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BET protein targeting suppresses the PD-1/PD-L1 pathway in triple-negative breast cancer and elicits anti-tumor immune response

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ABSTRACT

Therapeutic strategies aiming to leverage anti-tumor immunity are being intensively investigated as they show promising results in cancer therapy. The PD-1/PD-L1 pathway constitutes an important target to restore functional anti-tumor immune response. Here, we report that BET protein inhibition suppresses PD-1/PD-L1 in triple-negative breast cancer. BET proteins control PD-1 expression in T cells, and PD-L1 in breast cancer cell models. BET protein targeting reduces T cell-derived interferon-γ production and signaling, thereby suppressing PD-L1 induction in breast cancer cells. Moreover, BET protein inhibition improves tumor cell-specific T cell cytotoxic function. Overall, we demonstrate that BET protein targeting represents a promising strategy to overcome tumor-reactive T cell exhaustion and improve anti-tumor immune responses, by reducing the PD-1/PD-L1 axis in triple-negative breast cancer.

1. Introduction

Breast cancer was not originally considered for immunotherapy approaches because it is suspected to be immunologically silent. However, an increasing number of studies have established that some breast tumors are in fact sometimes heavily infiltrated by immune cells, including tumor-infiltrating lymphocytes (TILs), which indicates a robust, adaptive immune response. Particularly, in early stage triple-negative breast cancer (TNBC), the presence of TILs is associated with better outcomes and is a strongly favorable prognostic biomarker [1–4]. These developments have led to the evaluation of immunotherapy for treatment of TNBC.

Immune evasion is one of the major challenges to overcome for anti-tumor immunotherapy to be effective. Tumor cells can deploy a vast arsenal of molecules and pathways to escape immune surveillance and resist the cytotoxic effect of host T cells, such as by upregulating inhibitory molecules that comprise the immune checkpoints [5]. Among these molecules, PD-L1 (CD274), expressed by tumor cells and a variety of immune cells, engages its cognate receptor PD-1 (CD279), predominantly expressed on the surface of activated T cells, and inhibits T cell function [6]. Thus, targeting the PD-1/PD-L1 axis is considered to be a promising option to overcome T cell exhaustion and elicit effective anti-tumor immune responses. In this regard, several strategies targeting either PD-1 or PD-L1 have been tested in various cancer models [7]. TNBC is the most immunogenic subtype of breast cancer and is associated with elevated PD-L1 expression in the tumor, along with increased presence of TILs [8,9]. The main treatment for TNBC remains chemotherapy. However, several ongoing clinical trials are investigating anti-PD-1 or anti-PD-L1 antibodies in combination with chemotherapy in TNBC [10] and have shown mixed overall responses. Given the limited therapeutic options available to patients, new modalities are urgently needed, and promising results from these immunotherapy clinical trials could quickly reshape the treatment of TNBC. Recently, the IMpassion130 trial (NCT02425891 [11]) led to Food and Drug Administration approval of atezolizumab, an anti-PD-L1 antibody, in combination with nab-paclitaxel for patients with PD-L1-

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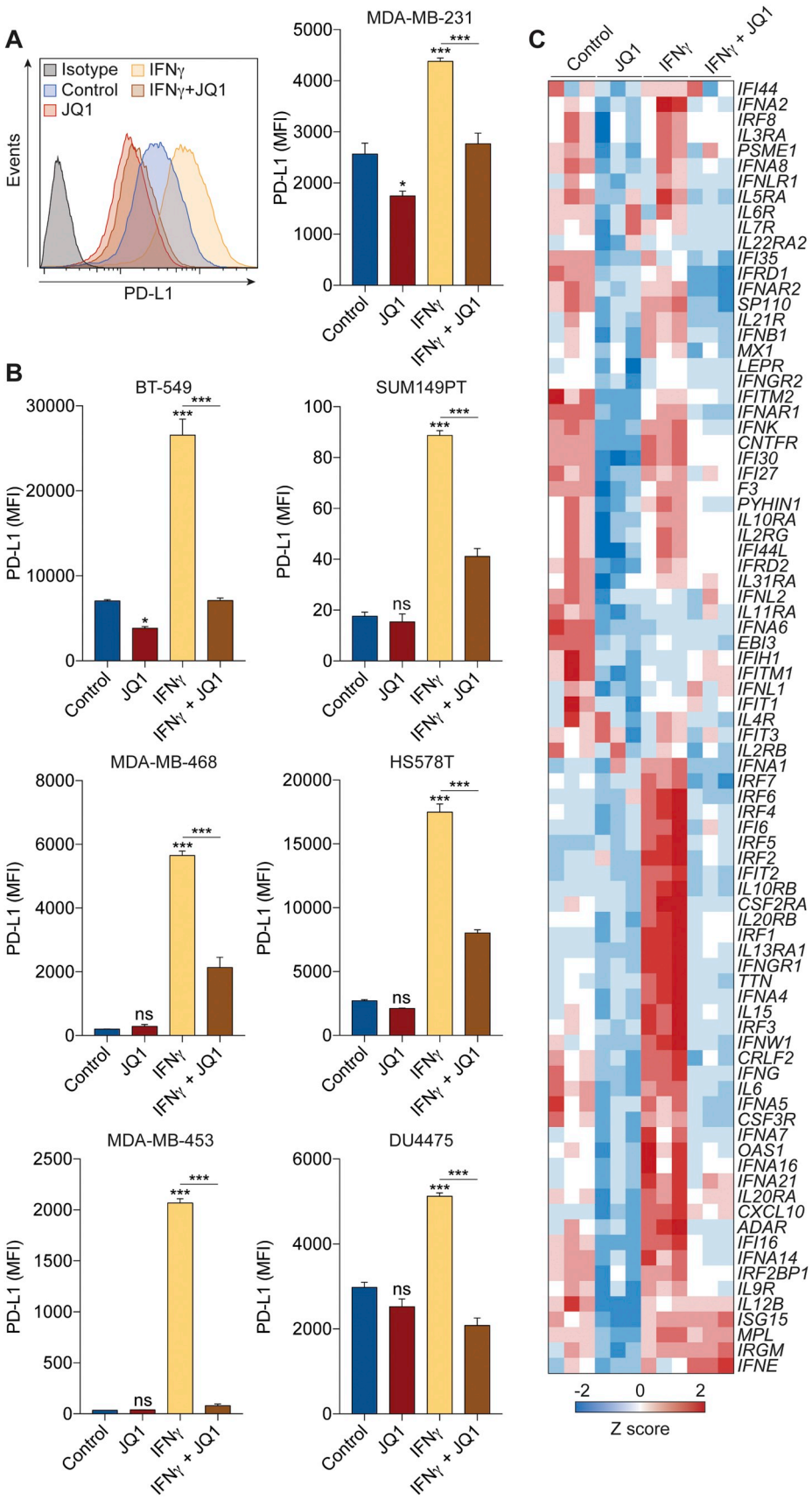

Fig. 1. BET protein inhibition suppresses PD-L1 expression in triple-negative breast cancer. (A) Representative PD-L1 expression determined by flow cytometry on MDA-MB-231 cells treated under the indicated conditions. A control isotype antibody (Isotype) was used to determine background signal. The inactive JQ1 enantiomer was used a control and is further indicated 'Control' in the figures. The histogram represents the quantification of three independent experiments. At least 1 x 10^5 viable cells were counted. (B) PD-L1 expression in the indicated TNBC cell lines was assessed upon 400 nM JQ1 treatment, stimulation with 5 ng/mL IFNγ or co-treatment with IFNγ and JQ1 for 24 h. (C) MDA-MB-231 cells were treated with either 5 ng/mL IFNγ, 400 nM JQ1 or a combination of both agents for 4 h prior to performing the Interferon RT^2 PCR array. Heatmap depicts the Z score hierarchical clustering analysis performed on 84 genes of the interferon responses. Errors bars represent SEM of three independent experiments. ns, $p > 0.05$; *, $p < 0.05$; ***, $p < 0.001$.

