Bromodomain inhibition exerts its therapeutic potential in malignant pleural mesothelioma by promoting immunogenic cell death and changing the tumor immune-environment

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ABSTRACT
Systemic treatment of malignant pleural mesothelioma (MPM) is moderately active for the intrinsic pharmacological resistance of MPM cell and its ability to induce an immune suppressive environment. Here we showed that the expression of bromodomain (BRD) proteins BRD2, BRD4 and BRD9 was significantly higher in human primary MPM cells compared to normal mesothelial cells (HMC). Nanomolar concentrations of bromodomain inhibitors (BBIs) JQ1 or OTX015 impaired patient-derived MPM cell proliferation and induced cell-cycle arrest without affecting apoptosis. Importantly, BBIs primed MPM cells for immunogenic cell death, by increasing extracellular release of ATP and HMGB1, and by promoting membrane exposure of calreticulin and ERp57. Accordingly, BBIs activated dendritic cell (DC)-mediated phagocytosis and expansion of CD8+ T-lymphocyte clones endowed with antitumor cytotoxic activity. BBIs reduced the expression of the immune checkpoint ligand PD-L1 in MPM cells; while both CD8+ and CD4+ T-lymphocytes co-cultured with JQ1-treated MPM cells decreased PD-1 expression, suggesting a disruption of the immune-suppressive PD-L1/PD-1 axis. Additionally, BBIs reduced the expansion of myeloid-derived suppressor cells (MDSC) induced by MPM cells. Finally, a preclinical model of MPM confirmed that the anti-tumor efficacy of JQ1 was largely due to its ability to restore an immune-active environment, by increasing intra-tumor DC and CD8+ T-lymphocytes, and decreasing MDSC. Thereby, we propose that, among novel drugs, BBIs should be investigated for MPM treatment for their combined activity on both tumor cells and surrounding immune-environment.

Introduction
Malignant pleural mesothelioma (MPM) is an asbestos-related cancer characterized by an extremely long latency. Current classification is based on three main histological subtypes, epithelioid, sarcomatoid and biphasic, having respectively better, worse and intermediate prognosis. Since MPM is usually diagnosed in advanced stages, chemotherapy usually remains the only therapeutic option, but it is only modestly effective, with a median overall survival of approximately 12 months. This limited efficacy is also ascribed to the immune-evasive attitude of MPM that is characterized by a low antigenicity and by an immune-suppressive environment. Molecular classification of MPM has lagged behind compared to other cancer types. Two recent high-throughput genomic analyses and provisional data from The Cancer Genome Atlas TCGA (https://tcga-data.nci.nih.gov) indicate that MPM has a generally low mutational burden. On the other hand, sporadic observations indicate that many genes involved in epigenetic modifications, such as BAP1, NF2, SPOP, NUTM1, LATS1/2 SMARCA4/1, SETDB1, SETD2, can be deleted, mutated or amplified, while druggable kinases are not generally altered, thus limiting the use of existing targeted therapies. BET-Bromodomain Inhibitors (BBIs) represent a new class of drugs that modulate the epigenetic and the transcriptional program of cancer cells exerting a very potent therapeutic action in several hematological and solid tumors. Interestingly, it has been recently demonstrated that the BBI OTX015 decreases MPM cell proliferation by reducing c-Myc expression and delays MPM tumor growth with an efficacy comparable to standard chemotherapy. However, the functional interactions...
between BBI and the host immune system of mesothelioma (MPM) tumors as well as the ability of BBIIs to alter the immunogenicity of MPM cells remain therapeutically unexplored. Here, we show that BBIIs act as multitasking agents that are able to interfere with MPM cell growth and to convert an immune-suppressive to an immune-active environment.

**Results**

**BRDs are amplified or overexpressed in primary MPM samples and BBI treatment reduces cell growth of patient-derived MPM cells**

We first interrogated through the cBioPortal\textsuperscript{13,14} publicly available TCGA data of 87 MPM samples (MESO). Interestingly, BRD2, BRD3, BRD4 and BRD9 were either amplified or up-regulated in 6, 2, 9 and 13 cases, respectively (n = 87; Fig. 1A). Collectively, BRDs were up-regulated in 28/87 (32%) MPM samples. Thereby we extended BRD expression analysis to our series of 15 primary MPM samples (Tables S1 and S2). BRD2, BRD4 and BRD9 were significantly upregulated in tumors compared to primary not-transformed human mesothelial cells (HMC; Fig. 1B). Consistently with the high expression of BRDs in MPM, both BBIIs JQ1 and OTX015 impaired cell proliferation in a dose-dependent manner in all histological subtypes of patient-derived MPM cells (Fig. 2A and B, Fig. S1A and B). Importantly, a concentration of 250 nM of BBIIs was sufficient to interfere with cell cycle progression (Fig. 2C, Fig. S1C, Fig. S2A and B). However, the anti-proliferative activity of JQ1 was not associated to apoptosis (Fig. 2D), and OTX015 treatment was accompanied by a modest increase in cell death (about 15%; Fig. S1D).

