI (Bethesda, MD), and NOD.Cg-Prkd<sup>scid</sup>Il2rg<sup>tm1Wjl</sup> /SzJ (NSG) mice were purchased from the University of Maryland Baltimore School of Medicine and bred in-house. B6.129-Hif1<sup>a</sup>d<sup>tm3Rjs</sup> /J and Tg(Cd4-cre)1Cwi/BfluJ mice were from Jackson Laboratory (Bar Harbor, ME). Human CTLA4 knockin mice were generated and bred in-house and have been previously described (30).

**Tumor Models** The details of each experiment are specified in the figure legends. Tumor cells were suspended in RPMI-1640 medium and injected into recipient mice at 0.5-1.0x10<sup>6</sup> cells/50 µl/mouse. 4T1 and E0771 cells were injected orthotopically into first (left) mammary fat pad of female recipients; MC38 cells were injected into the flank. On day 6 after transplantation, mice were assigned to treatment groups such that comparable initial mean tumor volumes between experimental and control groups were achieved. Tumor volumes were calculated using the formula \( V = \frac{ab^2}{2} \), where \( a \) is the longer diameter, and \( b \) is the shorter diameter. Echinomycin, or equivalent of empty liposomes as a vehicle control, were administered by intravenous (i.v.) injection into the lateral tail vein on the indicated days, at 0.15-0.25 mg/kg. Intraperitoneal (i.p.)
injection was used to deliver therapeutic antibodies 9D9, RMP1-14, or XMG1.2 at 0.2 mg/mouse/injection. The mice from different groups were sacrificed at the same timepoints for analyses.

**GI-irAE model** 10-day old CTLA4h/h mice received 0.1 mg of Ipilimumab i.p. on days 10, 13, 16, and 19 after birth and FITC-dextran assay was performed on day 32 to detect GI-irAE. On day 33, the mice were euthanized for flow cytometry and histological analyses described in the figures. LEM (10 µg/kg), Ipilimumab (0.1 mg/mouse/injection), RMP1-14 (0.2 mg/mouse/injection), and XMG1.2 (0.2 mg/mouse/injection) were administered i.p. according to the schedule in Figure 8A. See supplemental methods for additional details.

**Statistics** All experiments have been replicated at least twice with similar results. Appropriate statistical tests were selected on the basis of whether the data with outlier deletion was normally distributed by using the D’Agostino & Pearson normality test. Data comparing two groups were analyzed by unpaired two-tailed Student’s t-test. Unless otherwise noted in the figure legends, one-way analysis of variance (ANOVA) with Sidak’s posttest was used for multiple comparisons, and two-way ANOVA for analysis of tumor kinetics. The correlation coefficient and P-value of linear regression were calculated by Pearson’s method. Sample sizes were chosen with adequate statistical power on the basis of the literature and past experience. In the graphs, data are shown as mean ± SEM, indicated by horizontal line and y-axis error bars, respectively. Statistical calculations were performed using GraphPad Prism 8 software (GraphPad Software, San Diego, California). ns, not significant, *P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001.
Author contributions

CMB, YanL and YW designed and performed research as well as prepared the manuscript. ML, XD performed research and help with methodology. MD and PZ provided advice on experimental design, helped with histological experiments, and editing. YangL and YW designed the study, analyzed data, supervised the study and wrote the manuscript.

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Conflict of Interest Disclosure Statement

Yang Liu and Pan Zheng are co-founders of OncoC4, Inc. Other authors disclosed no potential conflicts of interest.

