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14. ABSTRACT

Relevance. This project addresses LCRP overarching challenges of understanding the molecular mechanisms of initiation and progression to clinically significant lung cancer, and identification of innovative strategies for prevention and treatment of lung cancer. Lung cancer is highly prevalent in both veterans and active-duty personnel due to exposures to mutagens in industrial substances, cigarette smoke, asbestos bearing materials, and battlefield air pollution. The cost of lung cancer to the VA has been suggested to be > $1 billion a year. This study will mechanistically dissect the IRE1α-XBP1 axis that constitutes a major immunosuppressive barrier that limits the efficacy of checkpoint blockade in NSCLC. Targeting the IRE1α has the immense potential to enhance the efficacy of PD-1 inhibition so that a larger cohort of NSCLC patients benefit.

Background. Mutant KRAS represents >30% NSCLC, and currently possess no effective therapeutic options. *KRAS* mutations typically predict a lack of response to conventional therapies and therefore, treatment of *KRAS* adenocarcinomas is an urgent unmet clinical need. We posit that targeting the ER stress IRE1α-XBP1 pathway has potential in the treatment of high-risk NSCLC patients.

Overarching challenges. Of the 1.8 million individuals diagnosed per year worldwide, approximately 1.6 million succumb to death. Non-small cell lung cancer (NSCLC) constitutes 85-90% of all lung cancer. KRAS is the most frequently occurring oncogenic mutation in NSCLC, representing ~20-30% of NSCLC. Moreover, KRAS mutations are associated with a poor prognosis, and reduced benefit from adjuvant chemotherapy, compared with the general NSCLC population. Despite this clinical significance, there is not a single effective FDA approved targeted therapy against KRAS. Given the unmet clinical need, there is an urgent requirement to develop targeted therapeutic approaches for effective treatment of mutant KRAS NSCLC.

Hypothesis /Objective. We hypothesize that interventions against IRE1α-XBP1 signaling either alone or in combination with immune checkpoint blockade can be developed into a viable therapeutic strategy for currently untreatable KRAS mutant patients. Using a combination of genetic and pharmacological approaches, we propose to achieve the following objectives: *1*) dissect the mechanisms by which cancer intrinsic IRE1α-XBP1 signaling generates immunosuppressive microenvironments in NSCLC, *2)* to assess whether pharmacological inhibition of IRE1α endoribonuclease can be used to target tumor progression, and *3*) to determine if pharmacological inhibition of IRE1α can used as a novel approach to enhance the effectiveness of immune checkpoint blockade in NSCLC. Our overall goal is to develop a mechanism-guided intervention against KRAS driven lung cancer.

Specific Aims. Using two independent but integrated aims, we propose*; 1*) To determine the mechanisms by which cancer cell-intrinsic loss of IRE1α-XBP1 signaling modulates anti-tumor immunity, and *2)* To evaluate the therapeutic potential of targeting the IRE1α-XBP1 pathway alone or in combination with PD-1 inhibition in NSCLC.

Study Design. Using a combination of genetic and pharmacological approaches, we propose to dissect the mechanisms by which IRE1α-XBP1 signaling in the tumor cells may elicit concomitant antitumor immunity in the tumor microenvironment through its role in activating tumor infiltrating lymphocytes, and simultaneously limiting immunosuppressive Tregs and MDSCs. Furthermore, the therapeutic potential of targeting the IRE1α-XBP1 axis in enhancing the efficacy of PD-1 blockade will be determined.

Innovation. This proposal is conceptually and technically innovative as it seeks to assess specific and direct inhibition of the IRE1α-XBP1 signaling pathway that has remained unexplored in lung cancer. A major conceptual innovation is that this study emphasizes that targeting cancer intrinsic IRE1α-XBP1 signaling has the potential to elicit concomitant antitumor immunity through its role in immune cell reprogramming. A variety of mouse genetic models, together with compartment-specific gene knockout strategies will be employed. In parallel, IRE1α inhibitors will be used to complement the genetic findings and enhance feasibility of clinical translation, and combined inhibition of IRE1α and PD-1 will be evaluated.

Impact. We expect that the mechanistic insights from the preclinical investigations will generate unique translational opportunities that may lead to the design of future clinical trials. The demonstration that targeting the IRE1α-XBP1 pathway may act synergistically with immune checkpoint blockade will allow future clinical trials to evaluate this new combination regimen in currently untreatable mutant KRAS lung cancer patients. Additionally, the XBP1 gene signature identified in human NSCLC has the potential to serve as a prognostic/diagnostic biomarker of the disease, and may allow monitoring efficacy of therapies targeting the IRE1α endoribonuclease.

