Reactive oxygen species modulate macrophage immunosuppressive phenotype through the up-regulation of PD-L1

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The combination of immune checkpoint blockade with chemotherapy is currently under investigation as a promising strategy for the treatment of triple negative breast cancer (TNBC). Tumor-associated macrophages (TAMs) are the most prominent component of the breast cancer microenvironment because they influence tumor progression and the response to therapies. Here we show that macrophages acquire an immunosuppressive phenotype and increase the expression of programmed death ligand-1 (PD-L1) when treated with reactive oxygen species (ROS) inducers such as the glutathione synthesis inhibitor, buthionine sulfoximine (BSO), and paclitaxel. Mechanistically, these agents cause accumulation of ROS that in turn activate NF-κB signaling to promote PD-L1 transcription and the release of immunosuppressive chemokines. Systemic administration of paclitaxel promotes PD-L1 accumulation on the surface of TAMs in a mouse model of TNBC, consistent with in vitro results. Combinatorial treatment with paclitaxel and an anti-mouse PD-L1 blocking antibody significantly improved the therapeutic efficacy of paclitaxel by reducing tumor burden and increasing the number of tumor-associated cytotoxic T cells. Our results provide a strong rationale for the use of anti-PD-L1 blockade in the treatment of TNBC patients. Furthermore, interrogation of chemotherapy-induced PD-L1 expression in TAMs is warranted to define appropriate patient selection in the use of PD-L1 blockade.

Significance

Immunotherapies targeting the programmed death-1 (PD-1) and its ligand PD-L1 have recently been combined with standard chemotherapy to potentiate the treatment of solid tumors, including triple negative breast cancer (TNBC). Reactive oxygen species (ROS) have been directly linked to the cytotoxic effects of chemotherapy. Here we report that ROS induced either by chemotherapy (paclitaxel) or antioxidant depletion induce PD-L1 expression in macrophages. PD-L1 positive macrophages have immune-suppressive and angiogenic properties that interfere with the efficacy of paclitaxel in vivo. Indeed, PD-L1 blockade reverts this effect and synergizes with paclitaxel to reduce tumor growth. Our work reveals a pathway that further supports the importance of combining taxane and PD-L1 inhibitors as promising anticancer strategy in TNBC.


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in cancer and stromal cells (11, 12). In TNBC, tumor-associated macrophages (TAMs) support tumor progression and are a potent regulator of therapeutic response in BC because they can suppress the immune-based mechanisms of cytotoxic chemotherapy (13, 14). Based on these considerations, we speculated that chemotherapy-induced ROS could affect the expression of PD-L1 in macrophages and the immune properties of the TME.

Here we report that ROS induced by the glutathione synthesis inhibitor, buthionine sulfoximine (BSO), positively regulates mRNA and protein surface expression of PD-L1 in human and mouse macrophages in vitro. These macrophages also produce immunosuppressive cytokines, including IL-4, IL-10, and IL-17 and the angiogenic factor, vascular endothelial growth factor-A (VEGF-A). Interestingly, the chemotherapeutic drug and ROS inducer, paclitaxel, reproduced all BSO-mediated effects in macrophages. Furthermore, in mouse BRCA1/p53-deleted mammary tumors, a model which resembles spontaneous TNBC, in vivo administration of paclitaxel induced PD-L1 expression in TAM as soon as 24 h after treatment, leading to an immunosuppressive TME. Consequently, the in vivo combination of paclitaxel and an anti–PD-L1 blocking antibody reduced mammary tumor burden and reverted the immune properties of TME. Our data show that ROS are regulators of PD-L1 expression, immune suppressive, and angiogenic features of macrophages. This study emphasizes the importance of evaluating PD-L1 expression in TAMs as a predictive biomarker of chemoinmunotherapy response in TNBC patients.

**Results**

**ROS Regulate PD-L1 Expression and Secretion of Immunosuppressive Cytokines in Macrophages In Vitro.** Recent work has shown that in tumor-bearing mice, TAMs expressed much higher surface levels of PD-L1 than circulating monocytes, implying up-regulation of PD-L1 by the tumor microenvironment (15). ROS generation and accumulation in the TME have important implications in the initiation and progression of cancer (12). To elucidate if ROS could regulate the expression of PD-L1 in macrophages, we treated bone marrow-derived macrophages (BMDMs) with BSO, which increases ROS by depleting reduced glutathione (GSH) (16). BSO positively induced *Pd1* mRNA levels in a ROS-dependent manner since its effect was reverted by cotreatment with the antioxidant and ROS
querence N-acetylcyesteine (NAC) (Fig. 1A). This change coincided with modulation of intracellular ROS levels as shown by quantification of CM-H2DCFDA (DCF-DA) staining by flow cytometry (SI Appendix, Fig. S1A). BSO-mediated effect on Pdll expression was greater when BMDMs were first treated with IL-4 and M-CSF that polarize them toward alternatively activated macrophages with features similar to TAM (17) (SI Appendix, Fig. S1B). The polarization of these BMDMs was confirmed by the elevated expression of arginase-1 (Arg1) (17) (SI Appendix, Fig. S1C).