**BBIs induce immunogenic cell death (ICD) along with adaptive immune response against MPM cells**

Since inhibitors of chromatin-associated enzymes and BRDs can exert their therapeutic action also by modulating tumor
we investigated this aspect in our primary patient-derived MPM cells under BBI treatment. Intriguingly, JQ1 and OTX015 increased the release of ATP (Fig. 3A, Fig. S3A) and High Mobility Group Protein 1 (HMGB1; Fig. 3B, Fig. S3B) in the extracellular supernatant of MPM cells, as well as the exposure of the "eat-me signals" calreticulin (CRT; Fig. 3C, Fig. S3C) and ERP57 (Fig. 3D, Fig. S3D), without affecting these parameters in non-transformed HMC. All these findings are typical of immunogenic cell death (ICD), a process that promotes an anti-tumor adaptive response followed by expansion of T lymphocytes with an increased percentage of cytotoxic CD8\(^+\)CD107\(^+\) cells and secretion of IFN-\(\gamma\). Accordingly, BBIs significantly increased the DC-mediated phagocytosis of patient-derived MPM cells, which were more resistant to phagocytosis than HMC in untreated condition (Fig. 3E, Fig. S3E). As we previously observed, proliferation of co-cultured CD8\(^+\) T-lymphocytes was negatively affected by MPM cells respect to normal HMC (Fig. 3F, Fig. S3F). This low expansion was associated with a lower IFN-\(\gamma\) secretion (Fig. 3G) and percentage of CD8\(^+\)CD107\(^+\) cells (Fig. 3H), after co-culture with DC that had phagocytized HMC or MPM cells. Conversely, BBIs increased all these parameters in patient-derived MPM cells, without significant differences across histotypes (Fig. 3F–H; Fig. S3F–H).
Figure 3. JQ1 primes MPM cells for immunogenic cell death and raises an adaptive anti-tumor immune response. (A, B) ATP release was measured by a chemiluminescent-based assay, HMGB1 release was measured by ELISA in the supernatant of cells incubated for 6 days in medium containing DMSO (ctrl) or 250 nM JQ1. Pooled data of MPM samples (epi: epithelioid; bip: biphasic; sar: sarcomatoid) are means±SEM. For both panels: *p < 0.05: JQ1-treated cells vs respective untreated cells. (C, D) Representative histograms of surface calreticulin (CRT) and ERp57, obtained by flow cytometry, in cells incubated as indicated in (A, B). The percentage of CRT/ERp57-positive cells treated with JQ1 is indicated. Mean fluorescence intensity±SEM of pooled data for CRT: HMC ctrl: 3.3±1.2; HMC JQ1: 15.9±2.9; epi MPM ctrl: 2.5±0.4; epi MPM JQ1: 15.9±2.9; bip MPM ctrl: 2.2±0.7; bip MPM JQ1: 25.7±2.5; sar MPM ctrl: 2.3±0.8; sar MPM JQ1: 21.3±2.1. Mean fluorescence intensity±SEM of pooled data for ERp57: HMC ctrl: 2.3±0.2; HMC JQ1: 3.3±1.0; epi MPM ctrl: 2.5±0.4; epi MPM JQ1: 21.4±2.8; bip MPM ctrl: 2.8±0.4; sar MPM JQ1: 18.9±3.4. Significance for both CRT and ERp57: p < 0.001: MPM cells vs HMC ctrl; p < 0.001: JQ1-treated cells vs respective untreated cells. Blank: non-immune isotypic antibody. (E) Cells were incubated as indicated in (A, B), then stained with PKH2-Green FITC, incubated at 1:1 ratio for 18 h at 37 °C with DC labelled with an anti-HLA-DR antibody (APC-conjugated). PKH2-Green FITC/HLA-DR APC-positive cells, i.e. DC that have phagocytosed MPM cells, were counted by flow cytometry. Pooled data of MPM samples are means±SEM. *p < 0.05; **p < 0.01; ***p < 0.001: MPM cells vs HMC ctrl; "p < 0.001: JQ1-treated cells vs respective untreated cells. (F) The amount of proliferating T-cells co-cultured 6 days with HMC or MPM cells, treated as reported in (A, B), was measured by labelling them with [3H]-thymidine. As positive control of proliferation, PBMC were treated with the anti-CD3 and anti-CD28 antibodies; as negative control, the PBMC were grown in medium alone. Pooled data of MPM samples are means±SEM. *p < 0.05; **p < 0.01; ***p < 0.001: MPM cells vs HMC ctrl; p < 0.001: JQ1-treated cells vs respective untreated cells. (G) After 10 days of co-culture with DC that have phagocytosed HMC or MPM cells, IFN-γ was measured by ELISA in the supernatant of CD3+CD8+ T-cells. Pooled data of MPM samples are means±SEM. *p < 0.05; **p < 0.01: MPM cells vs HMC ctrl; p < 0.001: JQ1-treated cells vs respective untreated cells. (H) Representative dot plots of CD8+CD107a+ T-lymphocytes, isolated from T-cells co-cultured with DC as reported in (G), determined by flow cytometry. Mean fluorescence intensity±SEM of pooled data: HMC ctrl: 2.4±0.2; HMC JQ1: 3.0±0.3; epi MPM ctrl: 1.3±0.4; epi MPM JQ1: 6.3±0.5; bip MPM ctrl: 1.5±0.3; bip MPM JQ1: 6.2±0.8; sar MPM ctrl: 1.4±0.1; sar MPM JQ1: 8.2±1.2. Significance: p < 0.001: MPM cells vs HMC ctrl; p < 0.01: JQ1-treated cells vs respective untreated cells.
Comparison of the immune phenotype of peripheral blood mononuclear cells (PBMC) co-cultured with HMC or MPM cells revealed that MPM cells decreased the number of CD8\(^+\) T-lymphocytes, and increased the amount of T-regulatory cells (Treg), granulocytic-derived (Gr-MDSC) and monocytic-derived myeloid derived suppressor cells (Mo-MDSC) (Fig. 4A–C, Table 1, Table S3). These data suggested that patient-derived MPM cells primed immune cell populations to an immune-suppressive rather than an immune-active environment. Interestingly, treatment with either JQ1 or OTX015 counteracted the immune-suppressive potential of MPM cells, increasing CD8\(^+\) T-lymphocytes (Fig. 4A, Table 1, Table S3) and decreasing Gr-MDSC and Mo-MDSC (Fig. 4B and C, Table 1, Table S3).

