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B7 immune-checkpoints as targets for the treatment of neuroendocrine tumors

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Abstract

The B7 family, and their receptors, the CD28 family, are major immune checkpoints that regulate T-cell activation and function. In the present study, we explore the role of two B7 immune-checkpoints: HHLA2 and B7x, in the progression of gastrointestinal and pancreatic neuroendocrine tumors (GINETs and PNETs). We demonstrated that both HHLA2 and B7x were expressed to a high degree in human GINETs and PNETs. We determined that the expression of B7x and HHLA2 correlates with higher grade and higher incidence of nodal and distant spread. Furthermore, we confirmed that HIF-1 α overexpression is associated with the upregulation of B7x both in our *in vivo* and *in vitro* models. When grown *in vitro*, islet tumor β -cells lack B7x expression, unless cultured under hypoxic conditions, which results in both HIF-1 α and B7x upregulation. In addition, we demonstrated that *Men1*/B7x double knockout (KO) mice (with loss of B7x expression) exhibited decreased islet β -cell proliferation and tumor transformation accompanied by increased T-cell infiltration compared with *Men1* single knockout mice. We have also shown that systemic administration of a B7x mAb to our *Men1* KO mice with PNETs promotes an antitumor response mediated by increased T-cell infiltration. These findings suggest that B7x may be a critical mediator of tumor immunity in the tumor microenvironment of NETs.

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Z. Yuan, J. Howe, E. Lattime, X. Zang, S. Libutti, designed experiments; Z. Yuan, E. Maggi, J. Gardiner, J. Howe, E. Lattime, X. Zang, S. Libutti, wrote the manuscript; Z. Yuan, J. Gardiner, S. Huang, A. Adem, G. Li, S. Lee, A. Exarchakis, performed experiments; Z. Yuan, S. Huang, G. Li, analyzed data.

Disclosure of Potential Conflicts of Interest: Dr. Zang is an inventor on patent number US 9447186 B2 which covers the topic of anti-B7x cancer immunotherapy and an inventor on two pending patents which cover the topic of HHLA2-directed immunotherapy.

Therefore, targeting B7x offers an attractive strategy for the immunotherapy of patients suffering from NETs.

Keywords

B7 immune-checkpoints; neuroendocrine tumors; tumor microenvironment; HIF-1 α ; immunotherapy

INTRODUCTION

Gastrointestinal (GI) and pancreatic neuroendocrine tumors (GINETs and PNETs) are increasing in incidence and yet remain an understudied tumor type with few effective treatments (Yao *et al.* 2008). Therefore, further understanding of their pathogenesis would be of high significance in developing new therapeutic approaches. The immune system and tumor cells have dynamic interactions, (Dunn *et al.* 2004, Grivennikov *et al.* 2010, Schreiber *et al.* 2011) which play crucial roles in tumor development and clinical outcome. New clinical data show that tumor-infiltrating lymphocytes, particularly CD4 and CD8 T-cells, are prognostic and predictive in human GINETs and PNETs, suggesting that T-cells play a major anti-tumor role during human NET progression (Epardaud *et al.* 2008, Katz *et al.* 2010, Sato *et al.* 2014). In humans, PD-1, PD-L1, and CTLA-4 are not expressed on resting T-cells but are induced after T-cell activation and expressed on regulatory T-cells (Scanduzzi *et al.* 2011). B7x and HHLA2 have been identified as being absent in most normal tissues and only HHLA2 is expressed on human monocytes without any stimulation (Zhao *et al.* 2013). However, B7x and HHLA2 are overexpressed in human tumors of the brain, thyroid, esophagus, lung, stomach, pancreas, kidney, gut, skin, ovary, uterus, prostate, and breast, often correlating with negative clinical outcomes (Hofmeyer *et al.* 2012, Janakiram *et al.* 2017, Lee *et al.* 2012, Wei *et al.* 2011, Zang *et al.* 2007, Zang *et al.* 2010). Both B7x and HHLA2 are involved in tumor development and progression by inhibiting T-cell function (Vigdorovich *et al.* 2013, Zang *et al.* 2003, Zhao *et al.* 2013). In the present study, we investigated the role of two B7 immune checkpoints, HHLA2 (HHLA2 is not present in mice) and B7x, in the tumor microenvironment of NETs. We then assessed the efficacy of a high-affinity antibody targeting the immune checkpoint B7x in reducing the growth of PNETs, using our previously described multiple endocrine neoplasia type 1 (MEN1) knockout (KO) mouse model (Shen *et al.* 2009).

During tumor development and progression, cancer and stromal cells often have restricted access to nutrients and oxygen. Hypoxia is a common feature of solid tumors, including NETs, and results in the aberrant vascularization observed in the tumor microenvironment (Kobayashi *et al.* 2016, Pinato *et al.* 2014, Pouyssegur *et al.* 2006, Sethumadhaven *et al.* 2017, Westendorf *et al.* 2017). The hypoxic response is mainly ascribed to hypoxia-inducible factors (including HIF-1, HIF-2, HIF-3). The activation of the HIF-1 α oxygen sensitive subunit is a critical effector in the hypoxic tumor microenvironment of common advanced tumors including NETs (Laitala *et al.* 2018, Maione *et al.* 2012, Soni *et al.* 2017,). Studies have shown an upregulation of HIF-1 α in neuroendocrine tumors (Pinato *et al.* 2014). Hypoxia has also been shown to suppress immune function within the tumor

microenvironment (Palazon *et al.* 2014, Palazon *et al.* 2017). Recent studies reported that hypoxia causes a rapid, dramatic, and selective upregulation of PD-L1, and it is important to note that HIF-1 α directly binds to the promoter of PD-L1 (Noman *et al.* 2014). In this study, we also explored the mechanistic relationship between the expression of HIF-1 α and the upregulation of B7x in the tumor microenvironment of PNET tumors.