15. SUBJECT TERMS XBP1, IREalpha, lung cancer, ER stress, immune checkpoint blockade, KRAS, immunosuppressive, T-cell, endoplasmic reticulum, immunophenotype, CRISPR					
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1. INTRODUCTION:

This proposal aims to dissect the mechanisms by which cancer cell specific IRE1α-XBP1 signaling generates an immunosuppressive microenvironment that promotes tumor progression by inactivating cytotoxic T lymphocytes and simultaneously increasing immunosuppressive Tregs and MDSCs. Overcoming immunosuppression in the tumor microenvironment is a fundamental pre-requisite for the success of clinically relevant immune checkpoint inhibitors including anti-PD-1 and CTLA4. We posit that treatment with IRE1α selective small molecule inhibitors will overcome major immunosuppressive barriers and boost anti-tumor immunity in NSCLC, which in turn may constitute a new approach in enhancing the efficacy of immune checkpoint inhibitors in NSCLC. This approach has the potential to increase the current objective response rates 17-20% with immune checkpoint inhibitors in NSCLC. We expect that the mechanistic insights from these investigations will generate unique translational opportunities that may lead to the design of future clinical trials for currently untreatable mutant KRAS patients.

2. KEYWORDS:

XBP1, IRE1α, lung cancer, ER stress, immune checkpoint blockade, KRAS, immunosuppressive, T-cell, endoplasmic reticulum, immunophenotype, CRISPR

3. ACCOMPLISHMENTS:

What were the major goals of the project?

Aim 1: Determine the mechanisms by which cancer cell- intrinsic loss of IRE1α-XBP1 signaling modulates anti-tumor immunity (1-24 months)

Subaim 1.1 Identify IRE1α/XBP1regulated immunomodulators. (1-6 months)

Task: Perform integrated pathway and gene set enrichment in order to identify known and novel candidate immunomodulators to be evaluated (1-6 months) **(See Fig. 1)**

Subaim 1.2: IRE1α-XBP1 mediated gene signatures via RNA-seq in human NSCLC. (4-18 months)

• identify differentially regulated genes in tumor epithelial cells compared to epithelial cells from matched adjacent lungs (**(See Fig. 2-3)**

• integrated cross-species analysis will be performed at the gene (focusing on orthologs) and pathway level to confirm HKP1 findings

Task: Use prospectively collected surgically resected fresh specimens to sort tumor epithelial cells from 20 KRAS patients

Subaim 1.3: Dissect the IRE1α-XBP1-PGE2 axis. (4-24 months)

Task 1.3.1: Evaluate XBP1s-mediated transcriptional regulation of Ptges and Ptgs2 (COX-2). Conduct (ChIP-qPCR) experiments to determine binding of XBP1s to the promoter of Ptges and Ptgs2 genes (samples from subaim 1.2). Validate the patient relevance of the XBP1s binding in vivo.

Task 1.3.2: Investigate the consequences of modulated PGE2 levels on the immune landscape of IRE1αKO HKP1 tumors. **(See Fig. 4)**
Collect BALF from IRE1αWT and IRE1αKO HKP1 tumors and perform immunophenotyping on the matched sets of lungs.
Control (n=10 mice) + Tumor bearing (n=10 mice) X 3 cell lines = 40 mice X 2 experiments= 80 BL6 mice

Task 1.3.3: Inducible expression of Ptges (mPGES1) in IRE1αKO HKP1 cells to rescue phenotype. Infect IRE1αWT and IRE1αKO cells with an inducible lentiviral Ptges1 cDNA.
Confirm the expression via qPCR for Ptges and ELISA for PGE2. Inject mice with Ptges+IRE1α WT or KO cell line. Evaluate in vivo tumor kinetics by BLI: survival experiment, immunophenotyping via flow cytometry Control (n=10 mice) + Tumor bearing (n=10 mice) X 3 cell lines = 40 mice X 2 experiments= 80 BL6 mice

Aim 1 Milestone(s) Achieved: Identify cancer cell intrinsic immunomodulators regulated by IRE1a-XBP1 signaling and evaluate the functional role of these modulators in reprogramming immune cells (6-24 months)

Local IRB/IACUC Approval (3 months)

Milestone Achieved: HRPO/ACURO Approval (4 months)

Aim 2: Evaluate the therapeutic potential of targeting the IRE1α-XBP1 pathway alone or in combination with PD-1 inhibition in NSCLC (6-24 months)

Subaim 2.1: Evaluate pharmacological inhibition of IRE1α or XBP1 as a therapeutic strategy in NSCLC. (3-18 months) **(See Fig. 5-6)**

Task: Determine the efficacy of single agent IRE1α targeting drugs. Administer B-I09 at 50mg/kg, 5 days a week for 3 weeks. Cohorts of KO and WT mice will be challenged with HKP1or CMT 167 tumor cells (n=10 per group). Tumor nodules, proliferation (Ki-67), apoptosis (cleaved Caspase 3) and microvessel density will be evaluated (IF staining, FACS-based immunophenotyping).
Control (n=10 mice) BL6 mice + Tumor bearing (n=10 mice) X 3 specific KOs = 40 mice X 2 experiments= 80 Bl6 mice

Subaim 2.2: Determine if targeting the IRE1α -XBP1 axis acts synergistically with PD-1 inhibition. (12-24 months)

