Differential effects of PD-L1 versus PD-1 blockade on myeloid inflammation in human cancer

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Introduction

Antibody-mediated blockade of PD-1 or PD-L1 has led to tumor regression and improved survival in a subset of patients with diverse tumor types (1). PD-L1–expressing tumor cells and antigen-presenting cells (APCs) engage PD-1+ T cells, leading to T cell dysfunction. In view of the dominant role of T cells in tumor immunity, blockade of PD-1 or PD-L1 has been studied interchangeably in clinical cancer immunotherapy.
as a strategy to activate T cells. However, both molecules have alternate ligands/receptors; it has also been suggested that PD-L1 can act as a receptor to back-transmit signals into T cells (2) and tumor cells (3). PD-L1 is constitutively expressed on a subset of myeloid APCs, including DCs, and prior studies in murine models have suggested a functional role for PD-L1 in myeloid cells or DCs (4–6). Direct comparison of signaling pathways altered in vivo following PD-1 or PD-L1 blockade in T cells and APCs in humans are limited and may help optimal design of combination therapies with these antibodies (Figure 1A). While the PD-L1 axis has been extensively studied in the context of immunotherapy of established cancer, data about the effects of PD-L1 blockade on premalignant states are limited.

Multiple myeloma (MM) is a common hematologic malignancy, which is preceded in all cases by well-defined precursor states, monoclonal gammopathy of undetermined significance (MGUS), and asymptomatic MM (AMM) (7). In spite of major therapeutic advances, there is an unmet need to achieve durable unmaintained responses in this malignancy, prompting the need to pursue strategies to engage long-term immunologic memory against tumor cells. Antibody-mediated blockade of PD-L1 as a single agent did not lead to tumor regression in relapsed MM (8). Prior studies have demonstrated immune recognition of preneoplastic MGUS cells by T cells (9, 10). In a prospective trial, the presence of preexisting T cell immunity to an embryonal stem cell antigen SOX2 was associated with reduced risk of progression to clinical MM (11). MM tumor cells commonly express PD-L1, and the expression of PD-L1 on MGUS/AMM cells correlated with an increased risk of transformation to clinical malignancy (11, 12). These considerations prompted us to initiate a clinical trial of single-agent anti–PD-L1 antibody (atezolizumab) in patients with AMM (Figure 1B).

Results

Prior studies have shown that therapy with anti–CTLA-4, anti–PD-1, or combination leads to distinct genomic signatures in purified human T cells and monocytes in vivo (13). In order to compare the genomic and proteomic profiles of anti–PD-1 and anti–PD-L1 therapies, we isolated T cells and CD14+ monocytes from peripheral blood before and after anti–PD-L1 therapy in patients with advanced non–small cell lung cancer and analyzed changes in gene expression using Affymetrix HTA v2.0 array (Figure 1A). In direct contrast to prior studies with anti–PD-1 therapy, which predominantly leads to gene expression changes in T cells (13), anti–PD-L1 therapy led to dominant gene expression changes in CD14+ monocytes (Figure 2A). Importantly, changes in gene expression following anti–PD-L1 therapy in both T cells and monocytes were nonoverlapping with those observed following anti–PD-1 therapy (Figure 2A). Top differentially expressed genes (DEG) in myeloid cells following PD-L1 blockade included inflammation-associated genes such as heparin-binding EGF-like growth factor (HBEGF), thrombospondin-1 (THBS), IL-1β, CXCL1/GROα, CXCL2, and NLRP3 (Figure 2B). Pathway analysis of DEGs (q < 0.01) in monocytes revealed pathways related to inflammation and inflammasome-associated cytokines (IL-1 and IL-18) (Supplemental Table 1; supplemental material available online with this article; https://doi.org/10.1172/jci.insight.129353DS1). In order to further validate these data in the context of samples analyzed together and determine if these signals were derived from only a subset of monocytes, we analyzed purified monocytes from patients before and after anti–PD-1/PD-L1 therapy using single cell RNA sequencing (RNA-seq). These data demonstrate that early changes in myeloid cells were again more prominent following PD-L1 blockade (Figure 2C) and involved nearly all classical monocytes (Figure 2D). Changes in gene expression in these monocytes were similar to those in earlier studies (Supplemental Figure 1A) and also revealed pathways consistent with myeloid activation (Supplemental Figure 1B). Analysis of sera before and after therapy demonstrated that, while both therapies led to an increase in IP-10 as a marker of immune activation, increases in serum IL-18, GROα, IFN-α2 typically derived from myeloid cells and sCD40L, are only observed following anti–PD-L1 therapy (Figure 3, A–E). Taken together, these data demonstrate that systemic immunologic changes following anti–PD-L1 therapy are surprisingly distinct from that following anti–PD-1 therapy, both at genomic and proteomic levels — in particular, with rapid activation of inflammation-associated genes in monocytes.

