Inhibiting tumor cell-intrinsic UBA6 by inosine augments tumor immunogenicity

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Abstract

Metabolic alteration influences cancer immunity. However, the role and mechanism of metabolic adaption on immune checkpoint blockade (ICB) responses remains ill-defined. Here, to identify metabolites that modulate ICB sensitivity, metabolomic profiling in mouse tumor models and cancer patients treated with ICB was performed. We identified that metabolite inosine was associated with ICB sensitivity in mice and humans, and overcame ICB resistance in several mouse tumor models. Notably, inosine sensitized tumor cells to T cell-mediated cytotoxicity by amplifying tumor-intrinsic immunogenicity. Chemical proteomics further identified that inosine directly bound and inhibited ubiquitin-activating enzyme UBA6. Tumor UBA6 loss augmented tumor immunogenicity and substituted the synergistic effect of inosine in combination with ICB. Clinically, tumor UBA6 expression negatively correlated with ICB response in cancer patients. Thus, we reveal an unappreciated function of inosine on tumor-intrinsic immunogenicity and provide UBA6 as a candidate target for immunotherapy.

Introduction

Despite the success of immune checkpoint blockade (ICB) including anti-PD-1 or anti-CTLA-4 therapies in advanced cancer ^{1,2}, a considerable proportion of patients remain unresponsive to these treatments and resistance can develop in patients who initially respond ³⁻⁷. Thus, the rational and combinatorial strategies to improve ICB response by overcoming these resistance mechanisms will be emerging. Interestingly, there is growing evidence that metabolic alterations modulate tumor immunity⁸⁻¹². However, the detailed understanding of the effect of the metabolic alterations on immunotherapy responses has remained exclusive. Here, we sought to dissect the impact and mechanism of the metabolic alterations on ICB responses in mounting a more effective immunotherapy response.

In this study, we employed untargeted metabolomics analyses of large-scale cancer patient' cohorts and mouse tumor models with ICB treatment to reveal that inosine enhances antitumor immunity. Unexpectedly, we specifically pinpointed that inosine inhibited UBA6 in tumor cells to overcome tumor-intrinsic resistance to ICB by augmenting tumor immunogenicity.

Results

A metabolic screen identifies inosine is associated with immunotherapy responses in mice and humans

To better understand the association of metabolic alterations and ICB responses, we performed untargeted metabolomics of plasma samples from B16-F0 tumor-bearing mice with vehicle or ICB (anti-PD1 plus anti-CTLA4) treatment (Supplementary Fig. 1a). The metabolic profiling revealed that the relative abundance of 5.3% (13/244) metabolites were significantly altered in the B16-F0 tumor-bearing mice with ICB treatment (Supplementary Fig. 1b, c and Table 1). Notably, 5 of these 13 changed metabolites were involved in purine metabolism including inosine, guanosine, hypoxanthine, and

xanthine (Supplementary Fig. 1a-c and Table 1). Interestingly, consistent with previous report^{13,14}, depletion of gut microbiota with an antibiotic cocktail (Abx) significantly compromised the efficacy of ICB in the B16F0 mouse model, and the levels of these purine metabolites were also significantly decreased in antibiotics-treated mice (Supplementary Fig. 1d-f), indicating that these purine metabolites might be partly dependent on gut microbiota and contribute to ICB responses.

Moreover, we reanalyzed the metabolic profiling of renal cell carcinoma (RCC) patients, among which 394 received nivolumab, a PD1 checkpoint blockade, and 349 received everolimus, an mTOR inhibitor (Phase III trial: CheckMate 025, NCT01668784)^{16,17}. The results showed that the plasma level of 37/202 metabolites was associated with overall survival (OS) of cancer patients treated with nivolumab ($P < 0.01$) (Fig. 1a and Supplementary Table 2). Notably, Venn diagram analysis demonstrated that among all identified metabolites, only inosine was significantly associated with ICB response in both mice and humans (Supplementary Fig. 1g). Specifically, the higher level of inosine was associated with the longer overall survival of cancer patients only in the setting of nivolumab treatment (High: mOS = 33 months; Low: mOS = 22 months), but not in cancer patients treated with everolimus (an mTOR inhibitor) (Fig. 1b), indicating that high inosine had durable benefit specifically for ICB treated patients. Interestingly, consistent with the mouse model (Supplementary Fig. 1c), the relative abundance of inosine was reduced after nivolumab treatment in RCC patients (Supplementary Fig. 1h), suggesting inosine consumption might be increased by ICB treatment. Collectively, these findings indicate that metabolite inosine is associated with immunotherapy responses in both mice and humans.

Inosine augments ICB immunotherapy responses in vivo

The strong association of inosine level and ICB responses suggests a potential role of inosine in enhancing immune response. This prompted us to investigate whether systemic administration of inosine could augment immunotherapy response *in vivo*. Although inosine has been used as a dietary supplement or immunomodulatory drug for several decades¹⁸, its application in cancer immune therapies remains exclusive. Indeed, inosine alone significantly reduced tumor growth in the B16-F0 model (Fig. 1c). Strikingly, mice in the combined inosine with ICB treatment regimen (Combo) had the best response in the B16-F0 model (Fig. 1c). Next, we moved forward to assess the efficacy of inosine in combination with ICB in the B16-GMCSF, which resistant to ICB¹⁹. Surprisingly, the combo treatment overcame the resistance to ICB and resulted in the elimination of more than 80% of ICB-resistant B16-GMCSF tumors (Fig. 1d and Supplementary Fig. 2a-c). Most importantly, the combo treatment increased the overall survival of B16-GMCSF tumor-bearing mice in comparison with either inosine or ICB alone (Fig. 1d).

To test whether the synergistic effect in the B16-GMCSF model also extends to other ICB-resistant models, we evaluated the role of inosine and ICB combination therapy in the 4T1 tumor model (murine triple-negative mammary carcinoma in Balb/c background), which was aggressive and highly resistant to ICB treatment. Consistent with the B16 melanoma models, inosine and ICB combination therapy can

synergize and promote long-term survival of 4T1 tumor-bearing mice, whereas the combination of inosine and ICB led to complete remissions in 50% of 4T1 tumor-bearing mice (Fig. 1e and Supplementary Fig. 2d-f). Moreover, we identified the synergistic efficacy of isoprinosine, an inosine derivative, in combination with ICB in the 4T1 model (Supplementary Fig. 2g-i). Collectively, given inosine is a safe, naturally occurring purine with non-toxic to humans, coupled with our preclinical evidence showing its synergic effect with ICB, it is worthwhile to repurpose the therapeutic potential of inosine for enhancing cancer patient response to immunotherapies.