Task: Mice bearing HKP1 lung adenocarcinoma (n=10 per group) will be administered vehicle or IgG antibody (control), B- I09 (50 mg/kg), anti-PD1 antibody (clone RMP1-14 from BioXCell, 250 ug/mouse twice a week for two weeks) or B-I09 plus anti-PD1 antibodies.
Control (n=10 mice) BL6 mice + Tumor bearing (n=10 mice) X 5 treatments x 2 dosages = 100 mice X 2 experiments= 200 Bl6 mice

Determine: i) the number and proportion of activated, antigen-experienced T cells infiltrating lung tumors, ii) the capacity of tumor-infiltrating T cells to effectively respond to tumor antigens, iii) the number and proportion of tumor-specific T cells exhibiting central memory-like markers (CD62L+CD44+), in lymphoid tissue and bone marrow, and iv) the number and proportion of tumor-infiltrating Treg cells in NSCLC tumors and associated lymphoid organs.

Memory T cells isolated from treated tumor-bearing mice will be adoptively transferred into different groups of naive mice, and animals will be challenged 24 hours later with wild-type HKP1 cells. Tumor growth will be analyzed
Total mice = 100 Bl6 mice

Milestone(s) Achieved: 1) Treatment with IRE1α selective small molecule inhibitors will boost anti-tumor immunity and impair tumor progression in NSCLC; 2) determine whether targeting IRE1α catalytic function has the potential in enhancing the efficacy of PD-1 blockade in NSCLC (12-24 months)

What was accomplished under these goals?

For the final report, we are summarizing major progress made in all the aims (Year 1-2 presented previously), and have laid more emphasize on new progress in Aim 2 made during the no cost extension period.

Aim 1: Determine the mechanisms by which cancer cell- intrinsic loss of IRE1α-XBP1 signaling modulates anti-tumor immunity.

Subaim 1.1 Identify IRE1α/XBP1regulated immunomodulators.
Task: Perform integrated pathway and gene set enrichment in order to identify known and novel candidate immunomodulators to be evaluated

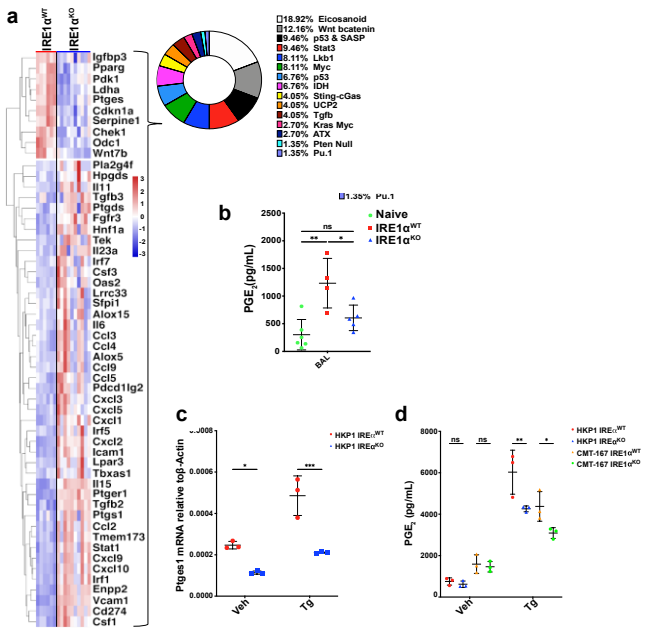

Fig. 1. IRE1α-XBP1 signaling drives immunosuppressive PGE_2 production
a, Heatmap of differentially expressed genes between *IRE1α*^{KO} vs. *IRE1α*^{WT} HKP1 cells from the Immunomodulator database.
b, BALF PGE_2 levels as measured by ELISA from tumor naïve (green, n=6), and *IRE1α*^{WT} (red, n=4), or *IRE1α*^{KO} (blue, n=5) tumor bearing lungs. Data are shown as mean ± SD. One-way ANOVA with Tukey's multiple comparisons test for ratios; *$P < 0.05$, **$P < 0.001$, ***$P < 0.0001$.
c-d. Reduced PGE_2 production in IRE1α^{KO} HKP1 and CMT-167 cells compared to IRE1α^{WT} counterparts *in vitro*. **c,** qPCR of *Ptges* from 12 hour 1 mM Tg treated IRE1α^{WT} (red) and *IRE1α*^{KO} (blue) HKP1. **d**, PGE_2 ELISA from 12 hour Vehicle or Tg treated *IRE1α*^{WT} HKP1 (red), *IRE1α*^{KO} HKP1 (blue), *IRE1α*^{WT} CMT-167 (orange), and *IRE1α*^{KO} CMT-167 (green) cells.

