Identification of regulators of cancer: immune interactions

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The purinergic receptor P2RY2 regulates B7-H3 expression in myeloid cancer cells

Meike E. W. Logtenberg[1,#], Zhaoqing Hu[2,#],
Ton N. Schumacher[1,3,*], and Chong Sun[2,3,*]

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[1] Division of Molecular Oncology & Immunology, Oncode Institute, The Netherlands Cancer Institute, Amsterdam, The Netherlands
[2] Immune Regulation in Cancer (D250), German Cancer Research Center, Heidelberg, Germany
[3] To whom correspondence should be addressed. Email: t.schumacher@nki.nl; c.sun@dkfz.de
[#,*] These authors contributed equally

ABSTRACT

B7-H3 (*CD276*) is an immunomodulatory molecule that is overexpressed on solid and hematological tumor cells and the targeting of B7-H3 has been shown to increase T cell cytotoxicity and inhibit tumor growth. However, the underlying mechanisms that regulate the (over)expression of B7-H3 on tumor cells remain unclear. Here we exploit flow cytometry-based CRISPR/Cas9 genetic screening to identify the purinergic receptor P2RY2 as a positive regulator of B7-H3 expression on myeloid cancer cells. Stimulation of P2RY2 by ATP, its major ligand, is shown to increase B7-H3 expression, whereas deletion of P2RY2 reduces B7-H3 transcription and cell surface protein expression in multiple myeloid cancer cell lines. Finally, we demonstrate that P2RY2 expression on tumor cells has a major impact on T cell function, at least partly through regulation of B7-H3 levels. Collectively, our data reveal that P2RY2 signaling increases B7-H3 expression in hematological cancer cells and indicate that the P2RY2 pathway may be exploited to enhance anti-cancer T cell immunity.

INTRODUCTION

The identification of immune checkpoint molecules has led to new therapeutic opportunities against cancer in recent years. In particular, the success of targeting the PD-1 - PD-L1 axis and the CTLA-4 immune checkpoint, has stimulated interest in other molecules that may enhance the activity of existing checkpoint therapies, or show activity in tumor types in which blockade of CTLA-4 and the PD-1/ PD-L1 pathway shows lower activity[1]. An immune checkpoint ligand of particular interest is the B7 family member B7-H3 (CD276). Similar to other B7 family members, B7-H3 has immune modulating functions, but its receptor on immune cells remains unknown. B7-H3 was initially identified as a co-stimulatory molecule that augments T cell activation *in vitro* and in autoimmune disease models[2,3]. However, subsequent work demonstrated that B7-H3 suppresses the effector function of both T cells and NK cells *in vitro* and in syngeneic mouse cancer models[4,5]. In addition, an immunosuppressive activity of B7-H3 has also been described in mouse models of graft-versus-host disease[6], cardiac allograft rejection[7] and autoimmune encephalomyelitis[8,9].

Higher levels of B7-H3 expression in cancer cells compared to healthy tissue have been reported in many solid human tumor types, including non-small-cell lung cancer (NSCLC), breast cancer, ovarian cancer, endometrial cancer, lung cancer, liver cancer and gastric cancer[10–14], and in hematological tumors, including diffuse large B cell lymphoma (DLBCL)[15,16], acute myeloid leukemia (AML)[16] and mantle cell lymphoma (MCL)[17]. In solid tumors, B7-H3 expression has also been detected on surrounding tumor blood vessels[10] and tumor-infiltrating immune cells, including myeloid cells[14]. Despite the widely observed aberrant expression of B7-H3 and the evidence for its role in immune evasion, the understanding of the mechanism(s) that regulate this immune checkpoint molecule remains limited. For example, whereas PD-L1 expression is increased in response to high interferon gamma (IFNγ) levels in the tumor microenvironment (TME)[18–20], no such danger or inflammation-related signals have been reported to regulate B7-H3.

RESULTS

To identify putative signals that may regulate B7-H3 levels in the TME, we set out to screen a genome-scale sgRNA library for regulators of B7-H3 cell surface expression, using the THP-1 myeloid cancer cell line that constitutively expresses B7-H3 (Extended Data Fig. 1a). Using fluorescence-activated cell sorting (FACS), sgRNA-modified cells that displayed particularly bright or particularly dull B7-H3 expression were isolated, and sgRNA abundance in both cell pools, was subsequently compared. As expected, sgRNAs targeting the *CD276* gene, encoding B7-H3 itself, were highly enriched in B7-H3^{low} cells,

underpinning the reliability of the genetic screen. In addition, this screen identified *P2RY2*, encoding the purinergic receptor P2RY2, as a positive regulator of B7-H3 (Fig. 1a). CRISPR/Cas9-mediated knock-out of P2RY2 resulted in a modest decrease in B7-H3 surface levels in THP-1 cells under non-inflammatory conditions (Fig. 1b, Extended Data Fig. 1b). The other way around, overexpression of P2RY2 increases steady-state B7-H3 levels, both in P2RY2-proficient and -deficient cells (Fig. 1c).