MATERIALS AND METHODS

Human tissue specimens

Thirty-seven human specimens from gastrointestinal (*GINET*, *N*=13) and pancreatic (*PNET*, *N*=24) tissues and *matched adjacent normal tissue* were obtained. All tissues were collected from individuals operated on at the Albert Einstein College of Medicine (Einstein) or Roy J and Lucille A. Carver University of Iowa College of Medicine (University of Iowa). All tissues were stored at -80°C and carefully evaluated by an experienced pathologist. Patient samples were obtained after informed consent regarding the use of specimens for research purposes and the nature of all procedures used. Unidentified specimens were used in these studies. This study was approved by the Institutional Review Board at both Einstein and the University of Iowa.

In this study cohort (*n*=37 patients), the mean age of the patient population was fifty-seven years. The known *American Joint Committee on Cancer (AJCC)* staging information (*n*=37), stage I, II, III, and IV, were 48.6% (18/37), 32.4% (12/37), 10.8% (4/37), 8.1% (3/37), respectively (Supplemental Table 1).

Men1 knockout (KO) mice and Non-*Men1* KO (*RIP-Tag*) mice

We utilized the Cre-lox system, driven by the tissue-restricted promoter *Pdx1*, to inactivate *Men1* in the pancreas of mice (C57BL/6 background). Mice with homozygous loss of *Men1* develop islet hyperplasia at 6 months and insulinomas at 12-months with increased vasculature in *Pdx1-Cre; Men1 floxed/floxed (Men1 KO)* mice (Shen *et al.* 2009). These *Men1* KO mice had elevated serum insulin levels starting at 4 months of age that persists throughout their lifetime (Shen *et al.* 2009). Since these mice are patterned after the human MEN1 familial cancer syndrome, they provide an *in vivo* tool for the study of neuroendocrine tumors of the pancreas (Shen *et al.* 2009, Smith *et al.* 2016). The tumor tissues from these *Men1* KO mice were used to characterize B7x expression and immune response, and they were used to assess the efficacy of the anti-B7x mAb in the treatment of PNETs. To explore whether the NET tumors with wild type menin also have high expression of B7x, we investigated the B7x expression in the PNETs from *rat insulin promoter-T antigen (RIP-Tag)* mice. This *RIP-Tag* transgenic mouse model expresses the SV40 T-antigen under control of the rat insulin promoter, resulting in pancreatic β -cell tumors without *MEN1* mutation and loss of menin (Contractor *et al.* 2016). All animal experiments were conducted under the approved animal protocols at Rutgers University.

B7x KO mice

The mice lacking *B7x (B7x KO)* on a C57BL/6 background are as previously described (Wei *et al.* 2011). *In our study, we did not find that the phenotype nor B7x expression have*

any differences between age and gender in the *B7x* KO mice. For our experiments, we used 50% female mice and 50% male mice. The *B7x* KO mice have been crossed to *Men1* KO mice with PNETs on the same C57BL/6 background. We tested if loss of *B7x* allowed for T-cell activation and prevents the formation of PNETs. 24 mice were assigned to 4 groups for each genotype (*Men1* KO/*B7x* KO, *Men1* KO/*B7x* wild type (WT), *Men1* WT/*B7x* KO, *Men1* WT/*B7x* WT). We monitored tumor growth by measuring serum insulin, a tumor growth biomarker of *Men1* KO mice, from 3 to 12 months of ages. All mice for each genotype were sacrificed at 12 months of age. Whole pancreatic tissues from the mice were formalin-fixed for further analysis. Genetic controls were age- and gender-matched littermates. All animal experiments were conducted under the approved animal protocols at Rutgers University.

Islet β -cell tumor cell line

Since our *Men1* KO mouse pancreatic β -cells fail to grow *in vitro*, we have adopted a transplantable line from the N134 cell model for *in vitro* studies. N134 is a pancreatic β -cell tumor cell line derived from a tumor from *RIP-Tag; RIP-Tva* transgenic animal (obtained from Dr. Nancy Du, Weill Cornell Medical College, New York) at 16 week of age, and maintained in dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Du *et al.* 2007). The N134 β tumor cells were cultured under hypoxic (1% O₂) and normoxic conditions at different time points.

Targeting *B7x* with anti-*B7x* mAb in *Men1* KO mice with PNETs

- a. The first protocol evaluated the effects of therapy on tumor growth by measuring serum insulin and tracking animal survival: Twelve *Men1* KO mice (12 months of age) with insulinomas (based on measurement of serum insulin levels, as outlined above) were assigned to two groups treated with either *1H3 anti-B7x mAb* (Jeon *et al.* 2014) ($n=6$) or *normal mouse IgG* ($n=6$) (control group). 200 μ g of anti-*B7x* mAb or normal mouse IgG were administrated by intraperitoneal injection (IP) on days 1, 3, 7, 11, and 14. Each mouse received a total of 1mg of antibodies during the course of the experiments. All mice were treated at 12 months of age and we followed the survival time in each group during and after treatment.
- b. The second protocol was designed to examine immune response and tumor cell growth: Eight *Men1* KO mice (12 months of age) with insulinomas (based on measurement of serum insulin levels, as outlined above) were assigned to two groups and treated with either *1H3 anti-B7x mAb* ($n=4$) or normal mouse IgG ($n=4$) (control group). 200 μ g of anti-*B7x* mAb or normal mouse IgG were administrated by IP injection on days 1, 3, 7, 11, and 14. Each mouse received a total of 1mg of antibodies during the *B7x* experiments. All mice were sacrificed at 1 month post-treatment. Whole pancreatic tissues from the mice were flash-frozen at -80°C then later formalin-fixed for further analysis. The tumor growth and immune response in the tumor immune microenvironment were assessed.