To identify IRE1α-XBP1-regulated genes, we performed RNA-seq analysis of IRE1α^{WT} and IRE1α^{KO} HKP1 cancer cells, which identified the Eicosanoid and WNT/β-catenin were the most enriched pathways in IRE1α^{WT} tumors (**Fig. 1a**). Evaluation of the candidate genes in the Eicosanoid biosynthetic pathway identified *Ptges* (encoding m-PGES1, prostaglandin E synthase), as one of the most significantly down-regulated genes in IRE1α^{KO} cancer cells. m-PGES1 is an inducible enzyme that rapidly converts prostaglandin H2 (PGH_2) to prostaglandin E2 (PGE_2), a potent lipid mediator that is known to promote differentiation of Tregs, enhance MDSC function, and block DC differentiation, infiltration and activation. Consistent with our RNA-seq findings, analysis of bronchoalveolar lavage fluid (BALF) showed that mice bearing IRE1α^{KO} HKP1 tumors had a marked decrease in PGE_2 levels, compared with their IRE1α^{WT} tumor-bearing counterparts (**Fig. 1b**). *Ptges* transcript and secreted PGE_2 levels were reduced in Tg-treated IRE1α^{KO} HKP1 cells compared with IRE1α^{WT} cells, confirming a direct link between IRE1α and PGE_2 biosynthesis. Consistent with the in vivo analysis, *Ptges* mRNA transcript and secreted

PGE_2 levels were reduced in *in vitro* Tg treated $IRE1\alpha^{KO}$ HKP1 cells compared with $IRE1\alpha^{WT}$ cells, confirming a direct link between IRE1α and PGE_2 biosynthesis (**Fig. 1c-d**).

Subaim 1.2: IRE1α-XBP1 mediated gene signatures via RNA-seq in human NSCLC.

To determine the clinical relevance of IRE1α-XBP1 signaling in NSCLC patients, we developed a new computational pipeline to specifically quantify the fraction of the spliced *XBP1* mRNA isoform (*XBP1s*) relative to the total *XBP1* transcript from RNA-seq data available in the TCGA database.

Next, we determined if differentially expressed genes identified from RNA-seq analysis of $IRE1\alpha^{WT}$ vs. $IRE1\alpha^{KO}$ tumor cells harvested from HKP1 tumors could be exploited to develop an IRE1α-dependent gene signature that could predict outcome in human NSCLC. To this end, we systematically evaluated a variety of statistical parameters including fold change (FC) and false discovery rate (FDR) to identify an optimal gene signature associated with survival in human NSCLC (**Fig. 2a)**. We posited that our pure mouse tumor cell signature could be applied to the TCGA LUAD dataset, as each human tumor sample has a minimum of 80% cancer cells. We performed single sample gene set enrichment analysis (ssGSEA) on a discovery cohort of >300 LUADs available in the TCGA database[29], ranking samples by their enrichment scores, and comparing outcomes for the top and bottom tertiles for each signature and evaluated for survival. We selected the log2FC>1 and FDR 1% gene signature ($IRE1\alpha^{KO}$ high comprised of 582 genes), as this appeared to comprise a robust number of genes for downstream analysis and provided marked survival benefits at both the quartile and tertile cutoff ranges (**Fig. 2b**).

Consistent with the HKP1 model, GO analysis (Log2FC>0.5, FDR<10%, p-value <0.05) highlighted alterations in immune mediated processes in the $IRE1\alpha^{KO}$ upregulated signature human NSCLC cohort (**Fig. 2c**). To determine if the $IRE1\alpha^{KO}$ upregulated high and low signature group