The expression of the immune checkpoints on CD8\(^+\) and CD4\(^+\) T-lymphocytes (e.g. PD-1, CTLA-4, TIM-3 and LAG-3), plays a key role in MPM-induced immune suppression.\(^4\) Indeed, CD8\(^+\) T-lymphocytes co-cultured with patient-derived MPM cells showed an increased expression of PD-1, CTL-4 and LAG-3 (Fig. 5A and B, Table 2). Notably, JQ1-treated MPM cells reduced the proportion of PD-1and LAG-3 positive CD8\(^+\) (Fig. 5A and B, Table 2) and CD4\(^+\) (Table S4) T-lymphocytes. Although PD-L1 and LAG-3 were expressed at higher levels in MPM than in HMC, JQ1 reduced both markers at levels comparable to HMC cells (Fig. 5C, Table 3). Treatment of MPM cells with OTX015 induced the same modulation of immune checkpoints on CD8\(^+\) and CD4\(^+\) T-lymphocytes (Tables S5 and S6), as well as in tumor cells (Table S7).

**JQ1 reduces tumor growth and immunosuppressive tumor-infiltrating cells in vivo**

The efficacy of BBI was finally evaluated against murine MPM AB1 cells, implanted in syngeneic immunocompetent or immunodeficient Balb/C mice. AB1-derived tumors grew more rapidly in nude Balb/C mice than in immunocompetent hosts (Fig. 6A and B). JQ1 was particularly effective in restraining tumor growth in immunocompetent animals (Fig. 6A and B), suggesting that BBI activity could be partly ascribed to modulation of the immune system. Accordingly, flow cytometry analysis of intra-tumor immune infiltrate revealed that JQ1 increased the amount of DC (Fig. 6C, Fig. S4A) and CD8\(^+\) T-lymphocytes (Fig. 6D, Fig. S4B), and reduced the amount of Gr-MDSC (Fig. 6E, Fig. S4C) and Mo-MDSC (Fig. 6F, Fig. S4C). The production of IFN-γ from draining lymph nodes was also increased (Fig. 6G). Notably, JQ1 did not elicit signs of myelosuppression or liver or kidney toxicity (Table S8).

**Discussion**

Resistance to conventional chemotherapy, lack of effective targeted therapies, low antigenicity of MPM and its ability to induce an immune-suppressive environment suggest that novel therapeutic strategies, including epigenetic drugs, should be explored to treat MPM patients. Histone deacetylase and DNA methyltransferase inhibitors have been evaluated in clinical trials in combination with chemotherapy, obtaining only a low rate of partial response associated to a high degree of toxicity.\(^21\)