Inosine inflames the tumor immune microenvironment (TIME)

To provide a more comprehensive and unbiased assessment of the effect of inosine on TIME, single-cell RNA sequencing (scRNA-seq) of CD45⁺ immune cells in the 4T1 model was performed. We obtained single-cell transcriptomes for 16,199 CD45⁺ cells in the control group, 9,842 in the Combo group. To define the intratumoral cell populations, we performed canonical correlation analysis to computationally combine data from two treatment groups, then conducted graph-based clustering and dimensionality reduction with UMAP to respectively identify and visualize transcriptionally homogeneous clusters of immune cells (Fig. 1f and Supplementary Fig. 2j, k). Using the SingleR package, we further annotated the clusters by directly comparing their transcriptional state with that of known populations and the assessment of cell-type-specific markers²⁰. We compared the immune microenvironment of combo and control 4T1 tumors and found a significant increase in Ki67⁺ CD8⁺ T cells in combo treated tumors that were infiltrated throughout the tumor with an increase in CD8⁺/Treg ratio following combinational treatments, as determined by manual gating analyses and reflect the induction of an effective immune response by inosine in combination with ICB (Supplementary Fig. 2k). Moreover, analysis of myeloid cells in the combination regimen of inosine with ICB showed that the addition of inosine reduces the immunosuppressive microenvironment by increased the M1/M2 ratio, resulting in improved T cell effector function in 4T1 tumors (Supplementary Fig. 2j, k). Notably, the addition of inosine with ICB treatment caused a striking shift of immunosuppressive to inflammatory TIME characterized by the decreased accumulation of M2 macrophages and Tregs, and the increased abundance of M1 macrophages and effector CD8⁺ T cells (Fig. 1f and Supplementary Fig. 2j, k). Specifically, a substantial increase in tumor-reactive gp70 tetramer-specific CD8⁺ T cells in 4T1-bearing mice with combo treatment was also identified by Flow Cytometric Analysis ($P < 0.01$; Fig. 1g), indicating the strong specific antitumor immunity after addition of inosine. Thus, these findings suggest that inosine in combination with ICB inflames TIME to provoke a strong antitumor immune response.

Inosine sensitizes tumor cells to T cell-mediated killing by enhancing tumor-intrinsic immunogenicity

To reveal the mechanism by which inosine influences antitumor immunity, we set up the different strategized *in vitro* co-culture platforms of T cell-mediated tumor cell killing assay to evaluate the effect of inosine on tumor cells and T cells simultaneously. Despite previous reports have indicated the multiple

immunomodulatory roles of inosine on immune cells under different conditions^{18,21}, we did not find the stronger T cell-mediated tumor killing in B16-GMCSF and 4T1 tumor cells when we pretreated T cells with inosine compared to untreated control (Fig. 2a-c). However, when we pretreated tumor cells with inosine and then co-cultured tumor cells with activated T cells, we found that both 4T1 and B16-GMCSF tumor cells were dramatically sensitive to T cell-mediated cytotoxicity, as indicated by the lower cell viability in the inosine-pretreated group compared to unpretreated control (Fig. 2d-e), suggesting the direct effect of inosine on tumor cells.

Notably, inosine didn't directly influence the proliferation and apoptosis of 4T1 or B16-GMCSF tumor cells (Fig. 2f-h). Importantly, we further identified that inosine markedly potentiated MHC-I upregulation (Fig. 2i). In addition, inosine treatment increased the expression of related genes involved in antigen processing/presentation and IFN- γ responses in 4T1 or B16-GMCSF tumor cells (Fig. 2j-k), establishing the functional importance of inosine on tumor cell immunogenicity. Thus, our data indicate that inosine renders tumor cells more sensitive to T cell-mediated tumor killing by directly modulating tumor cell immunogenicity.

Inosine binds and inhibits UBA6 activity of tumor cell

To directly explore by which inosine elicits the tumor immunogenicity, chemical proteomics screening following a LiP-small molecule mapping (LiP-SMap) workflow²² in 4T1 cell lysate was performed to identify the functional proteins potentially binding with inosine in tumor cells (Fig. 3a). Significant changes in the abundance of half-tryptic peptides (fold change > 2 or < 0.5, $p < 0.001$, > 2 peptides per protein) were a readout for structural changes induced by the binding of inosine. Out of 2470 proteins, only 23 proteins fulfilled these stringent criteria (Fig. 3b-c and Supplementary Fig. 3a, Table 3).

We further identified which candidates binding with inosine involved in immune cell-mediated tumor killing. The gene knockout phenotype from genetic screens profiling regulators of lymphocytes-mediated tumor-killing resistance based on several CRISPR genetic screen datasets was analyzed²³. Out of 23 candidates, only *Uba6* deletion in tumor cells enhanced the T cell²⁴ (Fig. 3d and Supplementary Fig. 3b) or NK cell-mediated tumor-killing²⁵ (Supplementary Fig. 3c). UBA6, ubiquitin-like modifier activating enzyme 6, is one of the ubiquitin-activating enzymes which activates and transfers the ubiquitin to the subsequent proteins to serve as the starting enzyme for the extensive downstream ubiquitination cascades²⁶. Besides, UBA6 also activate the ubiquitin-like proteins FAT10 and transfer FAT10 to its substrate proteins, leading to its proteasomal degradation independently from ubiquitin²⁷. Owing to the central role in UBA6-dependent post-translational modification, UBA6 participates in multiple pathogeneses of diseases. However, how inosine regulates UBA6 activity to modulate immunotherapy is unclear.

Considering the bispecific effect of UBA6 on ubiquitin and FAT10 using a similar mechanism with greater affinity for FAT10²⁸, we identified that the main effects of inosine on UBA6 activity likely is through FAT10 (Fig. 3e-f and Supplementary Fig. 3d-e). Inosine had a moderate effect on UBA6-mediated transfer

ubiquitin *in vitro* (Supplementary Fig. 3d-e). By contrast, we found that inosine reduced the interactions between UBA6 and USE1 in HEK293 cells and directly inhibited UBA6-mediated transfer of FAT10 *in vitro* (Fig. 3e-f), in which FAT10-dependent degradation machinery was linked to antigen processing pathway and inflammatory signaling pathway^{29,30}. Moreover, the deletion of UBA6's UFD domain, which is responsible for the interaction between UBA6 and USE1, led to the loss of function of UBA6 on USE1 ubiquitination and abolished the effect of inosine on the interaction between UBA6 and USE1 in HEK293 cells (Supplementary Fig. 3f-g). Functionally, loss of UBA6 in tumor cells sensitized tumor cells to the cytotoxicity of T cells and abolished the effect of inosine on T cell-mediated tumor killing (Fig. 3g). Collectively, our results indicate that inosine sensitizes tumor cells to T cell-mediated killing by directly inhibiting UBA6 activity.

Inosine and genetic inhibition of UBA6 increases tumor immunogenicity

To decipher the molecular mechanism of the UBA6 effect on tumor cells, we analyzed the transcriptome of *Uba6*-null 4T1 cells and WT 4T1 cells by RNA-seq (Supplementary Fig. 4a and Table 4). Remarkably, enrichment analysis revealed that *Uba6*-null tumor cells had a marked increase in gene expression profiles evoked by inflammatory cytokines, such as TNF- α , IFN α , and IFN γ (Fig. 4a-c and Supplementary Fig. 4b, Table 5). The qPCR analysis confirmed the upregulation of TNF- α and IFN response genes, and antigen presentation-related genes in *Uba6*-null 4T1 cells (Supplementary Fig. 4c-e).