Laser capture microdissection (LCM)

*In order to detect the mRNA expression level of target genes in the islets of the pancreas, we used a LCM technique to isolate the islets of the pancreas from mice in our study. The pancreatic tissues from *Men1* KO and WT mice were harvested and embedded in OCT. The OCT-embedded tissue sections were stained by an Arcturus Histogene Frozen Section Staining Kit (AB Applied Biosystems, CA, USA) and the islet tissues dissected using an Arcturus XT LCM system.*

Real-time RT-PCR analysis

*In human samples with PNETs, the endocrine tissues were isolated from primary tumors, metastases, and islets of adjacent normal tissues by LCM. For human samples from GINET patients, because we cannot isolate the endocrine cells from GI tissues, we used matched adjacent normal tissues as normal controls. Moreover, HHLA2 and B7x were reported not to be expressed in all cell types in normal GI organs. In addition, we also used LCM to isolate islet tissues of the pancreas from *Men1* KO and *Men1* WT mice. Furthermore, total RNA was extracted from isolated tissues using the RNeasy total RNA kit (Qiagen, Valencia, CA, USA). mRNA expression was detected by quantitative real-time RT-PCR. Measurements of fluorescent signal intensity after standardization with an 18S ribosomal RNA (rRNA) internal control allowed quantification. The primers for HHLA2 and B7x gene were purchased from Qiagen (NM_007072 and NM_001253849). The primers for Perforin, Granzyme B, and FasL genes are listed in Supplementary Table 2.*

Hematoxylin and eosin (H&E) staining

Mice were sacrificed and whole pancreas and other normal tissues (brain, lung, heart, liver, spleen, kidney, and muscle) were dissected, formalin-fixed, paraffin embedding, and sectioned (5 μ m) using a rotating microtome (Microm, Walldorf, Germany). Hematoxylin and eosin staining was performed according to standard procedures. For morphometric analysis, three 100 μ m sections were digitalized and pancreas cross-sectional area in the section with maximal tumor diameter was determined by computerized pixel counting.

Immunohistochemistry (IHC) staining

IHC staining was used to determine expression levels of HHLA2, B7x, CD4, CD8, CD11b, Ki67, PCNA, and Caspase 3 in pancreatic tissues from NET patients and *Men1* KO and WT mice. Purified mouse anti-HHLA2 (Zhao *et al.* 2013) and anti-B7x antibodies (obtained from Dr. Zang's laboratory, Einstein; 1:50 dilution)(Zang *et al.*, 2007), rabbit anti-CD4 (1:1000 dilution; Abcam, MA, USA), rabbit anti-CD8 (1:200 dilution; Abcam, MA, USA), mouse-anti-HIF-1 α (1:200 dilution; Abcam, MA, USA), rabbit anti-Ki67 (1:100 dilution; Abcam, MA, USA), rabbit anti-CD11B (1:400 dilution; Novus Biologicals, CO, USA), rabbit anti-proliferating cell nuclear antigen (PCNA) (1:200 dilution; Santa Cruz, CA, USA), and rabbit anti-Caspase 3 (1:200 dilution; BD Pharmingen, CA, USA) antibodies were used and incubated overnight at 4°C. A HEP/DAB (ABC) detection IHC kit (Abcam, MA, USA) was used for IHC analysis. *An IHC staining without primary antibody was used as a negative control and a recommended tissue for positive expression of the antibody was used as a positive control.*

Immunofluorescence (IF) staining

IF staining was used for the evaluation of the expression levels of *interferon- γ* (*IFN- γ*) and *tumor necrosis factor- α* (*TNF- α*) in pancreatic tissues from *Men1* KO and WT mice. The sections were incubated overnight at 4°C with primary antibodies: pig anti-insulin antibody (1:500 dilution; DAKO, CA, USA) and rabbit anti-IFN- γ (1:100 dilution; Abcam, MA, USA), rabbit anti-TNF- α (1:500 dilution; Abcam, MA, USA) antibodies. After washing, the slides were incubated with goat anti-rabbit Alexa Fluor 488 and goat anti-guinea pig Alexa Fluor 647 (both 1:200 dilutions; Thermo Fisher Scientific, NY, USA) for 45 min in the dark. The slides were assembled in Vectashield mounting medium with DAPI (Vector Laboratories, CA, USA). Images were obtained with an Epifluorescence microscope with an attached camera. *The quantification of IF staining was performed by counting cells that strongly expressed the target proteins in 4 randomly selected fields per slide under 10 and 20x magnification.*

Western Blot analysis

Total protein was extracted from pancreatic tissues of *Men1* KO and WT mice using Radioimmunoprecipitation assay (RIPA) lysis buffer (Pierce, Rockford, IL). The extracted proteins were quantified and equal amounts of protein were separated by SDS-PAGE and blotted. The blots were incubated overnight at 4°C with primary antibodies: rabbit anti-GAPDH (1:1000 dilution; Thermo Fisher Scientific, NY, USA), mouse-anti-HIF-1 α (1:400 dilution; Abcam, MA, USA), *rabbit-anti-Perforin (1:1000 dilution; Abcam, MA, USA), rabbit-anti-Granzyme B (1:1000 dilution; Abcam, MA, USA), mouse-anti-Fas L (1:1000 dilution; Santa Cruz Biotech, CA, USA)* antibodies, respectively. The secondary antibodies used were: 1:5000 dilution of anti-mouse IgG-HRP, 1: 2500 dilution of anti-rabbit IgG-HRP, and 1:2500 dilution of anti-goat IgG-HRP (Santa Cruz, CA, USA), respectively. The small differences in loading were corrected by comparison to the loading control (GAPDH).

Chromatin Immunoprecipitation (ChIP)-PCR assay—We used ChIP-PCR to validate the HIF-1 α -B7x promoter interaction in N134 tumor β -cells in vitro and MEN1 tumor tissues from *Men1* KO mice (Magna ChIP kit, Millipore, CA) according to the manufacturer's protocol. Briefly, cell lines and tumor tissues were collected and cross-linked with 1% formaldehyde for 10 minutes at room temperature. Cross-linking was stopped by adding glycine for 5 minutes then cells were washed three times with ice-cold PBS/proteinase inhibitor. The nuclear extract was prepared, and chromatin was sonicated to a size of 500–1000 bp. The HIF-1 α ChIP assay was performed by incubating with 10 μ l (1 μ g/ μ l) of HIF-1 α antibody (Abcam, MA, USA) overnight at 4°C. Rabbit IgG was used as a negative control. Protein G magnetic beads were used to collect the immunoprecipitates (IP). The HIF-1 α -ChIP DNA was assayed by PCR using primers corresponding to the promoter regions of the B7x gene, *and a known target of HIF-1 α , PFKFB4, also was used as a positive control* (Xia et al., 2009) (Supplementary Table 3).