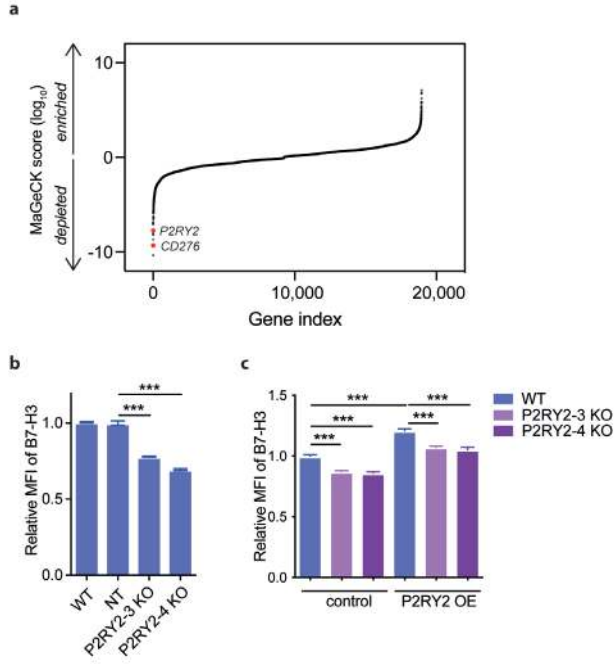

Figure 1. P2RY2 is a regulator of B7-H3 expression. a. Identification of modulators of B7-H3 expression by flow cytometry-based genetic screening. Dots represent individual genes; *y*-axis indicates Log_{10}-transformed MAGeCK robust ranking aggregation (RRA)-scores, based on the depletion or enrichment of sgRNAs in B7-H3^{high} vs B7-H3^{low} cell populations that were sorted from a genome-wide CRISPR/Cas9 (Brunello) library of THP-1 cells. **b.** B7-H3 cell surface expression on parental wild-type (WT) THP-1 cells, THP-1 cells transduced with a non-targeting sgRNA (NT), or with sgRNAs targeting *P2RY2* (P2RY2 KO), as determined by flow cytometry. Values indicate mean fluorescence intensity (MFI) relative to WT cells. **c**, B7-H3 cell surface expression on WT THP-1 cells, THP-1 cells transduced with sgRNAs targeting *P2RY2*, with the *P2RY2* open reading frame, or with the combination of the two, as determined by flow cytometry. Values indicate MFI relative to control-transduced WT cells. Data represent mean ± s.d. of triplicates (**b, c**). Data are representative of at least three (**b, c**) independent experiments and were analyzed by one-way ANOVA with multiple comparisons (**b, c**). ***P < 0.0001; n.s, not significant.

P2RY2 has previously been identified as a myeloid cell sensor for nucleotides that are released by apoptotic cells, directing their migration to enable phagocytic clearance[21]. As ATP and UTP are ligands of P2RY2[22], we next tested whether ligand-induced activation of the receptor influences B7-H3 surface expression. A dose-dependent increase of B7-H3 on THP-1 cells was observed following ATP or UTP exposure (Fig. 2a, Extended Data Fig. 2a). Furthermore, regulation of B7-H3 expression by P2RY2 was not restricted to THP-1

cells, but was observed in all other myeloid cancer cell lines tested, i.e., U937, K562, and KBM7 (Fig. 2b). ATP induced-B7-H3 expression was abrogated upon co-incubation with the ATP-diphosphohydrolase apyrase. Finally, neither apyrase nor ATP treatment influenced B7-H3 expression in P2RY2-deficient cells (Fig. 2b), indicating ATP-induced B7-H3 upregulation primarily occurs through P2RY2 in these cell lines.