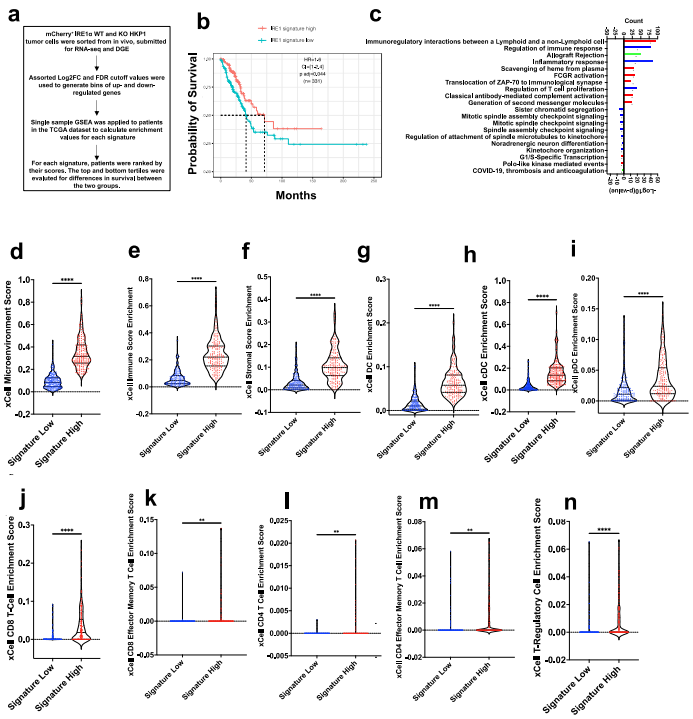

Fig. 2. $IRE1\alpha^{KO}$ gene signature enrichment is associated with human LUAD survival and immune infiltration.
a, Schematic depicting *IRE1α* signature generation and evaluation.
b, Kaplan-Meier survival plots depicting associations between overall survival (OS) and *IRE1α*^{KO} signature status in human TCGA-LUAD. High is top 1/3rd (n=166) and low is bottom 1/3rd (n=165) of the *IRE1α*^{KO} signature scores. HR is hazard ratio and p adj is log-rank p-value from the multivariate Cox proportional-hazards regression model.
c, Gene ontologies between the $IRE1\alpha^{KO}$ signature high and low tertile patients. Count of genes enriching the term on the left y-axis represented as a barplot, and the –log(10) p-value for the terms on the right y axis, represented as black symbol. Exact P values are in the Source Data file.
d-n Violin plots of xCell pipeline enrichment scores for microenvironment (**d**, *P* <0.0001), immune (**e**, *P* <0.0001), stromal **(f**, *P* <0.0001), DC (**g**, *P* <0.0001) cDC (**h**, *P* <0.0001), pDC (**i**, *P* <0.0001), CD8+ T cells (**j**, *P* <0.0001), Effector CD8 T cells (**k**, *P* = 0.0011), CD4 T cells (**l**, *P* = 0.0013),

were associated with an altered immune landscape, as was observed in the IRE1α^{KO} murine tumors, we used the xCell pipeline[30] to computationally estimate immune cell infiltration in the TME of the IRE1α^{KO} high signature patients from the top and bottom tertiles above. Patients enriched for IRE1α^{KO} high signature showed an increase in Microenvironment score (**Fig. 2d**), which is the sum of all immune and stromal cell scores (**Fig. 2e-f**), suggesting an overall enhanced immune milieu. Further evaluation of the XCell DC scores, showed that consistent with the murine data, there was enrichment of pan-DC (**Fig. 2g**), cDC (**Fig. 2h**) and plasmacytoid DC scores (**Fig. 2i**). Similarly, we observed enrichment in both CD8 and CD4 lymphocytes, and Effector/ Memory T cells (**Fig. 2j-m**). Compared to mouse tumors, IRE1α^{KO} high signature patients did not show an enrichment score in Foxp3 T regs (**Fig. 2n**).

To validate the findings from the analysis of the TCGA datasets, we applied the murine IRE1α gene signature to an independent collection of 44 human lung tumors (**Fig. 3a**), we had recently reported[31]. Consistent with the TCGA analysis, patients enriched for the IRE1α gene signature exhibited increased survival (**Fig. 3b**). Deconvolution of RNA-seq data set from these patients showed that IRE1α^{KO} high signature patients exhibited an increase in Microenvironment score, Immune and Stromal enrichment scores, together with enrichment of pan-DC, cDC and plasmacytoid DC, CD8 and CD4 lymphocytes and Effector/ Memory T cells (**Fig. 3c-o**). To experimentally validate the computational deconvolution data, we used IHC, and observed increased infiltration of T cell lymphocytes, associated with concomitant reduction in Tregs (**Fig. 3p-q**). Together, these findings suggest that IRE1α signature is associated altered immune landscape and predicts outcomes in human NSCLC.