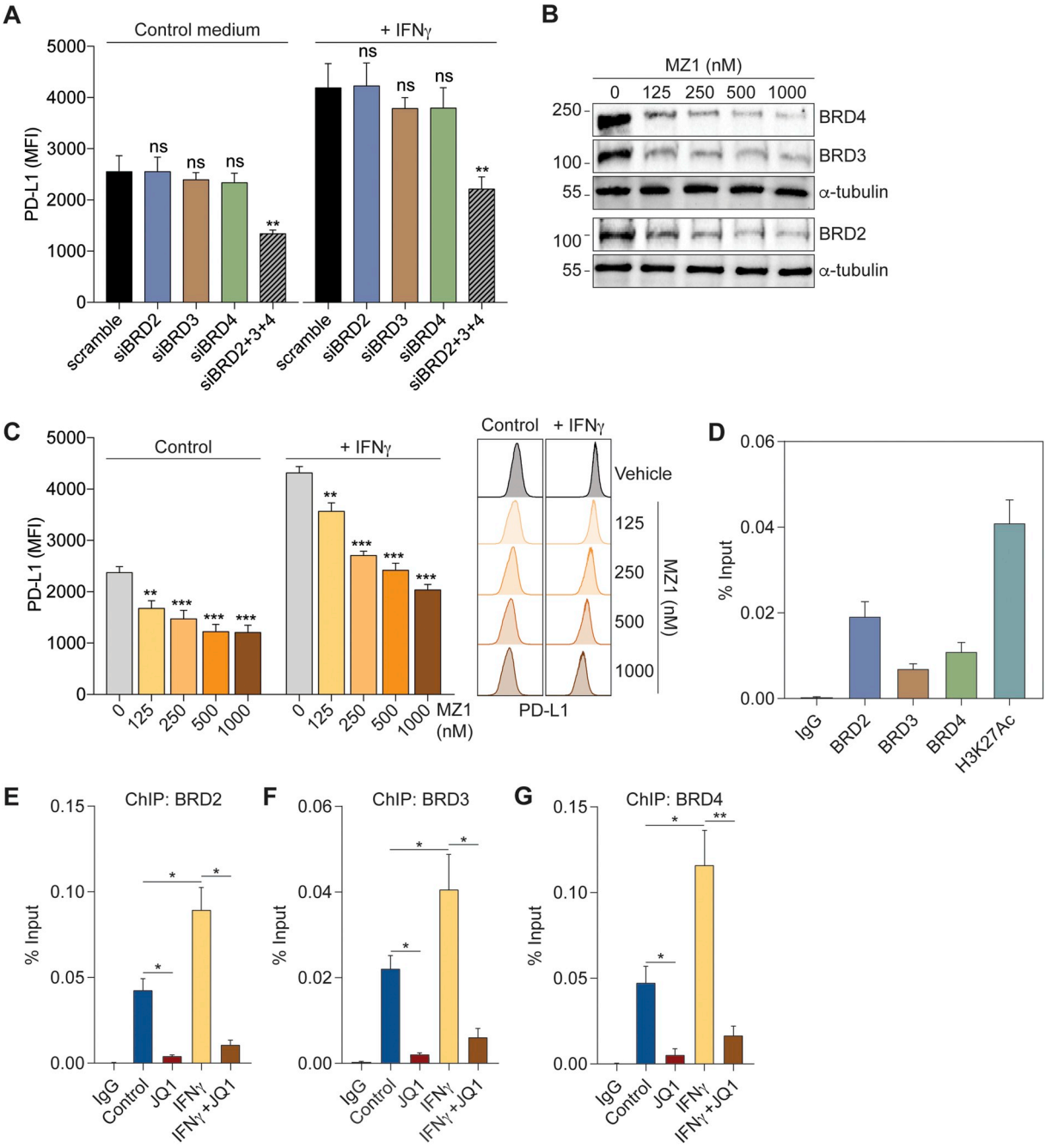

Fig. 2. BET protein inhibition reduces PD-1 expression in activated T cells. (A) MDA-MB-231 cells were transfected with control scramble or siBRD2, siBRD3, siBRD4 alone or in combination to achieve BET protein depletion as previously validated [28]. 48 h post-transfection, cells were or were not treated with 5 ng/mL IFNγ for 24 h prior to PD-L1 expression analysis by flow cytometry. Histograms show four independent experiments. (B) MDA-MB-231 cells were treated with the indicated doses of MZ1 for 24 h then BET protein expression was determined by immunoblot. The depicted blot is representative of the BET degradation obtained in three independent experiments. (C) MDA-MB-231 cells were treated with the indicated doses of MZ1 for 24 h alone or in the presence of IFNγ (5 ng/mL). PD-L1 expression was then analyzed by flow cytometry. Histograms show three independent experiments. (D) MDA-MB-231 cells were analyzed by ChIP using an isotope-matched control IgG antibody, or antibodies against BRD2, BRD3, BRD4 or the H3K27 acetylation mark (H3K27ac). The association with the *CD274* promoter region was quantified by qPCR. (E–G) MDA-MB-231 cells were either treated for 24 h with JQ1 (400 nM), IFNγ (5 ng/mL), alone or in combination, before ChIP analysis using an isotope-matched control IgG antibody, or antibodies against BRD2 (E), BRD3 (F), or BRD4 (G) as in (D). Errors bars represent SEM of three independent experiments. ns, $p > 0.05$; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

positive metastatic TNBC.

Recent studies revealed a promising role for Bromodomain and ExtraTerminal (BET) proteins in the control of the PD-1/PD-L1 axis in cancer [12–18]. These epigenetic readers interact with acetylated lysine residues on histone tails of nucleosomal chromatin present at target promoters, and recruit a functionally critical set of co-regulators to modulate transcription [19]. Here, we show that BET proteins modulate the PD-1/PD-L1 axis in a TNBC model. BET proteins control the expression of PD-1 in activated T cells and PD-L1 in TNBC cells. Moreover, we establish that BET proteins mediate interferon-γ (IFNγ) secretion by activated T cells, and IFNγ signaling in TNBC to induce PD-L1. Finally, we show that BET protein targeting, in reducing PD-1 and PD-L1 expression, overcomes T cell exhaustion and restores tumor cell-specific T cell cytotoxicity. These findings establish the BET proteins as genuine