In addition, proteomic profiles also confirmed that the higher engagement of the IFN signaling pathway and inflammatory response signaling in *Uba6*-null tumor cells, showing consistency between our proteomic and transcriptomic data sets (Fig. 4d and Supplementary Fig. 4f). The flow analysis proved the upregulated cell surface MHC-I protein expression in *Uba6*-null tumor cells (Supplementary Fig. 4g), which was consistent with the effect of inosine.

Notably, *Uba6* deletion in 4T1 cells reversed the effect of inosine on the expression of immune response-related genes (Fig. 4e), confirming the inhibitory effect of inosine on UBA6 in tumor cells. Functionally, *Uba6*-null 4T1 and B16-GMCSF cells showed markedly decreased cell viability when stimulated with TNF α and IFN γ (Fig. 4f and Supplementary Fig. 4h), which mimics the functional feature of inosine on T cell-mediated tumor killing by targeting tumor cells, as TNF and IFN γ are major cytolytic cytokines released by cytotoxic CD8⁺ T cells. Thus, UBA6 deletion in tumor cells primed tumor cell-intrinsic immune response and ablated the effect of inosine on gene expression of immune response signaling.

UBA6 loss substitutes the effect of inosine on immunotherapy response

We subsequently assessed the role of UBA6 for the synergistic efficacy of inosine in combination with ICB *in vivo*. *Uba6* deficiency in B16-GMCSF cells did not markedly affect tumor growth and survival in

NSG mice, and *Uba6*-null B16-GMCSF cells did not show any growth disadvantage *in vitro* (Fig. 5a and Supplementary Fig. 5a-c). By contrast, the *Uba6*-null B16-GMCSF tumor showed a reduced tumor volume and improved survival in WT mice (Fig. 5a and Supplementary Fig. 5a-b). However, the effect of inosine in combination with ICB on tumor growth was abolished in *Uba6*-null B16-GMCSF tumor-bearing WT mice, compared with that of ICB treatment (Fig. 5b and Supplementary Fig. 5d-e).

Consistent with the *Uba6*-null melanoma model, *Uba6*-null 4T1 tumors implanted in NSG mice showed a modest reduction in tumor volume and limited benefit in survival (Fig. 5c and Supplementary Fig. 5f-h), whereas *Uba6*-null 4T1 cells did not show any growth disadvantage *in vitro* (Supplementary Fig. 5f-h). In WT mice, *Uba6*-null 4T1 tumors were completely rejected within two weeks (Fig. 5c and Supplementary Fig. 5h). Notably, ICB or combination with inosine treatment did not exhibit further benefits (Fig. 5d and Supplementary Fig. 5i-j). The dramatic biology of *Uba6*-null 4T1 and B16-GMCSF tumor-bearing mice was consistent with the relatively higher expression of UBA6 in these two tumor cell lines (Supplementary Fig. 5k), indicating the potential application of UBA6 as a diagnostic or predictive biomarker for immunotherapy.

UBA6 expression predicts immunotherapy responses in clinical patients

Finally, we investigated the relationship between UBA6 expression and immunotherapy response in cancer patients. UBA6 was highly expressed in human tumors compared to normal tissues (Supplementary Fig. 6a) and low UBA6 expression was associated with improved overall survival of patients in several tumor types (Supplementary Fig. 6b-c). Using the computational TIDE datasets³¹, we found that a higher CTL level was associated with better survival in melanoma patients with a low expression of UBA6, but not a high expression (Fig. 6a). Moreover, this correlation was also obtained in cohorts with metastatic triple-negative breast cancer (TNBC) and lung cancer (Supplementary Fig. 6d-e). This observation indicates the potential important function of UBA6 in initiating immunotherapy resistance.

To directly evaluate the relationship of UBA6 expression and ICB responses, we analyzed the clinical dataset in a melanoma cohort treated with anti-CTLA4³² and observed that UBA6 expression was significantly predictive of the progression-free survival of ICB treated patients (Fig. 6b). Similar trends were observed in two additional independent cohorts of melanoma patients treated with anti-PD1^{33,34} (Supplementary Fig. 6f-g). In our cohort, we found that UBA6 expression in lung cancer patients who had clinical benefit from anti-PD1 based immunotherapy was lower than that in patients who did not respond to anti-PD1 treatment (Fig. 6c-d and Supplementary Fig. 6h-i). This finding suggested the predictive role of UBA6 expression for immunotherapy responses.

Discussion

Our results demonstrate that inosine overcomes tumor cell-intrinsic resistance to immunotherapy by inhibiting UBA6 in tumor cells to enhance tumor immunogenicity (Fig. 7). We identify UBA6 functions as a tumor-intrinsic checkpoint that limits antitumor immunity and implicate UBA6 as an attractive target for immunotherapy. Together with recent studies^{35,36}, our findings highlight the potential application of inosine in combination with ICB for cancer patients with high UBA6 expression.

The metabolic alterations in some cancer patients following treatment with ICB generate an immunosuppressive tumor microenvironment (TME) that orchestrates the resistance to immunotherapy response¹¹. Notably, the immunosuppressive TME is characterized by metabolic imbalance, such as nutrient shortage and abundant immunosuppressive metabolites adenosine³⁷. Inosine is a naturally occurring metabolite of adenosine and the circulating level of inosine is impacted by diet, genetic, and drugs^{38,39}. Gut microbiota may contribute to inosine level because it has been demonstrated that fecal microbiota transplantation or probiotics can reverse inosine depletion *in vivo*^{36,40,41}. Moreover, inosine is synthesized and secreted by cancer cells⁴². But how immunotherapy alters the circulating level of inosine will be further explored.

Despite emerging evidence indicating that inosine has potent immunomodulatory effects^{21,41}, the mechanisms underlying the effect of inosine remain incompletely understood. Recent studies demonstrated that inosine improves immunotherapy response by being an alternative carbon source for CD8⁺ T-cell function under glucose restriction³⁵ or directing the differentiation of Th1 cells in an A_{2A}R-dependent manner³⁶. Aside from the effect of inosine on T cells, we surprisingly identified that the increased tumor cell immunogenicity also contributed to the function of inosine for driving antitumor immunity and enhancing current immunotherapy. The complementary mechanisms of inosine on tumor cells in combination with ICB targeting T cells reasonably explain the superiority of combinational therapy in multiple murine cancer models. Thus, these findings in certain contexts indicate the complex and multiple action modes of the interactions between inosine and antitumor immunity.