Since BBIs have well documented activities on both tumor and immune system cells, we hypothesized that they could represent a novel potential therapeutic option in MPM. Data released from TCGA and analysis of our series of primary MPM samples indicate that several BRD members are overexpressed in MPM compared to HMC. In a screening of more than 650 cancer cell lines treated with JQ1, cells were classified as "JQ1-sensitive" if their IC\(_{50}\) was lower than 1 \(\mu\)M.\(^11\) Since JQ1 reduced MPM cell proliferation and induced cell cycle arrest at nanomolar concentrations, MPM cells may be reasonably considered sensitive to the drug. Differently from previous reports that tested JQ1 in the range of 0.5–5 \(\mu\)M,\(^10,11\) in this work the reduction of cell proliferation was not paralleled by increased apoptosis. Accordingly, also the treatment at nanomolar concentrations with OTX015 was associated to a very modest apoptotic index. It can be argued that the induction of apoptosis is a concentration-dependent event and that 250 nM of BBIs is below a putative "pro-apoptotic" threshold. Alternatively, it can be hypothesized that different mechanisms of cell death are involved. ICD is a process that makes dying tumor cells visible to the immune system, following stress events such as chemotherapy or radiotherapy that induce endoplasmic reticulum (ER) stress and/or alter autophagy mechanisms. This is associated to ATP and HMGB1 release in the extracellular environment, and to the exposure on the cell surface of ER-residing proteins, such as calreticulin and EBPs. All these signals contribute to recruitment and activation of local DC to remove dying cancer cells.\(^18\) MPM cells are known to be refractory to chemotherapy-induced ICD.\(^19\) Of note, both JQ1 and OTX015 BBIs overcame such refractoriness and induced a typical ICD signature in MPM cells, increasing DC-mediated phagocytosis and the subsequent expansion of anti-tumor CD8\(^+\) T-lymphocytes characterized by cytotoxic activity. It has already been reported that JQ1 activates antigen-presenting cells against melanoma,\(^22\) a tumor with high immunogenicity. Our results are particularly relevant because MPM is a poorly immunogenic tumor.\(^2,3\) Moreover, MPM-infiltrating DC are defective in presenting tumor antigens and inducing a CD8\(^+\)-mediated anti-tumor response.\(^23\) Interestingly, both BBIs spared not-transformed mesothelial cells from ICD. The differential expression of BRD between HMC and MPM cells may explain such selectivity, and may represent an advantage for using BBIs in MPM.

MPM is associated to an immune-suppressive rather than immune-active micro-environment, as documented by increased amount of anergic CD4\(^+\) and CD8\(^+\) T-lymphocytes, Treg and MDSC.\(^24–26\) Our experimental data from MPM/PBMC co-cultures well fit with the findings from the analysis of the immune infiltrate in murine models and MPM patients. Indeed, compared to HMC, patient-derived MPM cells decreased the amount of CD8\(^+\) T-lymphocytes, and increased the amount of Treg, Gr-MDSC and Mo-MDSC. Importantly, BBIs modified two critical cell populations in the immune-environment associated to MPM. First, BBI-treated MPM cells...
showed an increase in CD8\(^+\) T-lymphocytes. Second, BBIs significantly reduced the percentage of Gr-MDSC and Mo-MDSC that are critical in sustaining MPM progression. Since active anti-MPM CD8\(^+\) T-lymphocytes induce the apoptosis of MDSC, BBIs likely induced a virtuous circle: by increasing MPM cell immunogenicity and priming it for ICD, the drugs activate anti-tumor CD8\(^+\) clones that eliminate MDSC; in turn, the reduction of MDSC rescues the

**Figure 4.** JQ1 prevents the decrease of CD8\(^+\) T-lymphocytes and the increase of granulocytic-/monocytic-derived myeloid derived suppressor cells induced by MPM cells. (A) Representative dot plots of CD3\(^+\)CD8\(^+\) T-lymphocytes, isolated from PBMC after 6-days co-culture with HMC or MPM cells (epi: epithelioid; bip: biphasic; sar: sarcomatoid), in medium containing DMSO (ctrl) or 250 nM JQ1, as per flow cytometry. (B) Representative dot plots of Gr-MDSC, isolated from HLA-DR\(^-\)CD14\(^-\)CD11b\(^+\) PBMC co-cultured as in (A). To identify Gr-MDSC, first HLA-DR\(^-\)CD11b\(^+\) cells were gated (R1), then CD14\(^-\)CD11b\(^+\) cells (R2 on R1, not shown). This cell population was used to identify CD11b\(^+\)CD15\(^+\) cells. Gating of CD11b\(^+\)CD15\(^+\) is shown. (C) Representative dot plots of Mo-MDSC, isolated from HLA-DR\(^-\)CD15\(^+\)CD11b\(^+\) PBMC co-cultured as in (A). Specifically to identify Mo-MDSC, first HLA-DR\(^-\)CD11b\(^+\) cells (R1) were gated, then CD15\(^+\)CD11b\(^+\) cells (R2 on R1, not shown). This cell population was used to identify CD11b\(^+\)CD14\(^+\) cells. Gating of CD11b\(^+\)CD14\(^+\) cells is shown.
cytotoxic functions of CD8+ T-lymphocytes and restores an anti-tumor immune-environment. Recently, the expression of immune checkpoints on T-lymphocytes and of their respective ligands on MPM cells emerged as a critical mechanism at the basis of MPM-induced immunosuppression. Consistently, we found that both CD4+ and CD8+ T-lymphocytes co-cultured with patient-derived MPM cells had increased expression of PD-1, CTLA-4 and LAG-3 compared to lymphocytes co-cultured with HMC. On one hand, the disruption of this biological circuit may further contribute to overcome the immune anergy induced by MPM cells.

The high expression of BRD members may underlie the efficacy of BBIs in our series of primary MPM samples. Our results indicate that the broad activity of BBIs seen in MPM models is not related to specific clinical or histological features of MPM patients from which they were derived. However, we acknowledge that our MPM series, although representative of the three histotypes and of the main clinical and pathological features of MPM, are rather limited and can potentially lead to data overinterpretation. Expansion of this collection of MPM patient-derived models will help to identify potential unresponsive tumors and characterize the molecular bases of refractoriness to BBIs.