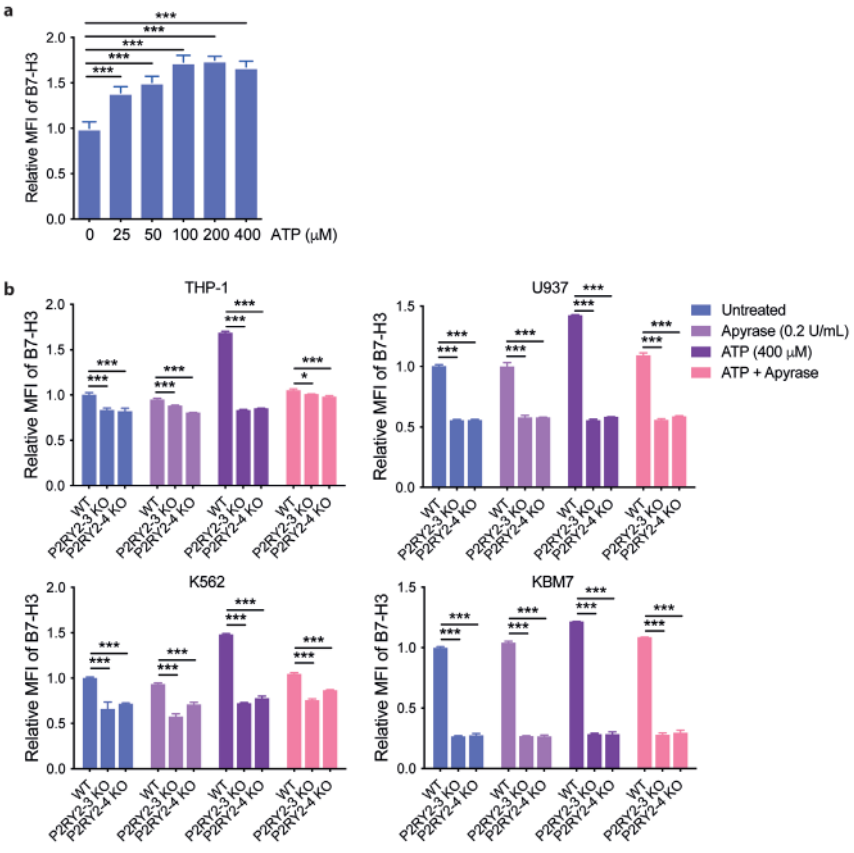

Figure 2. P2RY2 regulates ATP-mediated B7-H3 expression in myeloid cancer cell lines. a. Flow cytometry analysis of B7-H3 expression on THP-1 cells treated with the indicated concentrations of ATP, as determined by flow cytometry. Values indicate MFI relative to untreated cells. **b.** Flow cytometry analysis of B7-H3 expression in parental and P2RY2-deficient bulk THP-1, U937, KBM7, and K562 cells in the presence of ATP, apyrase, or the combination. Values indicate MFI relative to untreated WT cells. Data were collected in a single experiment (**a**), or are representative of three (THP-1, U937) or two (KBM7, K562) (**b**) independent experiments, and were analyzed by one-way (**a**) or two- way (**b**) ANOVA with multiple comparisons. ***$P \leq 0.0002$; **$P \leq 0.0021$; *$P \leq 0.0332$; n.s., not significant. Data represent mean ± s.d. of triplicates (**a, b**).

To elucidate the mechanism by which P2RY2 regulates B7-H3 cell surface expression, we assessed the effects of *P2RY2* disruption on total B7-H3 protein and mRNA levels. In line with the effect of P2RY2 deletion on cell surface levels of B7-H3, total B7-H3 protein levels were reduced by P2RY2 deletion (Fig. 3a). Furthermore, mRNA levels of B7-H3

were reduced by P2RY2 disruption, while ATP stimulation increased B7-H3 mRNA levels in a P2RY2 dependent fashion (Fig. 3b), and similar data were obtained in the U937 cell line (Extended Data Fig. 3a). Together, these data demonstrate that P2RY2 regulates B7-H3 expression at the transcriptional level, and that, through this mechanism, sensing of extracellular ATP levels influences B7-H3 expression on myeloid cancer cells.

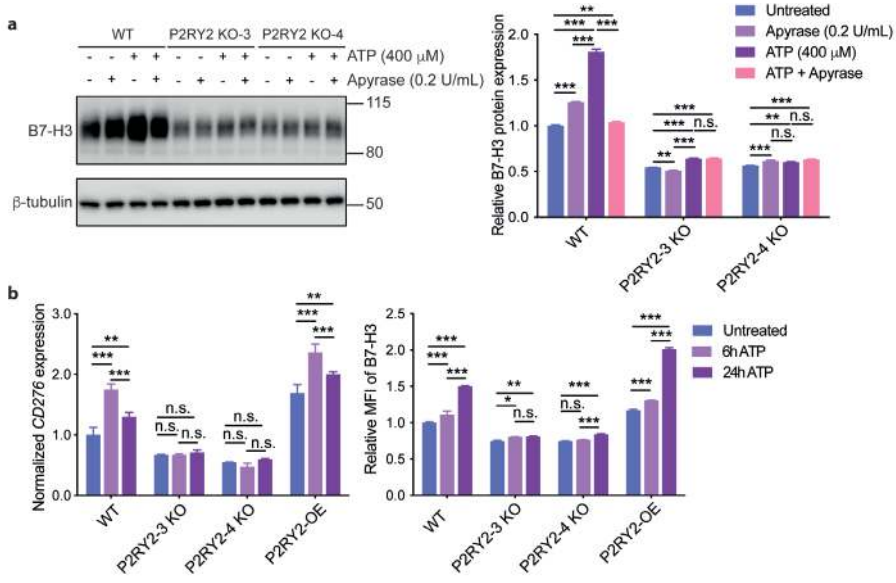

Figure 3. P2RY2 regulates B7-H3 expression at the mRNA and protein level. a. Western blot analysis of WT and P2RY2 KO cells left untreated (-) or treated with ATP, apyrase or the combination. Blot image has been cropped to show the relevant bands, and molecular mass markers are indicated (in kD). Right panel shows quantification of B7-H3 protein levels by Image J. Values are relative to untreated WT cells. **b.** *CD276* mRNA (left) and B7-H3 cell surface expression (right) in WT, P2RY2 KO or P2RY2 OE THP-1 cells, as determined by RT-QPCR and flow cytometry, respectively. Values are relative to untreated WT cells.

