IMMUNOLOGY

A deubiquitination module essential for Treg fitness in the tumor microenvironment

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The tumor microenvironment (TME) enhances regulatory T (Treg) cell stability and immunosuppressive functions through up-regulation of lineage transcription factor Foxp3, a phenomenon known as Treg fitness or adaptation. Here, we characterize previously unknown TME-specific cellular and molecular mechanisms underlying Treg fitness. We demonstrate that TME-specific stressors including transforming growth factor-β (TGF-β), hypoxia, and nutrient deprivation selectively induce two Foxp3-specific deubiquitinases, ubiquitin-specific peptidase 22 (Usp22) and Usp21, by regulating TGF-β, HIF, and mTOR signaling, respectively, to maintain Treg fitness. Simultaneous deletion of both USPs in Treg cells largely diminishes TME-induced Foxp3 up-regulation, alters Treg metabolic signatures, impairs Treg-suppressive function, and alleviates Treg suppression on cytotoxic CD8+ T cells. Furthermore, we developed the first Usp22-specific small-molecule inhibitor, which dramatically reduced intratumoral Treg Foxp3 expression and consequently enhanced antitumor immunity. Our findings unveil previously unappreciated mechanisms underlying Treg fitness and identify Usp22 as an antitumor therapeutic target that inhibits Treg adaptability in the TME.

INTRODUCTION

Tumors have long been recognized as having distinctive properties of growth, invasion, and metastasis, but their ability to evade immune recognition and destruction has recently attracted attention. While neoplastic cells have sufficient antigenicity to promote an antitumor immune response, tumors evade the immune system through a variety of mechanisms including the production of immunosuppressive mediators and cytokines, defective antigen presentation, and recruitment of immunoregulatory cells such as T regulatory (Treg) cells (1, 2). Furthermore, the disorganized vascular system and enhanced rate of proliferation observed in tumors create a hostile microenvironment depleted of oxygen, glucose, and amino acids while enriched with cytokines, metabolic toxins, and lactic acid (3). Many, if not all, of these alterations in the tumor microenvironment (TME) are known to inhibit antitumor immune responses through a variety of mechanisms. In particular, these TME-derived pressures favorably alter intratumoral Treg (iTreg) cells, resulting in heightened proliferative and suppressive abilities, while diminishing the antitumor effects of effector T (Tem) cells (4–7). Moreover, iTreg cells are known to aid in metastasis, and their increased number correlates with poor clinical outcomes (1, 5).

The presence of iTreg cells plays a pivotal role in inhibiting antitumor immunity and is a major hurdle for current tumor-targeting immunotherapies. To enhance the effects of immunomodulatory therapies, minimizing the heightened suppression by iTreg cells could significantly increase responses and overall survival across cancer types. iTreg cells display up-regulated expression of the lineage-defining Treg transcription factor, forkhead box P3 (Foxp3) (8), which is essential for proper Treg function and acts to enhance Treg fitness by augmenting cell stability and suppressive capabilities (9). However, the molecular mechanisms underlying which and how TME factors up-regulate Foxp3 expression to potentiate iTreg-suppressive function remain unknown.

Although Foxp3 is uniquely important for Treg identity and function, it is an intracellular protein whose targeting would require great care as complete inhibition would likely drive significant autoimmune (9). In addition, specifically targeting a transcription factor like Foxp3 remains technically challenging. So, as Treg depletion through a Treg-specific marker remains arduous (10, 11), the specific pathways that enhance Treg-suppressive capabilities within the TME are attractive candidates for new therapeutic targets to diminish iTreg-suppressive function. Therefore, superior therapeutic candidates will be those that control the expression and stability of Foxp3 specifically in the TME.

Foxp3 expression and stability can be regulated from the transcriptional to the posttranslational level, with each layer independently controlling the stability and overall function of Treg cells. In particular, a newly appreciated layer of Foxp3 regulation and Treg functional modulation is through ubiquitination (12, 13). Ubiquitination of histones on the Foxp3 promoter and conserved noncoding DNA sequence regions via E3 ubiquitin ligases results in chromatin condensation and lack of Foxp3 transcription (14). Furthermore, direct ubiquitination of the FOXP3 protein can result in proteasomal degradation. Ubiquitin may be removed from these sites by deubiquitinating enzymes (DUBs), functioning both to open the chromatin at the transcriptional level and to stabilize FOXP3 at the protein level (12). The balance between E3
ligases and DUBs on Foxp3 expression results in an equilibrium state that regulates Foxp3 levels within Treg cells. We and others have discovered three members of the ubiquitin-specific peptidase (USP) family as direct modulators of Foxp3 deubiquitination at the transcriptional and/or posttranslational level: Usps7, Usps21, and Usps22 (12–16). However, the broad environmental cues and cellular regulation of these deubiquitinases remain unknown. Here, we investigate the role of the TME on the USP–Foxp3 axis and identify specific TME factors that selectively induce Usps22 and Usps21 expression to control Treg stability and adaptation. Last, we develop the first Usps22-specific inhibitor capable of antitumor activity, demonstrating the importance of targeting Treg adaptability within the TME.

**RESULTS**

**Selective up-regulation of Foxp3 deubiquitinases Usps22 and Usps21 in iTreg cells**

To determine how the TME affects tumor immunity, we first characterized the tumor infiltrated Treg cells using multiple syngeneic tumor models including B16 melanoma, LLC1 Lewis lung carcinoma, and EG7 lymphoma. Both the percentages of and FOXP3 protein levels are increased in iTreg cells relative to splenic Treg cells within the same mouse as well as the control non-tumor mouse (fig. S1, A to C). Furthermore, iTreg cells in each tumor type exhibited increased surface expression of multiple known Treg-suppressive markers including CTLA-4 and PD-1 (fig. S1, D to G). These data suggest that iTreg cells have elevated immunosuppressive functions through the up-regulation of Foxp3 and surface inhibitory receptors, which is consistent with previous studies demonstrating that human iTreg cells display enhanced suppressive function (7).

As multiple Foxp3-stabilizing USPs have been recently identified (14–16), we hypothesized that modulation of their expression may drive the Foxp3 up-regulation in iTreg cells. The mRNA level of Usps22 was consistently increased within iTreg cells in comparison to the peripheral Treg cells harvested from the same mouse, but the Usps7 mRNA level was unchanged. In contrast, the Usps21 mRNA level was only increased under B16 but not LLC1 and EG7, suggesting that Usps21 up-regulation in Treg cells occurs only under certain TME conditions (fig. 1, A to C). None of the Usps levels were altered in intratumoral CD8 T cells compared to splenic CD8 T cells (fig. S2A). These data indicate that one or many factors in the TME up-regulate both Usps22 and Usps21 transcription to stabilize Foxp3 specifically for Treg fitness. We also detected an upward, but statistically yet to be significant, trend of Usps22 in Treg cells from human lung tumor tissues (LTu) in comparison to adjacent healthy lung tissue (AHL) (fig. S2B). This Usps22 up-regulation shows a positive association with FOXP3 up-regulation within the LTu patient samples (fig. S2C). Consistent with our observation from the syngeneic lung cancer model, Usps21 was not increased in human lung tumor iTreg cells, nor did it have a significantly positive correlation with Foxp3 (fig. S2, C and D), suggesting that Usps22 is the more dominant USP in Treg cells within the tumor in the context of lung cancer. Together with our recent discovery that Usps22 functions as a Foxp3-specific deubiquitinase, our data suggest that Usps22 promotes Foxp3 expression in iTreg cells (14).

**Tumor-derived TGF-β selectively induces Usps22 and Usps21 in Treg cells**

To identify the TME factors involved in inducing Usps22 and Usps21 expression in Treg cells, we began with testing whether any tumor-derived soluble factors are involved by coculturing in vitro induced Treg (iTreg) cells with tumor-conditioned medium (TCM). TCM from B16 and LLC1, but not EG7 cells, enhanced Usps22 and Usps21 mRNA levels (Fig. 2A). In contrast, the levels of Usps7 remained unchanged, recapitulating the results in Fig. 1. Similar to the mRNA levels, Usps22 and Usps21 protein levels were increased upon incubation with LLC1 TCM (fig. 2B). Consistently, the addition of EG7 cultured medium did not enhance any of the USPs at the protein level (fig. 2B), confirming that specific tumor types selectively induce Foxp3 deubiquitinases in Treg cells.