A limitation of this study may be related to the challenge of MPM cells with PBMC of healthy donors. On one hand, our results may provide useful indications about the alterations induced by MPM on a healthy immune system and about the rescuing activity of BBIs. However, our work cannot predict the effect of BBIs on the immune infiltrate of MPM patients that is known to change during MPM progression. To partially overcome this limitation, we measured the effects of JQ1 on local immune system in a preclinical model of MPM. JQ1 resulted significantly more effective against MPM growing in immunocompetent rather than in immunodeficient animals. These results suggest that a significant fraction of JQ1 effect was due to the restoration of a proper anti-tumor immune activity. Murine MPM growth is characterized by a first phase of progressive increase of Treg cells that suppress T-lymphocytes functions, followed by a second phase of progressive increase of MDSC. MDSC are well detectable within MPM of untreated Balb/C mice, suggesting that our model mirrors an advanced stage of MPM. The intratumor immune infiltrate profiling of JQ1-treated animals recapitulates the events induced by the drug in ex vivo assays, i.e. the increase of DC and CD8+ T-lymphocytes, and the reduction of Gr-MDSC and Mo-MDSC. The high ratio of CD8+ T-lymphocytes/MDSC observed in JQ1-treated animals indicates a clear shift from an immune-suppressive to an immune-active environment. Indeed, the higher production of IFN-γ from tumor-draining lymph nodes confirmed the presence of active cytotoxic CD8+ T-lymphocytes. Collectively, our results well reconcile with the experimental observation that JQ1, in combination with histone deacetylase inhibitors, fosters a T-cell-mediated anti-tumor immunity against non-small cell lung cancer.

In summary, we demonstrated that BBIs induce the reduction of MPM cell proliferation and the reversion of the MPM-induced immune-suppression. Strategies combining epigenetic drugs and immunotherapy are under development.

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**Table 1.** Immune phenotype analysis of immune cells co-cultured with HMC and MPM cells treated with JQ1.

| Cell type          | T-helper lymphocytes (CD3+CD4+) | T-cytotoxic lymphocytes (CD3+CD8+) | NK (CD56+CD335+) | Treg (CD4+CD127++CD25+) | Gr-MDSC (CD11b+CD4+CD15+HLA-DR-) | Mo-MDSC (CD11b+CD14+CD68+HLA-DR-) | Monocytes (CD14+) | Macrophages (CD14+CD68+) |
|--------------------|---------------------------------|----------------------------------|------------------|----------------------------|--------------------------------|--------------------------------|-------------------|------------------|
| HMC Ctrl           | 52.1 ± 7.5                      | 4.5 ± 1.3                        | 1.5 ± 0.3        | 2.4 ± 0.4                  | 2.3 ± 0.4                      | 7.4 ± 2.3                     | 22.4 ± 4.3        | 31.2 ± 4.5       |
| HMC JQ1            | 56.4 ± 3.5                      | 4.1 ± 0.5                        | 2.2 ± 0.4        | 3.4 ± 0.6                  | 2.5 ± 0.3                      | 8.4 ± 0.9                     | 24.4 ± 1.8        | 27.7 ± 4.2       |
| Epithelioid MPM Ctrl | 47.4 ± 6.7                    | 2.5 ± 0.3                        | 2.4 ± 0.2        | 4.1 ± 0.5                  | 10.8 ± 1.2                     | 27.9 ± 3.5                     | 27.8 ± 3.9        | 33.1 ± 2.8       |
| Epithelioid MPM JQ1 | 47.6 ± 8.9                    | 9.6 ± 0.7                        | 2.4 ± 0.2        | 4.4 ± 0.5                  | 3.4 ± 0.6                      | 13.9 ± 2.4                     | 23.8 ± 4.5        | 38.9 ± 7.3       |
| Bip MPM Ctrl       | 67.4 ± 9.7                      | 3.1 ± 0.2                        | 2.3 ± 0.1        | 4.5 ± 0.7                  | 12.4 ± 2.3                     | 24.5 ± 2.4                     | 24.8 ± 4.4        | 28.9 ± 3.8       |
| Bip MPM JQ1        | 55.3 ± 4.4                      | 11.4 ± 0.8                       | 2.6 ± 0.3        | 4.9 ± 0.6                  | 6.8 ± 1.1                      | 10.1 ± 2.4                     | 27.9 ± 1.9        | 23.4 ± 2.1       |
| Sar MPM Ctrl       | 42.5 ± 3.9                      | 2.3 ± 0.3                        | 2.4 ± 0.2        | 5.0 ± 0.5                  | 9.7 ± 0.9                      | 19.8 ± 1.9                     | 23.6 ± 4.9        | 22.9 ± 3.4       |
| Sar MPM JQ1        | 48.5 ± 7.7                      | 8.4 ± 0.3                        | 2.5 ± 0.4        | 5.8 ± 0.6                  | 4.2 ± 0.3                      | 7.9 ± 3.2                     | 24.7 ± 2.8        | 23.5 ± 8.4       |