Expression of PD-L1 was previously correlated with the risk of progression to MM (11). In order to evaluate the potential of targeting the PD-L1 axis to prevent MM, we enrolled AMM patients in a pilot trial of single-agent atezolizumab. The trial was closed prematurely based on FDA guidance after enrollment of only 2 patients due to safety concerns emerging in 2 clinical trials of pembrolizumab and lenalidomide in MM. Clinical data from these patients are summarized in Supplemental Table 2. Both patients had stable
disease at the time of study closure, after receiving 7 and 1 cycles, respectively, and remain progression-free off therapy with 23- and 18-month follow-up. Patient 1 developed grade 2 endocrinopathy with hypothyroidism and adrenal insufficiency after 7 cycles. Incidentally, this patient also experienced remission of prior gluten intolerance after enrolling in the study. Serial analysis of peripheral blood samples by mass cytometry revealed an early increase in blood monocytes and a decline in B cells in both patients, detected at 15 days after initiation of therapy (cycle 1 day 15; C1D15) (Figure 4A and Supplemental Figure 2A). Phenotypic analysis revealed an increase in CD16+CD40+HLADRhi monocytes (Figure 4B). In order to validate these findings in an independent data set, we analyzed early changes in blood monocytes from another clinical trial in MM (NCT02431208), wherein a cohort received single-agent atezolizumab. These data also corroborate our prior studies and demonstrate a similar pattern of rapid increase in circulating activated monocytes in vivo (Figure 4C). Taken together, data from both lung cancer and MM patients show that PD-L1 blockade leads to early activation of myeloid cells with a transient increase in activated circulating monocytes in vivo.

Evaluation of T cells in AMM patients treated with atezolizumab revealed an early increase in circulating CD8+ and CD4+ memory T cells detectable by C1D15 (Figure 5A and Supplemental Figure 2B). Single cell mass cytometry revealed proliferation of CD8+ and CD4+ effector memory (Tem) compartment, as well CD8+ central memory (Tcm) compartment, manifest as upregulation of Ki-67 (Figure 5B). In prior studies, we have shown that SOX2 is a common antigenic target of T cells in MGUS (10). Evaluation of antigen-specific T cells at C1D15 also revealed an increase in antigen-reactive IP10
production following stimulation with SOX2-peptide library (Figure 5C). However, therapy-induced changes in circulating T cells were transient and returned to baseline by cycle 2. Although the number of total B cells declined, therapy was also associated with an increase in the CD21<sup>+</sup> B cell subset implicated in autoimmunity (ref. 14 and data not shown). Analysis of serum cytokines also revealed early but transient changes in inflammatory cytokines (IL-18, IP-10, GRO<sub>α</sub>, and TNF-α), which returned closer to baseline by cycle 2 (Supplemental Figure 3). Together, these data show that atezolizumab leads to rapid but only transient systemic immune activation in vivo in AMM patients.