Enzyme-linked immunosorbent assay (ELISA)

Blood was collected via a retro-orbital bleeding technique from anti-B7x treated mice and IgG-treated control mice following an 18-h fast. Serum levels of insulin were measured by

ELISA with the Ultrasensitive Mouse Insulin ELISA Kit (Mercodia, NC, USA) as described previously (Shen et al., 2009). The expression levels of IFN- γ and TNF- α in tumor tissues from B7x treated mice and IgG control mice were measured by a mouse IFN- γ ELISA kit (Abcam, MA, USA) and a mouse TNF- α ELISA kit (Life Technologies, MD, USA) following the manufacturer protocols.

Terminal deoxyuridine triphosphate nick end labeling (TUNEL) assay

Apoptotic status in tumor tissues was measured in anti-B7x treated and IgG control mice by TUNEL assay. For quantification of apoptosis, the TUNEL assay was performed according to the manufacturer instructions with an *In Situ* Cell Death Detection Kit (Roche Diagnostic, IN, USA). The sections were labeled and stained with the TUNEL reaction mixture (label plus enzyme solutions) for 60 min at 37°C and washed twice with PBS in the dark. The slides were mounted in Vectashield mounting medium with DAPI (Vector Laboratories, CA, USA). The apoptotic fluorescent cells were counted under a fluorescent microscope and the numbers were expressed as the percentage of total cells $\pm$ standard deviation (SD). A negative control without enzyme treatment and a positive control with DNase I treatment were also performed.

Survival Analysis

We followed the survival in each group during and after treatment from our animals under the first protocol. Then we calculated the average survival rate for each group.

Statistical analysis

The two-tailed student's t-test and ANOVA were performed to assess the significance between experimental groups. The Pearson's correlation or linear regression analysis was used for correlations between parameters. Cumulative survival time was calculated by the Kaplan-Meier method and comparison between the groups was tested by the log-rank test. GraphPad software (GraphPad Software, Inc, version 5.02) was used for all statistical analyses. Statistically significant P values are indicated in the figures with asterisks: ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$ for tested samples vs controls.

RESULTS

Increased HHLA2 and B7x with decreased tumor infiltrating lymphocytes (CD4 and CD8) in human GINETs and PNETs

To determine if HHLA2 and B7x were overexpressed in human NETs, we performed IHC assays and quantitative real-time PCR on GINETs (N=13) and PNETs (N=24) and matched normal tissues. We demonstrated that both HHLA2 and B7x proteins were expressed to a high degree in GINET and PNET tissues compared with their matched normal tissues as measured by IHC (Figure 1). The pathological analysis was reviewed by a pathologist with double-blind reviewing. *The positive cell rate was scored by 0: < 1%, 1: 0–15%, 2: 15–30%, 3: 30–60%, 4: >60%, and the score in human NETs for IHC staining is also presented in the Supplemental Table 1 and 4.* Tumor infiltrating T cells (CD4 and CD8) were significantly decreased in the tumor microenvironment with high expression of HHLA2 and B7x compared with the tumor microenvironment with low

expression of HHLA2 and B7x (Figure 1). These results suggest an association between HHLA2 and B7x and tumor infiltrating lymphocytes. Furthermore, we demonstrated that the expressed positive cell rates of HHLA2 and B7x were statistically higher in non-functional (do not produce hormones) (expressed positive cell rate: $45.2 \pm 7.9\%$ and $42.6 \pm 12.8\%$) and functional (hormone-producing) GINETs and PNETs (expressed positive cell rate: $15.9 \pm 5.7\%$ and $22.8 \pm 4.8\%$) compared to nonfunctional case matched normal tissues, $4.9 \pm 2.3\%$ and $3.5 \pm 1.9\%$, respectively ($P < 0.001$), and functional case matched normal tissues, 4.2% and 1.8% , respectively ($P < 0.001$) as measured by IHC staining. The mRNA expression levels, as measured by qRT-PCR, of both HHLA2 and B7x, are also statistically higher in non-functional and functional GINETs and PNETs compared to case matched normal tissues (Supplemental Figure 1A). We also found a strong correlation between mRNA and protein expression of HHLA2 and B7x, correlation coefficient: 0.8998 ($P < 0.001$) and 0.8702 ($P < 0.001$), respectively. In addition, we determined that the mRNA and protein expression levels of HHLA2 and B7x are significantly higher in primary tumor tissues that resulted in metastasis ($90.3 \pm 8.2\%$ and $80.5 \pm 5.4\%$) compared to primary tumor tissues that did not result in metastasis ($P < 0.001$) (Supplemental Figure 1A) (Supplemental Table 1). These results revealed that both the mRNA and protein levels of HHLA2 and B7x were highly expressed in human GINETs and PNETs and that primary tumors with metastasis tended to have higher levels than primary tumors without metastasis, suggesting these two new immune checkpoints may play important roles in tumor progression and metastasis.

Expression levels of immune checkpoint proteins HHLA2 and B7x correlate with clinical and pathological parameters.

To evaluate the clinical utility of HHLA2 and B7x as informative markers, we performed correlation analysis between the expression levels of HHLA2 and B7x and clinical-pathological parameters using Pearson's Correlation. We found that there is a correlation between the expressed positive cell rate of HHLA2 with tumor size, correlation coefficient: 0.6488 ($P = 0.0002$) for nonfunctional NETs (Supplemental Figure 1B₁) and 0.5613 ($P = 0.0421$) for functional NETs (Supplemental Figure 1B₅), respectively, and tumor cell proliferation as measured by IHC using a Ki67 marker, correlation coefficient: 0.4222 ($P = 0.0064$) for nonfunctional NETs (Supplemental Figure 1B₃) and 0.5854 ($P = 0.0370$) for functional NETs (Supplemental Figure 1B₇), respectively. In addition, we demonstrated that a correlation between the expressed positive cell rate of B7x with tumor size, correlation coefficient: 0.6681 ($P < 0.0001$) for nonfunctional NETs (Supplemental Figure 1B₂) and 0.7818 ($P = 0.0082$) for functional NETs (Supplemental Figure 1B₆), respectively, and tumor cell proliferation as measured by IHC using a Ki67 marker, correlation coefficient: 0.5786 ($P = 0.0010$) for nonfunctional NETs (Supplemental Figure 1B₄) and 0.8621 ($P = 0.0025$) for functional NETs (Supplemental Figure 1B₈), respectively. Our results indicate that the expression of HHLA2 and B7x correlate with higher grade and higher incidence of nodal and distant spread of GINETs and PNETs (Supplemental Figure 1B)(Supplemental Table 1 and 4).