Recently, it is recognized that beyond their roles as energy sources, metabolites serve as signals that trigger adaptive responses by functional interactions between metabolites and proteins²². Notably, our chemical proteomics indicated the specific binding of inosine to UBA6, and *in vitro* biochemical assay validated the inhibitory effect of inosine on UBA6 activity. UBA6 is a ubiquitin-activating enzyme that activates ubiquitin and ubiquitin-like protein, FAT10²⁶. UBA6 plays an important role in embryogenesis and multiple pathogenesises of diseases^{27,28}, however, the impact of UBA6 on tumor-intrinsic immunogenicity has been never addressed before. Interestingly, a recent report indicates the important role of ubiquitin-proteasome system (UPS) dysregulation in human cancer and underscores the potential therapeutic utility of targeting the UPS⁴³. Despite abnormal expressions in UBA6 are found in several types of carcinomas, the function of UBA6 in antitumor immunity and immunotherapy is unclear. Here, a systematic series of genetic loss-of-function studies showed that loss of function of UBA6 in tumor cells led to tumor inflammation, and overcame resistance to ICB immunotherapy. These data for the first time indicate a critical role for UBA6 in the function of antitumor immunity and ICB therapy. However, the

detailed molecular mechanism of how inosine modulates UBA6 and further details about the UBA6-dependent cell-intrinsic effects remain to be defined in cancer patients.

Altogether, the findings of our proof-of-concept study not only provide molecular insight into how inosine triggers antitumor immunity but also suggest the application of inosine or targeting UBA6 for more effective immunotherapy.

Materials And Methods

Mice and cell lines

Female WT C57BL/6, BALB/c, and NOD-SCID IL2Rg^{null} (NSG) mice (6–8 weeks old) were purchased from Shanghai Jie Si Jie Laboratory or Beijing Biocytogen and allowed to acclimatize for 1–2 weeks before experimentation. All animal experimental procedures were approved by the Institutional Animal Care and Use Committee of Shanghai General hospital affiliated with Shanghai Jiao Tong University School of Medicine (2019-A012-01).

The cell lines B16-F0, B16-F10, and 4T1 were obtained from the American Type Culture Collection (ATCC). B16-GMCSF cells were generated by retroviral-mediated gene transfer, following the previously described⁴⁴. B16-GMCSF-OVA was ovalbumin (OVA)-transfected clone derived from B16-GMCSF cells which were transfected with the plasmid pCI-neo-mOVA (Cat. 25099, Addgene). Cells were cultured using RPMI-1640 (Corning) with 10% fetal bovine serum (Corning) and 1% Pen/Strep (GIBCO). Cells were incubated in an incubator maintained at 37° C and 5% CO₂.

Tumor challenge and treatment

For the B16 tumor challenges, 2×10^5 B16-F0 or B16-GMCSF tumor cells were resuspended in Hanks balanced salt solution (Gibco) and intradermally (i.d.) injected into the right flank of C57BL/6J mice on day 0. For the 4T1 model, 2×10^5 4T1 cells were orthotopically injected into the mammary fat pad of BALB/c mice on day 0. For studies in immune-compromised mice, the *Uba6*-null or control 4T1 cells were done in the NSG mice. Treatments were given as single agents or in combinations with the indicated regimen for each drug. Inosine (Cat. 4060, Sigma-Aldrich) was administered by oral gavage once a day at 400mg/kg. Control groups received vehicles (sterilized water). Treatment was initiated on day 4 and ended on day 21 post tumor implant. The combination of Rat monoclonal anti-CTLA4 antibody (100μg per mouse, clone 9H10, Bio X Cell) and anti-PD1 antibody (200μg per mouse, clone RPM1-14, Bio X Cell) (ICB) treatment were injected intraperitoneally (i.p.) on days 7, 10, 13 and 16 for the indicated tumor models. Rat IgG2a isotype control was used in control mice corresponding to the ICB treatment group. Each tumor was measured every 3 days with a caliper beginning on day 7 after the challenge until either the survival endpoint was reached, or no palpable tumor remained. Tumor volume was calculated using the formula: $(L \times W^2)/2$ and expressed as mm³. Mice that had no palpable tumors that could be measured on consecutive measurement days were considered complete regressions.

T cell-mediated cytotoxicity assays

CD8⁺ T cells were isolated from the spleen of Balb/c or C57BL/6 mice using a CD8a⁺ T cell isolation kit (Miltenyi Biotec, Germany) according to the manufacturer's protocol. and then cultured in complete RPMI 1640 media (10% FBS, 20 mM HEPES, 1 mM sodium pyruvate, 0.05 mM 2-mercaptoethanol, 2 mM L-glutamine, and 50 U/ml of streptomycin and penicillin). Freshly isolated CD8⁺ T cells were stimulated with anti-CD3/CD28 antibody (BioLegend, USA) to induce differentiation into an effector state. On day 3, recombinant mouse IL-2 (BioLegend, USA) was added to the culture at 20 ng/ml. For the generation of activated OT-1 T cells, splenocytes were harvested from the spleen of OT-1 transgenic mice and stimulated with 100 ng/mL of OVA peptide (SIINFEKL) for 24 hr to expand CD8⁺ OT-1 T cells. After washing to remove the peptide, cells were cultured in media for an additional 2 days before use in co-culture assays. 4T1 and B16-GMCSF cells were maintained in complete RPMI-1640 media. For the effect of inosine on T cells, isolated CD8⁺ T cells were pretreated with a serial dilution of inosine for 48 hours during T cell activation. After washed, in vitro-activated CD8⁺ T cells co-cultured with indicated tumor cells at a different effector to target ratios. For the effect of inosine on tumor cells, 4T1 cells or B16-GMCSF-OVA were seeded and pretreated with a serial dilution of inosine for 48 hours, after washed and then co-cultured with activated CD8⁺ T cells or OT-1 T cells respectively at a different effector to target ratios. Tumor cells were plated at equal density in all wells and activated CD8⁺ T cells were added at target-to-effector 1:0, 1:2, 1:5 ratio (Target: Tumor cells; Effector: activated CD8⁺ T cells). Cell viability is calculated as the quantification of the number of live cells and is also expressed as relative cell viability by calculating the fold change (FC) of remaining alive target tumor cells following the incubation with T cells at the indicated inosine treatment compared to that in the untreated control. After a two or three-day co-culture with T cells, the number of viable tumor cells was counted using the automated cell counting system.

RNA-seq transcriptome analysis of tumor cells

Total RNA of *Uba6*-null or sgCtrl 4T1 cells was extracted from cell pellets and libraries prepared with the NEB Next Ultra Directional RNA Library Prep Kit for Illumina (New England Biolabs, USA) were sequenced on an Illumina NextSeq 500 instrument. Clean reads obtained by filtering the raw reads with Cutadapt (v 1.9.1) were aligned to the mouse reference genome (assembly GRCm38) using the HISAT2 v2.1.0. and subsequently assembled using Stringtie (v 1.3.3). Cuffdiff (v1.3.0) was applied to calculate Fragments Per Kilobase of exon per Million fragments mapped (FPKMs) for coding genes in each sample, and differentially expressed genes calling was applied using DESeq2 (v 1.30.1), in which significance was assessed by Benjamini-Hochberg False Discovery Rate (FDR) to account for multiple hypothesis testing. ClusterProfiler (v 3.18.1) was used to annotate genes with gene ontology (GO) terms and perform GSEA using the Hallmark gene signature collection from mSigDB ⁴⁵. Ingenuity Pathway Analysis (QIAGEN) was used for ingenuity upstream regulator analysis ⁴⁶.