Study approval

All animal experiments were conducted according to guidelines established by the NIH Guide for the Care and Use of Animals (National Academies Press). All procedures involving experimental animals were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Maryland School of Medicine.
References


Figures and Figure legends

A

TSA  4T1  E0771

Hif-1α
β-actin

B

4T1  E0771

Isotype
Vehicle
EM

PD-L1

C

Isotype
Normoxia
CoCl₂

PD-L1

D

% of Maximum

EGFP

HRE (-)
HRE (+)
HRE (+)+CoCl₂

E

% of Maximum

PD-L1 MFI

Vehicle
LEM

PD-L1/DAPI

F

4T1  E0771  MC38

PDL1/DAPI

G

% of Maximum

PD-L1

Isotype
sh-Scr
sh-Hif1α

H

% of Maximum

PD-L1

Isotype
sh-Scr
EM

I

% of Maximum

PD-L1

Isotype
sh-Hif1α
EM

J

PD-L1 MFI

EM

sh-Scr
sh-Hif1α

Figure 1. Hif-1α drives PD-L1 expression in tumor cells.  A. Western blot of Hif-1α protein in murine breast cancer cells.  B. Effect of echinomycin on PD-L1 expression in 4T1 or E0771. Tumor cells were treated with echinomycin (EM, 0.45 nM) or DMSO (vehicle) for 48hrs (1:1000 dilution). Flow cytometry histograms for PD-L1 staining are shown. C. Effect of CoCl₂ on PD-L1 expression in E0771. E0771 cells were cultured as in (B) with CoCl₂ (250 µM) or PBS and PD-L1 was measured by flow cytometry. D. 4T1-HRE cells were treated for 24hrs with PBS or CoCl₂ (250 µM). Flow cytometry histograms for EGFP intensity are shown. E. BALB/c mice
received 1x10⁶ 4T1-HRE cells (day 0). On day 21, tumors were dissociated and stained for PDL1. The PDL1 MFI is plotted for the tumor cells (gated on Singlets/CD45 EGFP⁺) further divided into top/bottom 30 percentiles based on EGFP. The data are pooled from three experiments, presented as mean ± SEM, analyzed by t test. E. 4T1, E0771, or MC38 cells were transplanted into BALB/c or C57BL/6 mice, which received vehicle or echinomycin (LEM, 0.25 mg/kg) every other day for 5 doses. Representative PDL1 immunofluorescence staining is shown for tumor tissues (2 days after final dose). Blue, DAPI. G-J. Effects of Hif-1α shRNA on PD-L1 expression in E0771 in vitro. E0771 cells were transduced with lentivirus packaged with scrambled (sh-Scr) or Hif1α shRNA (sh-Hif1α) and cultured under normoxia for 48hrs with DMSO (-), or echinomycin (EM, 1.35 nM). Flow cytometry histograms of PD-L1 staining are shown, comparing effects of Hif1α knockdown (G), or effects of echinomycin between sh-Scr (H) and sh-Hif1α (I) cells. The data is summarized in (J), expressed as mean ± SEM of PD-L1 MFI for triplicate wells, analyzed by one-way ANOVA with Sidak’s posttest. Data are representative of three independent experiments.
Figure 2. Therapeutic effects of echinomycin on tumor growth in immunodeficient and immunocompetent mice. Three murine tumor lines were tested: 4T1, E0771, or MC38. For each, immunodeficient (NSG) and immunocompetent (BALB/c or C57BL/6) mice were inoculated (day 0), and treatment was initiated with control liposomes (vehicle) or echinomycin liposomes (LEM) on day 6 (blue arrows indicate a single treatment). Tumor growth kinetics were compared to deduce the role of adaptive immunity in the therapeutic effects of echinomycin. A. Diagram of experimental design. B. 4T1. NSG and BALB/c mice received 4T1 cells (1x10⁶/mouse) and were treated with vehicle or LEM, 0.15 mg/kg (n=10/group). Mean tumor volumes ± SEM are shown, analyzed by two-way ANOVA. The data are representative of two independent experiments. C. E0771. NSG and C57BL/6 mice received E0771 cells (0.7x10⁶/mouse) and were treated with vehicle or LEM, 0.25 mg/kg (n=5/group). Mean tumor