Evaluation of posttreatment BM specimens was planned after the completion of 2 cycles and, therefore, was obtained in only 1 patient. Posttreatment BM revealed a decline in T and B cells but clear increase in the proportion of CD14<sup>+</sup> myeloid cells (Figure 6, A and B), which also exhibited some evidence of activation manifest with upregulation of HLA-DR (Figure 6C). Although reduced in number, BM memory T cells from posttreatment biopsies did demonstrate an increase in granzyme and T-bet relative to baseline samples, particularly within the Tem subset (Figure 6D).
In order to understand the observed changes in immune cells in further detail, we analyzed single cell transcriptomes of circulating as well as BM mononuclear cells. In the BM, single cell RNA-seq (sc-RNA–seq) identified 6 major T/NK cell clusters (Figure 7, A and B). Of these, the proportion of T cell clusters 0 and 3 declined in the posttreatment biopsy, consistent with reduction in T cells detected by mass cytometry. In contrast, there was an increase in the proportion of cells in several myeloid clusters, including classical CD14+ myeloid cells (clusters 5), CD16+ myeloid cells (cluster 7), and DCs (cluster 8). Pathway analysis of DEGs in these clusters demonstrated an increase in TNF-α signaling and IFN-α response in myeloid cells, as well as other cell types, consistent with evidence of inflammatory signaling in posttreatment BM (Supplemental Table 3). sc-RNA–seq analysis of paired blood samples from baseline and C1D15 from both patients also demonstrated systemic changes in gene expression, particularly in cluster 2 (myeloid cells) and cluster 5 (B cells), consistent with prior results using mass cytometry (Supplemental Figure 4 and Supplemental Table 4). Pathway analysis revealed an enrichment of IFN response and inflammation-associated pathways after therapy in several major circulating cell types (T cells, B cells, monocytes, and NK cells), consistent with systemic immune activation and changes in serum cytokines at this time point (Supplemental Table 4).

Among myeloid cells, DCs constitutively express high levels of PD-L1. Prior studies have mostly focused on effects of PD-L1 blockade in the context of DC–T cell interactions (1). In order to test if PD-L1 may directly impact the biology of human monocyte-derived DCs (Mo-DCs) independent of DC–T cell interactions, we cultured purified Mo-DCs with anti–PD-L1 antibodies. Culture of Mo-DCs with anti–PD-L1, but not anti–PD-1, led to modest increases in CD80 and CD83 as markers of DC maturation (Figure 8A). This was associated with an increase in the secretion of several inflammatory cytokines — notably, IL-6, IL-8, TNF-α, and IL-1β — in the culture supernatants (Figure 8B), as well as rapid (within 4 hours) activation of caspase-1 (Figure 8C) and changes in cellular energetics associated with DC maturation, manifest as an increase in spare respiratory capacity (Figure 8D). In the setting of DC–T cell interaction, CD40L-mediated licensing of DCs is a critical regulator of antigen presentation (15).
Therefore, we examined the impact of PD-L1 blockade on DC maturation following suboptimal concentration of CD40L. PD-L1 blockade led to an increase in CD40L-driven DC maturation, as detected by the expression of CD80 and CD83 (Figure 9, A and B), but also greater expansion of influenza-matrix peptide–specific (Flu-MP–specific) T cells by Flu-MP–loaded DCs (Figure 9C). Expression of PD-L1 in human Mo-DCs can vary in a donor-dependent fashion. Expression of PD-L1 on DCs correlated with the observed synergy for DC maturation with CD40L and atezolizumab (Supplemental Figure 5A). In order to further evaluate the effects of PD-L1 blockade on naturally occurring BM myeloid cells, we cultured these cells with atezolizumab. Consistent with our in vivo data, atezolizumab also led to an increase in CD16+HLADR+CD14+ BM myeloid cells in culture (Supplemental Figure 5B).

Discussion

Together, these data demonstrate that PD-L1 blockade leads to a distinct genomic signature characterized by early activation and expansion of myeloid compartment in vivo. Therefore, while both PD-1 and PD-L1 blockade share well-studied effects in terms of reinvigoration of T cells, PD-L1 blockade also unleashes an underappreciated myeloid inflammatory checkpoint in vivo in humans. These findings are also consistent with recent data on PD-L1–mediated regulation of macrophage activation and proliferation in PD-L1–deficient mice (16).