BSO also triggered the expression of the NRF2 antioxidant targets, Gclc, Gclm, Nqo1, and Hmxo1 as a response to the intracellular redox imbalance (Fig. 1B and SI Appendix, Fig. S1D).  Data in mouse BMDMs were validated in human monocyte-derived macrophages treated with BSO with or without NAC. Human macrophages increased PD-L1 mRNA levels as well as the NRF2 target, NQO1, as a response to different ROS conditions (SI Appendix, Fig. S1 E and F).

Next, we investigated which population among BMDMs was mainly affected by BSO in terms of PD-L1 protein surface expression. In BSO-treated mouse BMDMs, defined as CD11b+ and F4/80 double-positive cells (CD11b+/F4/80+), PD-L1 expression was not affected by the presence of BSO (SI Appendix, Fig. S1G), but it completely coincided with a population characterized by high positivity of mannose receptor C type 1 (MRC1/Cd206) and the expression of major histocompatibility complex II (MHC-II) molecules (Fig. 1C and SI Appendix, Fig. S1 H and I). Of note, the CD206⁺MHC-II⁺ macrophages were defined as usually activated macrophages (14, 18). By contrast, PD-L1 expression was almost unchanged in a population highly expressing MHC-II (CD206⁺MHC-II⁺) (Fig. 1D and SI Appendix, Fig. S1 H and I). In human macrophages, PD-L1 surface staining increased in CD11b⁺ cells upon BSO and decreased when NAC was added to the culture (SI Appendix, Fig. S1U).

PD-L1 expression has been previously associated with the immune-suppressive features of macrophages (19). Therefore, we analyzed which cytokines were present in the media of BSO- and BSO ± NAC-treated mouse BMDMs by applying a mouse cytokine antibody array. We found that, compared with untreated cells, BSO stimulated production of interleukin-10 (IL-10), interleukin-17 (IL-17), interleukin-4 (IL-4), interleukin-1 beta (IL-1b), insulin-like growth factor-binding protein 3 (IGFBP-3), and insulin-like growth factor A ligand (CX3CL1) (Fig. 1F). IL-10, IL-4, IGFBP-3, and CXCL1 are usually associated with an immune-suppressive phenotype of macrophages (20–23). IL-1b is a well-known proinflammatory cytokine that has been associated with breast cancer progression and ability to metastasize, especially to extravasate when induced by neutrophils with metalloproteases and other proinflammatory cytokines (24, 25). IL-17 is another inflammatory cytokine but it can enhance immunosuppression in several systems, including macrophages (26). On the other hand, BSO-treated BMDMs produced low levels of interleukin-12 isoform (IL-12) p40/p70 heterodimer and p40 monomer, as well as CD30L (TNFRSF30), CD40, and C-X-C motif chemokine 10 (CXCL10) (Fig. 1E). Overall, these data suggest that ROS drive a phenotypic change in macrophages characterized by reduced antigen presenting function and costimulatory ability (27–29). In the same cells, ROS also upregulated the production of VEGF-A, indicative of angiogenic macrophages (30). Notably, NAC completely reverted the production of cytokines and VEGF-A induced by BSO, indicating a key role of ROS in these changes (Fig. 1E).

ROS induction is a key component of the cytotoxic properties of chemotherapy (11). We compared three chemotherapeutic drugs for their ability to increase ROS in BMDMs: the antimitotic agent paclitaxel, the polyADP-ribose polymerase (PARP) inhibitor, olaparib, and the platinum-based drug cisplatin (1). Compared with cisplatin and olaparib, paclitaxel induced the highest ROS levels in BMDMs (SI Appendix, Fig. S2A). In contrast to cisplatin and olaparib, paclitaxel did not cause any DNA damage as measured by intracellular accumulation of phosphorylated H2AX (γH2AX) (SI Appendix, Fig. S2B) (31). Paclitaxel-induced ROS levels were not cytotoxic, since BMDMs had similar cellular viability in both untreated and treated conditions as measured by the sulforhodamine B (SRB) assay (SI Appendix, Fig. S2C). Given its ability to elevate ROS, paclitaxel triggered the expression of Pdll compared with control cells, which was reverted when ROS were scavenged by NAC (Fig. 1F and SI Appendix, Fig. S2D). Similarly to BSO, the paclitaxel-mediated effect on Pdll expression was augmented by polarization of BMDMs toward alternatively activated macrophages (SI Appendix, Fig. S2E). NRF2-regulated antioxidant genes, Gclm, Gclc, and Hmxo1, were also elevated in paclitaxel-treated BMDMs (Fig. 1G and SI Appendix, Fig. S2F). Flow cytometry analysis also showed increased levels of PD-L1 specifically on the surface of CD206⁺MHC-II⁺ upon treatment with paclitaxel that was reduced by adding NAC (Fig. 1H and SI Appendix, Fig. S2G). Analysis of human macrophages recapitulated the elevation of both PD-L1 mRNA and surface protein after exposure to paclitaxel that was reverted by NAC (SI Appendix, Fig. S2 H and J). The expression of the NRF2 antioxidant enzyme NQO1 was similarly regulated (SI Appendix, Fig. S2J). Then, we analyzed the cytokine and growth factor production of BMDMs treated with paclitaxel with or without NAC. Interestingly, a profile of cytokines from paclitaxel-stimulated BMDMs contained the same profile of cytokines observed after BSO treatment (Fig. 1J). In addition, we detected Fas ligand (Fasl) and C-X3-C motif chemokine ligand (CX3CL1) (Fig. 1J). Fasl is a common mediator of apoptosis in T cells expressing the receptor Fas, whereas CX3CL1 functions as an adhesion molecule (32, 33). The production of these cytokines was significantly reverted by cotreatment with NAC (Fig. 1J).