volumes ± SEM are shown, analyzed by two-way ANOVA. The data are representative of two independent experiments. D. MC38. NSG and C57BL/6 mice received MC38 cells (1x10^6/mouse) and were treated with vehicle or LEM, 0.15 mg/kg (n=5/group). Mean tumor volumes ± SEM are shown, analyzed by two-way ANOVA. The data are representative of two independent experiments.
Figure 3. Effects of pharmacological and/or genetic targeting of HIF-1α on E0771 tumor growth in immunodeficient or immunocompetent mice. A. Experimental design. Three sublines of E0771 were generated by lentiviral transduction: scrambled shRNA (sh-Scr), or shRNA for Hif1a (sh-Hif1α) or Pdl1 (sh-Pdl1). For each, 0.5x10^6 cells were orthotopically transplanted into NSG or C57BL/6 mice (day 0), which received vehicle or echinomycin (LEM, 0.25 mg/kg) starting day 6. B. Effects of Hif1α or Pdl1 knockdown on E0771 growth among immunodeficient or immunocompetent recipients. C. Effects of vehicle or LEM on sh-Scr, sh-Hif1α, or sh-Pdl1 E0771 growth in immunocompetent recipients. D. Effects of vehicle or LEM on sh-Scr, sh-Hif1α, or sh-Pdl1 E0771 growth in immunodeficient recipients. In the graphs, tumor volumes are plotted as the mean ± SEM for each group (n=5/group), with statistics determined by two-way ANOVA, and the data shown are representative of 2 experiments.
Figure 4. Echinomycin potentiates therapeutic effect of anti-CTLA4 antibody. In 4T1, E0771, or MC38 syngeneic tumor models, the effects of echinomycin (LEM) + anti-CTLA-4 (9D9) on tumor growth were tested in comparison to either monotherapy or vehicle control. In E0771 and MC38 models, effect of 9D9 + anti-PD-1 (RMP1-14) was also assessed. Treatment was initiated on day 6 after tumor cell inoculation, with single treatments indicated by the blue (LEM) and red arrows (mAbs). In the graphs, mean tumor volumes ± SEM are shown for each group, analyzed by two-way ANOVA. A. Diagram of experimental design. B. Effects of 9D9 + LEM on syngeneic 4T1 tumor growth. BALB/c mice with 4T1 tumors received vehicle, LEM (0.15 mg/kg/dose), 9D9 (0.2 mg/mouse/dose), or combination (n=10/group). Data shown for one of three independent experiments. C. Effects of 9D9 + LEM on syngeneic E0771 growth. E0771 cells (0.5x10^6) were orthotopically transplanted into C57BL/6 mice, which received vehicle,
LEM (0.25 mg/kg) and/or various mAbs (0.2 mg/mouse/dose) (n=5/group). Representative data shown for one of three independent experiments. D. Effects of 9D9 + LEM on syngeneic MC38 growth. MC38 cells (1x10^6) were transplanted into the left flank C57BL/6 mice. Mice received vehicle, LEM (0.15 mg/kg), and/or various mAbs (0.2 mg/mouse/dose) (n=5/group). Representative data shown for one of three independent experiments.