Understanding differences between PD-1 and PD-L1 blockade will be essential for optimal design of rational combination therapies with these approaches and may differ for each of these targets. Differential effects of PD-1 versus PD-L1 blockade on myeloid cells in vivo may also help explain why PD-L1 expression on myeloid cells better predicts responsiveness to PD-L1 than PD-1 blockade in the clinic (17). PD-L1 blockade of human DCs led to rapid activation of caspase-1/inflammasome, with upregulation of NLRP3 and inflammasome-dependent cytokines such as IL-18. Inflammasome activation plays a complex and context-dependent protumor/antitumor role in tumor immunity (18). Activation of NLRP3 inflammasome in DCs was shown to be critical for induction of adaptive immunity to dying tumor cells following...
chemotherapy (19). Therefore, PD-L1 may play an important role in the afferent arm of tumor-immunity cycle in regulating antigen presentation. The finding that PD-L1 blockade may enhance CD40L/T cell–mediated DC maturation may provide the rationale for combinations of PD-L1 blockade with agents targeting agonistic CD40 signaling.

Effect of PD-L1 blockade on myeloid cells in vivo could, however, also have potential undesired effects. Enrichment and activation of myeloid cells following PD-L1 blockade may lead to T cell exclusion and resistance to PD-L1 blockade in myeloid-rich tumors (20). Effects on myeloid cells could also have contributed to the lack of persistent T cell activation following atezolizumab that we observed in AMM patients, and this suggests that combinations with therapies that inhibit enrichment of myeloid compartment may be explored to improve PD-L1 blockade. Recent studies have also suggested the potential for myeloid cells to mediate hyperprogression in some tumors (21); prior studies have, indeed, shown the capacity of myeloid cells to promote MM growth (22, 23).

While the small number of patients treated due to regulatory issues limits interpretation, the correlative immunologic data in this earlier stage do demonstrate the feasibility to achieve immune activation in the tumor bed. The bar for acceptable complications is lower in this setting than in clinical myeloma. Therefore, careful selection of patients more likely to respond to immune therapies would be important for future investigations in immune-prevention based on checkpoint blockade. In this regard, recent studies show that loss of stem-like and marrow-resident T cells is an early feature of MM, which may restrict the efficacy of checkpoint blockade in this setting (24). It is, however, notable that the finding of increase in inflammasome-dependent cytokines such as IL-18 has been prominently demonstrated in large cohorts of patients treated with anti–PD-L1 but not anti–PD-1 antibodies, which is consistent with our data (17).
An important limitation of these data is the small number of patients studied, due to early closure of the trial linked to regulatory concerns about PD-1 blockade in a different myeloma trial. In addition, comparison between effects of PD-1 and PD-L1 blockade is based on patients treated in different clinical studies and not as a part of a prospective randomized clinical trial directly comparing PD-1 and PD-L1 blockade. Such a clinical trial may now be feasible in malignancies wherein both PD-1 and PD-L1 blockade are now clinically approved and would be useful to dissect biologic differences between these therapies in humans.

The finding that PD-L1 blockade leads to rapid activation of inflammatory signatures on human myeloid cells in vivo suggests that the PD-L1 axis may be an important regulator of myeloid inflammation and impact emergency myelopoiesis and trained immunity in the clinic. While studies in human subjects described here are mostly correlative, a possible role of PD-L1 axis in regulating myeloid inflammation is also supported by emerging data from murine models, which is consistent with our studies. Engaging these pathways may be important for improving combination therapies with PD-L1 blockade, particularly harnessing the afferent arm of the cancer immunity cycle. Finally, differences in pharmacodynamic effects of PD-1 and PD-L1 blockade, as shown here, also have important implications for optimal combinations in the clinic, which may differ between these targets.

**Methods**

**Patients and samples.** For studies comparing genomic signatures of PD-1 and PD-L1 blockade, blood samples were obtained before and after 1 cycle of therapy from patients with advanced non–small cell lung cancer undergoing therapy with anti–PD-1 (nivolumab) (13) or anti–PD-L1 (atezolizumab).