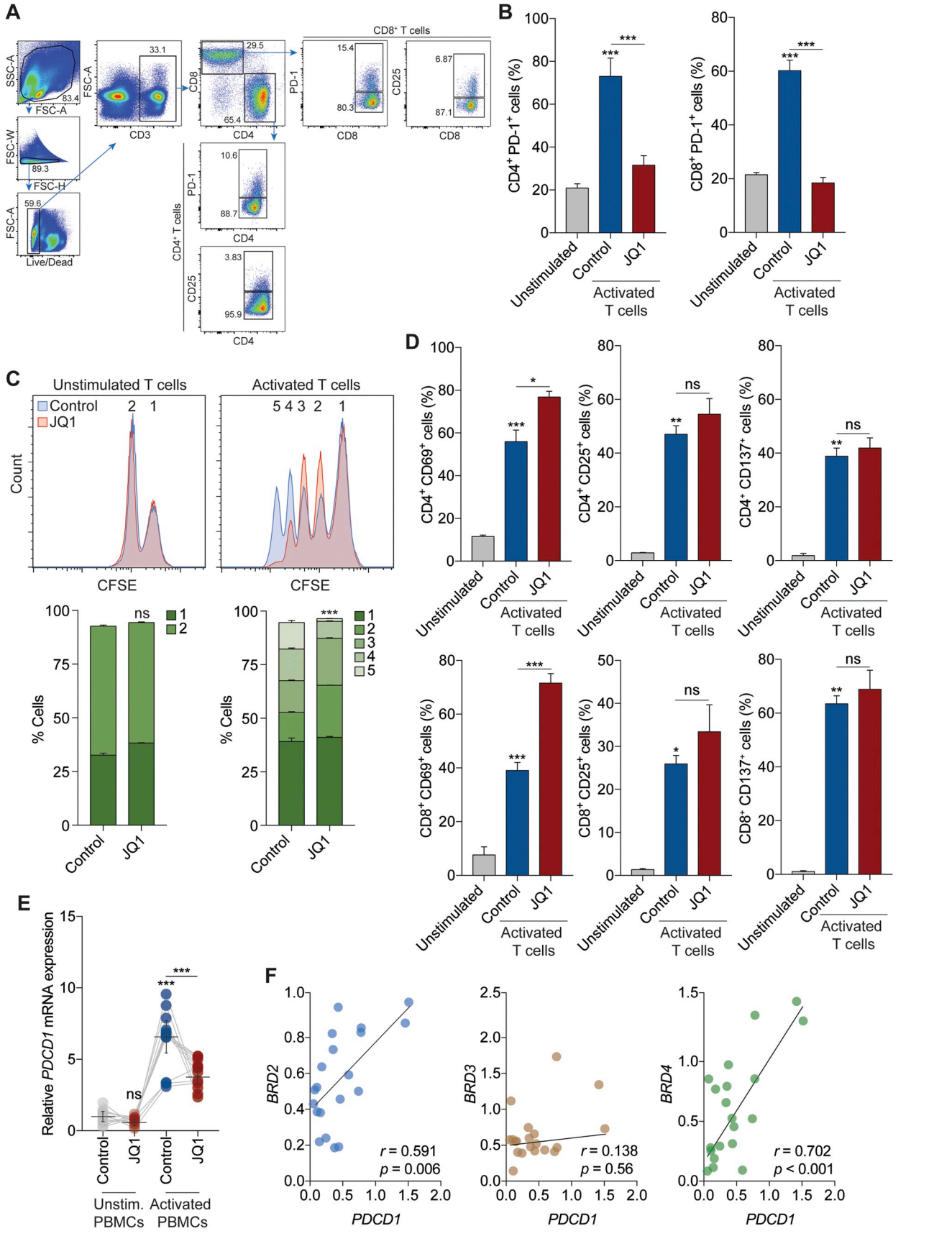

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Fig. 3. BET protein inhibition reduces PD-1 expression in activated T cells. (A) Flow cytometry experimental design and gating strategy to analyze PD-1 expression in $CD4^+$ and $CD8^+$ T cells. These experiments were repeated four times. At least 1 x 10^6 viable total T cells ($CD3^+$) were counted and PD-1^+ proportions were determined among the $CD4^+$ and $CD8^+$ T cell populations. (B) 1 x 10^7 total human PBMCs were cultured, either unstimulated or activated by anti-CD3/CD28 beads, in the presence of control or active JQ1 (400 nM) for 3 days prior to staining with our lineage and exhaustion panel (Fig. S3A) and flow cytometry analysis. (C) Unstimulated PBMCs or activated T cells were cultured as described above with CFSE to determine their proliferation in presence of control or active JQ1 (400 nM) for 3 days. The numbers above the peaks indicate the numbers of cell divisions. The histograms indicate the quantitation of three independent experiments. (D) $CD69^+$, $CD25^+$ and $CD137^+$ percentages were determined among the $CD4^+$ and $CD8^+$ T cell populations. (E) Unstimulated or activated PBMCs from 20 female patients were analyzed by qRT-PCR for *PDCD1* upon 400 nM JQ1 treatment for 24h. (F) Activated PBMCs from 20 female patients were analyzed by qRT-PCR for *PDCD1, BRD2*, *BRD3* and *BRD4* expression. Spearman's correlation was calculated for each pair of plotted genes and indicated in the corresponding plots along with the calculated *p*-values. Errors bars represent SEM of four independent experiments. ns, $p > 0.05$; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

regulators of the PD-1/PD-L1 axis in TNBC and advocate BET inhibition as a strategy to boost anti-tumor immune responses.

2. Materials and methods

2.1. Cell lines and reagents

Human triple-negative breast cancer MDA-MB-231 and SUM149PT cell lines, authenticated by and maintained at the NCI Office of Physical Sciences-Oncology Centers Network Bioresource Core Facility, were obtained from the American Type Culture Collection (ATCC, Manassas, VA). We thank Dr. Neil Ganem of Boston University for providing the following human triple-negative breast cancer cell lines: BT-549, DU4475, HS578T, MDA-MB-453, and MDA-MB-468. All these cell lines were cultured as specified by the ATCC and [20]. HS587T, MDA-MB-231, MDA-MB-453 cells were expanded in DMEM medium (Gibco) supplemented with 10% fetal bovine serum (FBS, Corning), penicillin (100 u/mL) and streptomycin (100 mg/mL) (Sigma). BT549 and DU4475 cells were propagated in RPMI-1640 (Gibco) + 10% FBS, penicillin (100 u/mL) and streptomycin (100 mg/mL). The SUM149PT cell line was cultured in F-12 medium supplemented with 10% FBS, 5 μg/mL insulin and 1 μg/mL hydrocortisone (Sigma), penicillin (100 u/mL) and streptomycin (100 mg/mL). Mycoplasma contamination was prevented by treating the cells with plasmocin (25 mg/mL for 2 weeks, Invivogen) after thawing, prior the experiments. Human peripheral blood mononuclear cells (PBMCs) and immune cells were cultured in RPMI-1640 medium supplemented with 10% FBS, penicillin (100 u/mL) and streptomycin (100 mg/mL). All human cells and cell lines were maintained at 37 °C in a humid 5% CO_2 atmosphere, except for the MDA-MB-468 cell line that requires expansion at 37 °C without CO_2. Co-cultures were realized using the Transwell system (0.4 μm pore size, Costar). Triple-negative breast cancer cells were seeded in the lower chamber prior to addition of T cells in the upper chamber (for a 10:1 T cell:tumor cell ratio). Conditioned media obtained from cell cultures were successively centrifugated at 500 × *g* then at 16,100 × *g* at 4 °C for 10 min to remove any cells and debris. Conditioned media were then either used for culture diluted 1:1 with fresh DMEM or snap-frozen and cryopreserved at −80 °C.

The inactive JQ1 enantiomer (referred to as 'control'), active JQ1 (referred to as 'JQ1') and MZ1 (Tocris) were dissolved in DMSO to a stock concentration of 10 μM prior to dilution in culture media. Recombinant human IFNγ and neutralizing anti-IFNγ antibody were purchased from R&D Systems.

2.2. Patients

Women were recruited during their initial consultation for elective reduction mammaplasty without cancer diagnosis in the Division of Plastic and Reconstructive Surgery clinic at Boston Medical Center [21]. Informed consent was obtained in writing from each participant at recruitment and blood samples were collected before the time of their surgery. The investigation was conducted in accordance with the principles expressed in the Declaration of Helsinki.

2.3. Flow cytometry

Single cell suspensions were washed after collection and stained in ice-cold flow cytometry buffer (Ca^{2+}/Mg^{2+}-free PBS supplemented with 2% FBS) with the appropriate conjugated antibodies for 30 min at 4 °C (listed in the Supplemental Methods). Cell suspensions were then washed once with ice-cold flow cytometry buffer, and resuspended in ice-cold flow cytometry buffer containing a viability dye (7-AAD, BD Pharmingen or Live/Dead Fixable Dead Cell Stain kit, Invitrogen) prior to flow cytometry analysis. Unstained and single-stained controls were used to calculate compensation and background staining for each channel. Data acquisition was performed on a LSRII flow cytometer (BD Biosciences) at the Boston University Flow Cytometry Core Facility. Data analysis was carried out using FlowJo Software (version 10.6.1, Tree Star).

2.4. Chromatin immunoprecipitation

MDA-MB-231 cells were collected after treatments and crosslinked in 1% formaldehyde for 10 min at room temperature. After quenching with addition of 250 mM glycine for 5 min, cells were washed in PBS and resuspended in lysis buffer (20 mM HEPES pH 7.6, 1% SDS supplemented with protease inhibitor cocktail, Sigma) for chromatin immunoprecipitation (ChIP). Cells were then sonicated with a Bioruptor Pico sonicator (30 cycles of sonication for 30s – rest for 30s; Diagenode) to obtain sheared fragments of 100–300 bp. Fragment sizes were verified on a Agilent 2100 Bioanalyzer (Agilent). Immunoprecipitation was performed in ChIP dilution buffer (20 mM Tris-HCl pH 8, 167 mM NaCl, 1.2 mM EDTA, 1% Triton-X) for at least 17 h at 4 °C under rotation. The following antibodies were used for ChIP assays: control IgG antibody (BD Pharmingen), anti-BRD2, -BRD3, -BRD4 (Bethyl Laboratories), anti-H3K27ac (Diagenode #C15410196). Equal volumes of Dynabeads Protein A magnetic beads (ThermoFisher Scientific) were added to samples for 1 h at 4 °C. Crosslink reversion was achieved by incubating immunoprecipitated chromatin samples with 100 μg/mL proteinase K (Sigma) and 200 mM NaCl at 65 °C for 4 h under agitation. DNA fragments were eluted using QIAquick MinElute PCR Purification kits (Qiagen). ChIP DNA were analyzed by qRT-PCR using 7500 Fast Real-Time PCR System (Applied Biosciences). The following primer pairs were used to target the *CD274* promoter region (Forward: 5′- AAGCC ATATGGGTCTGCTC-3'; Reverse: 5′- TTATCAGAAAGGCGTCCCCC-3′).