Data are representative of at least three (**a, b**) independent experiments and were analyzed by two-way ANOVA with multiple comparisons (**a, b**). ****P* ≤ 0.0002; ***P* ≤ 0.0021; **P* ≤ 0.0332; n.s., not significant. Data represent mean ± s.d. of triplicates (**a, b**).

Expression of B7-H3 has been shown to influence the effector functions of both T cells and NK cells, with higher levels of B7-H3 expression leading to an increased impairment of immune cell activity[23,24] . To determine the effects of P2RY2 on T cell recognition of tumor cells, we engineered human peripheral blood $CD8^+$ T cells with the MART-1 specific 1D3 TCR and assessed T cell activation in the presence of P2RY2 and B7-H3 proficient and deficient tumor cells. Notably, both P2RY2- and B7-H3 deletion in tumor cells resulted in a substantial increase in T cell activation, as evidenced by increased expression of the T cell activation markers CD25, CD69 and CD137, and also increased production of the cytokines IFNγ, IL-2 and TNFα (Fig. 4a-b). In contrast, overexpression of either P2RY2 or B7-H3 in tumor cells resulted in impaired T cell activation and cyto-

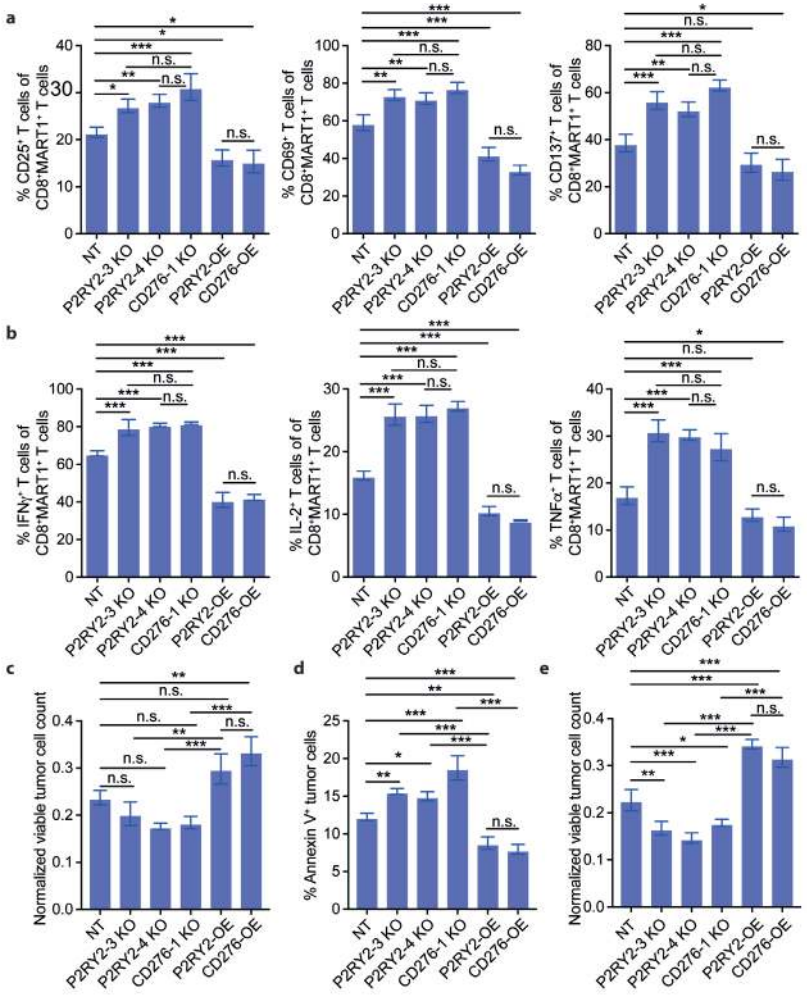

Figure 4. Regulation of T cell activity by P2RY2 and B7-H3 expression on tumor cells. a. Expression of CD25, CD69 and CD137 on MART1-specific $CD8^+$ T cells co-cultured with MART-1^+ NT, P2RY2 KO, B7-H3 KO, P2RY2 OE, or B7-H3 OE THP-1 cells for 21 h. **b.** Production of IFNγ, IL-2 and TNFα by MART1-specific $CD8^+$ T cells co-cultured with MART-1^+ NT, P2RY2 KO, B7-H3 KO, P2RY2 OE, or B7-H3 OE THP-1 cells for 21 h. **c.** Normalized cell count of viable $MART1^+$ NT, P2RY2 KO, B7-H3 KO, P2RY2 OE, or B7-H3 OE THP-1 cells upon co-culture with MART1-specific CD8+ T cells for either 21 h or 36 h. Cell counts were normalized to the number of THP-1 cells in cultures without T cells. **d.** Annexin V staining of MART-1^+ NT, P2RY2 KO, B7-H3 KO, P2RY2 OE, or B7-H3 OE THP-1 cells after co-culture with MART1-specific $CD8^+$ T cells for 6 h. **e.** Normalized cell count of viable MART1-expressing NT, P2RY2 KO, B7-H3 KO, P2RY2 OE or B7-H3 OE U937 cells upon co-culture with MART1-specific $CD8^+$ T cells for either 18 h or 36 h. Cell counts were normalized to the number of U937 cells cultured without T cells. Data are representative of at least three (**a-d**) independent experiments or were obtained in a single (**e**) experiment, and were analyzed by one-way ANOVA with multiple comparisons (**a-e**). $^{***}P \leq 0.0002$; $^{**}P \leq 0.0021$; $^{*}P \leq 0.0332$; n.s., not significant. Data represent mean ± s.d. of triplicates (**a-e**).

kine production (Fig. 4a-b). In line with these data, P2RY2 or B7-H3 deletion in THP-1 cells resulted in increased tumor cell killing by T cells (Fig. 4c-d), and similar results were observed in U937 tumor cells (Fig. 4e, Extended Data Fig. 4a-b). Together, these

data demonstrate that similar to B7-H3, P2RY2 expression on tumor cells inhibits both T cell activation and T cell-mediated cytotoxicity, thereby identifying P2RY2 as a novel regulator of T cell activity.