HMC and MPM cells (epithelioid: epi; biphasic: bip; sarcomatoid: sar; n = 4/each histotype), incubated for 6 days in fresh medium (ctrl) or with 250 nM JQ1, were co-cultured with PBMC. After this incubation time, PBMC cell populations were analyzed by flow cytometry. Results are expressed as means±SEM percentage of cells positive for the indicated markers. *p < 0.05; **p < 0.01; ***p < 0.001: vs HMC Ctrl; *p < 0.05 vs respective MPM Ctrl. NK: natural killer cells; Treg: T-regulatory cells; Gr-MDSC: granulocytic-derived myeloid derived suppressor cells; Mo-MDSC: monocytic-derived myeloid derived suppressor cells; Mon: monocytes; Mac: macrophages.
investigations, and several immunotherapy-based phase II clinical trials for MPM treatment are open (https://clinicaltrials.gov). BBIs are relatively well-tolerated compared to other epigenetic drugs and may be reasonably included among the novel agents to be further tested in combination therapy in MPM.

Figure 5. JQ1 reduces the levels of PD-1/PD-L1 and LAG-3 in CD8+ T-lymphocytes and in MPM cells. (A, B) Representative dot plots of CD8+ PD-1+ and CD8+ LAG-3+ T-lymphocytes, isolated from PBMC after 6-days co-culture with HMC or MPM cells (epi: epithelioid; bip: biphasic; sar: sarcomatoid), in medium containing DMSO (ctrl) or 250 nM JQ1, determined by flow cytometry. (C) Representative dot plots of PD-L1+ and LAG-3+ HMC or MPM cells, incubated for 6 days in medium containing DMSO (ctrl) or 250 nM JQ1.
mixture medium (primary MPM cells) or DMEM (AB1 cells), supplemented with 10% v/v fetal bovine serum (FBS), 1% v/v penicillin-streptomycin. Cells were checked for *Mycoplasma spp.* contamination by PCR every three weeks; contaminated cells were discharged.

### Immune-phenotype of primary MPM cells

The mesothelial origin of the isolated cells was confirmed by positive immune-staining, after cell centrifugation (1 200 × g for 5 min) and fixation in 4% v/v formalin at 4 °C overnight, using the following antibodies: calretinin (1:100, rabbit polyclonal #RB-9002-R7, Thermo Fisher Scientific), Wilms tumor-1 antigen (WT1, 1:10, mouse clone 6F2H2, Thermo Fisher Scientific), cytokeratin 5 (1:100, mouse clone D5, ImPath, Menarini Diagnostics), podoplanin (1:150, mouse clone D2-40, Dako), pancytokeratin (1:500, mouse clone AE1/AE3, Dako), epithelial membrane antigen (EMA, 1:6000, mouse clone E29, Dako), carcino-embryonic antigen (CEA, 1:15000, rabbit polyclonal #IRS2661-2, Dako). Only cells positive for at least one mesothelial antigen among calretinin, WT1, podoplanin and cytokeratin 5 or positive for pancytokeratin, were considered for subsequent experiments and included in the study.

### BRD expression

RNA was extracted from cells using TRIzol (Invitrogen). 1 μg of total RNA was used for reverse transcription with iScript cDNA Synthesis Kit (Bio-Rad). Real-time PCR was performed with iQ SYBR Green (Bio-Rad) using the following primers: BRD2: for:5′-GGAGTGGGGAGGAGGAGG-3′; rev:5′-TG GGCTTGATATTGACACC-3′; BRD3: for:5′-GAGACTCC- CAGCGACAG-3′; rev:5′-TCTCAACACCTCTGGAGGC- 3′; BRD4: for:5′-ATACCTGTCTAGATGTGTC-3′; rev:5′- GTTCCCATATCCCATAGGCGT-3′; BRD9: for:5′-GACGTG ATGAGAGGAGGAC-3′; rev:5′-GAGCTATGGCCAGCAGG- 3′; HuPO: for:5′-GGCTCTCTGGAGGTGTC-3′; rev:5′-GAGCTCGTTGACCCGT-3′. Real-time PCR parameters were as follows: cycle 1: 95 °C for 3 minutes; cycle 2: 95 °C for 15 seconds, 60 °C for 30 seconds (40 cycles). The 2-DDCT method was employed to analyze the data. HuPO expression was used to normalize the results.

### Cell proliferation analysis

For short-term proliferation assays, 2 × 10^3 cells were seeded in each well of a 24-well plate in complete growth media containing the indicated JQ1 or OTX015 concentrations. After 10 days, medium was aspirated, cells were fixed and stained with 5% w/v crystal violet solution in 66% v/v methanol, washed and analyzed under bright field Olympus IX73 microscope (Olympus Corporation), equipped with the CellSense Dimension imaging system (10 × objective; 10 × ocular lens). For short-term proliferation assay, cells were plated in 96-well plates at a density of 2 × 10^4 per well. Proliferation was evaluated by CellTitre-Glo (Promega). Proliferation at day 0 was considered as 100%; the results were expressed as percentage of proliferation vs day 0.
Cell cycle analysis

Cells were plated at a density of $6 \times 10^4$ in 6-well plates. Cells were harvested, washed with PBS, treated with RNAse (0.25 mg/ml) and stained for 15 min with propidium iodide (50 μg/ml). The cell-cycle distribution G0/G1, S, and G2/M was analyzed by FACScan cell sorter (Becton Dickinson) equipped with CellQuest software (Becton Dickinson).