The ChIP-seq datasets GSE102406 [22] and GSE63581 [23] were retrieved from the NCBI Gene Expression Omnibus database and visualized using Integrative Genomics Viewer (IGV [24]).

2.5. Multiplex array

Conditioned media human cytokine and chemokine concentrations were determined using the MILLIPLEX MAP Human Th17 Magnetic Bead Panel 25-plex kit (EMD Millipore). Quantitation was performed on a Luminex MAGPIX instrument using xPONENT 4.2 software (Luminex Corp) at the Boston University Analytical Instrumentation Core. Cytokine/chemokine concentrations were calculated using analyte standard curves.

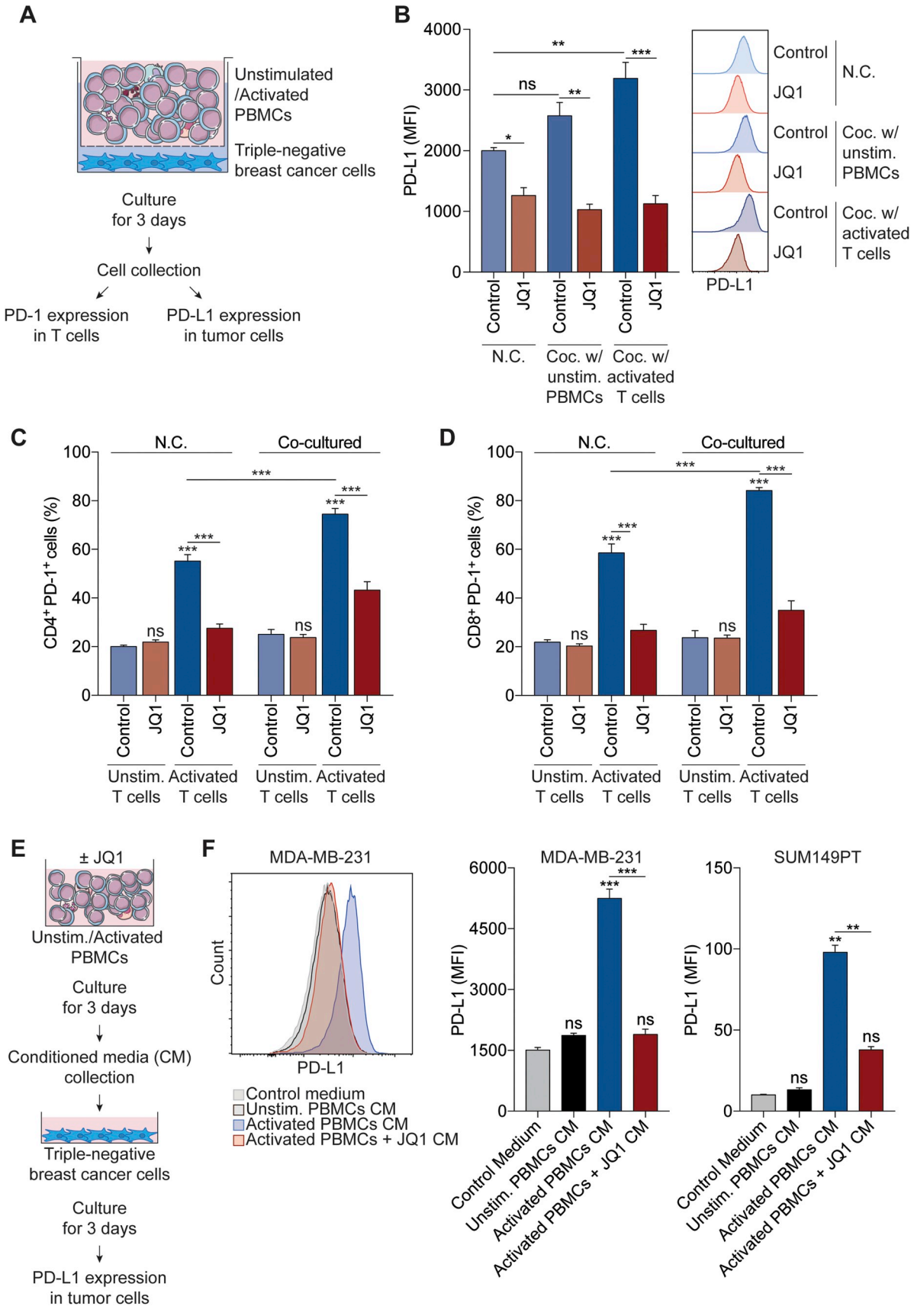

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Fig. 4. BET protein inhibition reduces the PD-1/PD-L1 axis in a T cell/triple-negative breast cell co-culture system. (A) Diagram of the co-culture experimental design. These experiments have been repeated three times. At least 1 x 10^5 viable tumor cells and 1 × 10^6 T cells were analyzed by flow cytometry. (B–D) MDA-MB-231 cells alone (N.C.) or co-cultured (Coc.) with unstimulated (Unstim.) PBMCs or activated T cells obtained from human normal blood donors at a ratio of 1:10 (tumor cells: T cells) were analyzed for PD-L1 expression (B). The histograms represent the quantification of the illustrated flow cytometry curves. (C–D) PD-1^+ proportions were determined among the $CD4^+$ (C) and $CD8^+$ T cell (D) populations. (E) Diagram of the conditioned media generation and subsequent experimental design. These experiments have been repeated three times. (F) MDA-MB-231 and SUM149PT cells were cultured with the generated conditioned media from (E) for 3 days prior to PD-L1 expression analysis. The histograms represent the quantification of the illustrated flow cytometry curves. Errors bars represent SEM of four independent experiments. ns, $p > 0.05$; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

2.6. Tumor-reactive T cell priming

T cells purified from donor peripheral blood (New York Biologics, Inc.) were primed with autologous, monocyte-derived dendritic cells that had been pulsed with antigens obtained from triple-negative breast cancer cells, using a protocol previously validated [12]. MDA-MB-231 cells were γ-irradiated (10,000 rad) then UV-treated for 30 min. Mature dendritic cells differentiated from monocytes were pulsed overnight with irradiated MDA-MB-231 cells (10:1 dendritic cell:tumor cell ratio). Human T cells were purified from peripheral blood samples by Ficoll extraction, followed by T cell isolation using a pan-T cell isolation kit (Miltenyi). Autologous T cells were then primed by co-culture with dendritic cells that had been pulsed with tumor cell antigens (10:1 T cell:dendritic cell ratio) in presence of interleukins 2 (IL-2, 10 u/mL, Gibco) and 7 (IL-7, 1 ng/mL, Invitrogen) for 7 days.

2.7. Hierarchical clustering and statistical analyses

Z scores calculated from normalized datasets were analyzed in MATLAB (MathWorks). Hierarchical clustering analyses were performed using Euclidean metrics and complete linkage clustering function. Statistical analyses were either performed with Student *t*-test or ANOVA as appropriate, using GraphPad Prism 7 software. The following symbols were used to indicate significant differences: ns, $p > 0.05$; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

Supplemental procedures are listed in the Supplemental Information. All the experiments executed for this study were conducted in accordance with NIH guidelines under the review of the Boston University Institutional Biosafety Committee.

3. Results

3.1. Pan-BET protein inhibition suppresses PD-L1 expression in triple-negative breast cancer

Recently, growing interest in BET proteins as therapeutic targets to treat cancer led to the discovery of new functions related to immune checkpoints. Several reports identified *CD274* as a BET protein-target gene [12–18]. Given the importance of the PD-1/PD-L1 axis for regulation of anti-tumor immune responses [6], and the therapeutic significance of targeting PD-L1 to leverage immunotherapy efficiency in triple-negative breast cancer (TNBC) [4,11], we sought to determine whether BET proteins also regulate transcription of PD-L1 in TNBC, as reported in these other models. Triple-negative breast tumors express elevated levels of PD-L1 due to genomic alterations [25]. In addition, the presence of inflammatory cytokines like IFNγ in the TNBC microenvironment promotes tumor cell PD-L1 expression, reducing anti-tumor immune responses [26]. We evaluated a panel of TNBC cell lines to determine if BET protein targeting suppresses PD-L1 expression. Among the tested cell lines, MDA-MB-231 cells express high levels of PD-L1 under basal conditions, as determined by flow cytometry analysis (Fig. 1A). We treated MDA-MB-231 cells with the pan-BET inhibitor JQ1 [27] for 24 h and observed a significant reduction in PD-L1 expression. Under these conditions, we have previously reported that JQ1 does not induce noticeable cell death [28]. We confirmed that JQ1 treatment did not provoke cell death (Fig. S1A), ruling out this possible confounder. Similar results were observed in several other TNBC cell lines: BT-549, SUM149PT, MDA-MB-468, HS578T, MDA-MB-453 and DU4475 (Fig. 1B). We further interrogated accessible RNA-seq datasets that report gene expression alterations upon JQ1 treatment in various cancer models to validate that BET proteins regulate PD-L1. We found that BET protein inhibition reduces PD-L1 mRNA expression in triple-negative breast cancer (GSE102407 [22]), which confirms our results, but also in melanoma (GSE94488 [29]), colorectal cancer (GSE95513 [30]), prostate cancer (GSE98069 [31]) and liposarcoma (GSE111252 [32]) (Fig. S1B).