ROS-Induced PD-L1 Expression Is Mediated by the Transcription Factor NF-κB. We noted that most of the cytokines induced by BSO and paclitaxel treatments have been previously characterized as transcriptional targets of the transcription factor NF-κB (https://www.bu.edu/nf-kb/gene-resources/target-genomes). Furthermore, it is known that activation of NF-κB can promote cell survival and prevent oxidative damage in response to ROS (34). Thus, we hypothesized that ROS might regulate PD-L1 expression through NF-κB activation.

NF-κB molecular forms are usually dimers and the dimer formation is necessary for DNA binding. The most abundant form of NF-κB dimer is p50/p65 heterodimer that mediates the canonical activation of the pathway (35). Upon phosphorylation on the two key residues, Ser276 and Ser366, p65 undergoes a conformational change that triggers its transcriptional activity (35). In BSO- and paclitaxel-treated BMDMs, we found an increased frequency of cells positive for the phosphorylation of p65 at Ser366 residue by immunofluorescence staining (Fig. 2A and SI Appendix, Fig. S3A). The number of these cells was reduced by cotreatment with either the ROS quencher NAC or the nuclear factor kappa-B kinase-2 (IKK-2) inhibitor SC514 (Fig. 2A and SI Appendix, Fig. S3A) (36). Treatment with lipopolysaccharides (LPS) was used as positive control of p65 phosphorylation in BMDMs (Fig. 2A) (37). These results were validated by an overall increase of P-p65 nuclear intensity in the same conditions (2B and SI Appendix, Fig. S3B). We further verified the activation of the NF-κB pathway by analyzing the expression of the NF-κB target gene, IkBa, IkBa mRNA was up-regulated by BSO and paclitaxel treatments and the effect was reverted by NAC and SC514 cotreatments (Fig. 2C). Strikingly, SC514 also reduced Pdll mRNA in BSO- or paclitaxel-treated BMDMs (Fig. 2D). Then we sought to validate that the SC514-mediated effect on PD-L1 was indeed mainly NF-κB dependent. To do so,
we analyzed the expression of IκBα, vascular endothelial factor-A (Vegfa) and Pdl1 in BMDMs treated with BSO and paclitaxel combined with an inhibitor of aryl-hydrocarbon receptor (AhR). AhR is a transcription factor involved in ROS detoxification and growth factor signaling and can cross-talk with the NF-κB pathway (38). AhR inhibition impaired BSO- and paclitaxel-regulated Vegfa as previously described (39, 40) but did not affect IκBα or Pdl1 increased levels (SI Appendix, Fig. S3C). SC514 also affected PD-L1 cell surface expression in CD206+ MHC-II+ BMDMs (Fig. 2E and SI Appendix, Fig. S3D). Our data showed a mode of regulation of PD-L1 by NF-κB via ROS. We found that ROS induced p65 phosphorylation at a level similar to LPS (Fig. 2A and B). PD-L1 has been previously identified to be regulated in BMDMs by LPS-induced NF-κB activation (41). By analyzing the same gene dataset, we confirmed that Pdl1 expression increased in LPS-treated BMDMs and positively correlated with Nfkb1/p65 and Rela/p50 mRNA levels (SI Appendix, Fig. S3E). Moreover, the link between NF-κB and mouse PD-L1 gene transcription was reinforced by the identification of a Nfkb1/p65 binding enhancer (I1551) through bioinformatics analysis of the inflammatory gene expression program in macrophages (42) (Fig. 2F). By chromatin immunoprecipitation followed by quantitative PCR (ChIP-qPCR) we found a significant enrichment of p65 at the I1551 site in the Pdl1 promoter at 1 h after paclitaxel treatment that was reverted by NAC (Fig. 2G). In the same conditions, p65 failed to bind the promoter of the NF-κB target, interleukin-6 (Il6), suggesting a specificity in gene transcription activation by NF-κB upon high ROS (SI Appendix, Fig. S3F).