Apoptosis

Cells were plated at a density of $6 \times 10^4$ in 6-well plates. After 3 days, floating and harvested cells were washed with PBS and stained for 15 min with 200 nM tetramethylrhodamine methyl ester perchlorate (TMRM). The percentage of apoptotic cells was analyzed by FACScan using the CellQuest Software.

ATP and HMGB1 release

The amount of extracellular ATP was measured with the ATP Bioluminescent Assay Kit (FL-AA, Sigma Chemicals Co.), using a Synergy HT Multi-Detection Microplate Reader (Bio-Tek Instruments). ATP was quantified as arbitrary light units; data were converted into nmoles/mg proteins. The extracellular release of the HMGB1 was measured with the High Mobility Group Protein 1 ELISA kit (Cloud-Clone Corp.). Results were expressed in pg/mg total cellular proteins.

Flow cytometry analysis

Cells were washed with PBS, detached with Cell Dissociation Solution (Sigma Chemicals Co.) and re-suspended at $5 \times 10^5$ cells/ml in culture medium containing 5% v/v FBS. Cells were incubated for 45 min at 4°C with anti-calreticulin (1:100, rabbit polyclonal, #PA3-900, Affinity Bioreagents) or anti-ERP57 (1:100, rabbit polyclonal, #PA3-009, Thermo Fisher Scientific) in 0.25% w/v bovine serum albumin (BSA)-PBS, washed, incubated with the secondary fluorescein isothiocyanate (FITC)-conjugated antibody (1:50, Sigma Chemicals Co.) for 30 min at 4°C, washed and fixed with 2.5% v/v paraformaldehyde. 1 × 10^6 cells were analyzed by a Guava easyCyte flow cytometer (Millipore), using the InCyte software (Millipore). Control experiments included incubation of cells with non-immune isotypic antibody, followed by secondary antibody.

Phagocytosis

DC were generated from peripheral blood samples obtained from healthy donors provided by Blood Bank of AOU Città della Salute e della Scienza, Torino, Italy (#DG-767/2015), as previously reported.19 The percentage of phagocytized cells at 4°C was less than 5% than the phagocytized cells at 37°C (not shown). Phagocytosis rate was expressed as phagocytic index, calculated as previously reported.37

T-lymphocyte proliferation in HMC/MPM co-cultures

1 × 10^6/ml human PBMC, isolated from buffy coats of healthy donors (Blood Bank, Città della Salute e della Scienza di Torino Hospital, Torino, Italy) by centrifugation on Ficoll-Hypaque density gradient, were treated with anti-CD3 (1:2 000, mouse clone OKT3, BioLegend) and anti-CD28 (1:500, mouse clone 37.51, BioLegend) antibodies, to induce the specific proliferation of T-lymphocytes. PBMC were co-cultured at an

Figure 6. JQ1 delays MPM growth and reduces the immune-suppressive tumor-infiltrating cells. (A, B) Six week-old female immunocompetent or nude Balb/C mice were inoculated subcutaneously with $1 \times 10^7$ AB1 cells and treated as detailed under Materials and methods. Tumor growth data are means±SEM (n = 15/group). **p < 0.01; ***p < 0.001: JQ1-treated vs. untreated animals; p < 0.01: JQ1-treated immunocompetent animals vs JQ1-treated nude animals. (C-F) After digestion of tumors from immunocompetent mice, single cell suspension was analyzed by flow cytometry to measure the percentage of DC, CD3⁺CD8⁺ T-lymphocytes, Gr-MDSC, Mo-MDSC. Data are means±SEM. **p < 0.01: JQ1-treated animals vs untreated animals. (G) IFN-γ was measured by ELISA in the supematant of tumor-draining lymph nodes of immunocompetent mice. Data are means±SEM. p < 0.05: JQ1-treated animals vs untreated animals.
effector/target (HMC or MPM cells) ratio of 10:1 for 6 days. The proliferation of T-lymphocytes was assessed by adding 1 μCi of [3H]thymidine (PerkinElmer) 18 h before the end of the co-cultures, then harvesting the plates and counting the radioactivity.

**T-lymphocyte activation**

After MPM cell phagocytosis, DC were washed and co-cultured 10 days at a 1:5 ratio with autologous T-lymphocytes, isolated by immuno-magnetic sorting with the Pan T Cell Isolation Kit (Miltenyi Biotec.). The percentage of CD8+CD107+T-lymphocytes, indicative of active anti-tumor cytotoxic T-lymphocytes, was determined by flow cytometry. IFN-γ in the culture supernatant of CD8+T-cells co-cultured with DC or in the supernatant of tumor-draining lymph nodes was measured with the Human IFN-γ DuoSet Development Kit (R&D Systems). Results were expressed as ng/ml cell proteins or pg/ml, according to the respective calibration curves.