IFNγ is a strong PD-L1 inducer. Reports have indicated that BET proteins mediate IFNγ signaling and that pan-BET inhibitors ablate IFNγ-induced PD-L1 in other cancer models [12,13,18]. We stimulated MDA-MB-231 cells with IFNγ for 24 h to induce PD-L1, or simultaneously challenged them with JQ1 to revert PD-L1 expression. We observed that IFNγ successfully induced PD-L1 expression and JQ1 ablated this expression (Fig. 1A). We confirmed that BET proteins transcriptionally regulate PD-L1. We found that JQ1 was able to reduce *CD274* mRNA expression, either basally or under IFNγ stimulation (Fig. S1C). We confirmed that this reduction was accompanied by ablation of the PD-L1 protein as well (Fig. S1D). Similar results were observed in the other tested TNBC cell lines (Fig. 1B). Although PD-L1 is not basally expressed in all the tested cell lines, IFNγ is able to induce PD-L1 expression. Treatment with JQ1 inhibited PD-L1 expression in all the tested cell lines, suggesting that BET protein inhibition alters IFNγ signaling and reduces the expression of IFNγ target genes including *CD274*.

We next explored whether pan-BET inhibition interferes with IFNγ signaling itself by monitoring the expression of IFNγ target genes by PCR array. Hierarchical clustering analysis revealed a JQ1-related inhibitory signature of IFNγ target genes (Fig. 1C). We determined that JQ1 down-regulates a subset of forty-nine IFNγ responder genes. The IFNγ response is functional in MDA-MB-231 cells as shown by the enrichment of twenty-eight IFNγ early response genes, including the interferon regulatory factors (IRFs) and other target genes. Co-treatment with IFNγ and JQ1 significantly down-regulated twenty-one IFNγ-induced genes, including the IRFs, suggesting that pan-BET protein inhibition ablates IFNγ signaling. These results are in agreement with the identification of *IRF1* as a key transcription factor that regulates IFNγ-induced PD-L1 expression [13] and suggest that BET protein targeting could neutralize PD-L1 function. Taken together, these data suggest that PD-L1 down-regulation observed in TNBC cells upon BET protein targeting can be attributed – at least partially – to neutralization of the IFNγ response.

We and others have reported that each BET protein plays individual functions, independent of the other family members, and sometimes act in opposition to each other. The misleading use of pan-BET inhibitors like JQ1 that often obscures the biological functions of BRD2, BRD3 and BRD4 has been discussed by our group and other investigators [33–35]. It is essential to establish the role of each BET protein independently, in addition to resorting to pan-BET inhibitor experiments and, more importantly, before considering pan-BET inhibitors for clinical usage [33]. We therefore tested whether depletion of individual BET proteins would recapitulate down-regulation of PD-L1 in a TNBC setting. Interestingly, single depletion of BRD2, or BRD3, or BRD4 failed to reduce PD-L1 in non-stimulated or IFNγ-treated cells (Fig. 2A). However, co-depletion of

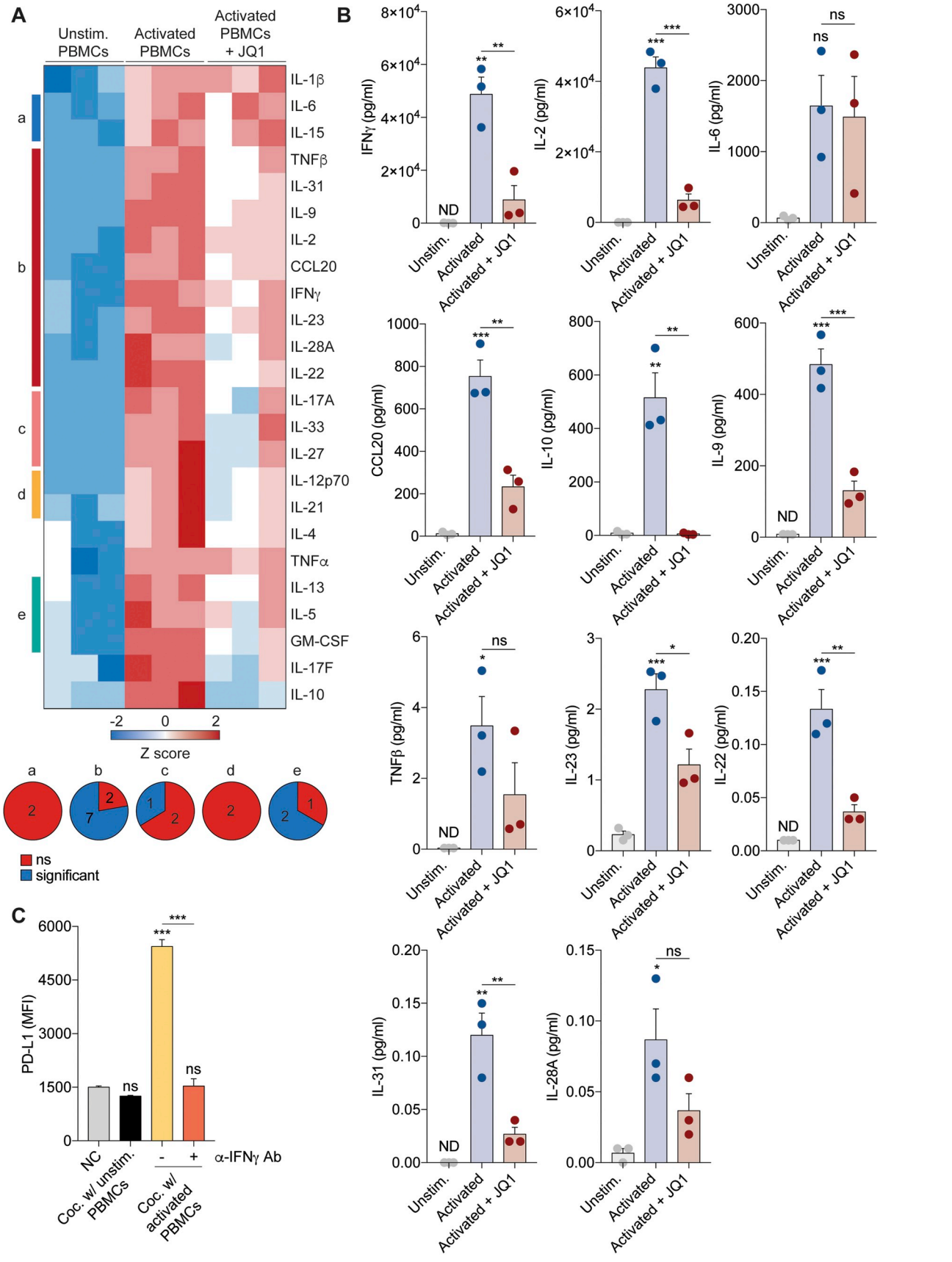

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Fig. 5. BET proteins regulate effector T cell interferon-γ secretion to induce PD-L1 expression in triple-negative breast cells. (A) Conditioned media generated from cultures of either unstimulated or activated PBMCs in presence of control or active JQ1 (400 nM) for 3 days were profiled for cytokine/chemokine expression by multiplex analysis. Heatmap shows the Z score hierarchical clustering analysis for each analyte. Five clusters identified by letters were identified. The number of significantly altered genes in each cluster is indicated. (B) Expression of Cluster B analytes is detailed. Each dot represents an experiment. (C) To validate the central role of IFNγ, MDA-MB-231 cells were co-cultured as previously described with unstimulated PBMCs or activated PBMCs under regular conditions or in the presence of a neutralizing anti-IFNγ antibody for 24 h prior to evaluation of PD-L1 expression by flow cytometry. Histograms represent three independent experiments. Errors bars represent SEM of three independent experiments. ns, $p > 0.05$; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ND, not detectable.