Many types of tumors secrete large amounts of transforming growth factor–β (TGF-β), which dampen immune responses and promote metastasis (17, 18). Together with the fact that TGF-β is particularly important for iTreg generation and stability (19), we speculated that TGF-β could enhance Foxp3 expression in iTreg cells through induction of Usps22 and Usps21. mRNA levels of both Usps22 and Usps21, but not Usps7, were increased when TGF-β was added to the medium of iTreg cells (fig. S3, A and B). This increase of both Usps22 and Usps21 expression was largely diminished by the addition of a TGF-β inhibitor (LY 3200882) (fig. S3C). The level of Foxp3 mRNA rose concurrently with the levels of Usps22 and Usps21 (fig. S3D), demonstrating that TGF-β can further enhance Foxp3 expression through Usps22 and Usps21 induction. To support this notion, we further demonstrated that the TGF-β inhibitor completely diminished the TCM-induced mRNA enhancement of Usps22 (fig. 2C), signifying that TGF-β is the primary factor in the B16 and LLC1 TCM that enhances Usps22 expression. The Usps21 mRNA level was diminished when the inhibitor was added to the LLC1 TCM, but not under B16 TCM condition (fig. 2C). It is possible that this difference could be due to the quantity of TGF-β secreted by the tumor cell lines into the medium. LLC1 cells secreted significantly higher amounts of TGF-β than both B16 and EG7 cells (fig. S3E), which positively correlates with observed increase in Usps22 and Usps21 mRNA expression (fig. S3, F and G). The levels of Usps7 remain unchanged under all treatment groups and displayed no correlation to the increasing level of TGF-β in the various tumor types (fig. 2C and fig. S3, B, C, and H).

**TGF-β signaling up-regulates Usps22 and Usps21 through distinctive pathways**

To uncover the mechanism by which TGF-β acts on Usps22 and Usps21 transcription, we first investigated the canonical TGF-β signaling pathway, which works through the coactivating SMAD (homologs of the Drosophila protein, mothers against decapentaplegic (Mad), and the Caenorhabditis elegans protein Smaj) transcription factors: SMAD2, SMAD3, and SMAD4 by specifically binding to the SMAD-binding element (SBE) (20, 21). We scanned along the promoter regions of both Usps22 and Usps21 for sequences of conserved SBE. Along the Usps22 promoter, we found promising regions for which we made primers and assessed the SMAD-binding capacity (fig. S4, A and B). Chromatin immunoprecipitation (ChIP) analysis detected that SMAD3 and SMAD4, but not SMAD2, bind to Usps22 promoter at around 300 and 1200 base pairs upstream of the transcription start site (fig. S4B). SMAD
binding at both sites was ablated upon the addition of the TGF-β inhibitor, demonstrating that SMAD3 and SMAD4 binding to the Usp22 promoter is due directly to TGF-β signaling (Fig. 2D). SMAD2 showed no binding capacity to any regions of the Usp22 promoter (Fig. 2D and fig. S3B), likely due to steric hindrance blocking its direct DNA interaction (22).

Although Usp22-null iTreg cells polarize normally with high levels of TGF-β, suboptimal polarization conditions with low TGF-β resulted in a significant decrease in FOXP3 mean fluorescence intensity (MFI) and percentage relative to the wild-type (WT) iTreg cells (14), implying that Usp22 perpetuates TGF-β signaling for iTreg polarization. Usp22-null iTreg cells display a significant reduction in both SMAD2 and SMAD4 protein levels compared to WT iTreg cells, but no difference in their mRNA levels (fig. S4, C and D), suggesting that Usp22 functions as a positive regulator for the TGF-β signaling pathway by stabilizing SMAD proteins. Usp22 interacts with and deubiquitinates both SMAD2 and SMAD4 (fig. S4, E, F, and H). Although Usp22 interacts with SMAD3, it does not act as a DUB of SMAD3 (fig. S4, E and G), suggesting that it acts specifically to regulate TGF-β pathway by stabilizing SMAD2 and SMAD4. In particular, Usp22-null iTreg cells displayed enhanced degradation of SMAD2 and SMAD4, which is rescued upon proteasomal inhibition with MG132 treatment (fig. S4, I and J). Therefore, our data suggest that Usp22 functions to reciprocally enhance TGF-β signaling through SMAD2 and SMAD4 protein stabilization. This effect ensures up-regulation of itself through a positive feedback loop, further ensuring FOXP3 expression in iTreg cells. The phenomenon induced by TGF-β in iTreg cells is not recapitulated in natural Treg (nTreg) cells, where development is independent of TGF-β (fig. S5, A and B), suggesting that the induction of Usps by TGF-β is unique but critical to iTreg cells. Consistently, SMAD2 degradation was not altered in nTreg cells, suggesting that the Smad feedback loop is most important in TGF-β-sensitive iTreg cells (fig. S5C).

Unlike with Usp22, no SBEs were found when scanning the Usp21 promoter. None of the regions showed binding capacity of any of the tested SMAD proteins as analyzed by ChIP assay, confirming that Usp21 expression is not induced through canonical TGF-β signaling (fig. S6A). However, the lack of Usp21 in iTreg cells results in reduced Foxp3 expression in vitro, leaving the opportunity for noncanonical TGF-β signaling to drive Usp21 induction and result in stabilization of FOXP3 (fig. S6, B to D). Inhibition of p38, a Smad-independent TGF-β-activated mitogen-activated protein kinase (MAPK), restrained TGF-β-mediated Usp21 induction (fig. S6E). Together, these data indicate that Usp22 and Usp21 are mediated through distinct TGF-β signaling pathways, and the TGF-β–induced Usp22 as positive feedback to stabilize SMAD2 and SMAD4 (fig. S6F).

**Hypoxia selectively induces Treg Usp22, which supports Foxp3 expression**

In addition to tumor-cell–secreted factors, tumor-driven hypoxia has been repeatedly implicated in Foxp3 stability and Treg cell function (23, 24). A known negative prognostic factor in solid tumors (3, 25), hypoxia preferentially down-regulates T cell proliferation, receptor signal transduction, and effector function while increasing Treg cell–suppressive capabilities (23, 26, 27). We, therefore, investigated the effects of hypoxia on Foxp3-stabilizing Usps levels in Treg cells. Unexpectedly, only Usp22 expression was enhanced under hypoxic conditions at both the mRNA and protein levels (Fig. 3A and fig. S7A). Therefore, we speculated that Usp22 could function as a stabilizer of Foxp3 under the hypoxic conditions in the TME and performed a Foxp3 stability assay. Usp22-deficient Treg cells show a reduced ability to sustain Foxp3 expression under hypoxic conditions (Fig. 3B and fig. S7B), suggesting that Usp22 is required for Foxp3 stabilization under the hypoxic conditions found within the TME.

In hypoxic conditions, hypoxia-inducible factor–α (HIF-α) is stabilized, resulting in the activation of a transcriptional program that promotes cellular adaptation to low oxygen levels (28). HIF-α is known to have two functional binding sites on the Usp22 promoter (29), suggesting that hypoxic induction of Usp22 may be HIF-α dependent. Incubation with hypoxia-independent HIF-α activator, dimethyloxalylglycine (dMOG), increased Usp22 mRNA levels in Treg cells (Fig. 3C), indicating that HIF-α–induced Usp22 expression is involved in Foxp3 stabilization. To support this, we further showed that Usp22-deficient Treg cells displayed decreased stability of Foxp3 following treatment with dMOG, confirming that Usp22-dependent Foxp3 stabilization under hypoxic conditions is HIF-α dependent (fig. S7, C and D). These results demonstrate the

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**Fig. 1. Intratumoral Treg cells have increased mRNA expression of Usp22 and Usp21.** (A to C) mRNA level of YFP+ sorted Treg cells from control mice spleens and tumor-challenged mice spleens (Sp) and tumor cells (Tu). All mRNA values were calculated relative to WT Treg cell levels of unchallenged mice. Spleens: B16: Usp22: n = 5 to 6, Usp21: n = 3 to 5, and Usp7: n = 3 to 5; LLC1: Usp22: n = 5 to 6, Usp21: n = 3 to 6, and Usp7: n = 3 to 6; EG7: Usp22: n = 4 to 5, Usp21: n = 3 to 4, and Usp7: n = 3 to 7. (A to C) Two-tailed unpaired t test was done to determine statistical significance. All data are presented as means ± SD. ns, not significant. *P < 0.05, **P < 0.01.
importance of Usp22 in hypoxia-regulated Foxp3 expression within the TME.