Figure 5. Echinomycin suppresses PD-L1 on tumor cells and tumor-infiltrated myeloid cells and expands the IFNγ-producing CD8 and CD4 T cells with or without anti-CTLA-4 antibodies. C57BL/6 mice received E0771 cells (0.5x10^6/mouse) on day 0 followed by treatment with vehicle, echinomycin (LEM, 0.25 mg/kg/dose), anti-CTLA-4 (9D9, 0.2 mg/mouse/dose), or 9D9 + LEM on days 6, 8, 10, and 12. On day 14, the tumors were analyzed by flow cytometry. A-D. PD-L1 expression on tumor and tumor-associated myeloid cells. PD-L1 expression was analyzed on tumor cells (gated on Singlets/Live_CD45-) (A), M-MDSCs (gated on Vehicle, LEM, 9D9, 9D9+LEM (B), PMN-MDSCs (gated on Vehicle, LEM, 9D9, 9D9+LEM (C), and TAMs (gated on Vehicle, LEM, 9D9, 9D9+LEM (D)). E, F. Frequency of CD8+ (E) and CD4+ (F) TILs with or without 9D9 + LEM.
on Singlets/Live_{CD45^+}/CD11b^+CD11c^-Ly6C^{high, Ly6G^-} (B), PMN-MDSCs (gated on Singlets/Live_{CD45^+}/CD11b^+CD11c^-Ly6C^{int, Ly6G^-}) (C), or CD11c^+ TAMs (gated on Singlets/Live_{CD45^+}/CD11b^+CD11c^+) (D). Upper panels show representative histograms of PD-L1. In the lower panels, dot plots show the PD-L1 MFI for individual mice from three independent experiments (n=5 mice/group/experiment). The data is presented as the mean ± SEM of PD-L1 MFI, analyzed by one-way ANOVA with Sidak’s multiple comparisons test (A), or by two-tailed unpaired t-tests (B-D). E-F. Frequency of TILs producing IFNγ (E-F). The frequencies of CD8^+IFNγ^+ (Tc1) among total CD8^+ TILs (E) and CD4^+IFNγ^+ (Th1) among total CD4^+ TILs (F) are shown. The tumor cell suspensions were cultured for 4 hr in presence of PMA + ionomycin and GolgiStop prior to staining. The dot plots show the Tc1 or Th1 cell frequencies for individual mice from two independent experiments (n=5 mice/group/experiment), analyzed by one-way ANOVA with Sidak’s multiple comparisons test.
Figure 6. Echinomycin improves TIL function in anti-CTLA-4 treated mice and CD8 TILs are critical for combination efficacy. A-H. C57BL/6 mice received E0771 cells (0.5x10^6/mouse) on day 0 followed by treatment with vehicle, echinomycin (LEM, 0.25 mg/kg/dose), 9D9 (0.2 mg/mouse/dose), or 9D9 + LEM on days 6, 8, and 10. On day 14, tumors were analyzed by flow cytometry. Each graph shows the frequencies of CD8+ or CD4+ subsets among CD8 or CD4 TILs (gated on Singlets/Live_CD45+/CD3+/CD8+CD4- or Singlets/Live_CD45+/CD3+/CD8 CD4+, respectively). Data are presented as the ± SEM of each group (n=5/group), analyzed by unpaired two-tailed Student’s t-test and are representative of two
A-B. Frequencies of TILs expressing PD-1. C-D. Frequencies of annexin V+ TILs. E-F. Frequencies of CD8 TILs expressing granzyme B or perforin. G-H. Granzyme B and perforin expression in CD8 TILs. I-J. Granzyme B and perforin expression in CD4 TILs. J. Effect of depletion of CD4, CD8, or NK cells on tumor growth inhibition by 9D9 + LEM in syngeneic E0771 model. C57BL/6 mice received E0771 cells (0.5x10⁶/mouse) on day 0. On day 5, the mice were randomized to receive depletory antibodies (500 µg of anti-CD4 (GK1.5), anti-CD8 (YTS169.4), anti-NK1.1 (PK136), or isotype ctrl). All groups received 9D9 (200 µg) on day 6, and LEM (250 µg/kg) on days 6, 8, and 10. Mice received supplemental dose of depletory antibodies (200 µg) on days 8 and 10. The mean ± SEM tumor volumes are plotted on the y axes for each group (n=5/group) and analyzed by two-way ANOVA. Representative data shown for one of two experiments.
Figure 7. Echinomycin stimulates PD-L1 expression in irAE target organs to limit the infiltration of T cells caused by anti-CTLA-4 mAbs by an IFNγ-dependent mechanism.