Proteomics analysis of tumor cells

About 10^7 of *Uba6*-null and sgCtrl 4T1 cells were suspended in a solution of 9.5 mol/L urea, 1% DTT, 40 ml/ml protease inhibitor cocktail, 0.2 mmol/L Na₂VO₃, and 1 mmol/L NaF. The mixture was centrifuged at 40000 x g at 15°C for 1 h and the supernatant was collected. The sequencing-grade trypsin was added to the supernatant containing about 1.5 mg of protein at an enzyme-to-protein ratio of 1:50 and incubated at 37°C for 14 h. The peptides were desalted using a 1.3 ml C18 solid-phase extraction column (Sep-Pak® Cartridge) (Waters Corporation, Milford, USA) and analyzed by two-dimensional (2D) strong cation-exchange, (SCX)/reversed-phase (RP) nano-scale liquid chromatography/mass spectrometry (2D-nanoLC/MS). Proteins and peptides were identified using a target-decoy approach with a reversed database and queried against the Mouse UniProt FASTA database. The quantification of peptides and proteins with “label-free quantification” (LFQ) was performed by MaxQuant.

Identification of proteins interacting with inosine

Chemical proteomics by LiP-SMap approach was performed as in previous studies²². At first, 4T1 cells were lysed by bead-beating in PBS at 4°C. After centrifugation at 16,000 g for 10 min at 4°C, the supernatant was collected and aliquoted in equivalent volumes containing 100 µg proteins each. To identify the proteins that interacted with inosine, 0.33 nmol/µg (total protein) of inosine was added to each aliquot and incubated at 25°C for 10 min. Limited proteolysis was conducted by adding protein kinase K (Sangon Biotech, China) at a 1:100 enzyme/substrate ratio. The generated protein fragments were digested by trypsin with a 1:50 trypsin/substrate ratio to generate peptides for mass spectrometry analysis. Peptide fragments were analyzed by Nano Acuity Ultra High-Pressure liquid chromatography coupled with Thermo Q Exactive mass spectrometer (Thermo Fisher, USA). Proteins and peptides were identified using a target-decoy approach with a reversed database and queried against the Mouse UniProt FASTA database. The quantification of peptides and proteins with “label-free quantification” (LFQ) was performed by MaxQuant.

Thioester Assay

Recombinant human ubiquitin and FAT10 were purchased from Boston Biochem. Plasmids pEnter-UBA6 and pEnter-USE1 were used for the expression of Flag-UBA6 and Flag-USE1. The two plasmids were transfected into HEK293, respectively, using Lipofectamine 2000 reagent. Purification of Flag-UBA6 and Flag-USE1 were carried out using Anti-FLAGM2 Affinity Gel (Sigma) as described by the manufacturer's instructions. Flag-UBA6 (0.5µM) and Flag-USE1 (0.5µM) were incubated with vehicle, inosine, or TAK243 at room temperature for 15min. Then, ubiquitin (5µM) or FAT10 (2µM) with ATP (250µM) were added. The reaction mixtures were incubated at 37°C for 30 min before 2×Lammi sample loading buffer was added to quench the reaction. The thioester detection was fractionated by SDS-PAGE under nonreducing conditions and immunoblotted with anti-UBA6 antibody (Proteintech, 1:1000) and anti-USE1 antibody (ABclonal, 1:1000).

Generation of CRISPR-edited tumor cell lines

Uba6 was deleted in Cas9-expressing 4T1 and B16-GMCSF mouse tumor cell line for validation experiments using a lentiviral delivery system (*lentiCRISPR v2*, Addgene) to express sgRNAs, and

puromycin selection. For determining the knockout efficiency of the *Uba6* gene, Western Blotting was used to measure the protein expression of UBA6 in sgCtrl control and sg*Uba6* 4T1 or B16-GMCSF cells. The *Uba6*-null 4T1 or B16-GMCSF cells were selected for experiments.

CRISPR sgRNA sequences:

Control sgRNA 1: GCGAGGTATTGGCTCCGCG

Control sgRNA 2: ATGTTGCAGTTGGCTCGAT

Uba6 sgRNA 1: AAGTCCTGTGTCTTCTTAAG

Uba6 sgRNA 2: AAATCGATGATGGATTATAC

Metabolomic analysis in tumor-bearing mice

Plasma metabolites in B16-F0 tumor-bearing mice or Abx-treated mice were measured. A total of 244 metabolites in plasma were detected by ultra-high-performance liquid chromatography (HPLC) coupled with a tripleTOF 5600 plus mass spectrometer (Applied Biosystems, USA). The metabolomic data were analyzed by pattern recognition analyses (principal component analysis and Heat-map).

Antibiotic treatments

Six-week-old C57BL/6J mice were treated with a cocktail of broad-spectrum antibiotics (1 g/L ampicillin, 1 g/L neomycin, 1 g/L metronidazole, and 0.5 g/L vancomycin) in drinking water for 3 weeks. The mice were allowed 3–4 days to recover before tumor implants. For measuring the levels of purine metabolites, the fresh fecal pellets, and plasma were collected at day 0 after two hours in collection cages with a paper liner. For evaluating the effect of ICB on tumor growth, IgG2a or a combination of anti-CTLA4 and anti-PD1 Abs (ICB) treated B16-F0 tumor-bearing mice as the indicated time points.

The correlation analysis between survival and metabolites in human cancer patients

We reanalyzed the public dataset ¹⁷ regarding the metabolic profiles of 743 renal cell carcinoma (RCC) patients (Phase III trial: CheckMate 025, NCT01668784), among which 394 patients received nivolumab and 349 patients received everolimus. The overall survival in nivolumab or everolimus-treated RCC patients grouped by metabolite level (the upper half was as a high-level group; the lower half was as a low-level group defined by the median value of individual metabolites) were measured using Kaplan–Meier plot.

Quantitative real-time PCR (qPCR)

Total RNA of indicated tumor cells was extracted by using RNeasy pure Tissue Kit (Qiagen Biotech, China), according to the manufacturer's protocol. RNA (2 µg) was reverse transcribed using the PrimeScript™ RT reagent Kit with gDNA Eraser (Takara, China). Quantitative RT-PCR was performed using

PowerUp™ SYBR™ Green Master Mix with QuantStudio 6 Flex System (Thermo Fisher, USA). Relative mRNA expression was determined by the $\Delta\Delta C_t$ method and normalized to *Gapdh*. All qPCR primers used are listed in Supplementary Table 6.