DISCUSSION

B7-H3 can provide inhibitory signals to immune cells and may thus potentially be exploited in cancer immunotherapy. In this work, we identify the ATP and UTP sensing purinergic receptor P2RY2 as a positive transcriptional regulator of B7-H3 expression on myeloid cancer cells. Comparison of mononuclear cells from acute myeloid leukemia (AML) patients and healthy donors has shown that B7-H3 expression was 2- to 3-fold higher in AML cells than in healthy donor cells[23]. Interestingly, the level of ATP, a major ligand of P2RY2, has been found to be increased in bone marrow niches of mice with AML[25]. Combined with our findings, these data suggest that P2RY2 signaling may facilitate immune escape in leukemia via induction of the B7-H3 immune checkpoint. Furthermore, prior work has established that cell surface expression of B7-H3 is substantially increased upon tumor formation in mouse solid tumor models[5]. Considering the fact that increased levels of extracellular ATP have been widely observed in the tumor microenvironment[26–29], ATP-induced signaling may be proposed to also play an immunosuppressive role via B7-H3 induction in solid tumors. However, regulation in non-myeloid cancer cells might be different, as P2RY2 deletion had no effect on ATP-induced B7-H3 regulation in pancreatic, melanoma and lymphoid leukemia cells tested (data not shown). This could potentially be explained by redundancy in the purinergic receptor family.

We note that genetic disruption or overexpression of B7-H3 and P2RY2 on tumor cells affects T cell activation and cytotoxicity toward tumor cells to a similar extent. Since P2RY2-deficient tumor cells still display residual expression of B7-H3 on the cell surface (Fig. 1c and Fig. 2b), these results suggest that either the ability of B7-H3 to modulate T cells is dependent on a threshold of expression, or that P2RY2 regulates additional molecules or pathways in tumor cells that affect T cells. In the future, it will be of interest to determine whether P2RY2 signaling affects B7-H3 levels on primary leukemic tumor cells, and whether P2RY2 and B7-H3 influence immune recognition through only partially overlapping pathways. Finally, based on the current data the development of antibody- or small molecule-based inhibitors of P2RY2 appears attractive.

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AUTHOR CONTRIBUTIONS

M.E.W.L. and C.S. conceived the project, designed and performed experiments, interpreted data and co-wrote the manuscript. Z.H. designed and performed experiments, interpreted data and co-wrote the manuscript. T.N.S. conceived the project, designed experiments, interpreted data and co-wrote the manuscript.

COMPETING INTEREST

M.E.W.L, Z.H., T.N.S., and C.S. are inventors on a patent application covering the use of P2RY2 as a therapeutic and diagnostic target. M.E.W.L. is a consultant for Third Rock Ventures, outside of this work. T.N.S. is consultant for Third Rock Ventures, and stockholder in Allogene Therapeutics, Asher Bio, Merus, Neogene Therapeutics and Scenic Biotech, all outside of this work.