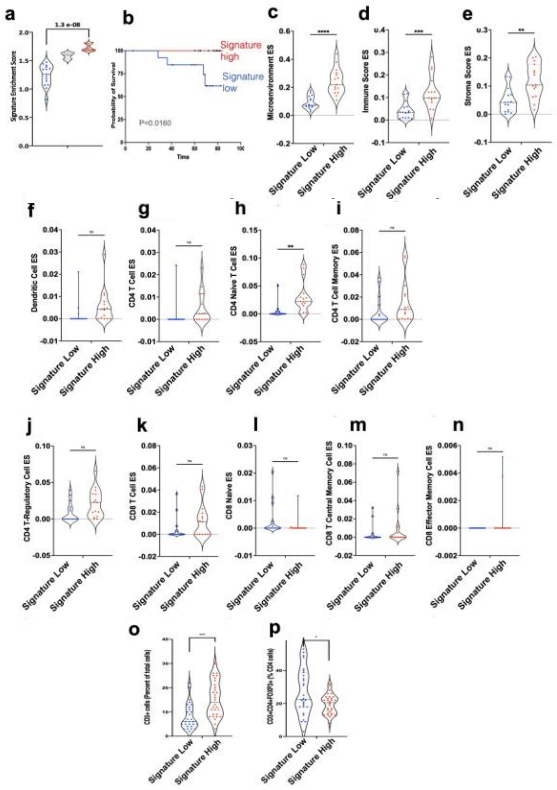

Fig. 3. Validation of the IRE1a signature and RNA-seq deconvolution.
a, Violin plots of enrichment scores (ssGSEA) for IRE1a^{KO} gene signature (Log2 Fold change>1 and FDR 0.01) low (n=15, blue), mid (grey, n=14) and high (red, n=15) NSCLC patients. One-way ANOVA with Tukey's multiple comparisons test for ratios; *P < 0.05, **P < 0.001, ***P < 0.0001.
b, Kaplan-Meier survival plots depicting associations between overall survival (OS) and *IRE1α*^{KO} signature status of NSCLC patients stratified into the top and bottom tertiles (15 patients in each) for the IRE1a^{KO} gene signature.
c-n, Violin plots of xCell pipeline enrichment scores for microenvironment (**c**, P <0.0001), immune (**d**, P=0.001), stromal (**e**, P= 0.001), DC (**f**, P=ns), CD4+ T cells (**g**, P=ns), CD4+ naïve (**h**, P=0.0027), CD4 memory (**i**, P=ns), T-regulatory (**j**, P = NS), CD8+ T cell (**k**, P=ns), CD8+ Naïve (**l**, P=ns), CD8+ TCM (**m**, P=ns), and CD8+ effector memory (**n**, P= ns) from the validation set of lung cancer patients from the IRE1α signature high (red, n=15) and low (blue, n=15) groups for enrichment of various immune cells. Unpaired, two-tailed, Student's t-test. *P < 0.05, **P < 0.001, ***P < 0.0001.
o-p, IF analysis showing quantitation of CD3T cells (left panel) and Tregs (right panel) in tumor nests of validation set of lung cancer patients from the

1c. Dissect the IRE1α-XBP1-PGE2 axis.

a) **Evaluate XBP1s-mediated transcriptional regulation of Ptges (mPGES1) and Ptgs2 (COX-2).**

b) **Investigate the consequences of modulated PGE_2 levels on the immune landscape of $IRE1\alpha^{KO}$ HKP1 tumors.**

c) **Inducible expression of Ptges (mPGES1) in $IRE1\alpha^{KO}$ HKP1 cells to rescue phenotype.**

Subaim 1.3: Dissect the IRE1α-XBP1-PGE2 axis. (4-24 months)

Task 1.3.1: Evaluate XBP1s-mediated transcriptional regulation of Ptges and Ptgs2 (COX-2). This direct link is reinforced by our recent demonstration that XBP1s can directly transactivate *COX2* and *PTGES* genes in human leukocytes to enable PGE_2 production in the context of inflammatory pain. Hence, we posited that PGE_2 secreted by the tumor cells via IRE1-XBP1 activation may modulate the tumor immune microenvironment.

Task 1.3.3: Inducible expression of Ptges (mPGES1) in IRE1αKO HKP1 cells to rescue phenotype. To directly establish the role of the IRE1α-PGE_2 axis in malignant progression, we stably reconstituted *Ptges* in $IRE1\alpha^{KO}$ HKP1 cells. Normal tumor growth kinetics was observed in mice with $IRE1\alpha^{KO}$ tumors reconstituted with *Ptges* (**Fig. 3a,** p=0.002). Furthermore, *Ptges*-induced tumor growth in $IRE1\alpha^{KO}$ mice was associated with decreased survival with a median survival of 28 days in $IRE1\alpha^{WT}$, 56.5 days in $IRE1\alpha^{KO}$ and 32 days in $IRE1\alpha^{KO}$ *Ptges* (32 days) (**Fig. 3b**).

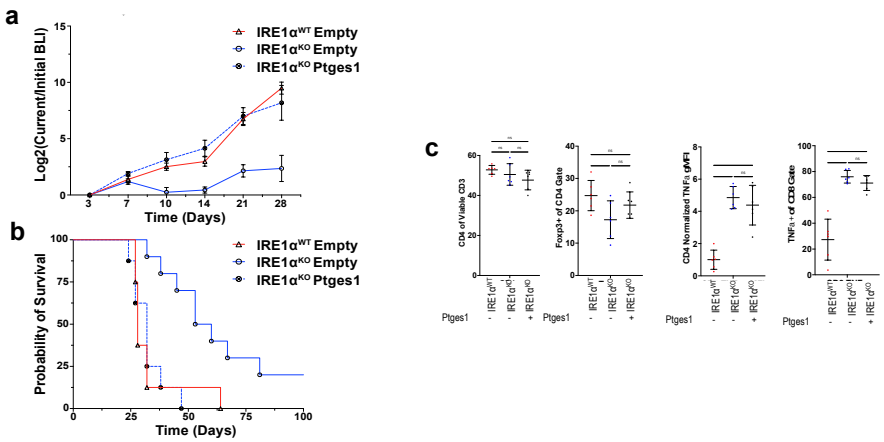

Fig. 3. IRE1α-XBP1 signaling drives immunosuppressive PGE_2 production that promotes NSCLC progression.
a, BLI plots of longitudinally tracked *in vivo* $IRE1\alpha^{WT}$ empty vector (red, n = 10), $IRE1\alpha^{KO}$ empty vector (blue, n = 10) and $IRE1\alpha^{KO}$ *Ptges* cDNA (blue broken line, n = 10) HKP1 tumors. Data are shown as mean ± SEM of biological replicates. Analyses of different time points in tumor progression were performed using 2-way ANOVA with Tukey's multiple comparisons test ; **P* < 0.05, ***P* < 0.001, ****P* < 0.0001.
b, Kaplan-Meier plots showing probability of overall survival in $IRE1\alpha^{WT}$ (red) vs $IRE1\alpha^{KO}$ (blue) and $IRE1\alpha^{KO}$ *Ptges* cDNA HKP1 tumor bearing mice (*P*< 0.001). Tumors were allowed to progress until endpoint and survival evaluated using Mantel-Haenszel Log rank-test).
c, Flow cytometry data from $IRE1\alpha^{WT}$ (red, n=6) vs $IRE1\alpha^{KO}$ (blue, n=6) and $IRE1\alpha^{KO}$ with Ptges1 (black, n=6) HKP1 tumor bearing lungs. Box and whisker plots are presented as mean ± SD.