E0771 cells (0.5x10⁶) were transplanted into C57BL/6 mice (day 0), which were divided into 6 treatment groups (n=5/group): vehicle, echinomycin (LEM), anti-CTLA-4 (9D9), 9D9 + LEM, 9D9 + LEM + anti-IFNγ (XMG1.2), or 9D9 + anti-PD-1 (RMP1-14). LEM (0.25mg/kg) or mAbs (0.2mg/mouse/dose) were given days 6, 8, 10, and 12. On day 14, the mice were perfused. Dissociated spleens were stimulated 4hrs with PMA + ionomycin + GolgiStop prior to flow cytometry. Liver and kidney tissues were fixed and immunofluorescence stained for indicated markers and DAPI (blue). A. PD-L1 expression in the tumor-bearing mice treated with different therapies. Representative PD-L1 immunofluorescence staining shown for kidney and liver tissues from indicated treatment groups. B-D. T cell infiltration in the liver and kidney of tumor-bearing mice. B. Representative CD3 immunofluorescence staining depicting T cell infiltration in kidney and liver tissues. C-D. T cell infiltration was scored (scale of 0-4) in the kidney (C) and liver (D) tissues as follows: normal/none; 1, minimal; 2, mild; 3, moderate; 4, severe. E. Frequency of splenic Tc1 cells among CD8⁺ T cells (gated on Singlets/Live_CD45⁺/CD3⁺/CD8⁺CD4⁺). F. Frequency of splenic Th1 cells among CD4⁺ T cells (gated on Singlets/Live_CD45⁺/CD3⁺/CD8⁻CD4⁺). G. Frequency of splenic T cells (gated on Singlets/Live_CD45⁺/CD3⁺/CD8⁻CD4⁺) among total hematopoietic cells (gated on Singlets/Live_CD45⁺). H. CD3 and cleaved-caspase 3 staining in kidney and liver. Representative immunofluorescence images shown for kidney (upper) and liver tissues (lower) of mice that received 9D9 or 9D9 + LEM. All data is representative of at least two independent experiments. In C-G, data are presented as the mean ± SEM with each dot representing an individual mouse, analyzed by one-way ANOVA with Sidak’s posttest.
Figure 8. Echinomycin induces PD-L1 to counter Ipilimumab-induced GI-irAEs by an IFN-γ-dependent mechanism. **A.** Experimental design. Ipilimumab (Ipi.) was used to induce GI-irAE in CTLA4h/h pups (detailed in methods). Single dose of Ipilimumab and other agents are indicated in the diagram by arrows (red, mAbs; blue, vehicle/LEM). **B-C.** Representative immunofluorescence images showing T cell infiltration (B) or PD-L1 expression (C) in jejunum of vehicle- or Ipilimumab-treated mice. **D.** Association of intestinal PD-L1 expression with GI-irAE determined by FITC-dextran assay. Intestinal PD-L1 expression was scored as negative/low
(n=18) or high (n=21) based on immunofluorescence, and serum FITC-dextran intensity presented as the mean ± SEM for each group, analyzed by t test. Aggregate data shown from 4 experiments. **E-G.** Effects of LEM, anti-PD-1 (RMP1-14) and anti-IFNγ (XMG1.2) in GI-irAE model. Mice were grouped as follows to receive therapies based on the experimental design depicted in (A): vehicle(n=33), LEM(n=27), Ipilimumab(n=33), Ipilimumab+LEM(n=26), Ipilimumab+RMP1-14(n=27), Ipilimumab+LEM+RMP1-14(n=28), Ipilimumab+LEM+XMG1.2(n=15). E. Serum FITC-dextran intensity shown as mean±SEM for individual mice pooled from 3 independent experiments. GI-irAE incidence corresponding to each group are annotated (percentages); dotted line represents the threshold for GI-irAE+/-.

Statistics were determined by unpaired t tests (two-tailed). **F.** Representative H&E images from intestines of mice receiving different therapies. Panel iv marks cellular debris and necrosis in lamina propria and epithelium (arrow). **G.** Representative immunofluorescence images showing PD-L1 staining in jejunum of mice from different treatment groups. **H.** Flow cytometry analysis of PD-L1 expression in intestinal epithelial cells (gated on Singlets/Live/CD45−/Cytokeratin+) from mice treated with Ipilimumab (n=6) or Ipilimumab + LEM (n=8). Data shown as means±SEM of the PD-L1 MFI for each mouse, analyzed by unpaired two-tailed Student’s t test.