MATERIALS AND METHODS

Flow cytometry-based CRISPR-Cas9 screen

To generate a whole genome mutant library of THP-1 cells, a Cas9 expressing THP-1 clone (cl35) was lentivirally transduced with the Human Brunello CRISPR Genome-Wide Knockout Library in the Lenti-Guide-Puro vector (Addgene) at a coverage of 1,500-2,000x, with an infection rate of approximately 40%, and cells were selected with 1.0 ug ml^{-1} of puromycin for at least 10 days. To identify genetic modulators of B7-H3 expression on THP-1 cells, as measured by binding of the anti-human B7-H3 antibody MIH42 (αhB7-H3-MIH42), library cells ($3x10^8$ cells in total) were washed once with PBS and stained with Near-IR Dead Cell Stain (ThermoFisher) in PBS for 10 min at 4 °C while protected from light. After 2 washes in PBS containing 0.5% (w/v) BSA (Sigma) and 0.2% (w/v) sodium azide (Sigma) (FACS buffer), cells were stained with unconjugated αhB7-H3 MIH42 (Bio-

legend) at a dilution of 0.1 μg ml^{-1} for 30 min in FACS buffer at a concentration of $1x10^7$ cells/mL, at 4 °C while protected from light. After 2 washes with FACS buffer, cells were stained with AF488-conjugated goat anti-mouse IgG antibody (A28175, ThermoFisher) at a dilution of 2 μg ml^{-1} for 30 min in FACS buffer at 4 °C while protected from light. After 2 washes with FACS buffer, cells were fixed with IC Fixation Buffer (eBioscience), according to the manufacturer's protocol. Next, cells were passed through a 40 μm strainer (BD Falcon) and B7-H3-MIH42^{high} and B7-H3-MIH42^{low} cell populations, both constituting approximately 5-10% of the total cell pool, were isolated by FACS.

Cell lines

THP-1, KBM7, K562 and U937 cells were obtained from the American Type Culture Collection (ATCC). THP-1, K562 and U937 cells were cultured in RPMI supplemented with 10% FCS (Sigma-Aldrich) and 100 U ml^{-1} penicillin–streptomycin (Thermo Fisher Scientific). KBM7 cells were cultured in IMDM (Thermo Fisher Scientific) supplemented with 10% fetal calf serum (FCS, Sigma-Aldrich), 100 U ml^{-1} penicillin–streptomycin (Thermo Fisher Scientific). All cells were cultured at 37 °C and 5% CO_2.

Flow cytometry analysis

Cell suspensions were labeled with conjugated antibodies as listed in "compounds and antibodies". Dead cells were excluded based on 4,6-diamidino-2-phenylindole (DAPI) incorporation, or using the LIVE / DEAD ™ Fixable Near-IR Dead Cell Stain Kit (L10119). All washes and reagent dilutions were performed in PBS containing 2% FCS and 0.09% sodium azide (NaN_3). Data acquisition was performed on a BD LSR Fortessa HTS Wolfgang cytometer (BD Biosciences) interfaced to the FACS-Diva software system.

Compounds and antibodies

The following antibodies were used: anti-CD276-PE (MIH42, Biolegend), anti-mouse AF488 IgG (A28175, ThermoFisher), anti-CD276-APC (FM276, Miltenyi Biotec), unconjugated anti-CD276 (D9M2L, Cell Signaling Technology), unconjugated anti-β-Tubulin (9F3, Cell Signaling Technology), anti-CD137-BV421 (4-1BB, Biolegend), anti-CD69-APC/Fire™ 750 (FN50, Biolegend), anti-CD25-AF700 (BC96, Biolegend), anti-CD3-APC (UCHT1, Biolegend), anti-CD3-AF700 (SK7, Biolegend), anti-CD8a-PE (RPA-T8, Biolegend), anti-mouse TCR β chain-AF488 (H57-597, Biolegend), anti-IL-2-PE/Cy7 (MQ1-17H12, Biolegend), anti-IFN-γ-BV421 (4S.B3, Biolegend), anti-TNF-α-BV785 (MAb11, Biolegend), anti-CD25-APC (MEM-181, ImmunoTool). The following reagents were used: adenosine-5'-triphosphate disodium salt (ATP) (987-65-5, ROTH CARL; R0441, ThermoFisher or A3377-1G, Sigma-Aldrich), uridine-5'-triphosphate (UTP) (R-471, ThermoFisher), apyrase (A6132, Sigma Aldrich).

Vector generation

sgRNA sequences targeting B7-H3 and P2RY2 were extracted from the Brunello library[30], synthesized, and cloned into the lentiviral sgRNA expression vectors pLentiGuide-puro and pLentiGuide-puro-2A-BFP. P2RY2 expression and B7-H3 overexpression vectors were generated by introduction of the relevant open reading frames into the lentiviral pCDH-blast vector.