**Immune phenotyping**

PBMC isolated from buffy coats as indicated above, were incubated for 6 days with HMC or MPM cells, then harvested, washed and re-suspended in PBS containing 5% v/v FBS. A three- and four-color flow cytometry was performed on 1 × 10⁶ cells, with the appropriate combinations of antibodies (all diluted 1:10, Miltenyi Biotec.) for: CD3 (mouse clone REA613), CD4 (mouse clone M-T466), CD8 (mouse clone BW135/80) for T-lymphocytes; CD56 (mouse clone AF127H3), CD335/NKp46 (mouse clone 9E2) for Natural Killer (NK) cells; CD4 (mouse clone M-T466), CD25 (mouse clone 4E3), CD127 (mouse clone MB1518C9) for Treg cells; CD11b (rat clone M1/70.15.11.5), CD14 (mouse clone TÜK4), CD15 (mouse clone VIMC6), HLADR (mouse clone AC122) for Gr-MSDC and Mo-MDSC; CD14 (mouse clone TÜK4) and CD68 (mouse clone Y1/82A) for macrophages and monocytes. The analysis of immune infiltrate of murine MPM was performed by using antibodies recognizing: CD11c (hamster clone N418, Miltenyi Biotec.) for DC; CD3 (mouse clone REA641, Miltenyi Biotec.) and CD8 (rat clone 53–6.7, Miltenyi Biotec.) for T-lymphocytes; CD11b (rat clone M1/70.15.11.5, Miltenyi Biotec.), Ly6C (rat clone AL-21, BD Biosciences), Ly6G (rat clone 1A8, BD Biosciences) for Gr-MSDC and Mo-MDSC. Cells were analyzed using a Guava® easyCyte flow cytometer (Millipore), equipped with the InCyte software.

**Immune check-point detection**

CD3+ cells were isolated from 1 × 10⁶ PBMC co-cultured with HMC or MPM cells for 6 days, with the Pan T Cell Isolation Kit (Miltenyi Biotec.), washed and re-suspended in PBS containing 5% v/v FBS. The detection of immune checkpoints on T-lymphocytes and/or immune checkpoint ligands on MPM cells were performed using antibodies for CD279/PD-1 (mouse clone PD1.3.1.3), CD223/LAG-3 (mouse clone REA351), CD366/TIM-3 (mouse clone F38-2E2), CD152/CTLA-4 (mouse clone BN13; all diluted 1:10, Miltenyi Biotec.), CD274/PD-L1 (1:1 000, mouse clone 29E.2A3, BioLegend). 1 × 10⁵ cells were analyzed by as reported above.

**In vivo tumor growth, immune-environment analysis and hematochemical parameters**

1 × 10⁷ AB1 cells, mixed with 100 μL Matrigel, were injected subcutaneously in 6 weeks-old female immunocompetent or nude Balb/C mice (Charles River Laboratories), housed (5 per cage) under 12 h light/dark cycle, with food and drinking provided ad libitum. Tumor growth was measured daily by caliper and was calculated according to the equation (LxW²)/2, where L = tumor length; W = tumor width. When the tumor reached the volume of 50 mm³ (day 15 after injection), mice were randomly divided into 2 groups, treated intraperitoneally twice a week for 3 consecutive weeks, as follows: 1) control group, treated with 0.1 ml saline solution; 2) JQ1 group, treated with 0.1 ml JQ1 (in 1:10 sterile saline solution/DMSO solution; final dosage: 50 mg/kg). Tumor volumes were monitored daily by caliper and animals were euthanized with zolazepam (0.2 ml/kg) and xylazine (16 mg/kg) at the end of treatment. Tumor were excised, cut into 1 mm³-pieces and digested (in DMEM medium containing 1 mg/ml collagenase and 0.2 mg/ml hyaluronidase) for 1 h at 37°C. The material was filtered in a syringe using a 70 μm-cell strainer to obtain a single cell suspension, and washed in DMEM. Infiltrating immune cells were collected by centrifugation on Ficoll-Hypaque density gradient and subjected to immune phenotyping by flow cytometry. Draining lymph nodes were collected, homogenized for 30s at 15 Hz, using a TissueLyser II device (Qiagen) and centrifuged at 12 000 x g for 5 minutes. The supernatant was used to measure the amount of IFN-γ. The hemocromocytometric analyses were performed with a UniCel DxH 800 Coulter Cellular Analysis System (Beckman Coulter Inc.) on 0.5 ml of blood collected immediately after mice sacrifice; lactate dehydrogenase, aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, creatinine, creatine phosphokinase were measured using kits from Beckman Coulter Inc.

Animal care and experimental procedures were approved by the Bio-Ethical Committee of the Italian Ministry of Health (#122/2015-PR).

**Statistical analysis**

All data in the text and figures are provided as means±SEM. The results were analyzed by a one-way analysis of variance (ANOVA), using Statistical Package for Social Science (SPSS) software (IBM SPSS Statistics v.19). p < 0.05 was considered significant.

**Disclosure of potential conflicts of interest**

No potential conflicts of interest were disclosed.

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