Western blot

ORF of human UBA6 and USE1 in pEnter, with C terminal Flag, were purchased from Vigene Biosciences (JiNan, China). Moreover, UBA6 with the UFD domain (residues 949–1052) deletion (UBA6 Δ UFD) was generated by PCR. The amplified DNA fragment was cloned into pEnter. The human embryonic kidney cell line HEK293 was purchased from ATCC and was cultured in DMEM supplemented with 10% of FBS and 50 U/ml of penicillin/streptomycin. Cells were transfected using Lipofectamine 2000 reagent as described by the manufacturer's instructions. 500 μ M of inosine or vehicle was added 24 h after transfection. At 48h, cells were harvested and lysed in lysis buffer (50 mM Tris HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% TRITON X-100). Cleared lysates were subjected to anti-FLAG immunoprecipitation using Anti-FLAGM2 Affinity Gel (Sigma, USA) overnight at 4°C. Samples were washed three times with TBS. Proteins were separated on 8% or 12% Laemmli SDS gels and subjected to western blot analysis using an anti-UBA6 antibody (Proteintech, 1:1000), anti-USE1 antibody (ABclonal, 1:1000), and anti- β -Actin antibody (Sangon Biotech, 1:1000).

Prepare the single-cell suspension from 4T1 tumor tissues

4T1 cells (2×10^5) were implanted in BALB/c female mice. Mice were treated with IgG2a (Ctrl) or a combination of inosine and ICB (anti-CTLA4 and anti-PD1 Abs) (Combo) as described above. On day 13, tumors were harvested and minced with scissors before incubation with collagenase A (2 mg/ml, Roche) and DNase I (50 μ g/ml, Roche) in RPMI-1640 completed medium (10%FBS, 1% P/S) for 30 min at 37°C. Tumor samples were homogenized by repeated pipetting and filtered through a 70 μ m nylon filter (BD Biosciences) in FACS staining buffer (PBS/0.5% albumin) to generate single-cell suspensions. After red blood cell (RBC) lysis (RBC Lysing Buffer, Biolegend), all samples were washed and re-suspended in FACS staining buffer for further single-cell RNA sequencing (scRNA-seq) or flow cytometry.

Analysis of tumor-infiltrating immune cells by scRNA-seq

Tumor-infiltrating immune cells from 4T1 tumor-bearing mice with IgG2a (Ctrl, n = 2) or a combination of inosine and ICB (anti-CTLA4 and anti-PD1 Abs, n = 2) (Combo) treatment were enriched using CD45⁺ MicroBeads kit (Miltenyi Biotec, Germany). 2 biological replicates in the vehicle and inosine + ICB groups were performed. The single-cell RNA-seq was performed as described⁴⁷. Briefly, cells were counted and loaded into the 10x Genomics device. After reverse transcription, barcoded cDNAs were purified, amplified, end-repaired, and ligated with Illumina adapters to generate a single multiplexed library according to the manufacturer's protocol. All resulting libraries were sequenced on the Illumina Novaseq 6000 platform (Illumina, USA).

Preliminary sequencing results were de-multiplexed the cellular barcodes and aligned reads to the transcriptome GRCm38 (mouse) using the Cell Ranger v2.1.1 pipeline. Mean and dispersion values were

calculated for each gene across the remaining 16,199 cells (Ctrl group) and 9,842 cells (Combo group), and variably expressed genes were selected for principal component analysis (PCA). Then, t-SNE was performed using default parameters for visualization in two dimensions. All CD45⁺ immune cells were clustered as described⁴⁸. Unsupervised clustering using a shared nearest neighbor modularity optimization-based algorithm identified 32 distinct clusters. 14 major clusters were identified by mapping canonical marker genes in the two-dimensional tSNE map. Detailed descriptions of the immune cell subsets and their marker genes are included in the figures and main text of the relevant sections.

Flow cytometry assay

For flow cytometry analysis of *in vivo* experiments, tumor single cells were isolated from mouse 4T1 tumors as described above and pre-incubated (15 min, 4°C) with an anti-CD16/32 monoclonal antibody (clone 93, Biolegend) to block nonspecific binding and then stained (30 min, 4°C) with appropriate dilutions of various combinations of the following fluorochrome-conjugated antibodies: anti-CD45-AF700 (clone 30-F11), anti-CD11b-PE (M1/70.15), anti-F4/80-APC (clone BM8), anti-MHC Class II-FITC (clone M5/114.15.2), anti-CD206-PE (clone 19.2), anti-CD8-Percp-Cy5.5 (clone 53 – 6.7), anti-CD4-PE (clone RM4-5) antibodies, all purchased from Biolegend or ThermoFisher. For tetramer staining in the 4T1 model, tumor antigen-specific CD8⁺ T cells were detected with PE-conjugated H-2L^d tetramer to peptide SPSYVYHQF (MuLV env gp70, 423 to 431) was purchased from MBL International. Antibodies were used at 5 ug/ml, and tetramer staining was performed in FACS buffer for 20 min at room temperature and followed by surface staining on ice for 20 min. Dead cells and doublets were excluded based on forward and side scatters and Fixable Viability Dye eFluor 506 (Thermo Fisher, USA).

For in vitro analysis of the effect of inosine on MHC Class I antigen expression, B16-GMCSF or 4T1 cells were seeded and treated with a serial dilution of inosine for 48 hours. Cells were non-enzymatically detached from the wells, washed with FACS staining buffer, and then incubated with FITC-conjugated anti-mouse H-2K^d antibody (clone SF1-1.1, BioLegend) for 30 min on ice. After washing, cells were resuspended in FACS staining buffer, and then >2000 cells were analyzed by flow cytometry. Acquisition and analysis were performed on Canto II Flow Cytometer using BD FACSDiva software (BD Biosciences, USA) and all analyses were performed with FlowJo software v10 (BD).

Tumor cells viability and apoptosis assays

For the effect of inosine on tumor cell growth in vitro assay, 4T1 or B16-GMCSF cells were seeded in 96-well plates (1,000 cells per well) and allowed to seed for 24 h, after which they were treated with inosine. For in vitro cytokine stimulations and growth inhibition assays, sgCtrl or UBA6-*null* 4T1 or B16-GMCSF tumor cells were plated in media containing the indicated combinations of cytokines: 10 ng/ml IFN γ (PeproTech, USA), 10 ng/ml TNF α (PeproTech, USA), or 10 ng/ml IFN γ + 10 ng/ml TNF α . Treatment was given only once at the beginning, after the seeding of cells. Subsequently, every 24 h, MTT reagent (Sigma, USA) was added to the cell culture media for 3 h at 37°C. The supernatant was then discarded and lysed with DMSO to dissolve the formazan product. Absorbance was measured by a spectrophotometric plate reader.

For flow cytometry analysis of apoptosis, 4T1 or B16-GMCSF cells were treated inosine for 48h, and following trypsinization and washes in FACS staining buffer, tumor cells were stained for 20 min on ice using the manufacturer's recommended concentrations of Annexin-V PE and 7-AAD from the PE Annexin V Apoptosis Detection Kit 1 (BD Pharmingen, USA) according to the manufacturer's instructions. The staining of cell surface markers was then analyzed using the Canto II flow cytometry system (BD Biosciences, USA). The analysis was carried out using FlowJo software.