Paclitaxel Promotes PD-L1 Expression in Tumor-Associated Macrophages in Vivo. Through bioinformatics analysis of The Cancer Genome Atlas (TCGA) human database of both basal BC and BC with homologous recombination DNA repair defects (HR-defective BC, see Materials and Methods for additional details), we found that cancer-associated PD-L1 positively correlated with an elevated infiltration of mononuclear cells (monocytes and macrophages) in the TME (SI Appendix, Fig. S4A) (43). To test if paclitaxel could induce PD-L1 expression in TAMs, we took advantage of a mouse mammary tumor cell line carrying BRCA1/Trp53 deletion and resembling human TNBC (K14cre BRCA1–/– p53−/−, hereafter referred to as KBP) (44). These cells form palpable tumors once transplanted in the mammary fat pad of immune-proficient female mice, allowing analysis of both tumor and immune cell populations in the TME, including TAMs. We administrated vehicle and paclitaxel i.v. at 20 mg/kg to mice bearing tumors at a palpable and measurable size (70 mm3). Tumors...
were harvested and dissociated for flow cytometry analysis both at 24 h and 5 d posttreatment. In CD206+/MHC-II(low) TAMs, PD-L1 surface expression did not change at 24 h posttreatment but showed a significant increase at 5 d after paclitaxel injection, even if we noticed a slight increase of PD-L1 in tumors from vehicle-treated mice, likely due to the tumor mass progression from 70 mm³ to about 200–300 mm³ (Fig. 3, A and D, and SI Appendix, Fig. S4B). Indeed, in vitro cocultured BMDMs with KBP cells displayed an increase in Pdil and Arg1 expression after being in contact with tumor cells (SI Appendix, Fig. S4 C and D). These results postulate that TAMs are targeted in situ by tumor cells to express Pdil during tumor progression. We found that circulating monocytes in tumor-bearing mice either untreated or paclitaxel treated expressed very low to undetectable levels of PD-L1 (Fig. 3B and SI Appendix, Fig. S4E).

Then, we asked the question if PD-L1 expression correlated with ROS levels in CD206⁺ TAMs as found in BMDMs. At 5 d posttreatment, we stained CD206⁺ TAMs for the presence of PD-L1 and during TAMs as found in BMDMs. At 5 d posttreatment, we stained CD206⁺ TAMs for DCF-DA to measure intracellular ROS. Strikingly, we observed an increased positivity for DCF-DA in the PD-L1⁺ macrophages, validating the link between cellular redox status and PD-L1 levels found in vitro (Fig. 3C). It is reported that paclitaxel treatment is also able to induce PD-L1 expression in tumor cells, including the TNBC cell line MDA-MB-231 and a panel of ovarian cell lines (45–47). Therefore, we investigated PD-L1 levels in CD45⁺CD49f⁻ KEB mammary tumor cells as we did in TAMs. We did not observe any increased PD-L1 positivity within mammary tumor epithelial cells, either 24 h or 5 d posttreatment (Fig. 3D and SI Appendix, Fig. S4F). In vitro treatment of the same KBP cells with increasing doses of paclitaxel induced a very marginal increase in PD-L1 surface expression after 24 h (SI Appendix, Fig. S4G). Consistent with the in vitro results from the BMDM cytokine array, TAMs from paclitaxel-treated tumors produced higher levels of IL-10 and IL-17 and lower amount of IL-12 (Fig. 3 E–G and SI Appendix, Fig. S4H).

To investigate the involvement of NF-κB in PD-L1⁺ TAMs in vivo, we analyzed CD206⁺ PD-L1⁺ TAMs for the presence of phosphorylated p65 in both paclitaxel- and vehicle-treated KEB allografts. At the 5-d time point, when PD-L1 surface expression was high, these macrophages also showed an increase in p65 phosphorylation, suggesting activation of the NF-κB pathway in the same cellular compartment (Fig. 3H and SI Appendix, Fig. S4I). Overall, both in vitro and in vivo data elucidate a link between paclitaxel, ROS accumulation, and NF-κB activation in macrophages. We corroborated this signaling pathway by bioinformatics analysis of TCGA basal and HR-defective BC cohorts. In these datasets, we looked for correlation between expression signature of human BC-infiltrating TAMs (48, 49), our key genes of interest (i.e., PD-L1 and NFKB1/p65) and a comprehensive ROS-induced gene signature (50). We investigated gene correlations in the expression profiling of both M1 and M2 compartments identified in the two published studies (48, 49). These studies elucidated that macrophages express M1- and M2-type gene modules simultaneously and M1 and M2 genes positively correlate in macrophages, contrary to models supporting mutually exclusive M1 and M2 subsets (49). We found that in both basal and HR-defective BC cohorts, M1 and M2 signatures positively correlated with expression of PD-L1, NFKB1/p65, and activation of the ROS signaling pathway (Fig. 3I and SI Appendix, Fig. S4J).

**PD-L1 Blockade Potentiates Antitumor Effects of Paclitaxel in Vivo.**

Several ongoing clinical trials in TNBC patients are currently exploring the effectiveness of combining paclitaxel treatment with...
with immune checkpoint inhibitors compared with single agent therapy (7, 51, 52). We hypothesized, based on our in vitro and in vivo observations, that inhibition of PD-L1 could revert the immune-suppressive and tumorigenic properties of TAMs to enhance the antitumor activity of paclitaxel. We first assessed if the use of anti–PD-L1 antibody (αPD-L1) could affect the viability of BMDMs whether alone or in combination with paclitaxel in vitro by SRB assay. Isotype-treated cells were included as control. We did not notice any difference in cell viability in both groups over a 5-d treatment (SI Appendix, Fig. S5 A and C). αPD-L1 also did not affect paclitaxel-induced Pdil mRNA increase (SI Appendix, Fig. SSD).