Upregulation of B7x in the PNETs of Men1 KO mice.—Previously, we developed a genetically-engineered mouse model of PNETs (*Pdx1-Cre; Men1 floxed/floxed*) that

provides an *in vivo* tool for the study of PNETs that closely mimics human disease (Shen *et al.* 2009). This model relies on the tissue-specific deletion of the *Men1* gene in the pancreas, resulting in development of PNETs that secrete insulin. Using this model, we demonstrated that the expression level of B7x is upregulated in the pancreatic endocrine tumor tissue from *Men1* KO mice compared to the normal pancreatic endocrine tissue from *Men1* WT mice (Figure 2). Additionally, B7x is not expressed in control tissues (brain, heart, liver, spleen, kidney, and muscle) from *Men1* KO mice or in any tissues of *Men1* WT mice (Supplemental Figure 2).

Further, to determine whether the increased B7x expression is caused by *Men1* KO or not, we demonstrated that PNET tissues with WT menin from *RIP-Tag* mice also have high expression of B7x (Supplemental Figure 3).

HIF-1 α overexpression coincides with the upregulation of B7x in the tumor microenvironment of *Men1* KO mice and in the *Rip-Tag* β -cell line.

By analyzing our *Men1* KO model, we have demonstrated that the proliferating β -cells result in the overexpression of HIF-1 α in the tumor microenvironment at 6 months (hyperplasia) and at 12 months (insulinoma) but not at earlier time points (4 months) (Figure 3). Furthermore, we have demonstrated that HIF-1 α overexpression coincides with the upregulation of B7x in the tumor microenvironment during β -cell proliferation at 6 months (hyperplasia) and at 12 months (insulinoma) but not at earlier time points (4 months) without β -cell proliferation and not in islets of WT mice (Figure 3).

Given that explants from our *Men1* KO mice fail to grow *in vitro*, we have adopted a transplantable line from the N134 model for *in vitro* studies. When grown *in vitro*, *RIP-Tag* islet tumor β -cells (N134) lack B7x expression, presumably due to the lack of influence of the tumor microenvironment. When cultured under hypoxic conditions (1% O₂), N134 cells expressed B7x and HIF-1 α (Figure 4A₁). However, HIF-1 α and B7x were not expressed in N134 cells transduced with HIF-1 α siRNA (200 nM, 48 hrs, Santa Cruz Biotechnology) under hypoxic conditions (1% O₂) (Figure 4A₂), providing a direct association between HIF-1 α and B7x expression.

HIF-1 α interacts with the B7x promoter

To expand our mechanistic characterization, we investigated whether HIF-1 α interacts with the promoter region of B7x in both N134 tumor β -cell line *in vitro* and in fresh *Men1* tumor tissues from *Men1* KO mice. We identified that HIF-1 α can bind to the B7x promoter in N134 β -cells under hypoxic conditions (1% O₂) and in tumor tissues from *Men1* KO mice by using a ChIP-PCR analysis (Figure 4B).

Loss of the immune checkpoint protein B7x results in T-cell infiltration associated with reduced tumor burden.

We have crossed our *Men1* KO mice with mice lacking B7x (*B7x* KO). We observed a significant decrease in serum insulin, a tumor biomarker, in the *Men1* KO/*B7x* KO mice with loss of B7x expression from 4 to 12 months when compared to *Men1* KO/*B7x* WT mice (P<0.001) (Figure 5). The *Men1* KO/*B7x* KO mice had a significant decrease in islet

size compared with the *Men1* KO/*B7x* WT mice ($1477 \mu\text{m}^2 \pm 441$ vs $27382 \mu\text{m}^2 \pm 827$; $P < 0.001$), but the islet size in *Men1* KO/*B7x* KO mice had a slight increase compared with the *Men1* WT/*B7x* KO and *Men1* WT/*B7x* WT control mice ($1477 \mu\text{m}^2 \pm 441$ vs $1037 \mu\text{m}^2 \pm 211$ and $988 \mu\text{m}^2 \pm 177$; $P < 0.05$) at 12 months of age (Figure 6A). Furthermore, we demonstrated that the *Men1* KO/*B7x* KO mice had significantly increased T-cell infiltration compared with *Men1* KO/*B7x* WT mice, but both negative control groups (*Men1* WT/*B7x* KO and *Men1* WT/*B7x* WT) did not exhibit any T-cell infiltration in the islets of the pancreas as measured by IHC (Figure 6C and 6D). These results indicate that loss of *B7x* allows T-cells activation and prevents the formation of PNETs.

Blockade of B7x with anti-B7x mAb decreases serum insulin levels and improves survival in *Men1* KO mice

Based on the therapeutic potential of inhibiting *B7x* overexpression, we treated PNET-exhibiting *Men1* KO mice using a high-affinity anti-*B7x* antibody (*B7x* mAb 1H3) (Jeon *et al.* 2014). Since *Men1* KO mice develop insulinomas, which are clinically defined by a measurable increase in serum insulin levels, serum insulin can be used as a surrogate for tumor presence and size. In two groups of *Men1* KO mice, after two weeks of treatment with either anti-*B7x* mAb ($N=6$) or normal mouse IgG ($N=6$), the group treated with anti-*B7x* mAb exhibited a strong and sustained decrease in serum insulin as measured by ELISA ($P < 0.001$) (Figure 7A) and an enhanced survival rate of 83.3% (5/6) compared to the group treated with control IgG 0% (0/6) ($P < 0.001$) (Figure 7B). These results support the conclusion that blockade of *B7x* can decrease serum insulin and improve survival in *Men1* KO mice.