Task 1.3.2: Investigate the consequences of modulated PGE2 levels on the immune landscape of IRE1αKO HKP1 tumors.

Compared to $IRE1\alpha^{KO}$ tumors, $IRE1\alpha^{KO}$ tumors reconstituted with *Ptges* showed a trend towards an increase in Foxp3+ T regs. There was also a trend towards a decrease in cytokine producing CD4 and CD8 T cells (**Fig. 3c**). Together, these results establish the role of IRE1α-mPEGS-1-PGE_2 axis in experimental NSCLC progression and host survival.

Aim 2: Evaluate the therapeutic potential of targeting the IRE1α-XBP1 pathway alone or in combination with PD-1 inhibition in NSCLC (6-24 months)

Subaim 2.1: Evaluate pharmacological inhibition of IRE1α or XBP1 as a therapeutic strategy in NSCLC. (3-18 months)

Task: Determine the efficacy of single agent IRE1α targeting drugs. Administer B-I09 at 50mg/kg, 5 days a week for 3 weeks. Cohorts of KO and WT mice will be challenged with HKP1or CMT 167 tumor cells (n=10 per group). Tumor nodules, proliferation (Ki-67), apoptosis (cleaved Caspase 3) and microvessel density will be evaluated (IF staining, FACS-based immunophenotyping). Control (n=10 mice) BL6 mice + Tumor bearing (n=10 mice) X 3 specific KOs = 40 mice X 2 experiments= 80 Bl6 mice

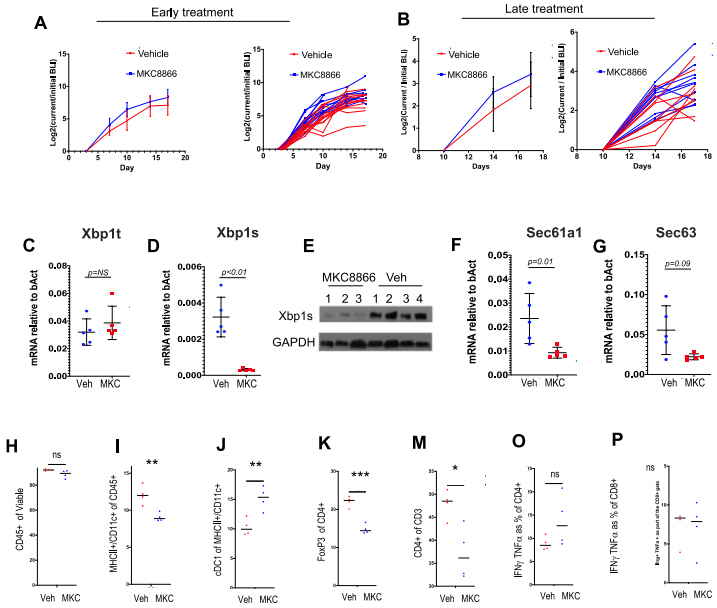

Fig. 4. **IRE1a inhibition *in vivo***. (**A**) Tumor growth in Veh and MKC8866 treated HKP1 cohorts (Daily treatment initiated 1 day before tumor innoculation, n=10 per group). Right panel shows BLI plots of individual mice. **(B)** Tumor growth in Veh and MKC8866 treated HKP1 cohorts (Daily treatment initiated at day 10 of tumor growth,, n=10 per group). Right panel shows BLI plots of individual mice. Data are shown as mean ± SEM of biological replicates. (**C-D)** RT-PCR for Xbp1t, Xbp1s, **(E)** Western blot of XBP1s in HKP1 tumors treated with Veh or MKC8866. **(F-G)** RT-PCR for XBP1s targets Sec61a1 and Sec63. Values calculated as the 2^-delta CT relative to b-actin. Mann-Whitney U nonparametric test. **(H-P)** Flow cytometry data from vehicle (Veh) and MKC8866 treated HKP1 tumor bearing lungs, showing percent viable immune cells. ns, not significant.