BRD2, BRD3 and BRD4 together recapitulated JQ1 treatment, suggesting that all the three BET proteins contribute in PD-L1 regulation. In this context, small-molecule pan-BET inhibition appears to be justified as an appropriate strategy to inhibit PD-L1 expression. We also tested MZ1, a newly developed small molecule that degrades BET proteins in a dose-dependent manner [36]. At low concentrations, MZ1 tends to degrade BRD4 first, and then targets BRD2 and BRD3 as the dose increases (Fig. 2B). We found that MZ1-induced BET protein degradation reduces PD-L1 expression in control and IFNγ-treated cells, with maximal reduction achieved when all the BET proteins are degraded (Fig. 2C and Fig. S2A), confirming that they all participate in PD-L1 regulation.

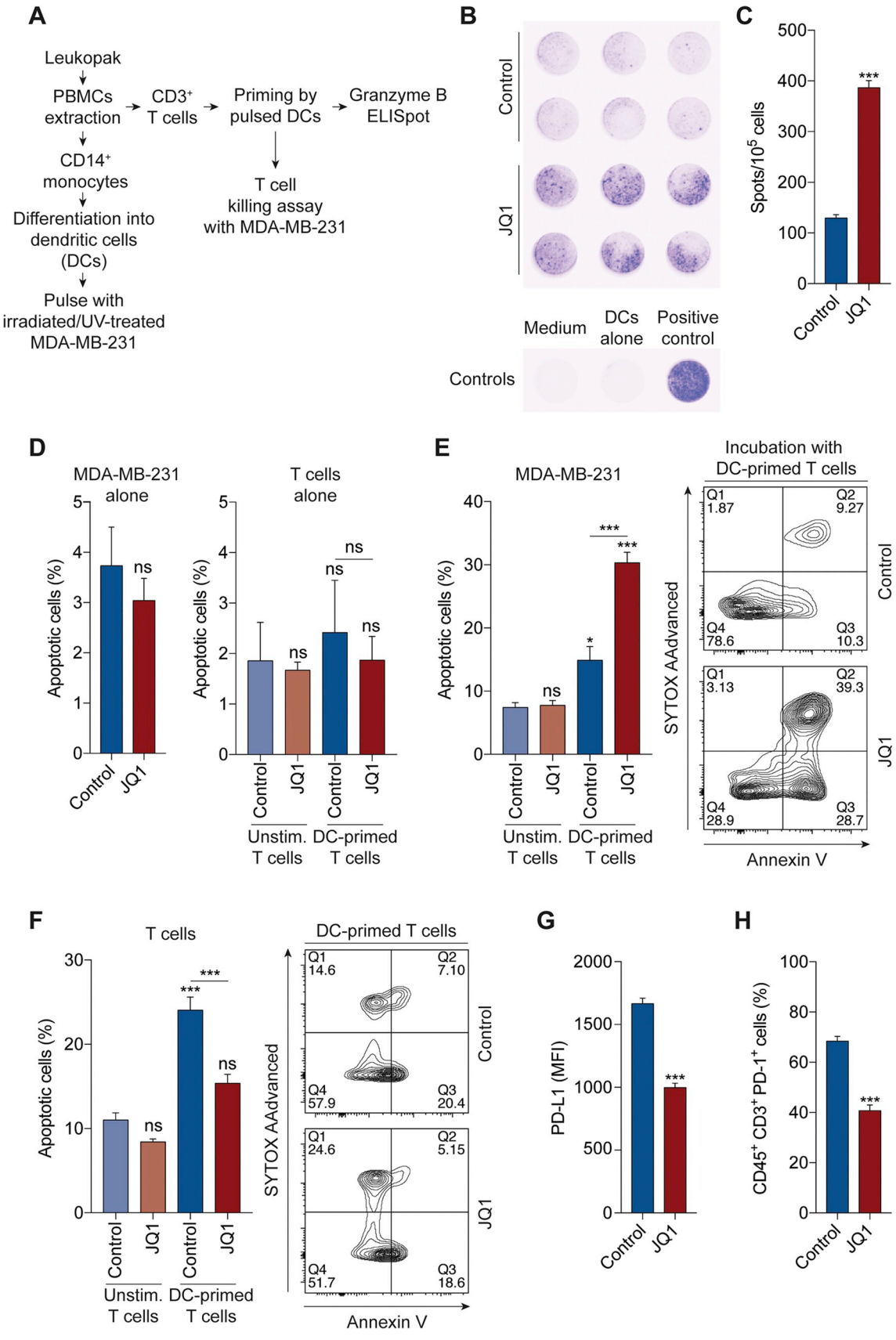
In the tested TNBC cell lines that express high endogenous levels of PD-L1, like MDA-MB-231 and BT549 cells, it is possible that the BET proteins directly regulate PD-L1 transcription, independently of IFNγ signaling, as it has been reported that BRD4 associates with the *CD274* promoter [12,13]. Thus, we envisaged that the BET proteins and their associated co-regulators exert a direct control of the *CD274* promoter that might be amplified upon IFNγ signaling to induce PD-L1. We found that all three BET proteins can be endogenously detected at the *CD274* promoter by chromatin immunoprecipitation (ChIP) (Fig. 2D). Consistent with this result, we observed that this locus was enriched in the histone H3K27Ac epigenetic mark read by the BET proteins. As expected, JQ1 treatment displaced BET proteins from the *CD274* promoter (Fig. 2E–G). We interrogated public data repositories for ChIP-sequencing datasets on BET proteins in TNBC cells (GSE102406 [22] and GSE63581 [23]). BRD4 is detected with RNA Pol II and the H3K27ac mark upstream, and at the *CD274* promoter (Figs. S2B–C). Notably, JQ1 treatment displaced BRD4 without altering the promoter epigenetic landscape, in accordance with our data and previous reports [12,13]. We then wondered if IFNγ-induced PD-L1 expression was mediated by dynamic recruitment of BET proteins to the promoter. Short treatment with IFNγ rapidly induced significant enrichments of the three BET proteins at the *CD274* promoter region (Fig. 2E–G). Importantly, JQ1 prevented these enrichments. Similarly, studies have reported that BRD4 is recruited at the *CD274* promoter upon IFNγ stimulation, and displaced by JQ1 in other models [12,13]. Altogether, our data demonstrate that *CD274* is a direct target gene of the BET proteins in TNBC. Moreover, we confirmed their central role in the mediation of IFNγ signaling as previously reported [12,13,18].

3.2. Pan-BET protein inhibition reduces PD-1 expression in effector T cells

Upon activation, T cells express exhaustion markers like PD-1 which, when engaged by their ligands, exert a negative feedback to limit T cell responses. Tumor cells that express PD-L1 can therefore ablate the cytotoxic function of PD-1-expressing, reactive T cells to escape immune cell surveillance. We wondered if BET proteins could control PD-1 expression by activated T cells. Normal peripheral blood mononuclear cells (PBMCs) were obtained from healthy blood donors and T cells were physiologically activated *ex vivo* by incubation with beads coated with anti-CD3/CD28 antibodies. We designed a flow cytometry staining strategy adapted from an established immune exhaustion panel [37] to investigate PD-1 expression in effector T cells (Figs. 3A and S3A). Whereas only a few unstimulated T cells appear to be PD-1$^+$, T cells activated by CD3/CD28 stimulation strongly expressed PD-1 as expected. JQ1 treatment does not change PD-1 expression in unstimulated T cells (data not shown). Conversely, when cells are activated in presence of JQ1, PD-1 expression is dramatically reduced in both CD4$^+$ and CD8$^+$ T cells (Fig. 3B). We verified that this reduction was not due to a defect in T cell activation. T cell proliferation upon activation was evaluated by carboxyfluorescein succinimidyl ester (CFSE) staining (Fig. 3C). Unstimulated T cell proliferation was not affected by JQ1 treatment. Upon activation, T cells treated with JQ1 underwent fewer cycles of cell division. However, when we analyzed T cell activation by measuring the expression of CD69, CD25 and CD137 markers, no significant decrease was observed upon JQ1 or MZ1 treatment, indicating that, despite a moderate inhibitory impact on T cell proliferation, BET protein targeting did not alter T cell activation (Fig. 3D and S3F-G). Conversely, in JQ1-treated cells, we detected more CD69$^+$ cells in both the CD4$^+$ and CD8$^+$ compartments, consistently with reduced PD-1 expression. We were able to reproduce these data using the BET protein degrader MZ1. BET protein degradation in activated T cells was efficient (Fig. S3B). PD-1 expression was reduced in both CD4$^+$ and CD8$^+$ T cells upon MZ1 treatment (Figs. S3C and S3D). Similarly, we confirmed that MZ1 did not affect T cell activation (Figs. S3E–G). Moreover, we measured *PDCD1* mRNA expression in PBMCs obtained from 20 female patients without breast cancer treated *ex vivo* with JQ1 (Fig. 3E). As reported, BET protein inhibition did not reduce *PDCD1* mRNA in unstimulated PBMCs. However, *PDCD1* induction upon activation was reverted by JQ1, consistent with our previous results. Finally, we evaluated correlations between BET gene expression and PD-1 gene expression in activated T cells. We found that *PDCD1* and *BRD2* or *BRD4* mRNA expression, but not *BRD3*, were positively correlated, suggesting that both BRD2 and BRD4 regulate PD-1 expression (Fig. 3F). Taken together, our data indicate that PD-1 is a target of BET proteins in effector T cells.