**Metabolic alterations in the TME induce Usp22 and Usp21 to promote Foxp3 stability**

In addition to oxygen, glucose levels in the TME are often decreased, in part, through its enhanced uptake by tumor cells, which compete with the glucose necessity of the highly glycolytic T$_{eff}$ cells (30, 31). Conversely, Foxp3 promotes oxidative phosphorylation over glycolysis in T$_{reg}$ cells, potentially giving them a functional advantage within the TME (4, 32, 33). Therefore, we hypothesized that the observed T$_{reg}$ cell advantage in nutrient-deprived environments could exist partially by inducing Foxp3-stabilizing USP expression. The mRNA and protein levels of Usp22, but not Usp21 and Usp7, were increased in T$_{reg}$ cells upon glucose deprivation (Fig. 3D and fig. S4E). In addition, Usp22-deficient T$_{reg}$ cells have significantly lower Foxp3 stability under glucose deprivation compared to WT T$_{reg}$ cells, demonstrating that Usp22 maintains Foxp3 levels under glucose-restricted conditions (Fig. 3E and fig. S7F).

In addition, a scarcity of amino acids within tumors also alters immune cell function (31), and amino acid starvation is known to enhance T$_{reg}$ cell induction (34). To investigate the role of USPs in amino acid starvation–induced Foxp3 expression, we cultured T$_{reg}$ cells in medium lacking amino acids. Amino acid starvation led to increased expression of both Usp22 and Usp21, but not Usp7, in T$_{reg}$ cells (Fig. 3F). Furthermore, the stability of Foxp3 in amino acid–starved T$_{reg}$ cells is reduced by the deficiency of Usp22 or Usp21 (Fig. 3G and fig. S7G).

In environments depleted of both glucose and amino acids, activation of adenosine monophosphate–activated protein kinase (AMPK) suppresses anabolic metabolism while up-regulating oxidative metabolism to promote cellular survival (35), prompting us to uncover whether AMPK activation is involved in Usp22 or Usp21 up-regulation. We therefore treated T$_{reg}$ cells with two AMPK activators: an inhibitor of mitochondrial adenosine triphosphate (ATP) synthase, oligomycin A, and a direct AMPK activator, AICAR. While oligomycin A treatment increased both Usp22 and Usp21, but not Usp7, in T$_{reg}$ cells, AICAR treatment was unable to up-regulate any Usps (Fig. 3H and fig. S7H). Oligomycin A functions to

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**Fig. 2. Tumor cell–secreted TGF-β increases Usp22 and Usp21 level in T$_{reg}$ cells.** (A) USP mRNA level in T$_{reg}$ cells in control T cell medium compared to addition of tumor cell–treated medium at 50:50 with T cell medium for 24 hours. Usp22: control: n = 14, B16: n = 10, LLC1: n = 5, and EG7: n = 4. Usp21: control: n = 12, B16: n = 8, LLC1: n = 3, and EG7: n = 3. (B) USP protein level in T$_{reg}$ cells in control T cell medium compared to addition of tumor cell–treated medium at 50:50 with T cell medium for 24 hours. The relative protein expression levels were quantified using Bio-Rad Image software and normalized with the loading controls. (C) USP mRNA level in T$_{reg}$ cells with the addition of a TGF-β inhibitor in tumor cell medium. Usp22: control: n = 22, B16: n = 15, B16 + inhibitor (inh): n = 5, LLC1: n = 10, LLC1 + inh: n = 5, EG7: n = 7, and EG7 + inh: n = 5. Usp21: control: n = 20, B16: n = 13, B16 + inh: n = 5, LLC1: n = 8, LLC1 + inh: n = 4, EG7: n = 7, and EG7 + inh: n = 5. Usp7: control: n = 14, B16: n = 10, B16 + inh: n = 5, LLC1: n = 8, LLC1 + inh: n = 3, EG7: n = 8, and EG7 + inh: n = 6. (D) SMAD2, SMAD3, and SMAD4 binding capacity along the Usp22 promoter under TGF-β inhibition. SMAD2: n = 4 to 5, SMAD3: n = 3, and SMAD4: n = 3. (A to D) Ordinary one-way ANOVA with multiple comparisons was performed to determine significance. All data are presented as means ± SD. *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001.
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through Usps22, and to a lesser extent Usps21, in T<sub>reg</sub> cells (fig. S7I) (35, 37–39).

**Usps22 and Usps21 modulate T<sub>reg</sub> fitness through distinct pathways**

Our discoveries thus far have suggested that Usps22 and, to a lesser extent, Usps21 are important in maintaining Foxxp3 expression in the TME through multiple pathways. To study their combined functionality in vivo, we generated a strain of T<sub>reg</sub>-specific Usps22 and Usps21 double knockout (dKO) mice by breeding Usps21<sup>+/f</sup> mice with Usps21<sup>+/f</sup>→Usps3<sup>YFPcre</sup> single KO mice. This breeding strategy gave us the T<sub>reg</sub>-specific KO of Usps22 (22KO), Usps21 (21KO), and the dKO, all of which were confirmed via quantitative polymerase chain reaction (qPCR) (Fig. 4A). Deletion of either Usps22, Usps21, or both in T<sub>reg</sub> cells did not alter the frequency of either B or T cells in the spleens of 6-week-old mice (fig. S8, A and B). While the mice display similar weights early in life, by 24 weeks of age, the 22KO and dKO animals are consistently smaller in size compared to WT (Fig. 4B).

Unsurprisingly, all three KO groups showed a significant increase in CD44<sup>hi</sup>CD62<sup>lo</sup>-activated splenic Teff cells in comparison to age-matched WT mice, consistent with the development of low-level, progressive inflammation with age (Fig. 4C). Of note, only the 22KO and dKO mice showed decreases in Foxxp3 expression and significant reductions in T<sub>reg</sub>-cell–associated suppressive markers (Fig. 4, D and E). Consistently, a previous study reported that the 21KO mice develop age-related impairments in T<sub>reg</sub> cell function and number secondary to impaired Foxxp3 expression, whereas 8-week-old young mice showed no changes in Foxxp3 expression (16). Transcriptional profiling revealed that more T<sub>reg</sub> cell–suppressive markers were differentially expressed in the dKO mice than in either single KO mice when compared to WT gene expression (Fig. 4E), suggesting synergism between the loss of Usps22 and Usps21 on T<sub>reg</sub> cell stability and function. Furthermore, differentially expressed genes (DEGs) between the 21KO and 22KO mice were relatively distinct (Fig. 4F). Although gene set enrichment analysis (GSEA) of both single KO mice showed changes in several cell cycle and proliferative pathways, such as G2-M checkpoints and E2F targets, as well as changes in oxidative phosphorylation (Fig. 4G), there were only a total of 32 overlapping DEGs between the 21KO and 22KO mice (Fig. 4F). T<sub>reg</sub> cells from the dKO mice displayed a GSEA and bulk gene expression signature that merged the changes found in each of the single KO mice, suggesting that the loss of both Usps22 and Usps21 synergizes to diminish T<sub>reg</sub> cell function (Fig. 4G) in both Foxxp3-dependent and Foxxp3-independent manner.