Work performed: We tested the IRE1α inhibitor MKC8866 that has been shown to exhibit potent IRE1α inhibition activity, together with favorable pharmacokinetics and safe toxicity profiles (Zhao et al. JCI 2018; Logue et al. Nat. Comm 2018). Although MKC8866 significantly reduced the levels of *Xbp1s* in the lung, treatment of HKP1-bearing mice with this inhibitor failed to control tumor progression (**Fig. 4A-G**). Western blot analyses confirmed decreased XBP1s protein (**Fig. 4E**), while RT-qPCR assays demonstrated that canonical XBP1s-target genes, including *Sec61a1* and *Sec6*, were downregulated upon MKC8866 treatment (**Fig. 4F-G**). Similar to IRE1α^{KO} tumors, MKC8866 administration did not alter the number of CD45+ cells, but it significantly increased the proportion of MHCII^{+}CD11c^{+} antigen presenting cells (APCs), and type 1 conventional DCs (cDC1:CD11b^{-}, CD11c^{+}, MHCII^{+}, CD64^{Low} CD24^{High} CD103^{+}), while decreasing immunosuppressive Tregs (**Fig. 4H-K**). Similar to our genetic data, MKC8866 treatment did not affect the number of CD3, CD4 or CD8 T cells **(Fig. 4L-N)**. However, unlike IRE1α^{KO} tumors, MKC8866-treated mice did not show an increase in the infiltration of T cells expressing effector cytokines (TNFα and IFNγ) at tumor sites **(Fig. 4O-P)**. Given that IRE1α-XBP1 pathway has been shown to be simultaneously activated in multiple tumor-infiltrating immune cells, including dendritic

cells (Cubillos-Ruiz Cell 2015, PMID 26073941; Chen and Cubillos-Ruiz Nat Rev. Cancer 2021), MDSCs (Condamine et al. JCI 2014, PMID: 24789911) Macrophages (Yan et al. Cell Rep 2016, PMID: 27626662) T cells (Song et al. Nature 2018, PMID: 30305738) and NK cells (Dong et al. Nat Immunology 2019, PMID: 31086333), it is likely that global IRE1α inhibition in NSCLC-bearing mice may induce confounding effects on overall anti-tumor immunity and disease progression. Therefore, these observations underscore the importance of targeting cancer cell specific IRE1α in the specific setting of NSCLC. Indeed, efforts to develop pharmacological inhibitors that selectively target IRE1/XBP1s in malignant cells are underway (Shao et al. Molecular Pharmaceutics 2022, PMID: 35253431).

Subaim 2.2: Determine if targeting the IRE1α -XBP1 axis acts synergistically with PD-1 inhibition. (12-24 months)
Since we did not observe marked immunomodulation with the IRE1a inhibitor, we did not pursue combination treatment with PD-1 inhibitor.

What opportunities for training and professional development has the project provided?

Nothing to Report

How were the results disseminated to communities of interest?

Nothing to Report

What do you plan to do during the next reporting period to accomplish the goals?

Nothing to report

4. **IMPACT:**

What was the impact on the development of the principal discipline(s) of the project?

We have showing a new function of tumor intrinsic IRE1-XBP1 regulates the tumor microenvironment through PGE2.

What was the impact on other disciplines?

Nothing to report this period.

What was the impact on technology transfer?

Nothing to report

What was the impact on society beyond science and technology?

Nothing to report for this reporting period.

5. CHANGES/PROBLEMS:

Changes in approach and reasons for change
None.

Actual or anticipated problems or delays and actions or plans to resolve them

We have achieved majority of tasks proposed.

Changes that had a significant impact on expenditures

N/A

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Significant changes in use or care of human subjects

N/A

Significant changes in use or care of vertebrate animals

Significant changes in use of biohazards and/or select agents

N/A

6. PRODUCTS:

- **Publications, conference papers, and presentations**

Journal publications.

Manuscript accepted in Nature Communications

Michael J.P Crowley, Bhavneet Bhinder, Geoffrey J. Markowitz, Mitchell Martin, Akanksha Verma, Tito A. Sandoval, Chang-Suk Chae, Shira Yomtoubian, Yang Hu, Sahil Chopra, Diamile A. Tavarez, Paolo Giovanelli, Dingcheng Gao, Timothy E. McGraw, Nasser K. Altorki, Olivier Elemento, Juan R. Cubillos-Ruiz and Vivek Mittal. Tumor-intrinsic IRE1α signaling controls protective immunity in lung cancer. (Nature Communications, accepted)

Books or other non-periodical, one-time publications.

Nothing to Report

Other publications, conference papers and presentations.

Nothing to report

- **Website(s) or other Internet site(s)**

Nothing to report

- **Technologies or techniques**

Nothing to report

- **Inventions, patent applications, and/or licenses**

Nothing to report

- **Other Products**

Nothing to report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name: Vivek Mittal, PhD
Project Role: PI
Months worked: 1.2
Contribution to project: Dr. Mittal oversees all aspects of the proposal as PD/PI.

Name: Juan Cubillos-Ruiz, PhD
Project Role: Co-Investigator
Months worked: 0.6
Contribution to project: Dr. Cubillos-Ruiz collaborators with Dr. Mittal to investigate XBP1 regulated gene signatures

Michael Crowley, MS
Project Role: Graduate Student
Months worked: 6
Contribution to project: Mr. Crowley performed all in vitro work and data analysis.

Name: Sharrell Lee, MS
Project Role: Technician
Months worked: 4
Contribution to project: Ms. Lee assisted Mr. Crowley on all experiments.

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Nothing to report

What other organizations were involved as partners?

Nothing to report

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS:

QUAD CHARTS:

9. APPENDICES:

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