Then, we investigated if PD-L1 blockade could influence the production of cytokines in BMDMs. Interestingly, we found that PD-L1 inhibition restored all of the paclitaxel-induced cytokines to control levels (Fig. 4A). Vegfa mRNA levels were also negatively affected by αPD-L1/paclitaxel combination compared with isotype/paclitaxel (Fig. 4B)

These in vitro data prompted us to investigate the antitumor effect of αPD-L1 in the KBP mouse model in combination with paclitaxel. We administrated paclitaxel (intravenously) and αPD-L1 (intraperitoneally) either in combination or as single agents. First, mice were treated with paclitaxel (to induce PD-L1) and then with αPD-L1 as summarized in Fig. 4C. Since paclitaxel was administrated once a week, we performed an additional injection of αPD-L1 to guarantee PD-L1 blockade. Both compounds were administrated when mammary tumors reached a palpable and measurable volume (70 mm³). Tumor-bearing mice were monitored until they reached a humane endpoint (tumor volume ≤2 cm³). Combinatorial treatment of KBP mice with paclitaxel and αPD-L1 significantly reduced both tumor volume and weight compared with control mice (Fig. 4D and E). On the contrary, the administration of either paclitaxel or αPD-L1 as monotherapy did not show any effect (Fig. 4D and E). Haematoxylin and eosin (H&E) staining of KBP allografts showed that all treated tumors had a reduced mitotic index compared with tumors from the vehicle + isotype mouse group (Fig. 4F). We did not find any difference in blood vessel density based on immunohistochemical staining of the platelet endothelial cell adhesion molecule PECAM1/CD31 (SI Appendix, Fig. S5E). Compared with paclitaxel alone, cotreatment with PD-L1 blockade significantly impaired P-p65 signal and increased cleaved caspase 3 (Fig. 4G and H and SI Appendix, Fig. S5 F and G).

Paclitaxel Combined With PD-L1 Blockade Leads to Antitumor Immune Activation. To evaluate the immune response in the KBP allografts, we collected and analyzed tumors from all mouse groups at endpoint for immune cell infiltrates. We observed a moderate reduction in the percentage of F4/80⁺CD11b⁺ TAMs in the αPD-L1/paclitaxel group, probably due to reduced infiltration in the TME rather than increased TAM cell death (Fig. 5A). Then, we looked at the impact of the αPD-L1/paclitaxel regimen on different T cell populations. αPD-L1/paclitaxel-treated tumors restored the number of CD4⁺ T cells that were reduced by paclitaxel alone (SI Appendix, Fig. S6 A and B).

**Fig. 4.** PD-L1 blockade potentiates antitumor effects of paclitaxel in vivo. (A) Levels of indicated chemokines in BMDMs after treatment with vehicle, paclitaxel (100 nM) with αPD-L1 antibody (10 µg/mL), or isotype control (10 µg/mL) for 24 h. Values are the mean of biological duplicates and are represented as ratio to control cells treated with DMSO and isotype. (B) Vegfa mRNA levels in BMDMs treated as in A. (C) Schematic representation of paclitaxel and αPD-L1 treatment schedule for KBP tumor-bearing mice. Control group received vehicle and isotype. (D) Volume measurement of mammary tumors over time in mice transplanted with KBP cells and treated according to the regimen described in C. (E) Weight of tumors isolated at humane endpoint posttreatment (day 14). n = 10–15 per group. (F) Mitotic index by direct counting of mitotic cells in H&E-stained tumor sections. n = 5 per group. (G) Mean of P-p65 signal calculated as optical density by ImageJ assessed by immunohistochemistry of KBP tumors. n = 5 per group. (H) Quantification of cleaved caspase 3 staining performed by immunohistochemistry of KBP tumors. n = 10 per group. Data in B–H are presented as mean ± SEM of biological replicates. *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001.
population, we found a reduced percentage of immune-suppressive CD4+ regulatory cells (as defined by FoxP3 and CD25 marker staining) in the tumors isolated from the αPD-L1/paclitaxel-treated group. CD4+FoxP3+ T cells were dramatically affected by αPD-L1 alone as previously published (53) (SI Appendix, Fig. S6C). While paclitaxel alone did not affect the CD8+ population, these cells were moderately reduced in tumors from the αPD-L1/paclitaxel cohort (SI Appendix, Fig. S6D). To better characterize the phenotype of CD8+ T cells, we stained them for CD44/CD62L markers. αPD-L1/paclitaxel-treated tumors contained a higher percentage of CD8 effector (CD8+CD62L−) cells than all other tumors (Fig. 5 B and C and SI Appendix, Fig. S6E). In the same tumors, CD8+ T cells presented the highest expression of IFN-γ (IFN-γ*), granzyme-B (GrnzB), CD107a, and PD-1 as indicated in control or treated cohorts (n = 10–15 per group). Data in A–G are presented as mean ± SEM of biological replicates. *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001.