Integrative gene knockout screening platform and survival analysis based on TIDE

We collected cancer data sets with both patient survival durations and tumor gene expression profiles from The Tumor Immune Dysfunction and Exclusion (TIDE) website and tools^{23,31}. Candidate genes were plotted based on mean log₂ fold change (logFC) of gRNA counts compared to control selection and normalized z-score generated using the pheatmap R package and presented as the expression level of the individual gene was standardized to zero mean and one standard deviation. The normalized logFC and Z-score in CRISPR screens help identify regulators/genes whose knockout can mediate the efficacy of lymphocyte-mediated tumor killing in cancer models. Higher logFC and Z-score mean that knockout of gene resistant to lymphocyte-mediated tumor cell killing, contrast, lower logFC, and Z-score mean that knockout of gene mediates the enhancement to lymphocyte-mediated tumor cell killing. Kaplan–Meier plots of overall survival (OS) or disease-free survival (DFS) of cancer patients treated with ICT who had high UBA6 vs. low UBA6 in the tumors as respectively defined by the median expression levels were accessed by TIDE program. To test the association between UBA6 gene expression level and patient survival, Kaplan-Meier survival analysis was performed using the program described in the Gene Expression Profiling Interactive Analysis (GEPIA2)⁴⁹.

Human Tissue Samples collection and histological evaluation of UBA6 expression

Human tumor tissues were collected from individual participants following the guidelines approved by the Institutional Review Board at Beijing Friendship Hospital affiliated to Capital Medical University (2017-P2-141-02). A total of 22 human tissue samples were obtained from patients with cancers of esophageal (n = 7), stomach (n = 4), lung (n = 3), colon (n = 3), and others (n = 5) (gender: 12 males and 10 females; mean ± SD age, 62.4 ± 8.6 years; median age, 63 years; range, 39–77 years). Pearson correlation test was utilized to confirm an insignificant association of patient age and sex with UBA6 expression (p = 0.62). The disease control rate and the objective response rate (n = 22 of this cohort with immunotherapy response rate available) were comparable to previously reported in unselected patients⁵⁰. Standard immunohistochemical (IHC) assays were performed for UBA6 evaluation as described previously⁵¹. In brief, tumors were harvested before immunotherapy and fixed in 10% neutral-buffered formalin. After deparaffinization and rehydration, 4µm tissue sections were subjected to heat-induced epitope retrieval. Slides were processed with the VECTASTAIN Elite ABC HRP Kit and DAB Substrate Kit (Vector Laboratories). Slides were then incubated with anti-UBA6 antibody (Proteintech, 1:1500). Five visual fields

from different areas of each slide were independently evaluated by 2 pathologists who were blinded to the group allocation during the staining and when assessing the outcomes. Necrotic areas in the tumors were excluded from the evaluation. IHC intensity scores of UBA6 were ranked into 4 groups: negative (-), positive-low (+), positive-medium (++), and positive-high (+++). In the IHC scoring of patient samples, the score “low” corresponded to negative (-) to positive-low (+), while the score “high” corresponded to the range from ++ to +++.

ICB treatment and assessment of ICB response in cancer patients

This clinical study was approved by the institutional ethics committee of Beijing Friendship Hospital affiliated with Capital Medical University and was conducted following clinical practice guidelines. The study was designed by the authors in collaboration with the sponsors, and all 22 cancer patients are recruited for evaluation of Anti-PD1 Ab combined with paclitaxel treatment. Anti-PD1 Ab (Sintilimab, Innovent Biologics) was provided by the sponsor or procured as commercial products, and paclitaxel was procured as commercial products. Anti-PD1 Ab was administered at a dose of 200mg/per time as a 60-minute intravenous infusion every 3 weeks. Paclitaxel was administered at 175 mg/m² intravenously daily for 3 weeks. Disease assessments were performed with the use of computed tomography (CT) or magnetic resonance imaging at baseline, every 8 weeks until disease progression or discontinuation of treatment. Imaging data were evaluated by the investigators to assess tumor response. The clinical objective response was determined as the investigator-assessed best response based on immune-related response evaluation criteria in solid tumors (irRECIST) ⁵² using unidimensional measurements (CR: complete response, PR: partial response, SD: stable disease, PD: progressive disease). The assessment of responses for patients was conducted independently in a double-blind fashion from the time of randomization to objectively documented disease progression or subsequent therapy.

Bioinformatics and statistical analysis

Statistical tests employed with the number of replicates and independent experiments are provided in the figure legends or text. Unless mentioned otherwise, all graphs with error bars are presented as mean ± s.e.m. GraphPad Prism (v.8) is used for basic statistical analysis and plotting. Statistical significance is determined by one-way ANOVA with Tukey and Dunnett’s posttests and two-way ANOVA with a Bonferroni test for multiple comparisons, or an unpaired Student’s t-test for pair-wised comparison. Multiple hypothesis testing corrections were applied where multiple hypotheses were tested and are indicated using FDR. Kaplan-Meier survival curves are graphed and analyzed using the log-rank test for multiple comparisons. *P*-value < 0.05 was indicated as statistically significant.

Material availability

Materials that are not available commercially can be requested from the corresponding author.

Data availability

Data are available within the Article, Supplementary Information, or available from the authors upon request.

Declarations

Disclosure of Potential Conflicts of Interest

The authors declare that they have no competing interests.

Author contributions

B.H. and X.Y. conceived the project, guided the experimental design, and wrote the manuscript. Y.G., D.Y., and D.X. guided the experimental design and help to write the manuscript. L.Z., L.J., L.Y., Q.L., X.T., J.H., C.W., Y.W., X.J., X.G., W.D., A.J.S., Q.G., J.L., and Y.Y. performed all the experiments and data analysis. All authors contributed to the editing of the manuscript.

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Figures

Figure 1

Inosine overcomes resistance to immunotherapy by inflaming tumor immune microenvironment. (a) Volcano plot showing the hazard ratios (HR) of high/low level of serum metabolites ($n = 202$, represented as points, high and low are stratified by the median of each metabolite) in CheckMate 025 renal cell carcinoma (RCC) patients with nivolumab (anti-PD1 Ab) treatment ($n=392$). Overall survival is using as a Cox proportional hazards model. A cutoff at $P = 0.01$ is shown as a horizontal line and HR (high/low) = 1 is shown as a vertical line (HR: high/low < 1 indicates that a high level of metabolite is a benefit for ICB patients). (b) Kaplan–Meier plot of overall survival in RCC patients with nivolumab (anti-PD1 Ab) (High, $n=196$, mean OS=33; Low, $n=195$, mean OS=21) or everolimus (mTOR inhibitor) (High, $n=174$, mean OS=20; Low, $n=174$, mean OS=20) grouped by the inosine level at the median of baseline level. (c–e) Tumor volume and survival analysis of B16-F0 (c), B16-GMCSF (d), or 4T1 (e) tumor-bearing mice treated with IgG2a (Ctrl), 400mg/kg of Inosine (Ino), anti-CTLA4+anti-PD1 (ICB) or ICB+Inosine (Ino+ICB)

treatment (n=6-8). (f) t-SNE plot of single-cell RNA sequencing of CD45+ immune cells from 4T1 tumors treated with Ctrl (n=16199 cells) or Ino+ICB treatment (n=9842 cells). (g) The frequency of gp70-specific CD8+ T cells in 4T1 tumor with Ctrl, Ino, ICB, or Ino+ICB treatment for 15 days (n=6-8). Data are presented as Mean $\pm$ s.e.m. Statistical significance was determined by ANOVA (c, d, e, g) or log-rank (Mantel-Cox) test (a, b, c, d, and e). *P < 0.05, **P < 0.01, ***P < 0.001.