3.3. BET proteins regulate the PD-1/PD-L1 axis in a breast tumor Cells-T cells Co-Culture system

We demonstrated that PD-1 and its ligand PD-L1 are both regulated by BET proteins. Within the tumor microenvironment, numerous factors originating from either the tumor cells or the stromal cells can influence tumor cell-T cell interactions and reshape the anti-tumor immune response. We sought to reproduce some of these interactions using a simplified co-culture system to determine how this crosstalk affects both PD-1 and PD-L1 expression in this setting. TNBC cells were co-cultured with either unstimulated or activated T cells in a Transwell system that allowed soluble factors to diffuse from one chamber to another (Fig. 4A). We then examined PD-1 and PD-L1 expression in T cells and tumor cells, respectively. We found that JQ1 was able to diminish PD-L1 expression in MDA-MB-231 cells cultured alone (Fig. 4B). TNBC cells co-cultured with unstimulated PBMCs showed similar PD-L1 expression to TNBC cells cultured alone. Interestingly, when tumors cells were co-cultured with activated T cells, we detected higher levels of PD-L1 than in control cells. JQ1 addition to the co-culture system inhibited this PD-L1 induction. This result suggests that activated T cells secrete soluble factors capable of inducing PD-L1 in TNBC cells, and these signals can be blocked by pan-BET protein inhibition. We also examined PD-1 expression in effector T cells upon co-culture. Consistent with our previous data, JQ1 treatment reduced PD-1 expression in activated T cells cultured alone (Fig. 4C–D). Co-culture with MDA-MB-231 did not change PD-1 expression in unstimulated T cells;



(caption on next page)

Fig. 6. BET protein inhibition restores T cell cytotoxic activity against triple-negative breast cells. (A) Diagram of the experimental design. These experiments have been repeated three times. At least 1 x 10^5 viable tumor cells and 1 × 10^6 T cells were analyzed by flow cytometry. (B) Granzyme B ELISpot assay performed on tumor cell-reactive T cells. 1 × 10^5 T cells were primed with 1 x 10^4 DCs pulsed with tumor cell antigens and analyzed for Granzyme B production. (C) Quantification of (B). (D) MDA-MB-231 cells, unstimulated T cells and DC-primed T cells cultured alone were treated with JQ1 (400 nM for 24 h) and apoptosis was evaluated. Histograms show three independent experiments. (E–F) MDA-MB-231 cells were co-cultured with tumor cell-reactive T cells (1:10 tumor cell:T cell ratio) for 24 h prior to collection, staining and analysis of apoptosis in both tumor cells and T cells (see Fig. S3D). Representative contour plots illustrate the flow cytometry analysis. Histograms show quantifications from three independent experiments. (G–H) PD-L1 expression in the tumor cells (G) and PD-1 expression in T cells (H) were determined. Errors bars represent SEM of three independent experiments. ns, $p > 0.05$; ***, $p < 0.001$.

however, we noted a higher proportion of PD-1$^+$ T cells when T cells were activated in presence of MDA-MB-231 cells. This result suggests that tumor cells secrete factors that elicit PD-1 expression by effector T cells. JQ1 treatment did reduce PD-1 expression in co-cultured effector T cells, reinforcing the concept that BET proteins control PD-1 expression. To test the hypothesis that activated T cells secrete factors modulated by BET proteins that can trigger PD-L1 expression in TNBC cells, we collected conditioned media from unstimulated or activated PBMCs, treated or not with JQ1, added to cultured TNBC cells and measured PD-L1 expression (Fig. 4E). Conditioned media obtained from unstimulated PBMCs did not affect PD-L1 expression, whereas media collected from activated PBMCs induced a 3-fold increase in PD-L1 expression in two cell lines (Fig. 4F). Importantly, conditioned media generated from activated PBMCs in the presence of JQ1 failed to induce PD-L1, suggesting that BET proteins control the production of soluble factors from activated T cells that induce PD-L1 in TNBC cells.

3.4. BET proteins control IFNγ secretion by activated T cells to modulate PD-L1 expression in triple-negative breast cancer cells

To identify the BET protein-dependent soluble factors produced by activated T cells that may regulate PD-L1 expression, we profiled the collected conditioned media using an antibody-capture, multiplex assay to quantify the expression of twenty five human cytokines and chemokines in a panel. Hierarchical clustering revealed five different clusters of factors based on their expression in activated T cells, with or without JQ1 (Fig. 5A). The most important cluster contains six cytokines and one chemokine that were significantly induced in activated T cells and repressed upon BET protein inhibition (Fig. 5B). Among these analytes, IFNγ was identified as the predominant cytokine, based on its expression. Highly secreted by activated T cells, IFNγ is almost completely absent in conditioned media of activated T cells in presence of JQ1. Given its importance for PD-L1 expression, these data suggest that IFNγ is the main T cell-derived factor that regulates PD-L1 in TNBC cells. Of note, we also found that interleukin-2 (IL-2) was strongly produced by activated T cells. IL-2 is an essential factor for T cell expansion upon activation and drives T cell proliferation. Interestingly, we found that IL-2 secretion was significantly inhibited by JQ1, possibly explaining the reduced proliferation we observed when T cells were activated in presence of JQ1. To confirm the major role played by IFNγ in our setting, we treated MDA-MB-231 cells with conditioned media as previously described, or in the presence of an IFNγ neutralizing antibody (Fig. 5C). PD-L1 induction was completely rescued by IFNγ blockade, strongly suggesting that IFNγ released by the activated T cells is the critical driver of PD-L1 expression.

3.5. Pan-BET protein inhibition improves anti-tumor cell immunity through suppression of the PD-1/PD-L1 axis

We have demonstrated that pan-BET protein inhibition reduces the PD-1/PD-L1 axis in TNBC. We further examined if this down-regulation potentiates anti-tumor immunity. To this end, we designed an *in vitro* strategy to generate human primary T cells that are reactive to TNBC cell lines (Fig. 6A). Dendritic cells (DCs) were differentiated from monocytes obtained from the peripheral blood of normal donors, following a validated protocol (Figs. S4A–B) [12]. DC maturation was evaluated by flow cytometry (Fig. S4C). Once maturation was confirmed, the mature DCs were pulsed with γ-irradiated and UV-treated MDA-MB-231 cells. Autologous T cells were then primed by the pulsed DCs to obtain tumor cell-reactive T cells. We observed that, when exposed to JQ1, T cells primed by pulsed DCs secrete more Granzyme B, detected by enzyme-linked immunospot (ELISpot) (Fig. 6B–C). We then examined the anti-tumor cell cytotoxic capacity of the educated T cells in the presence of JQ1. As a control, we did not observe the induction of cell death in MDA-MB-231 cells, or T cells cultured alone upon JQ1 treatment (Fig. 6D). Primed T cells were cultured in the presence of MDA-MB-231 cells, and apoptosis of both tumor and T cells was assayed by flow cytometry (Fig. S4D). Under these conditions, we observed functional, T cell-mediated cytotoxicity on tumor cells defined by a moderate yet significant increase in apoptosis of tumor cells (Fig. 6E). When co-culture was performed in the presence of JQ1, we observed a strong induction of tumor cell death, consistent with the observed increase in Granzyme B secretion (Fig. 6C–D). Interestingly, control tumor cell-reactive T cells exhibit elevated levels of apoptosis when cultured in the presence of MDA-MB-231. This apoptosis was probably due to engagement of PD-1 by PD-L1 expressed by the TNBC cells, and the secretion of other immunosuppressive factors. T cell apoptosis was rescued by JQ1 treatment. Consistently, we noted that both PD-L1 and PD-1 levels were reduced in MDA-MB-231 cells and T cells, respectively, in our settings upon JQ1 treatment (Fig. 6G–H). Taken together, these data suggest that pan-BET protein inhibition improves T cell-mediated, anti-tumor cell immunity by reducing the PD-1/PD-L1 axis in TNBC.