As we demonstrated that both Usps22 and Usps21 are regulated by metabolic alterations in the TME, it was particularly interesting to identify disruption of multiple metabolic pathways in each of the KO groups. T<sub>reg</sub> cells from dKO mice had profound changes in lipid metabolic processes, one-carbon metabolism, and ribosomal biogenesis (fig. S8, C to E). When compared to the DEGs in the dKO T<sub>reg</sub> cells, Usps22-null T<sub>reg</sub> cells, but not Usps21-deficient cells, displayed similar alterations in both lipid metabolism and one-carbon metabolism to the dKO T<sub>reg</sub> cells (fig. S8, C and D). In contrast, T<sub>reg</sub> cells from the 21KO and dKO mice showed profound decreases in ribosomal gene expression, which was not identified in the Usps22-null T<sub>reg</sub> cells (fig. S8E), suggesting distinct pathways by which Usps22 and Usps21 modulate T<sub>reg</sub> fitness. Our in vitro metabolic flux analyses further demonstrate that, unlike the 21KO mice,
Fig. 4. Loss of *Usp22* and *Usp21* in T<sub>reg</sub> cells differentially impairs Foxp3 expression and cell function. (A) *Usp22* and *Usp21* levels in WT, 21KO, 22KO, and dKO mice (n = 5 to 8). All mRNA values were calculated relative to WT T<sub>reg</sub> cells. (B) Mice weights over a 2-month period (n = 2 to 9). (C) Peripheral activation of CD4<sup>+</sup> and CD8<sup>+</sup> T cells as measured by CD44<sup>hi</sup>CD62L<sup>lo</sup> expression (n = 7 to 9). (D) Representative histogram (left) and quantification (right) of Foxp3 MFI in splenic T<sub>reg</sub> cells of WT and KO animals (n = 6 to 8). (E) Heatmap of T<sub>reg</sub> cell signature genes (*significance is adjusted P < 0.01) in 21KO (n = 2), 22KO (n = 3), and dKO (n = 3) versus WT (n = 3) mice. Genes ordered by decreased to increased differential expression in dKO mice. (F) Venn diagram of DEGs (adjusted P < 0.01) between 22KO, 21KO, and dKO (n = 2 to 3). (G) Normalized enrichment scores from GSEA (FDR < 25%) from the hallmark gene set in the Molecular Signatures Database comparing the gene set generated from RNA sequencing of WT, 21KO, 22KO, and dKO mice (n = 2 to 3). (H) Basal mitochondrial OCR and basal ECAR of 21KO (n = 5), 22KO (n = 5), and dKO (n = 4 to 5) relative to WT (n = 5) T<sub>reg</sub> cells. (A to C) Two-way ANOVA with multiple comparisons between rows was performed to determine statistical significance. (D and H) One-way ANOVA with multiple comparisons between rows was performed to determine statistical significance. All data are presented as means ± SD. *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001.
both the 22KO and dKO mice display enhanced mitochondrial oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) (Fig. 4H), suggesting that Usp22 may play a dominant and essential role in modulating the metabolic state of T_{reg} cells.

As Usp21 seemed to have a Foxp3-independent role in T_{reg} function, we compared the DEGs from the 22KO and dKO mice to determine the contribution of Usp21 to the dKO phenotype. We noticed significant changes in cell cycle pathways and effector differentiation pathways (fig. S8F), suggesting a loss of homeostasis in the T_{reg} compartment. An increase in proliferation of the 21KO and dKO T_{reg} cells in comparison to WT T_{reg} cells, but not in the 22KO T_{reg} cells (fig. S8G), suggests that the normally highly suppressive effector T_{reg} population has an increased proliferative capacity with a down-regulation of functional genes, generally suggestive of a dysfunctional suppressive capacity (40). Collectively, these data imply that both Usp22 and Usp21 modulate T_{reg} cell metabolism, although seemingly through unique pathways to maintain T_{reg} stability and function.

**Usp22 and Usp21 deletion in T_{reg} cells synergizes to enhance antitumor immunity**

To test the importance of T_{reg} cell Usp22 and Usp21 in tumor conditions in vivo, we used the B16 melanoma syngeneic tumor model. Mice with T_{reg}-specific ablation of Usp22 showed increased tumor rejection compared to the deletion of Usp21. Importantly, though, mice harboring the joint deletion of both Usp22 and Usp21 in T_{reg} cells grew the smallest tumors (Fig. 5A). In addition, the dKO and 22KO animals showed greater proportions of effector memory CD8^+ T cells in the spleens (Fig. 5B). In contrast, deletion of Usp21 in T_{reg} cells was insufficient to enhance the B16 tumor rejection (Fig. 5A). Consistently, 21KO mice effecter memory populations and cytokine levels were on par with WT mice, while the 22KO mice displayed an increase of CD8^+ granzyme B (GZMB)-producing T cells in the spleens (Fig. 5B). Notably, the tumor-bearing dKO mice had significant increases of both interferon-γ (IFN-γ)– and GZMB-producing CD8^+ T cells in the spleens, and each cytokine was enhanced even in comparison to single KO animals (Fig. 5, B and C). Furthermore, both the 22KO and dKO mice had significant drops in Foxp3- and T_{reg}-suppressive marker MFI in peripheral T_{reg} cells, which was not observed in 21KO T_{reg} cells (Fig. 5, D to G). Collectively, these data suggest that the combined loss of Usp22 and Usp21 in T_{reg} cells results in enhanced activation of T_{eff} cell effect compared to individual loss of Usp21 or Usp22 alone.

Further analysis of tumor-infiltrating lymphocytes indicated a significant increase in CD4^+ and CD8^+ T cell frequencies in the dKO mice, with each compartment in the dKO secreting higher amounts of both IFN-γ and GZMB than WT mice (Fig. 5, H to J). Notably, the dKO mice had significantly higher levels of T_{eff} cell infiltration than both the 22KO and 21KO mice, as well as having the highest levels of IFN-γ secretion. Consistently, itT_{reg} cells in the 22KO and dKO mice had significantly lower T_{reg} infiltration and Foxp3 MFI than in the WT and 21KO mice (Fig. 5, K and L). Impairment in T_{reg}-suppressive functions, such as by Usp22 and Usp21 loss, perpetuates antitumor immunity by presumably alleviating T_{reg} suppression on cytotoxic CD8^+ T cells. The enhanced tumor rejection in the Usp22 and Usp21 dKO mice was largely abolished by antibody-induced CD8 depletion (Fig. 5M). Collectively, these data suggest that Usp22 and Usp21 cooperate to maintain T_{reg} cell function in the TME.

**Identification of a Usp22-specific small-molecule inhibitor**

Although our discovery that dual Usp inhibition delivers the strongest antitumor effect, Usp22 presents as the dominant Foxp3-stabilizing Usp, and Usp22 deletion alone was also sufficient in diminishing tumor burden. Responding consistently to TME factors, Usp22 demonstrates potential as an antitumor immune therapeutic target. Therefore, we first aimed to identify Usp22-specific inhibitors. It has been suggested that in vitro purified Usp22 protein lacks catalytic activity (41, 42), leading to difficulties for high-throughput screening. Therefore, we used the computer-aided drug design (CADD) to develop a Usp22-specific small-molecule inhibitor (fig. S9A). As Usp22 contains a highly conserved putative catalytic domain (Cys, His, and Asp) from yeast to human, a homology modeling study was performed to obtain a model of human Usp22 for use in structure-based virtual screening (fig. S9B). Of three validated structural models of Usp22, the yeast UBP8 structure [Protein Data Bank (PDB) code 3MHS] was chosen as a template protein to construct the Usp22 model by Swiss Model (Usp22-m) (fig. S9, C and D). To obtain conformation at the lowest potential, the structure of Usp22-m was further subjected to molecular dynamics (MD) simulation and clustering analysis using GROMACS 5.15, and the distance between Cys^185 and His^{279} was increased from 3.6 to 4.8 Å in the position of catalytic site of Usp22 (Usp22-md) (fig. S9, C and D). We further compared the predicted amino acid sequence of Usp22 with 150 homologous full sequences. The conservation grades are mapped onto the structure and show that the Cys domain was highly conserved. This study provides not only the basis for the accuracy of homology modeling but also favorable conditions for drug selectivity screening.

We then used both Lipinski’s rule and Veber’s rule to filter through the Specs database and found a total of 240,000 compounds binding to the catalytic pocket of our Usp22 model. We then filtered the top 100 compounds ranked by docking affinity by MD and molecular mechanics/Poisson-Boltzmann surface area (MM/PBSA) methods and were left with 25 compounds (table S1). This limited number of compounds allowed us for further biological screening. As Usp22 suppression leads to marked reduction in Foxp3 expression levels, we used Foxp3 MFI reduction as a readout for the biological validation of Usp22 inhibitory efficacy by each of the 25 chemicals. As indicated in table S1, the chemical S02 (11-anilino-7,8,9,10-tetrahydrobenzimidazo[1,2-b]isoquinoline-6-carbonitrile) showed strong efficacy in down-regulating Foxp3 expression. The compound S02, structure shown in Fig. 6A, bound stably in the Usp22 catalytic domain pocket shown by the root mean square deviation (RMSD) trajectory (fig. S9E), with strong binding energies to our Usp22-md model (fig. S9F). Furthermore, analysis of S02 interaction with each residue of Usp22 indicated that the side chain–negative residues (Glu and Asp) make a favorable contribution to the binding of the inhibitor and protein; however, the positively charged residues, such as Arg and Lys, play a detrimental role (fig. S9G). Therefore, this Usp22 inhibitor stably bind not only to the hydrophobic residues but also to the charged and polar residues on the surface of the binding pocket of Usp22 protein, implying its potent efficacy in suppressing Usp22 catalytic functions.
Usp22i-S02 down-regulates FoxP3 in a Usp22- but not Usp21-dependent manner