Blockade of B7x with a B7x mAb inhibits tumor cell proliferation and induces tumor cell apoptosis in *Men1* KO mice

To identify the effects of targeting *B7x* with anti- *B7x* mAb treatment on tumor growth in *Men1* KO mice, we compared the tumor size of the treated group and the IgG control group. We demonstrated that the tumor size was significantly reduced in the anti-*B7x* mAb treated group when compared to the IgG control group ($1928 \mu\text{m}^2 \pm 661$ vs $13196 \mu\text{m}^2 \pm 1525$; $P < 0.0001$) (Figure 7C). Furthermore, we demonstrated that tumor cell proliferation was significantly inhibited as measured by PCNA (Figure 7D₁) and Ki67 staining (Figure 7D₂). In addition to decreased proliferation, apoptosis was significantly increased as measured by Caspase 3 staining (Figure 7D₃) and a TUNEL assay (Figure 7D₄) in *B7x* mAb treated mice when compared with IgG control mice. These results indicate that blockage of *B7x* can inhibit tumor growth and induce apoptosis in *Men1* KO mice.

Blockade of B7x enhances T-cell response, decreases infiltrating immune suppressor cells MDSCs, and induces the production of IFN- γ and TNF- α in the tumor microenvironment

We demonstrated that anti-*B7x* mAb treatment robustly increases tumor infiltrates of both CD4 (Figure 8A₂ and 8B) and CD8 (Figure 8A₄ and 8B) T-cells, in the PNET tumor microenvironment when compared to the control group (Figure 8A₁, 8A₃, and 8B). Furthermore, we investigated the effect of *B7x* antibody treatment on *immunosuppressive myeloid-derived suppressor cell* (MDSC) infiltrates and demonstrated that they are significantly decreased in the treated group (Figure 8A₆ and 8B) compared to control (Figure 8A₅ and 8B). We also observed that INF- γ and TNF- α are significantly increased

following anti-B7x treatment, suggesting that *cytotoxic T-lymphocytes (CTL)* may be activated and act to enhance the anti-tumor effect in B7x mAb treated mice. This was determined by IF staining (Supplemental Figure 4) and ELISA (Figure 8C). These results indicate that blockade of B7x can enhance anti-tumor immune responses by increasing CTL and decreasing immune suppression.

Blockade of B7x facilitates anticancer immunity through increased expression of Perforin, Granzyme B, and FasL in tumors of *Men1* KO mice

To further characterize the signaling pathways involved in the immune response with blockade of B7x, we determined the expression levels of perforin, granzyme B, and FasL mRNA and proteins in tumors using LCM-quantitative real-time PCR and Western blot. We demonstrated a significant upregulation of perforin, granzyme B, and FasL in the tumors of *Men1* KO mice with anti-B7x antibody treatment compared to IgG controls (Figure 9). These results indicate that blockage of B7x induces apoptosis in tumor cells by upregulation of perforin/granzyme B and FasL pathways.

DISCUSSION

The immune system is capable of playing an important role in controlling and eradicating cancer. However, immunosuppressive processes that occur in the tumor microenvironment limit the normal function of the immune system in multiple malignancies. It is thought that the main function of tumor cell-associated immune checkpoint molecules is the modulation of antitumor immune responses. To further explore this paradigm, we characterized the immunological role of two unique immune checkpoints, HHLA2 and B7x, in the development of NETs in human NET samples and our *Men1* KO PNET mouse model. We observed that increased expression of HHLA2 and B7x is associated with human GINETs and PNETs. The expression of the HHLA2 and B7x proteins is also higher in non-functional tumors, which are generally identified later in disease progression compared to functional tumors. Our studies further demonstrated that the expression of HHLA2 and B7x is higher in primary tumors with associated metastases compared to the those without metastases. This correlation between the expression of HHLA2 and B7x with detrimental clinical features also extends to tumor size and tumor cell proliferation (Ki67 staining). In addition to being present in human samples, upregulated B7x was also confirmed in our *Men1* KO mouse model, which serves as an excellent surrogate for human disease progression. Our results indicate that primary tumor cells may up-regulate the expression of HHLA2 and B7x, thereby equipping these cells with an increased capability to grow and metastasize, eventually resulting in the advanced progression of NETs. The over-expression of these B7 immune checkpoints by human NETs may provide the essential mechanism by which NET cells avoid destruction mediated by tumor-reactive T-cells and other immune cells. The high level of B7x expression may facilitate tumor progression, therefore providing a valuable prognostic marker to predict outcome based on a suitable drug target.

In addition, to explore whether the expression of B7x was only upregulated in the PNETs of *Men1* KO mice, we investigated B7x expression in the PNETs from *RIP-Tag* mice. This *RIP-Tag* transgenic mouse model expresses the SV40 T-antigen under control of the rat

insulin promoter, resulting in pancreatic β -cell tumors without *MEN1* mutation nor the loss of menin. We demonstrated that the B7x upregulation was also observed in the PNETs from *RIP-Tag* mice (Supplemental Figure 3). Our results showed that increased B7x expression is not caused by *Men1* KO.

Previous studies have reported upregulated expression of immune checkpoints that inhibit T-cell function and increase immunosuppressive MDSCs within the tumor microenvironment (Assal *et al.* 2015), and these reports support what we have shown in our current study. Tumor-infiltrating lymphocytes, particularly CD8 and CD4 T-cells, are prognostic and predictive in NETs, suggesting that T-cells are a significant anti-tumor population during NET progression (Epardaud *et al.* 2008, Katz *et al.* 2010, Sato *et al.* 2014). However, tumor cells may over-express T-cell inhibitory B7 molecules as they “evolve” to evade the immune system. Our experimental findings demonstrate that the overexpression of B7x in the tumor cells from our *Men1* KO mouse model was associated with a decrease in T-cells and an increase in MDSCs within the tumor microenvironment, an occurrence we reasonably believe to be occurring in human tumors as well. These findings suggest that the B7x molecule is mediating immune inhibition in NETs, by both inhibiting T-cell activation and increasing the MDSC immunosuppressive function.