Figure 2

Inosine sensitizes tumor cells to T cell-mediated killing by modulating tumor immunogenicity. (a) Experimental strategy to evaluate the ability of inosine to enhance T cell-mediated tumor killing by modulating T cells. (b) The relative cell viability of B16-GMCSF-OVA cells was shown. OT-1 T cells were pretreated with indicated concentrations of inosine or vehicle for 24h, then co-cultured with B16-GMCSF-OVA tumor cells at a 2:1 E: T (Effector: T cells; Target: Tumor cells) ratio for 48h (n=5). (c) The relative cell viability of 4T1 cells after incubation with activated CD8+ T cells pretreated with the indicated concentration of inosine at a 5:1 E: T ratio for 48h (n=5). (d) Experimental strategy to evaluate the ability of inosine to enhance T cell-mediated tumor killing by modulating Tumor cells. (e) The relative cell viability B16-GMCSF-OVA cells (left) and 4T1 cells (right) with Ctrl or inosine treatment following the method in (A) (n=5). (f) Experimental strategy to evaluate the direct effect of inosine on tumor cells. (g) The relative cell viability (left) and apoptosis (right) of B16-GMCSF cells following inosine treatment at indicated concentrations for 48h in vitro (n=5). (h) The relative cell viability (left) and apoptosis (right) of 4T1 cells following inosine treatment at indicated concentrations for 48h in vitro (n=5). (i) Representative flow analysis (left panel) and quantify (left panel) the intensity of cell surface MHC-I expression in B16-GMCSF cells treated with vehicle and inosine (100μM) upon IFN-γ (20ng/ml) treatment (n=3). (j & k) Selective represented antigen processing/presentation and interferon-responsive gene expression in B16-GMCSF (j) and 4T1 (k) and tumor cells treated with inosine at indicated concentrations (n=3). Data are presented as Mean ± s.e.m. Statistical significance was determined by ANOVA, Tukey's test, or Two-sided Student's t-test. NS, no significant; **P < 0.01, ***P < 0.001, ****P < 0.0001.

Figure 3

Inosine directly inhibits tumor UBA6 to sensitize T cell-mediated killing. (a) Scheme of chemical proteomics for target identification. (b) Volcano plots of LiP-SMap experiments with inosine treatment. (c) Heat-map of the top 23 proteins changed with inosine treatment identified by LiP-SMap. (d) The effect of deletion of the top 23 genes in (E) on OT-1 T-cell mediated tumor cell killing. (e) The effect of inosine on the interaction between UBA6 and USE1 in HEK293 cells. (f) The effect of inosine on USE1~S~FAT10 thioester in vitro. (g) The relative cell viability of WT (sgCtrl), Uba6-null (sgUba6) 4T1 or B16-GMCSF-OVA tumor cells with vehicle (Ctrl) or inosine (100μM) following the method in (a) (n=5). Data are presented as Mean ± s.e.m. Statistical significance was determined by ANOVA. NS, No Significant; **P < 0.01.

Figure 4

Genetic and inosine inhibition of UBA6 stimulates tumor cell-intrinsic immune response signaling. (a) Volcano plots of upstream regulator analysis of UBA6 dependent genes in 4T1 tumor cells by IPA. (b) Top-ranked GO terms in the transcriptome of Uba6-null 4T1 tumor cells. (c) The upregulated GSEA signatures in the Uba6-null 4T1 tumor. (d) Heat-map of proteins for differential signaling pathways (Red, upregulated; blue, downregulated). The expression level of these proteins in sgCtrl and sgUba6 4T1 cells is measured by the whole proteomics (n=3). (e) The relative mRNA expression of IFN γ response-related genes in sgCtrl and sgUba6-4T1 tumor cells without or with inosine (100 μ M) treatment for 48h (n=3). (f) Cell viability of Uba6-null and control 4T1 or B16-GMCSF tumor cells following stimulation with 10ng/ml TNF α + 10ng/ml IFN γ treatment for 48h (n=4). Data are presented as Mean $\pm$ s.e.m. Statistical significance was determined by ANOVA. NS, No Significant; *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

Figure 5

UBA6 deletion substitutes the effect of inosine on antitumor immunity in vivo. (a) Tumor volume and survival analysis of sgCtrl and sgUba6 B16-GMCSF tumors in NSG, wild-type (WT) mice (n=8). (b) Tumor volume and survival analysis of sgCtrl and sgUba6 B16-GMCSF tumors in WT mice with Ctrl, ICB, or ICB + Ino treatment (n=8). (c) Tumor volume and survival analysis of sgCtrl and sgUba6 4T1 tumor-bearing NSG mice (left) or wild-type (WT) mice (right) (n=10). (d) Tumor volume and survival analysis of sgCtrl and sgUba6 4T1 tumor-bearing WT mice with Ctrl, ICB, or ICB + Ino treatment (n=10). Data are presented as mean $\pm$ SEM. Statistical significance was determined by ANOVA or log-rank (Mantel-Cox) test. NS, no significant. **P < 0.01; ***P < 0.001; ****P < 0.0001.

Figure 6

UBA6 in tumor cells predicts patient outcomes to immunotherapy. (a) The association between CTL and OS of melanoma patients with distinct UBA6 levels. (b) Kaplan–Meier plots of PFS of 42 melanoma patients with anti-CTLA4 and OS of 47 melanoma patients with anti-PD1 based on UBA6 level. (c) Representative UBA6 staining tumor sections (top: 100x, bottom: 400x) (left) and CT scans (right) of lung cancer patients with anti-PD1 treatment. CT scans of tumors (top) and mediastinal lymph nodes (bottom) of patient 1 and left pleural effusion (top) and pericardial effusion (bottom) of patient 2 are highlighted by red arrows. (d) Pie charts of response fractions for each group of patients with UBA6 low and high expression in tumor cells. Data are presented as Mean $\pm$ s.e.m. Statistical significance was determined by a log-rank test.

Figure 7

A summary model linking inosine and immunotherapy responses. The metabolic imbalance, especially inosine, is associated with immunotherapy responses in mice and humans. Inosine overcomes tumor-intrinsic resistance to immunotherapy by inhibiting UBA6 and increasing tumor immunogenicity in tumors with UBA6 high expression.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [SupplementaryTable16.xlsx](#)
- [SupplementaryData.pdf](#)