We then determined the dose response of compound S02, now dubbed Usp22i-S02, in both WT and Usp22-null iTreg cells (Fig. 6, A and B, and fig. S10, A and B). At a concentration of 10 μg/ml, Usp22i-S02 decreased Foxp3 MFI to a level comparable in Usp22-null iTreg cells with little effect on viability, indicating a near-complete suppression of Usp22 activity in stabilizing Foxp3 (fig. S10A). The effect of Usp22i-S02 on Foxp3 was further confirmed by Western blot (fig. S10C). Lower doses of Usp22i-S02 administration to human iTreg cells significantly decreased Foxp3 MFI with little effect to cell viability, showing the relevance of this inhibitor to human cells (fig. S10, D to F). In contrast, Usp22i-S02 had minimal effect on Foxp3 levels in murine iTreg cells already lacking Foxp3.
Usp22 both in vivo and in vitro (Fig. 6, B to D) while having full effect on T<sub>reg</sub> cells lacking Usp21 (fig. S10G). Functionally, Usp22i-S02 administration had similar effects to Usp22 deletion in T<sub>reg</sub> cells, resulting in enhanced Foxp3 degradation, increased Foxp3 ubiquitination, and decreased Foxp3 transcription (fig. S10, H to J). Furthermore, Usp22i-S02–mediated Foxp3 degradation was halted by MG132 protease inhibition, indicating that Usp22i-S02 enhances proteasomal-specific degradation of Foxp3 (fig. S10K). Therefore, these results indicate that Usp22i-S02 is a potent Usp22–specific small-molecule inhibitor that down-regulates Foxp3 expression in T<sub>reg</sub> cells in a Usp22- but not Usp21-dependent manner.

Usp22-S02 administration enhances antitumor immunity with low toxicity in mice

An important aspect of a potential immunotherapeutic is its antitumor functionality paired with low immune toxicity. We used the LLC1 Lewis lung carcinoma syngeneic tumor model to test the efficacy of Usp22i-S02 in enhancing antitumor immunity. Upon LLC1 challenge, WT mice administered Usp22i-S02 at 20 mg/kg for 5 days showed a marked reduction in tumor volume and weight compared to untreated mice (fig. S11, A and B). As expected, Usp22i-S02 treatment resulted in a significant increase in CD8<sup>+</sup> T cell tumor infiltration (fig. S11, C and D). Further analysis of tumor-infiltrating lymphocytes indicated a less exhausted phenotype in CD8<sup>+</sup> T cells, with an increase in CD44<sup>+</sup> cells and a decrease in exhausted Foxp3<sup>+</sup> T<sub>reg</sub> cells.
in T-bet+, Blimp1+, and annexin V+ cells (fig. S11, E to H). Like in the T_{reg}-specific Usp22 KO mice, intratumoral Foxp3+ T_{reg} percentage significantly decreased following administration of Usp22i-S02 (fig. S11I). Therefore, specifically targeting Usp22 with a small-molecule inhibitor enhances antitumor immunity with the potential to complement existing immunotherapies.

As tumor rejection may be due to Usp22i-S02 toxicity or non-T_{reg}-specific immune cell modulation, we determined its effects in naïve mice. We found little alteration in the body weights, B cell and T_{eff} cell percentages and proliferation, and T_{eff} cell activation of treated mice at 20 mg/kg body weight compared to dimethyl sulfoxide (DMSO)–treated controls (fig. S11, J to M). Unlike T_{eff} cells, T_{reg} cell death was significantly increased (fig. S11N), resulting in a decrease in T_{reg} percentage (fig. S11K). T_{reg} proliferation was also increased (fig. S11L), potentially indicating a dysfunctional effector T_{reg} (eT_{reg}) population by Usp22i-S02 treatment. Furthermore, a comprehensive tissue panel showed no pancreatic, liver, or kidney organ toxicity form Usp22i-S02–treated mice (fig. S11, O to Q). These data indicate that administration of Usp22i-S02 results in a T_{reg}-specific phenotype in naïve mice with little effects on other immune cell types and tissue toxicity.

**Usp22i-S02 holds great therapeutic efficacy in treatment of the preestablished tumor**

To further evaluate the efficacy of Usp22i-S02 in antitumor immunotherapy at a clinically relevant setting, we tested the inhibitor on established tumors. Following initial LLC1 tumor establishment 15 days after tumor cell implantation, mice administered Usp22i-S02 at 20 mg/kg for 3 days showed marked tumor rejection compared to untreated mice (Fig. 6E). Analysis of tumor-infiltrated immune cells revealed a significant increase in both CD4 and CD8 T_{eff} cell tumor infiltration (Fig. 6, F and G). Foxp3+ MFI levels in the intratumoral, but not splenic, T_{reg} cells were significantly decreased following administration of Usp22i-S02 (Fig. 6H). Consequently, iT_{reg} cells had lower levels of GITR and PD-1 and expressed significantly higher levels of IFN-γ (Fig. 6, I and J). Our data indicate that Usp22i-S02 treatment partially impairs the suppressive functions of T_{reg} cells specifically within the tumor, potentially leading to T_{reg} dysfunction and fragility (40). Therefore, Usp22i-S02 holds great potential as an antitumor immunotherapy at a clinically relevant setting.

As Usp22 is also an important oncogene (43, 44), we were interested in the potential dual-therapeutic function of Usp22i-S02. Administration of Usp22i-S02 to LLC1 cells in vitro resulted in decreased tumor cell counts, viability, and growth (fig. S12, A to C). Furthermore, treatment of Rag−/− mice with established tumors resulted in slight but significant tumor rejection (fig. S12D). Together, our data show the critical role of Usp22 in T_{reg} cell stability and adaptation within the TME, and that specifically targeting Usp22 with a small-molecule inhibitor not only enhances antitumor immunity but also has a dual role as a chemotherapeutic agent.

Collectively, our study illustrates a previously unappreciated role of Foxp3-specific DUBs, Usp22 and Usp21, as environmentally sensitive factors that enhance Foxp3 stability in the TME. We identified several TME factors that up-regulate Usp22 and Usp21, ultimately stabilizing Foxp3: (i) tumor-secreted TGF-β, (ii) hypoxia, (iii) glucose restriction, and (iv) amino acid deprivation (Fig. 6K). These findings unveil new mechanisms behind the metabolic and functional uniqueness of iT_{reg} cells, providing evidence on how these cells adapt in response to environmental cues within solid tumor to support their function. In addition to our previous work demonstrating that loss of Usp22 in the T cell compartment results in only a T_{reg}-specific impairment (15), our data provide a rationale for Usp22-targeted therapy to enhance antitumor immunity. Last, our novel Usp22 inhibitor, Usp22i-S02, successfully reduced tumor burden in a T_{reg}-specific manner (Fig. 6K).

**DISCUSSION**

Emerging data suggest that the TME, often deprived of nutrients and oxygen, likely offers a metabolic advantage to T_{reg} cells, further promoting an immunosuppressive microenvironment. However, the TME-specific factors and their cellular targets that potentiate T_{reg} cell–suppressive function and adaptation remain largely unidentified.

It has been well documented that iT_{reg} cells are more suppressive and often have high Foxp3 expression (7, 8, 45), and we first confirmed these results in various murine tumor models. Our findings that iT_{reg} levels of Usp22, and occasionally Usp21, are up-regulated in these models concurrently with Foxp3 suggest that TME factors selectively induce these USPs to stabilize Foxp3 expression. We observed increased Usp22 levels in correlation with Foxp3 in human iT_{reg} cells, broadening the relevance of this pathway to human tumors. Although Usp7 in T_{reg} cells is known to control Foxp3 expression and T_{reg}-suppressive function in a model of colitis, we did not observe an increase in Usp7 expression in iT_{reg} cells, suggesting that Usp7 may primarily regulate T_{reg} function during homeostatic conditions.

TGF-β is a major player in iT_{reg} conversion and stability and is broadly secreted by many tumor types. We found that tumor-secreted TGF-β is sufficient in up-regulating Usp22 through canonical TGF-β signaling and Usp21 through noncanonical signaling. Furthermore, Usp22 partakes in a feedback loop to further up-regulate itself and Foxp3 through SMAD protein stabilization. The complexity of the p38 and MAPK pathway offers wide-ranging possibilities for downstream regulation of Usp21. p38 stabilizes GATA3 and STAT3 (signal transducer and activator of transcription 3), potentially leading to downstream Usp21 up-regulation (46–50). As TGF-β is widely implicated in Foxp3 expression and stability and iT_{reg} function, our data add a new level of complexity to already known systems (19, 51). These novel mechanisms potentially function to ensure T_{reg} cell stabilization through alternate pathways, strengthening their ability to maintain their suppressive capacity in diverse environments.