The molecular mechanism(s) behind B7x upregulation have not been entirely elucidated. We found that the *Rip-Tag* tumor cells lacked B7x expression, presumably due to the lack of influence of the tumor microenvironment. Hypoxia in the tumor microenvironment is a common feature of solid tumors as well as NETs. The hypoxic tumor microenvironment is caused by increased oxygen consumption due to hyperplasia and decreased oxygen delivery due to increased diffusion and is primarily driven by the hypoxia inducible transcription factors. Hypoxia causes the upregulation of PD-L1 and there are reports of HIF-1 α binding directly to the promoter of PD-L1 (Noman *et al.* 2014). In order to further our mechanistic understanding of B7x upregulation, we evaluated whether the rapid proliferation of tumor cells drives HIF-1 α overexpression resulting in upregulation of B7x in the tumor microenvironment of PNETs. We have demonstrated that HIF-1 α overexpression is associated with the upregulation of B7x both in our *in vivo* and *in vitro* models. Furthermore, we identified that HIF-1 α can bind the B7x promoter in tumor β -cells cultured *in vitro* under hypoxic conditions and MEN1 tumor tissues from *Men1* KO mice. This suggests that a potential molecular mechanism of B7x upregulation is due to the expression of HIF-1 α following the relative hypoxia resulting from the rapid growth of tumor cells.

We have demonstrated that B7x is expressed to a high degree in patient-derived GINETS and PNETs. To further investigate the role that B7x plays in the maintenance of the tumor phenotype seen in *Men1* knockout mice, we have crossed our *Men1* KO mice with mice lacking B7x (*B7x* KO). We observed a significant decrease in serum insulin, a tumor biomarker, in the *Men1* KO/*B7x* KO mice from 4 to 12 months when compared to *Men1* KO/*B7x* WT mice ($P < 0.001$). Furthermore, we demonstrated that the *Men1* KO/*B7x* KO mice with loss of B7x expression inhibited tumor growth in the islets of the pancreas and increased T-cell infiltration compared with *Men1* KO/*B7x* WT mice.

Immune checkpoint inhibitors have demonstrated considerable promise with their recent approval for the treatment of melanoma, non-small cell lung cancer, and other cancers (Shih *et al.* 2014). Studies have shown that targeting the PD-1 and PD-L1 molecules yields a robust antitumor response in the treatment of melanoma, non-small cell lung cancer, head and neck squamous cell cancer, urothelial cancer, Hodgkin lymphoma, and renal cell cancer (Bellmunt *et al.* 2017, Hodi *et al.* 2014, Reck *et al.* 2016,). In this study, our cohorts of mice administered with the anti-B7x antibody showed a strong and sustained decrease in serum insulin (a surrogate for tumor response) over the experimental timeframe compared with animals that were administered the IgG control. Importantly, mice treated with anti-B7x antibody also exhibited a significant increase in overall survival compared with mice treated with the IgG control. Furthermore, we also demonstrated that the beneficial outcome of the blockade of B7x was not limited to preventing insulin secretion but also included antiproliferative effects and apoptotic induction. This is of particular clinical relevance because the B7x antibody may not only alleviate symptoms in patients with insulinomas or other functional NETs but might actually lead to tumor regression and prolonged survival.

Blocking the interaction between an immune checkpoint molecule and its ligand(s) has been shown to result in impressive antitumor responses by altering the tumor immune microenvironment, including increasing T-cell activation and inhibiting infiltration by MDSC. We analyzed immune cells and cytokines to better understand how targeting B7x may transform immune suppression into immune stimulation in the tumor microenvironment. As evidence of the therapeutic mechanism of the anti-B7x mAb treatment, we observed that mice treated with the anti-B7x mAb had a higher percentage of CD4 and CD8 T-cell infiltration and a lower percentage of MDSC immune suppressing cells than control IgG treated mice. The production of IFN- γ and TNF- α were also significantly increased, suggesting that cytotoxic T-lymphocytes were activated. Furthermore, we have shown that targeting B7x activates the Perforin/Granzyme B/FasL pathways in the tumor microenvironment of *Men1* KO mice. This marks a significant finding because Perforins and Granzyme B are critical players in tumor cell destruction by CTL. CTL kills tumor cells through Perforins and Granzymes upon contact between the T-cell receptor and presented self- major histocompatibility complex molecules on the tumor cell or antigen presenting cells (Chowdhury *et al.* 2008). Granzyme B activates intrinsic cell death proteases and caspases in tumor cells leading to caspase mediated degradation of hundreds of cellular protein substrates, which promotes fast and efficient apoptosis. By functioning through both reduction of proliferation and increasing apoptosis, B7x blockade exhibits significant anti-tumor effects.

In the present study we demonstrated that B7x is a critical mediator of tumor immunity in the tumor microenvironment of NETs. Furthermore, we found that B7x upregulation is a result of the expression of HIF-1 α following the relative hypoxia resulting from rapid growth of tumor cells. Blockade of B7x with an anti-B7x antibody can inhibit tumor cell proliferation and induce apoptosis, both of which improve the tumor immune microenvironment and result in increased survival in the preclinical setting. This work reveals important insights that can be rapidly translated to the clinic by the targeting of B7x for the treatment of patients with NETs. Additionally, given the strong likelihood of the

presence of this mechanism in other cancer types, our anti-B7x mAb may have additional potential for the treatment of other types of cancers.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Expression of HHLA2 and B7x in human pancreatic neuroendocrine tumors (PNETs). Immunohistochemistry (IHC) staining of HHLA2 (A) and B7x (B) are shown in human PNET tissue; Negative staining of HHLA2 (E) and B7x (F) is also shown in the matched normal tissues by IHC staining. Furthermore, negative staining of CD4 (C) and CD8 (D) are shown in the human PNET tissue with high expression of HHLA2 and B7x; Positive staining of CD4 (G) and CD8 (H) is also shown in the human PNET tumor with low expression of HHLA2 and B7x by IHC staining. The images were taken with the 40X objective.