Discussion

The recent success of the PD-1/PD-L1 blockade has renewed interest in immunotherapies and in combining them with chemotherapy to achieve additive or synergistic clinical activity. Clinicians are currently exploring these combinatorial strategies for the treatment of TNBC, a very aggressive form of BC with poor prognosis. In TNBC, the expression of PD-L1 is almost undetectable in ductal carcinoma in situ (DCIS) tumor epithelial cells but increases to a higher extent in invasive ductal carcinoma (IDC) with the amplification of the CD274 locus encoding PD-L1 in about 30% of the cases (53). This important finding suggests that in TNBC, TME immune-suppressive functions progressively change during tumor evolution.

Together with anthracyclines, taxanes (including paclitaxel) are currently used as first-line therapy with variable success and frequent cases of relapse (1). The expression of PD-L1 in tumor and tumor-infiltrating cells in TNBC patients suggest that PD-L1 blockade may be a useful strategy to potentiate the antitumor effects of taxanes. Indeed, several clinical trials are currently exploring the combination of taxanes with PD-L1 inhibitors in TNBC (52). Very recently, the primary results of one of these trials, IMpassion130, a phase III trial of an anti-PD-L1 antibody, have been reported in patients with metastatic TNBC (54). Although the study has not reached statistical significance yet, numerical increases in median overall survival were clearly observed in both the randomized population and in the subgroup where PD-L1 expression was assessed in tumor-infiltrating cells (54). One of the most important findings of this work is that PD-L1 expression levels in the tumor-infiltrating cells should be taken into consideration to guide treatment strategies in TNBC. Our data reporting the regulation of PD-L1 expression by paclitaxel in macrophages and TAMs align with this result. Although PD-L1 expression has been generally considered to be induced at the transcriptional level after exposure to IFN-γ released by T effector cells (55), novel ways of transcriptional regulation of PD-L1 are emerging in both immune and tumor cells (19, 46). Here we have shown that in macrophages, PD-L1 levels respond to intracellular redox imbalances caused by both metabolic alterations such as deprivation of antioxidants and chemotherapy such as taxane. Overall, these data suggest a scenario where any intracellular or extracellular stresses affecting TME redox status can influence the communication between tumor cells and the surrounding immune system. In these settings, TAMs respond to chemotherapy-induced ROS by...
up-regulating PD-L1, releasing VEGF-A to promote angiogenesis and suppressing T cell-mediated antitumor response. This suggests that administration of immunetherapy could potentiate paclitaxel efficacy by interfering with the immunosuppressive abilities of macrophages established by paclitaxel itself. Indeed, combinatorial ap-DL1 and paclitaxel therapy promotes the antitumoral properties of the TME by significantly increasing the percentage of tumor-infiltrating effector and cytokotoxic CD8+ T cells. Given the broad expression of PD-L1 in the TME, PD-L1 blockade may affect a wide range of cells, including tumor cells, T and B cells, natural killer, dendritic cells, and macrophages (56). However, in our in vivo tumor model, ROS specifically increase PD-L1 in the macrophage compartment. Remarkably, it has been reported that TAMs interfere with the cytotoxic activity of paclitaxel and TAM depletion potentiates the antitumor effect of the paclitaxel (57–59). In conclusion, our work has revealed a unique scenario that further supports the combination of PD-L1 blockade with taxane for the treatment of TNBC patients.

Materials and Methods

Mice. KPB mice were provided by J. Jonkers, Netherlands Cancer Institute, Amsterdam, The Netherlands, and were on the FVB background. KPB tumor cells were obtained and used for in vivo transplantation studies as previously described. Human PBMCs were isolated from peripheral blood using lymphocyte separation medium (density 1.077 g/mL). Cells were washed twice with 1× PBS and resuspended in RPMI 1640 medium (Gibco) supplemented with 10% FBS, 2 mM glutamine, 100 units/mL penicillin, 100 μg/mL streptomycin, 0.05 mM β-mercaptoethanol, 0.2 μg/mL heparin (Sigma), and 20 units/mL DNase I (Sigma). Tumor cells were mechanically processed using a gentleMACS Octo dissociator with heaters (Miltenyi Biotec). Processed samples were filtered once through a 70-μm strainer (Falcon), followed by a 40-μm strainer (Falcon). Filtered samples were collected in 15-mL Falcon tubes and centrifuged at 278 × g for 8 min at 4 °C. Pellets were incubated with red blood cell lysis buffer for 7 min at room temperature (RT), and then centrifuged at 278 × g for 8 min at 4 °C before resuspension in 1× PBS containing 1% BSA plus 2 mM EDTA. Cell suspensions were subjected to fluorescence-activated cell sorting (FACS) flow cytometry as described below.

Flow Cytometry. For mouse tumor-associated macrophages and BMDMs, cell surface marker staining for flow cytometry analysis was performed using the following antibodies (Abs), all from BioLegend unless indicated: anti-CD49f (AF488 GoH3; 1/200), anti-CD45.1 (AF700 A20; 1/400), anti-CD11b (Pacific Blue M1/70; 1/400), anti-F4/80–PE (BM8; 1/400), anti-F4/80–PerC–Cy5.5 (BM1/70; 1/200), anti-MHC II (I–PE)–MS (51/14; 1/1600; Thermo Fisher Scientific), anti-CD206–AF647 (C068C2; 1/400), anti-CD1–PE–Cy7 (10F.9G2; 1/400), anti-CD4–PerC–Cy5.5 (RM4-5; 1/800), anti-CD25–PE (PC6; 1/200), anti-IFN–γ–APC (XM1G2; 1/200), anti-Granulocyte B–PE (GB11; 1/200), anti-TNF–α–PE–Cy7 (MP6–XT22; 1/800), anti-CD107a–BV421 (1D4B; 1/2000), anti-CD62L–PE–Cy7 (MEL-14; 1/800; Thermo Fisher Scientific), anti-CD4–APC–Cy7 (IM7; 1/200) and anti-CD1–PE (RMP1–30; 1/200; Thermo Fisher Scientific).