However, tumor-secreted TGF-β is not the only factor capable of up-regulating USPs, because T_{reg} cells treated with EG7 TCM could not recapitulate the increase of Usp22 seen in iT_{reg} cells isolated from EG7 tumors. Therefore, we hypothesized that other environmental factors within the TME are also implicated in T_{reg} stabilization through USPs. As hypoxia is a major hallmark of solid tumors (3, 25), we investigated how low oxygen conditions influence Usp22 levels in T_{reg} cells. Our findings that hypoxia induced Usp22 in a HIF-dependent manner, and loss of Usp22 resulted in diminished Foxp3 stability, imply that hypoxia can stabilize T_{reg} cells through Usp22-mediated Foxp3 stabilization. Our conclusion is in line with previous data that demonstrate heightened proliferation and suppressive capabilities of T_{reg} cells under hypoxic conditions and
is strengthened by the two active HIF binding sites along the Usp22 promoter (23).

Along with a decrease in oxygen availability, the competition for nutrients that occurs within the TME influences immune cell growth, survival, and function. Classically, Treg cells are thought to have a significantly lower reliance on glycolysis than Teff cells, potentially providing another advantage in the TME (4, 30, 33). Our data identify Usp22 and, to a lesser extent, Usp21 as important mediators in this process, functioning to stabilize Foxp3 under glucose and amino acid deprivation. Nutrient deprivation in T cells is generally accompanied by a shift toward oxidative metabolism following AMPK activation and mTOR inhibition, and the balance of AMPK and mTOR signaling functions as an environmental sensor for nutrient availability (31, 52). Foxp3 stabilization, in part, appears secondary to this metabolic shift (37, 53). As protein translation is severely reduced under cellular nutrient deprivation, our data revealing mTOR inhibition as a major player in Usp22 and Usp21 up-regulation describe a potential mechanism by which Treg cells stabilize important proteins through targeted up-regulation of deubiquitinases. By up-regulating Foxp3-specific deubiquitinases Usp22 and Usp21, Treg cells enhance their function, stability, and survival under energy stress.

The metabolic status of an immune cell is highly important within the TME for their cell survival and function. As Treg cells can adapt to low-oxygen, low-nutrient environments, they have a metabolic advantage compared to Teff cells. Foxp3 is essential to this process as it is known to promote oxidative phosphorylation within Treg cells. We show that Usp22- and Usp21-deficient Treg cells have significantly altered expression of metabolic genes and impaired OCR and ECAR. In addition, RNA sequencing analysis demonstrated that the loss of Usp22 and Usp21 in Treg cells resulted in the up-regulation of multiple pathways associated with cell growth and proliferation. Collectively, these data raise the intriguing possibility that Usp22 and Usp21 work to promote Treg cell fitness in nutrient-restricted environments in part by modulating Treg cell metabolic programs through Foxp3 stabilization. Ultimately, our data indicate that microenvironmental stress within the TME up-regulates Treg USP levels, which then function to stabilize Foxp3. Enhanced Foxp3 stability further supports Treg cell adaptation to the TME, thus identifying Usp22 and Usp21 as important environment-sensitive factors that regulate Treg cell identity, metabolism, and function in the TME.

As loss of Usp22 in Treg cells resulted in enhanced antitumor immunity relative to the loss of Usp21, there is a suggested dominance of Usp22 in iTreg cells. Therefore, specifically targeting Usp22 may be sufficient in eliminating the advantage Treg cells have over Teff within the TME. To test this, we developed and tested the first ever Usp22-specific inhibitor. Administration of the inhibitor resulted in a marked decrease in iTreg number, resulting in strong in vivo antitumor effects.

In addition, we and others have demonstrated that Usp22 is up-regulated in many cancer types, such as gastric carcinoma, pancreatic cancer, and melanoma, and has been correlated with poor prognosis (54). Usp22 promotes oncosgenic c-Myc activation as well as indirectly antagonizes the tumor-suppressive function of p53, and clearly diminishes tumor growth in vitro and in vivo LLC1 models (55). Usp22 also functions to maintain Foxp3 expression through DUB function at the transcriptional and posttranslational levels. This duality makes Usp22 highly attractive potential therapeutics that can target both tumor-cell intrinsic and immunosuppressive pathways simultaneously. Our data demonstrate that Usp22 is a targetable protein and that the inhibitor Usp22i-S02 has the potential of being incorporated into tumor immune therapies.

As approaches to specifically inhibiting Treg-suppressive functions are yet to be identified, our discovery that Usp22 deletion impairs iTreg-suppressive activity without promoting extensive inflammatory response indicates that Usp22 inhibition holds a great therapeutic potential in antitumor immune therapy. To enhance the effects of immunomodulatory therapies, minimizing the heightened suppression by iTreg cells through Usp22 targeting while concurrently enhancing Teff antitumor immunity through dual therapeutics could significantly increase response rates and overall survival across cancer types.

MATERIALS AND METHODS
Animal strains
Usp22fl/flFoxP3YFP-Cre mice were generated previously as described (14). Frozen sperm of C57BL/6N-A^tm1Brd^{Usp21}tm1La(UCCOMM)Wtsi/ WtsiBai1^{Usp22} were purchased from Emma Mouse Repository (EMMA ID: 07280). Blastocyst injections resulted in several chimeric mice with the capacity for germline transmission. Treg-specific Usp21-null mice were generated by breeding Usp21^fl/fl mice with Foxp3^{YFP-Cre} mice (obtained from The Jackson Laboratory). Usp22^fl/flUsp21^fl/flFoxP3^{YFP-Cre} mice were generated by breeding Usp22^fl/flFoxP3^{YFP-Cre} with Usp21^fl/flFoxP3^{YFP-Cre}. This breeding strategy produced the Treg-specific KO of Usp22 (Usp22^K0) and Usp21 (Usp21^K0) and a dKO of both. These mice were maintained and used at the Northwestern University mouse facility under pathogen-free conditions in accordance with institutional guidelines and following animal study proposals approved by the institutional animal care and use committees (IACUCs). Unless stated otherwise, all figures are representative of experiments with 6- to 8-week-old mice. All animal experiments followed IACUC protocols: IS00001629 and IS00015611.

Syngeneic tumor models
EG7 lymphoma, LLC1 lung carcinoma, and B16-F10 melanoma cell lines were provided by the Zhang laboratory at Northwestern and used for tumor models as previously reported (14). The cell lines were cultured in Dulbecco’s modified Eagle’s medium (DMEM) with 10% fetal bovine serum (FBS) and were tested for mycoplasma using a LookOut Mycoplasma PCR detection kit (Sigma-Aldrich, MP0035-1KT). Cultured cancer cells were trypsinized and washed once with phosphate-buffered saline (PBS). LLC1 lung carcinoma tumor cells were subcutaneously administered to the right flank of 8- to 10-week-old mice at 1 × 10^6 tumor cells per mouse, and B16 melanoma at 5 × 10^5 tumor cells per mouse. Tumors were measured every 2 to 3 days by measuring along three orthogonal axes (x, y, and z), and tumor volume was calculated as (xyz/2). The tumor size limit agreed by the Institutional Review Board (IRB) was 2 cm^3. Mice were intraperitoneally treated with anti-CD8 (Bio X Cell, 53-6,72) or isotype (Bio X Cell, 2A3) (200 mg) every 3 days starting on day 5.
In vitro iT<sub>reg</sub> cell generation
Splenic CD4<sup>+</sup>CD25<sup>-</sup>CD44<sup>-</sup>CD62L<sup>-</sup> naïve T cells were isolated using a mouse naïve CD4 isolation kit (STEMCELL Technologies, catalog no.19765) and cultured in 24-well plates at 5 × 10<sup>4</sup> cells per well for 3 days. Cells were cultured in T cell medium made of complete RPMI 1640 medium containing 10% FBS, 1% penicillin/streptomycin (MediaTech), 50 μM β-mercaptoethanol (Gibco), and 1% L-glutamine (Gibco). Wells were coated in anti-CD3 (3 μg/ml) and anti-CD28 (5 μg/ml) antibodies. Culture medium was supplemented with interleukin-5 (IL-2) (5 ng/ml), anti-CD28 (5 μg/ml) antibodies. Culture medium was used to avoid any TGF-β contamination found in serum. Cells were then plated in a 96-well plate at 5 × 10<sup>4</sup> cells per well with 2000 U of IL-2 and CD3/CD28 beads (from a T<sub>reg</sub> expansion kit, Miltenyi Biotec, catalog no.130-095-925) and added to T cell medium following the manufacturer’s instructions.