ATP and UTP treatment

Cells were washed once with PBS and plated in 96-well U-bottom plates at a concentration of $5x10^5$ cells ml^{-1} in medium with the indicated ATP or UTP concentrations. After 24 h, cells were harvested and analyzed for B7-H3 cell surface expression by flow cytometry.

Western Blot analysis

Cells were washed with PBS and lysed with RIPA buffer supplemented with freshly added protease inhibitor cocktail (Roche) and Phosphatase Inhibitor Cocktails II and III (Sigma) on ice for 30 min. Next, cell lysates were centrifuged at 14,000 rpm for 10 min at 4 °C. Supernatants were processed using the NuPAGE Gel Electrophoresis System, according to the manufacturer's instructions (Thermo Fisher Scientific).

Reverse transcription quantitative polymerase chain reaction (RT-QPCR)

RNA was isolated according using the RNeasy Plus Mini Kit (QIAGEN), according to the manufacturer's instructions. cDNA synthesis was carried out using the Maxima First Strand cDNA Synthesis Kit (K1641, Thermofisher Scientific). mRNA transcripts were amplified using the gene-specific primers listed below and SYBR Green master mix (Thermofisher Scientific, A25742) and measured on a ViiA 7 Real-Time PCR System (ABI). For quantification, the relative mRNA levels of each gene were normalized to ACTB.

B7H3-1F: 5'-ACAGGGCAGCCTATGACATT-3'
B7H3-1R: 5'-CTGCATTCTCCTCCTCACAG-3'
ACTB-1L: 5'-GTTGTCGACGACGAGCG-3'
ACTB-1R: 5'-GCACAGAGCCTCGCCTT-3'

Co-cultures of MART1-specific CD8+ T cells and MART1-expressing tumor cells

MART-1 specific CD8+ T cells were generated as described[31]. A gene fragment encoding the MART-1 ELA mutant epitope was cloned into the lentiviral vector pCDH-RFP, and this vector was used to generate MART-1^+ tumor cells. MART1-specific CD8+ T cells and MART1-expressing tumor cells were mixed at a ratio of 1:8 in 96-well U-bottom plates. Next, cells were spun down at 1,600*g* for 3 min and incubated at 37 °C and 5% CO_2 for the

indicated times. For samples intended for intracellular staining, brefeldin A (Biolegend, 420601) was added to the cell culture medium 4 h before cells were harvested.

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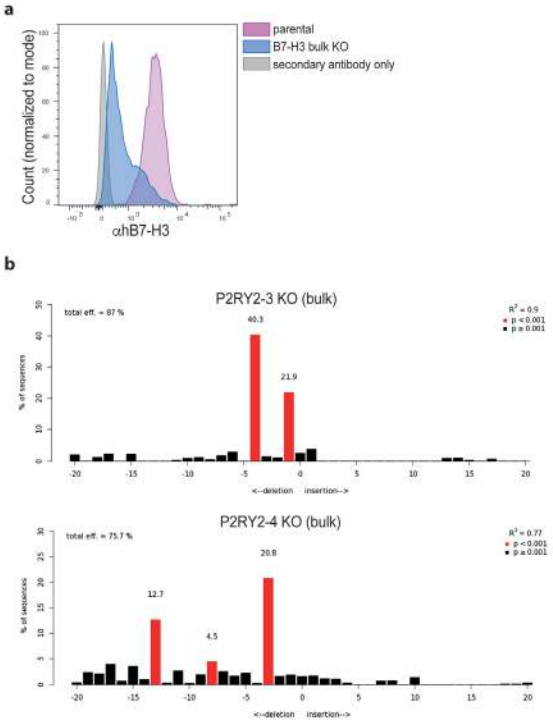
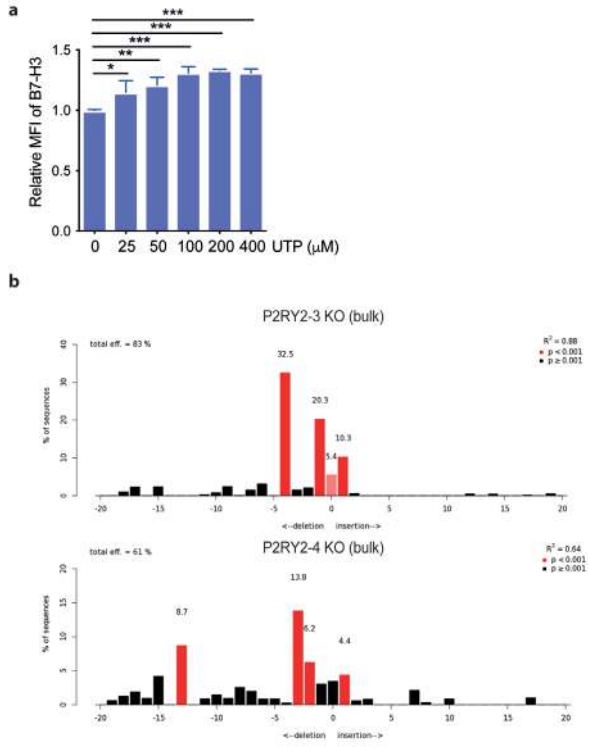
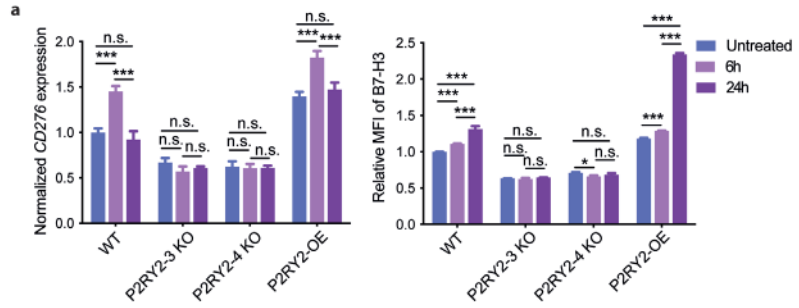