For IFN–γ and Granulocyte-μ B–staining, cells were stimulated with phorbol myristate acetate (PMA) (20 ng/mL; Sigma) and ionomycin (1 μg/mL; Sigma) in presence of the intracellular protein transport inhibitor Brefeldin A (eBioscience). Cells were harvested 5 h later and stained for surface markers as follows: Cells were washed twice with 1× PBS−/+, fixed, and permeabilized on ice for 30 min with the Intracellular Permeabilization kit (Thermo Fisher Scientific). After washing, permeabilization buffer, cells were stained for IFN–γ and Granulocyte-μ on ice for 30 min.

Mouse macrophages were identified as CD49f+CD45+CD11b+F4/80+ and mouse T cells as CD49f−CD45+CD11b−F4/80−. Mouse macrophages were cultured with Mouse BD Fc Block (anti-CD16/CD32 2.4G2; eBioscience) at 1/100 dilution for at least 10 min prior to staining with appropriate Abs. For human macrophages, cell surface marker staining was performed with PDL1 BV421 (29E.2A3; 1/400) and CD11b BV510 (ICRF44; 1/400), both from BioLegend.

Human macrophages were treated with 1× PBS−/+ containing 5% BSA and 2 mM EDTA for 30 min on ice prior to staining with Abs. For both mouse and human macrophages, Abs were prepared at the indicated dilutions in 1× PBS−/+ containing 1% BSA and 2 mM EDTA for 30 min on ice. Dead cells were excluded by adding 5 μL of 7-AAD (BioLegend) during the last 10 min of staining with the Abs. Cells were then washed twice and further analyzed.

Analysis of Peripheral Blood Monocytes. Peripheral blood monocytes were identified by flow cytometry of human monocytes (CD14+) gated on CD19−/− cells. Monocyte populations were analyzed by flow cytometry and used in all of the reported experiments.

KPB Mammary Tumor Induction and Treatment. KPB cells (3 × 105) were transplanted into no. 4 mammary fat pads of syngeneic female FVB recipient mice (10 wk old). Diameters of developing tumors were measured in duplicate using digital calipers starting on day 14 posttransplantation. Tumor volume (mm3) was calculated as 0.5 × Width × Height × Depth. Female inter- esters were measured, and volumes calculated as above, three times per week. For experiments with paclitaxel, anti–PD-L1 antibody (10F.9G2; BioXcell) or isotype control (LTF-2; BioXcell) antibodies, KPB transplanted female mice were monitored until tumors reached a volume of 70 mm3 and randomized. Mice were injected i.v. with paclitaxel (20 mg/kg; Medkoo) or vehicle (saline) once a week. When needed, the same mice were adminis- trated with anti–PD-L1 antibody (200 μg per mouse) or its isotype control (200 μg per mouse) twice a week intraperitoneally. Paclitaxel was purchased in a powder form and dissolved in a solution of ethanol/cremo- phor EL/1× PBS (1:1:18). Both anti–PD-L1 and isotype antibodies were diluted in appropriate dilution buffers that were provided by the manufacturer.

Mouse Mammary Tumor Dissociation. Tumors were resected from no. 4 mammary fat pads of transplanted mice, cut into 2- to 3-mm3 pieces, and placed into a 15-mL conical tube containing 5 mL ice-cold modified Dulbecco’s medium (IMDM) supplemented with 10% FBS, 2 mM l-glutamine, 100 units/mL penicillin, 100 μg/mL streptomycin, 0.05 mM β-mercaptoethanol, 0.26 units/mL Liberase TM (Sigma), and 20 units/mL DNase I (Sigma). Tumors were mechanically processed using a gentleMACS Octo dissociator with heaters (Miltenyi Biotec). Processed samples were filtered once through a 100-μm cell strainer (Falcon), and the corresponding C-tubes were rinsed with 5 mL cold IMDM and passed through the same strainer. Cells were filtered once using a 70-μm strainer (Falcon), followed by a 40-μm strainer (Falcon). Filtered samples were collected in 15-mL Falcon tubes and centrifuged at 278 × g for 8 min at 4 °C. Pellets were incubated with red blood cell lysis buffer for 7 min at room temperature (RT), and then centrifuged at 278 × g for 8 min at 4 °C before resuspension in 1× PBS−/+ containing 1% BSA plus 2 mM EDTA. Cell suspensions were subjected to fluorescence-activated cell sorting (FACS)flow cytometry as described below.