Figure 2. B7x expression in the tumor microenvironment of *Men1* knockout (KO) mice. Tumors are observed in pancreatic endocrine tissues of *Men1* KO mice (B1) but not in *Men1* wild type (WT) mice (A1) as shown by *H&E* staining. B7x expression is upregulated in tumor tissues from *Men1* KO mice (B2) compared to pancreatic tissue from *Men1* WT mice (A2) by *immunohistochemistry (IHC)* staining. The A1 and B1 images were taken with the 2.5X objective and A2 and B2 images were taken with the 20X objective.

Figure 3. HIF-1 α overexpression is associated with the upregulation of B7x in tumor microenvironment of *Men1* knockout (KO) mice.

The proliferated β -cells resulting in the overexpression of HIF-1 α in the tumor microenvironment of *Men1* KO mice at 12 months (insulinoma) (A1 and C1) and at 6 months (hyperplasia) (A2 and C2) are associated with the upregulation of B7x (E1 and E2) but not in islets of *Men1* KO mice at 4 months (A3, C3, and E3) and islets of *Men1* wild type (WT) mice (B1, D1, F1, B2, D2, F2, B3, D3, and F3) by immunohistochemistry (IHC) staining. The images were taken with the 20X objective.

Figure 4. HIF-1α regulates the B7x expression and HIF-1α binds to B7x promoter.

In vitro, when cultured under hypoxic conditions (1% O₂) after 48h and 72h, N134 isle tumor β cells that lack B7x expression were found to induce the upregulation of HIF-1α and B7x (A1); however, the expression levels of HIF-1α and B7x were not induced in the islet tumor β cells following HIF-1α siRNA treatment after 48 hours by western blot (A2). HIF-1α can bind to the B7x promoter in N134 isle tumor β cells under *in vitro* hypoxic conditions (B1) and tumor tissues of *Men1* KO mice (B2) using a *chromatin immunoprecipitation (ChIP)-PCR analysis*. The HIF-1α *immunoprecipitation (IP)* products were amplified using quantitative real-time RT-PCR with primers corresponding to the promoter regions of the B7x gene and the relative amount of DNA was calculated using fold enrichment compared to the IgG Ab control. *Statistically significant P values are indicated in the figures with asterisks: ***, P < 0.001.*

Figure 5. Loss of B7x decreases serum insulin level.

There was a significant decrease in serum insulin in the *Men1* knockout (KO)/B7x KO mice compared to *Men1* KO/B7x wild type (WT) mice ($P < 0.01$) by enzyme-linked immunosorbent assay (ELISA). Both normal negative control groups: *Men1* WT/B7x KO and *Men1* WT/B7x WT mice didn't observe any changes. Statistically significant P values are indicated in the figures with asterisks: ***, $P < 0.001$; **, $P < 0.01$.

Figure 6. Loss of B7x promotes T-cell infiltration in islets infiltration and associated with reduced tumor burden in *Men1* knockout (KO)/B7x KO mice.

H&E and immunohistochemistry (IHC) staining showed that the *Men1* KO/B7x KO mice have loss of B7x expression in islets of pancreas (B1) inhibited tumor growth in islets of pancreas (A1) and demonstrated increased T-cell infiltration in the islets (C1 and D1) compared with *Men1* KO/B7x wild type (WT) mice (A2, B2, C2 and D2). Both normal negative control groups: *Men1* WT/B7x KO and *Men1* WT/B7x WT mice didn't observe any changes (A3, B3, C3, D3, A4, B4, C4, and D4).

Figure 7. B7x mAb treatment induces an antitumor response and improved survival in Men1 knockout (KO) mice.

The mice were treated with B7x Ab and IgG control at 12 months old. There was a significant decrease in serum insulin in the B7x treatment group but no change in the IgG control group in Men1 KO mice ($P < 0.001$) (A). Increased survival was also observed in the B7x antibody treatment cohort compared with mice treated with IgG control ($P < 0.001$) (B). H&E staining shows that the B7x mAb treated mice had a significant decrease in tumor size compared with IgG control mice (C). The images were scanned with the 0.5X (C1 and C3) and 3X objective (C2 and C4). Tumor cell proliferation is significantly decreased by PCNA and Ki67 analysis (D1 and D2) and apoptosis is significantly increased by Caspase 3 and Terminal deoxynucleotidyl transferase (TUNEL) analysis (D3 and D4) in the B7x mAb treatment group compared with the IgG control group. The average across all samples $\pm$ SD were used for statistics in the histograms. Statistically significant P values are indicated in the figures with asterisks: ***, $P < 0.001$; **, $P < 0.01$.

Figure 8. Anti-B7x mAb induces cytotoxic T lymphocyte response and increased expression of cytokines in the tumor microenvironment with anti-B7x mAb treatment.

Immunohistochemistry (IHC) staining shows that the CD4+ (A2) and CD8+ (A4) T cells are increased and CD11b+ (MDSCs) (A6) are decreased in the B7x mAb treatment group compared with the IgG control group (A1, A3, and A5); quantification was achieved by counting CD4+, CD8+, and CD11b+ (B). The levels of IFN- γ and TNF- α are also significantly increased in the tumor microenvironment in the B7x treatment group compared with the IgG control as shown by enzyme-linked immunosorbent assay (ELISA) (C). The average across all samples $\pm$ SD was used for statistics in the histograms. Statistically significant P values are indicated in the figures with asterisks: ***, $P < 0.001$; **, $P < 0.01$.

Figure 9. Blockade of B7x increased expression of Perforin, Granzyme B, and FasL in the tumor microenvironment with anti-B7x mAb treatment.

The mRNA expression of perforin, granzyme B, and FasL were quantified by real-time PCR and we observed a significant upregulation of perforin, granzyme B, and FasL in the tumors of Men1 knockout (KO) mice with B7x antibody treatment compared to IgG controls (A). Furthermore, we demonstrated that the protein levels of perforin, granzyme B, and FasL were significantly increased in the tumors of Men1 KO mice with B7x antibody treatment compared to IgG controls (B) by Western blot. The average across all samples $\pm$ SD was used for statistics in the histograms. Statistically significant P values are indicated in the figures with asterisks: ***, $P < 0.001$; **, $P < 0.01$.