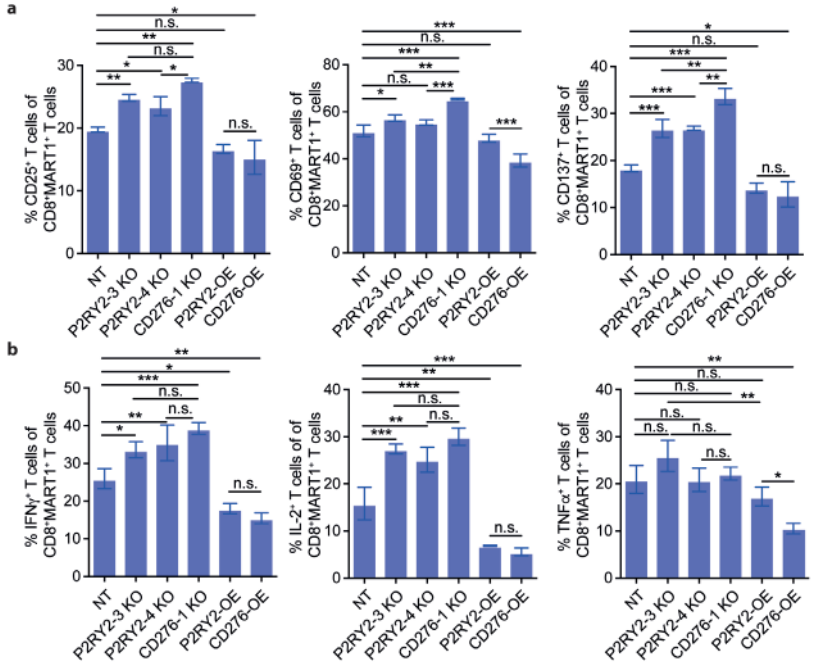
Extended Data Figure 1. a. Flow cytometry plot depicting αhB7-H3 binding to parental and bulk B7-H3 KO THP-1 cells. B7-H3 KO cells were generated using a Cas9-expressing THP1 clone (cl35). **b.** Indel characterization of P2RY2-3 and P2RY2-4 bulk KO THP-1 cells, as analyzed by TIDE.



Extended Data Figure 2. a. Flow cytometry analysis of B7-H3 expression on THP-1 cells left untreated or treated with the indicated concentrations of UTP, as determined by flow cytometry. Values indicate MFI relative to untreated cells. Data represent mean ± s.d. of triplicates. **b.** Indel characterization of P2RY2-3 and P2RY2-4 bulk KO U937 cells, as analyzed by TIDE.



Extended Data Figure 3. a. *CD276* mRNA expression and B7-H3 cell surface expression in WT, P2RY2 bulk KO, or P2RY2 OE U937 cells, as determined by RT-QPCR and flow cytometry, respectively. Values are relative to untreated WT cells. Data were collected in a single experiment and were analyzed two-way ANOVA with multiple comparisons. Data represent mean ± s.d. of triplicates.



Extended Data Figure 4. a. Expression of CD25, CD69 and CD137 on MART1-specific $CD8^+$ T cells co-cultured with MART-1^+ NT, P2RY2 KO, B7-H3 KO, P2RY2 OE, or B7-H3 OE U937 cells for 18 h. **b.** Production of IFNγ, IL-2 and TNFα by MART1-specific $CD8^+$ T cells co-cultured with MART-1^+ NT, P2RY2 KO, B7-H3 KO, P2RY2 OE, or B7-H3 OE U937 cells for 18 h. Data were collected in a single experiment (**a, b**) and were analyzed by one-way ANOVA with multiple comparisons (**a, b**). ***$P \leq 0.0002$; **$P \leq 0.0021$; *$P \leq 0.0332$; n.s., not significant. Data represent mean ± s.d. of triplicates (**a**).