Analysis of Peripheral Blood Monocytes. Peripheral blood monocytes (15 μL) was first collected from mouse tail vein into heparinized capillary tubes and then transferred into a 5-mL polystyrene tube containing 100 μL 1× PBS−/+ plus 20 mM EDTA. After blocking with anti-Cd16/CD32 Abs (1:100) for 10 min, samples were stained with anti–CD11b–Pacific Blue and anti–PD-L1–PE–Cy7 as described above. Samples were washed twice with 1× PBS−/+ containing 1% BSA and 2 mM EDTA. Red blood cells were lysed at room temperature for 10 min with 1 mL of FixLyse solution (Thermo Fisher Scientific). Cells were washed twice with 1× PBS−/+ and analyzed with a Fortessa instrument (BD Biosciences). Data were processed with FlowJo (Tree Star, Inc.) and FlowJo software.

Phospho-p65 Staining by Flow Cytometry. Tumors were dissociated according to a mouse mammary tumor dissociation method. The 106 cells were suspended in 0.5 mL of 1× PBS and immediately fixed with 0.5 mL of 4% formaldehyde (final concentration 2%) at 37 °C for 10 min. Cells were

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Roux et al.
Phospho-p65 Staining by Immunofluorescence. For analysis of p65 S536 phosphorylation, 2 × 10⁶ BMDMs were seeded in a 12-well plate containing glass coverslips. The day after, cells were treated with BSO (200 μM) and paclitaxel (100 nM) in the presence or absence of SC514 (50 μM) for 3 h. Cell treatment with LPS at 1 μg/mL for 30 min was included as positive control of p65 S536 phosphorylation. After treatment, cells were fixed with 4% PFA for 15 min at 37 °C. Cells were then stained overnight at 4 °C with primary antibody (93H1, 1:1,600; Cell Signaling) or isotype control (rabbit IgG, 1:1,600; Thermo Fisher Scientific) diluted on ice for 1 h. For 1 h after two washes, cells were resuspended in 100 μL of secondary antibody (goat anti-rabbit APC conjugated; Thermo Fisher Scientific) diluted 1/500 and incubated on ice for 1 h. Cells were then washed twice and analyzed with a Fortessa instrument (BD Bioscience) and data were processed with FlowJo software (Tree Star, Inc.).

Cell Lines and Treatments. Mouse K8P cells were cultured in DMEM/F12 medium containing 10% FBS (Thermo Fisher Scientific), 1-glutamine, 1 μg/mL hydrocortisone (Sigma), 5 μg/mL insulin (Sigma), 5 ng/mL epidermal growth factor (EGF) (Sigma) and Pen-Strep (Thermo Fisher Scientific). Mouse macrophages were cultured in DMEM supplemented with 10% FBS (Thermo Fisher Scientific), 1-glutamine, and Pen-Strep (Thermo Fisher Scientific). The glutathione synthesis inhibitor, BSO (Sigma) was used at 200 μM (BMDMs) or 1 mM (human macrophages) for specific periods of time as indicated above. Chemotherapeutic drugs were dissolved in DMSO (Sigma) and reconstituted with DAPI (360 μM; Pacific Science), 0.5 μM (olaparib, Medko), and 2 μM (ispilatin, Medko). Anti-PD-L1 mouse or isotype control antibodies were both used at 10 μg/mL (BioXCell). For ROS scavenging, BSO- or paclitaxel-exposed cells were cotreated with 1 mM NAC (Sigma). The NF-κB antagonist ST514 (Sigma) was applied to cells at 50 μM. The AhR inhibitor, CH-223191 (Cell Signaling) was used at 1 μM for 3 h. The day before, cells were treated with BSO (200 μM) and paclitaxel (100 nM) in the presence or absence of SC514 (50 μM) for 3 h. Cell treatment with LPS at 1 μg/mL for 30 min was included as positive control of p65 S536 phosphorylation. After treatment, cells were fixed with 4% PFA for 15 min at 37 °C. Cells were then stained overnight at 4 °C with primary antibody (93H1, 1:1,600; Cell Signaling). The following day, cells were washed three times and subjected to FITC-conjugated secondary antibody (A-11008; Thermo Fisher Scientific) at 1:1,000 for 2 h at RT. Nuclei were counterstained with DAPI (360 μM; Pacific Science) to quantify P-p65 nuclear fluorescence intensity, we randomly selected 100 nuclei for each sample and analyzed them with ImageJ software as follows. First, we applied an Otsu threshold to the DAPI channel to generate a mask marking the nuclear area. Then, with the tracing tool, we transposed each mask to the FITC-positive channel to calculate the mean intensity in the nuclear region.

RT-PCR. RNA was isolated using the Nucleospin RNA Plus kit (Macherey-Nagel) and reverse transcribed using the Script cDNA Synthesis Kit (Bio-Rad) according to the manufacturer's instructions. Quantitative RT-PCR was performed using SYBR Green primers (Applied Biosystems). Mouse and human ribosomal proteins S9 (rps9) were used as housekeeping genes to calculate relative mRNA expression. All mouse and human primer sequences are described in SI Appendix, Table 51.

Statistical Analyses. Data are reported in bar graphs as the mean or median ± SEM, with P values calculated using Student's t test (P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001).
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