Ex vivo nT<sub>reg</sub> cell culture conditions
nT<sub>reg</sub> cells were isolated from mouse spleens using a CD4<sup>+</sup> T cell negative isolation kit (STEMCELL Technologies, catalog no.17952), followed by yellow fluorescent protein–positive (YFP<sup>+</sup>) flow cytometry sorting on FACSaria (BD Biosciences) at 99% efficiency. Cells were then plated in a 96-well plate at 1 × 10<sup>4</sup> cells per well with 2000 U of IL-2 and CD3/CD28 beads (from a T<sub>reg</sub> expansion kit, Miltenyi Biotec, catalog no.130-095-925) and added to T cell medium following the manufacturer’s instructions.

In vitro iT<sub>reg</sub> cell TCM and TGF-β assays
Previously generated iT<sub>reg</sub> cells were washed and rested for 7 hours in Opti-MEM containing IL-2 (5 ng/ml) to maintain survival. Opti-MEM was used to avoid any TGF-β contamination found in serum. After resting, the cells were incubated in Opti-MEM containing IL-2 with or without the addition of TGF-β (20 ng/ml) or the various tumor cell media (B16, LLC1, and EG7). TCM was obtained by plating B16, EG7, or LLC1 cell lines at 50% confluency for 16 hours. TCM was then mixed 50:50 with fresh Opti-MEM and incubated on iT<sub>reg</sub> cells for 24 hours. The TGF-β inhibitor LY 3200882 (MedChem Express, catalog no. HY-103021) was added at 25 μg/ml where indicated.

In vitro T<sub>reg</sub> cell hypoxia culture
nT<sub>reg</sub> cells were isolated as described above and cultured at 37°C under a normoxic (21% O<sub>2</sub>) or hypoxic condition (1% O<sub>2</sub>) for 24 hours. Hypoxia was induced using the O<sub>2</sub> Control InVitro Glove Box from Coy Laboratory Products. T cell medium was incubated at 37°C at normoxia or hypoxia for 3 hours before use. Cells were then collected, and RNA was extracted as described above. For iT<sub>reg</sub> cells, cells were isolated and polarized as described above. Subsequently, cells were rested in Opti-MEM overnight and then plated in Opti-MEM containing IL-2 (5 ng/ml) under a normoxic or hypoxic condition. Opti-MEM was incubated at 37°C at normoxia or hypoxia overnight before use. Hypoxia stability assay was conducted as described above, but cells were cultured in normoxia or hypoxia for 72 hours, then collected, and stained for Foxp3 for flow cytometry.

Glucose and amino acid restriction assays
nT<sub>reg</sub> cells were isolated as described above and cultured in either normal T cell medium, T cell medium lacking glucose (Thermo Fisher Scientific, catalog no. 11879020), or T cell medium lacking amino acids including glutamine (US Biological, catalog no. R9010-02) substituted with dialyzed FBS (Gibco, catalog no. A3382001) for 24 hours at 1 × 10<sup>5</sup> cells per well. T cell medium included 2000 U of IL-2 and CD3/CD28 beads as described above. iT<sub>reg</sub> cells were isolated and polarized as described above for 3 days. Following polarization, iT<sub>reg</sub> cells were cultured in normal T cell medium or T cell medium lacking glucose or amino acids for 24 hours. Both nT<sub>reg</sub> and iT<sub>reg</sub> cells were then collected, and RNA was extracted as described above. For stability assays, cells were cultured as described above for 48 hours, then collected, and stained for Foxp3 for flow cytometry.

Cell isolation and flow cytometry for analysis of splenic and intratumoral T<sub>reg</sub> cells
T cells were isolated from mouse spleen using a CD4<sup>+</sup> T cell negative isolation kit (STEMCELL Technologies, catalog no.17952) per the manufacturer’s instructions. To isolate murine and human tumor-infiltrating lymphocytes, subcutaneous tumors were cut into small fragments and digested by collagenase D (Sigma-Aldrich) and deoxyribonuclease (DNase) (Sigma-Aldrich) for 1 hour at room temperature before isolation with a CD45<sup>+</sup> positive selection kit (STEMCELL Technologies, catalog no. 100-0350). Murine-enriched cells were further sorted for YFP<sup>+</sup> (Foxp3<sup>+</sup>) using FACSaria (BD Biosciences). Human-enriched cells were further sorted for CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>-</sup> cells. Purity of sorted cells was >99%. Flow cytometry analysis of cells was done using FACSCanto II. Samples were initially incubated with CD16/32 antibodies to block antibody binding to Fc receptor. Single-cell suspensions were stained with relevant antibodies (table S2) and subsequently washed twice with cold PBS containing 3% FBS. For intracellular staining, cells were fixed, permeabilized, and stained for transcription factors using the Foxp3 Transcription Factor Staining Buffer Set (eBioscience, 00-5523-00) following the manufacturer’s instructions. For cytokine staining, cells were first stimulated for 4 to 5 hours with phorbol 12-myristate 13-acetate (PMA; 20 ng/ml) plus 0.5 μM ionomycin (Sigma-Aldrich) in the presence of monensin (10 μg/ml) (eBioscience) before staining. Data were analyzed with FlowJo software.

Cell lines, plasmids, antibodies, and reagents
Human embryonic kidney (HEK) 293 cells were purchased from the American Type Culture Collection (ATCC) and stored in the Fang laboratory. EG7 Lymphoma, LLC1 lung carcinoma, and B16-F10 melanoma cell lines were provided by the Zhang laboratory at Northwestern and used for tumor models as previously reported (14). The cell lines were cultured in DMEM with 10% FBS and were tested for mycoplasma using a LookOut Mycoplasma PCR detection kit (Sigma-Aldrich, MP0035-1KT). MYC–Usp22, Flag–Smad2, Flag–Smad3, Flag–Smad4, and hemagglutinin (HA)–ubiquitin pCMV expression plasmids and their tagged vectors were constructed by the Fang laboratory and stored at −20°C. All Western blot, coimmunoprecipitation, and flow cytometry antibodies are listed in table S2. PMA, ionomycin, and cycloheximide were purchased from Sigma-Aldrich. Monensin was purchased from eBioscience. Antibodies are listed in table S2.

RNA extraction for RNA sequencing and qPCR
RNA was extracted using the RNeasy Micro Kit (Qiagen, catalog no. 74004) from sorted Foxp3<sup>+</sup> (YFP<sup>+</sup>) T<sub>reg</sub> cells from mouse organs. qPCR was performed following the manufacturer’s protocol using gene-specific primer sets (table S3). RNA sequencing was
performed by the Northwestern RNA sequencing core. Elaboration is found in the Supplementary Materials.

**RNA sequencing and qPCR analysis**

RNA was extracted using the RNeasy Micro Kit (Qiagen, catalog no. 74004) from sorted Foxp3+ (YFP+) Treg cells from mouse organs. qPCR was performed following the manufacturer’s protocol using gene-specific primer sets (table S3). Real-time PCR data were analyzed using the ΔΔCt method with actin as the housekeeping gene. Gene expression was normalized to WT, untreated Treg cells unless otherwise noted. RNA sequencing was performed by the Northwestern RNA sequencing core. The quality of DNA reads, in fastq format, was evaluated using FastQC. Adapters were trimmed, and reads of poor quality or those aligning to ribosomal RNA (rRNA) sequences were filtered. The cleaned reads were aligned to the Mus musculus genome (mm10) using STAR (56). Read counts for each gene were calculated using htsseq-count (57) in conjunction with a gene annotation file for mm10 obtained from UCSC (University of California Santa Cruz; http://genome.ucsc.edu). Differential expression was determined using edgeR (58, 59). The cutoff for determining significantly differentially expressed genes was a false discovery rate (FDR)–adjusted P value of less than 0.05. Following differential expression analysis, GSEA was performed using GSEA v4.03 (https://pubmed.ncbi.nlm.nih.gov/16199517/). For each separate KO mouse (Usp21, Usp22, and dKO), an inclusion cutoff of FDR-adjusted P value of <0.01 was set. Lists of genes, ordered by log2(fold change), were then run with the GSEA preranked tool under standard parameters against the Hallmarks MSigDB 7.2 gene sets (www.ncbi.nlm.nih.gov/pmc/articles/PMC4707969/). Normalized enrichment scores were then plotted for gene sets enriched at an FDR q value of <0.25.