4. Discussion

We have demonstrated that BET proteins are genuine regulators of the PD-1/PD-L1 axis in TNBC cells. Our results indicate that BET protein targeting constitutes a promising strategy to overcome PD-1/PD-L1-mediated, T effector cell exhaustion within the tumor microenvironment, to improve the anti-tumor T cell immune response. These results are in agreement with several recent reports establishing a functional link between BET proteins and the PD-1/PD-L1 axis in cancer [12–18]. The use of pan-BET inhibitors fails to resolve the individual biological functions of each BET protein [33]. Studies using pan-BET inhibitors or that focus solely on BRD4, neglecting BRD2 and BRD3, risk overlooking the complex interplay among the three BET family members and oversimplify the biological mechanisms. Our data indicate BRD2, BRD3 and BRD4 are functionally critical for PD-L1 regulation in TNBC cells (Figs. 2 and S2). Additionally, we have shown that the three BET proteins are found enriched at the *CD274* promoter in TNBC cells (Figure (Fig. 2D–G and S2B–C), consistent with other studies [12,13], demonstrating that they all control PD-L1 expression. Similarly, we found that both BRD2 and BRD4 expression correlate with PD-1 mRNA in activated T cells. Therefore, we postulate that all of the somatic BET proteins are functionally critical for regulation of the PD-1/PD-L1 axis in TNBC.

The TNBC microenvironment is highly inflamed and immunosuppressive. The presence of diverse immune infiltrates, combined with the secretion of numerous cytokines and chemokines by the tumor cells, reprogram the surrounding stroma that adopts a reactive state [38]. As a result, the TNBC microenvironment is enriched in

immunosuppressive factors that reduce the anti-tumor immune responses [39]. The prognostic value of the overall detectability of TILs in TNBC is still in dispute, but it is becoming increasingly clear that a high proportion of intratumoral $CD8^+$ TILs associates with a better outcome for patients presenting a basal-like TNBC subtype [9,40]. Yet, due to the significant secretion of immunosuppressive factors in TNBC, we speculate that neutralization of the immunosuppressive factors in TNBC would mobilize cytotoxic TILs function and improve tumor elimination. We have profiled the secretome of TNBC cell lines to identify BET protein-regulated cytokines and chemokines. Consistent with several previous reports [41,42], we found a panel of eight immunomodulatory factors that are secreted by TNBC cells and ablated by BET protein inhibition (Fig. S5). IL-6, IL-12p70, IL-15 and IL-21 are known to induce PD-1 expression in T cells [43–45]. IL-10 is known to block intratumoral $CD8^+$ T cell expansion and $CD4^+$ T cell function [46]. Thus, in addition to their direct regulation of PD-1 and PD-L1, BET proteins can regulate the secretion of important immunosuppressive factors by the tumor that lead to T cell anergy.

PD-L1 can be expressed by various immune cell types within the TNBC microenvironment, including DCs, monocytes and macrophages. Importantly, BET protein inhibition has been shown to reduce PD-L1 expression by antigen-presenting cells (APCs) [12], and improve APC efficacy to prime T cells and restore the responsiveness of anergic $CD4^+$ T cells [14]. Notably, in presence of JQ1, APCs exhibited enhanced antigen-presenting capacities and reduced PD-L1 expression, leading to better T cell priming. BET protein inhibition also reverts the expansion of immunosuppressive, mesenchymal-derived suppressor cells (MDSCs), while increasing intratumoral infusion of $CD4^+$ and $CD8^+$ T cells in a model of malignant pleural mesothelioma [16]. Finally, BET proteins have been linked to T cell response persistence in a model of adoptive immunotherapy [47]. JQ1 treatment elicited the expansion of T cells with central memory features with superior persistence and antitumor effects. We investigated how T cell co-culture with TNBC cells in presence of JQ1 could modulate memory T cell populations (Fig. S7). We did not observe any significant modification of $CD4^+$ or $CD8^+$ transitional memory (Tm), central memory (Cm), effector memory (Em), other memory (Om), terminally differentiated effector memory $CD8^+$ (TEMRA), naïve and terminally differentiated (Td) T cells upon co-culture with MDA-MB-231 cells, with or without JQ1. However, it is possible that our experimental model omits important features for memory T cell regulation; therefore, we cannot rule out a role for BET proteins in memory T cell function. Taken together, BET protein targeting may reshape the TNBC microenvironment to leverage the anti-tumor immune response, enhancing T cell priming by APCs, cytotoxic T cell function and T cell persistence in the microenvironment, while reducing T cell anergy.

Several studies have investigated the BET protein inhibitors in combination with anti-PD-1/PD-L1 antibodies in cancer, and shown promising results. Cooperative effects between JQ1 and PD-1 antibody have been reported in *Kras*-driven non-small cell lung cancer [48]. PD-L1 has been reported to be a target of JQ1 and a prognosis biomarker in neuroblastoma [49]. Moreover, an elegant mathematical model has established the efficacy of a combination therapy with BET and CTLA-4 inhibitors in breast cancer [50]. Based on these findings, several trials are testing BET protein inhibitors in combination with anti-PD-1 or anti-PD-L1 antibodies in the clinic, including TNBC patients (NCT03059147, SignalRx; NCT02419417, BMS; NCT03292172; Roche; the Roche trial was terminated in July 2019 due to 'portfolio prioritization').

Besides the PD-1/PD-L1 axis, several inhibitory pathways compose the immune checkpoints for maintaining tolerogenic responses. Cytotoxic T-lymphocyte-associated antigen-4 (CTLA-4), which ligates to CD80 or CD86 to dampen T cell activation, was the first antigen targeted to ablate the immune checkpoints. Other co-inhibitory receptors like LAG-3, a TCR co-receptor, or TIM-3 that binds to GAL9 can also reduce T cell activity [6]. We have explored whether BET protein inhibition also reduces the expression of immune checkpoint molecules at the T cell surface. As expected, we found that when both activated effector $CD4^+$ and $CD8^+$ T cells were predominantly positive for PD-1, CTLA-4, LAG-3 or TIM-3, BET protein inhibition with JQ1 effectively reduces the expression of these inhibitory receptors (Fig. S6). These data suggest that BET proteins control the expression of a broad range of T cell immune checkpoint molecules; thus, their targeting appears to be a viable option to target the immune checkpoints and overcome T cell anergy.

Tumor cells can also escape the immune system by hijacking the metabolism of the surrounding immune cells. For instance, nutrition competition driven by proliferative, highly glycolytic TNBC cells can deplete the tumor microenvironment of nutrients, acidify the microenvironment and limit T cell function [51,52]. Given the metabolic requirements of T cells to proliferate and exert their cytotoxic activity, this decreased accessibility to glucose, essential amino acids and other nutrients can lead to T cell anergy [53]. BET proteins play important roles in metabolism. BRD2 acts as a transcriptional co-repressor of peroxisome proliferator-activated receptor-γ and prevents fatty acid oxidation [34,54]. Conversely, BRD4 seems to repress oxidative phosphorylation (OXPHOS), as its genetic deletion induces key OXPHOS genes and restores the levels and activity of OXPHOS protein complexes [55]. More investigation is required to disentangle BET protein regulation of the complex metabolic interplay occurring in the tumor microenvironment, but it is highly apparent that metabolism is a critical factor for an effective anti-tumor immune response.

Our study demonstrates that the three somatic BET proteins function as central regulators of the PD-1/PD-L1 axis in TNBC and integrators of pertinent regulatory signals, including inflammatory cytokines. BET protein targeting successfully overcomes T cell exhaustion and restores tumor-specific cytotoxic activity, in agreement with important previous reports in other models [12–14,16]. Therefore, BET protein inhibition emerges as a promising adjuvant strategy in cancer immunotherapy.

Conflicts of interest

The authors declare that they have no conflict of interest. The funding agency played no role in the preparation of the manuscript or the decision to publish.

Author contributions

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Declaration of interests

No potential conflicts of interest were disclosed.

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Appendix A. Supplementary data

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