**ChIP-qPCR sample preparation**

iTreg cells (as described above) were used for immunoprecipitation to allow for ample cell number. Three million iTreg cells per immunoprecipitation were fixed in 37% formaldehyde for 10 min at 37°C. Glycine was then added to a final concentration of 0.125 M, and the samples were incubated for an additional 5 min at room temperature. The cells were subsequently washed with ice-cold PBS with 1× protease inhibitor cocktail (Roche, catalog no. 36363600). The ChIP assay was done using the Millipore ChIP Assay Kit (lot no. 3154126) following the protocol as described previously (14).

**Immunoprecipitation and Western blots**

Coimmunoprecipitation and Western blots were performed as previously described (14). For overexpression, plasmids were transfected into HEK293 (ATCC) cells for 24 hours before being resuspended in radioimmunoprecipitation assay (RIPA) buffer with 1× protease inhibitor cocktail. Primary cells were collected and directly resuspended in RIPA buffer with 1× protease inhibitor cocktail. Antibodies and plasmids used are listed in table S2. Membranes were then developed with enhanced chemiluminescence. The relative protein expression levels were quantified using Bio-Rad Image software and normalized with the loading controls unless otherwise noted.

**Ubiquitination assay**

For overexpression, Flag-Smad proteins and HA-ubiquitin plasmids were cotransfected into HEK293 cells using TurboFect Transfection Reagent (Thermo Fisher Scientific, R0532) along with either Myc empty vector or Myc-Usp22. After 24 hours, the cells were collected and immunoprecipitated with anti-Flag to pull down the relevant SMAD antibody and immunoblotted for HA-ubiquitin to assess SMAD protein ubiquitination in the presence or absence of functional Usp22. Whole-cell lysates controls were immunoblotted with horseradish peroxidase (HRP)–conjugated Myc and HRP-conjugated Flag to show transfection efficiency. For endogenous ubiquitination assay, iTreg cells were cultured as previously described, and cells were directly used for the assay. Either ubiquitin or Foxp3 antibodies were used for immunoprecipitation.

**Assessment of cellular metabolism**

The rates of mitochondrial oxygen consumption and glycolysis were measured using the Seahorse XF-96 analyzer (Agilent). Splenic Treg cells (YFP+) (250,000) were isolated by flow sorting and adhered to XF96 cell culture plates using Cell-Tak (Corning) per the manufacturer’s instructions. Cells were plated in XF RPMI medium (Agilent) supplemented with 1% FBS, 11 mM glucose, 2 mM glutamine, and 1 mM pyruvate to match normal concentrations of those metabolites in base RPMI. The basal mitochondrial OCR was determined by subtracting the antimycin A– and piericidin A–sensitive oxygen consumption from the basal mitochondrial oxygen consumption. The basal glycolytic rate was calculated by subtracting the 2-deoxyglucose–sensitive ECAR from the basal ECAR. Antimycin A and piericidin A purchased from Sigma-Aldrich were both used at a final concentration of 1 μM. 2-Deoxyglucose purchased from Sigma-Aldrich was used at a final concentration of 25 mM.

**Homology modeling of human Usp22**

The amino acid sequence of human Usp22 was retrieved from the sequence database of UniProt (www.uniprot.org/uniprot/ UniProt ID: Q9UPT9). UBP8 structure (PDB code 3MHS) was chosen as a template protein to construct the human Usp22 model. The homology modeling of the human Usp22 model was performed by three different homology modeling programs including SWISS MODEL, 1-TASSER, and MODELLER. Subsequently, the quality of the Usp22 models was checked with structure validation programs including PROCHECK and Verify_3D programs. Last, the best model was subjected to energy minimization for removing clashes between side chains using GROMACS 5.1.5 and subsequently applied in structure-based virtual screening.

**Virtual screening**

Specs (www.specs.net) database containing 212,558 compounds was first filtered by lead-like properties (180 < MW < 480, −0.5 < ClogP < 5.5, PSA < 140 Å, 0 < rotational bonds < 10, 0 < donor and acceptor < 13) (MW=molecular weight; PSA=polar surface area) and led to a total of 102,442 compounds using DrulITo software. The three-dimensional geometry of the ligands was optimized, minimized, and prepared using Open Babel. The virtual screening was performed using AutoDock Vina program. The docking grid was created with the grid points 16, 18, and 16 and defined as coordinates of the center of binding site with 37.64, 11.33, and 70.33 for x, y, and z dimensions, respectively. The Genetic Algorithms were selected to perform the molecular docking and keeping other docking parameters in default. The
top 100 compounds ranked by docking affinity were selected for further study. Moreover, residual interactions at the protein-drug interface were evaluated using LigPlot.

**Molecular docking mechanism**

Molecular docking result showed that compound S02 stably binds in the Usp22 catalytic domain pocket, mainly in the hydrophobic effect provided by the amino acid residues His471, Gln261, Gly478, Glu476, Leu475, Asp262, Arg419, Tyr480, and Phe412. It is worth noting that not only the benzene ring on compound S02 forms a hydrophobic interaction with the Tyr480 residue but also the hydrogen atom on the anilino group of ligand acts as hydrogen donor to form a hydrogen bond with the hydrogen acceptor on the Tyr480 residue. Binding stability indicates that the anilino functional group may play an important role in the inhibitory activity of Usp22 (Fig. 1D).

**MD simulation**

The conformation of the complexes formed between ligands and Usp22 receptor protein was predicted using AutoDock Vina program, and all of the MD simulations were performed by GROMACS 5.1.5 with Amber99sb force field. After MD simulation, the molecular mechanics energies combined with the MM/PBSA methods were used to calculate the binding free energy of ligands with Usp22 receptor protein. Structural stability of complexes was compared by analyzing RMSD and the hydrogen-bonding interactions throughout the trajectory.

**In vitro inhibitor assays**

All nTreg and iTreg were plated as described above. dMOG (Sigma-Aldrich, catalog no. D3695) was administered to the cells in relevant experiments at 1 mM for 24 hours. Oligomycin (Sigma-Aldrich, catalog no. 75351) was administered at 1 μM to the medium of the cells in relevant experiments for 24 hours. AICAR (Sigma-Aldrich, catalog no. 52871) was administered to relevant cells for 24 hours. Torin1 (Millipore, catalog no. 475991) was administered to the relevant cells at 250 nM for 24 hours. Foxp3 protein level was assessed via flow cytometry following 48 hours of treatment of inhibitors described above. In vitro administration of Usp22i-S02 was at 10 μg/ml.

**Usp22i-S02 in vivo inhibitor experiments**

LLC1 cells were transplanted into 6- to 8-week-old C57BL/6 or RAG−/− male mice. Subcutaneous injections were performed in the right flank of mice in a final volume of 100 μl using 1 × 10^6 cells per injection. The Usp22i-S02 was injected intraperitoneally at a concentration of 20 mg/kg per time, in 100 μl of oil, twice a day for 3 to 5 days beginning on either the first day of the LLC1 cell injection or when tumors reached 100 mm^3. Control animals received 100 μl of oil alone. Subcutaneous tumor diameters were measured daily with calipers until any tumor in the mouse cohort reached 2.5 cm in its largest diameter. Cells were processed and analyzed as stated above.

**Sample definition and in-laboratory replication**

Each graphed dot/n represents a biological replicate. Each n is stated after the corresponding figure legend section. In-laboratory replication is described for each experiment in table S4.

**Statistics and data availability**

No statistical methods were used to predetermine the sample size. The experiments were not randomized. The investigators were not blinded to allocation during experiments and outcome assessment. All statistical analyses were computed with GraphPad, and tests used for each experiment are listed in the figure legends. Analyses of variance (ANOVA s) with multiple comparisons between rows were corrected with Tukey’s test to determine statistical significance. Two-tailed unpaired t tests were performed with Welch’s correction.

**Supplementary Materials**

This PDF file includes:
- Figs. S1 to S12
- Tables S1 to S4

View/request a protocol for this paper from Bio-protocol.

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