**Study design and monitoring.** In the pilot study of atezolizumab in AMM (NCT02788843), patients were eligible if they met criteria for AMM based on BM clonal plasmacytosis of > 10% and/or levels...
of monoclonal immunoglobulin > 3 g/dL. Patients were also required to have an abnormal serum free light chain ratio (but < 100) and absence of end organ damage based on CRAB criteria (hypercalcemia, renal dysfunction, anemia, bone disease), < 60% BM plasma cells, and no more than 1 known focal lesion on MRI. Other key eligibility criteria included the presence of measurable disease and adequate hematologic and organ function. Patients with any prior therapy for plasma cell disorder and history of active autoimmune disease were excluded. All eligible patients received atezolizumab 1200 mg i.v. every 3 weeks. Blood samples for immune monitoring were collected at baseline, C1D15, and then before each cycle of therapy while in the study. BM biopsies were planned at baseline and after completion of 2 cycles of therapy.

Gene expression profiling of purified T cells and monocytes. Gene expression profiling of purified monocytes and T cells was performed as previously described (13). Briefly, CD14+ monocytes cells were isolated from PBMCs using immunomagnetic separation with anti–human CD14 microbeads (Miltenyi Biotec, 130-050-21), and T cells were isolated with human Pan–T cell isolation kit (Miltenyi Biotec, 130-096-535) following manufacturer instructions. RNA isolated from purified cells was analyzed using Affymetrix GeneChip Human Transcriptome Array (v2.0) as described (13).

Mass cytometry. Peripheral blood and BM mononuclear cells were immunophenotypically characterized using mass cytometry as described (25). The panel of antibodies used is shown in Supplemental Table 5. Data were acquired on Helios instrument (Fluidigm Sciences Inc.) and analyzed using Cytobank software (Cytobank Inc., Fluidigm).

sc-RNA–seq. sc-RNA–seq of peripheral blood or BM mononuclear cells was performed using the 10× Genomics platform chromium single cell 3’ kit following manufacturer’s protocol as described (14, 26). Libraries were sequenced. Reads were aligned, filtered, deduplicated, and converted into a digital count...
matrix using Cell Ranger 1.2 (10× Genomics). All downstream quality control and analyses were performed using Seurat (27). Cells with ≥ 200 expressed genes were used for analysis.

For analysis of sc-RNA–seq from lung cancer patients treated with either anti–PD-1 or PD-L1, pre- and posttreatment samples for each patient were merged, and gene expression for each cell was log-normalized to total expression per cell. To reduce noise due to batch effects and interpatient heterogeneity, each patient was aligned and integrated with all other patients receiving the same treatment (anti–PD-L1, n = 3; anti–PD-1, n = 4) via canonical correlation analysis (CCA) using the Seurat FindIntegrationAnchors and IntegrateData functions. Gene expression data were then scaled such that each gene had a mean expression of 0 and a variance of 1 across all cells using the ScaleData function, and principal component analysis was performed using the RunPCA function. Data were visualized in 2
dimensions using uniform manifold approximation and projection (UMAP) based on the first 20 principal components. Significant DEGs were identified by the Wilcoxon rank-sum test with a Bonferroni’s correction ($P < 0.05$). Cluster identity was determined by inspection of canonical marker genes (e.g., CD14 for monocytes), and identity was confirmed by automated cell type determination with SingleR (https://bioconductor.org/packages/release/bioc/html/SingleR.html) via comparison with the Human Primary Cell Atlas (http://biogps.org/dataset/BDS_00013/primary-cell-atlas). Pathway analysis was performed using Gene Set Enrichment Analysis (GSEA) software (https://www.gsea-msigdb.org/gsea/index.jsp) and the Molecular Signature Database (MSigDB; https://www.gsea-msigdb.org/gsea/msigdb/index.jsp) from the Broad Institute.

Whole transcriptome analysis. Gene expression profiles from monocytes and T cells before and after anti–PD-1 and anti–PD-L1 treatment were obtained using Affymetrix Human Transcriptome Array 2.0, and gene-level signal intensities were used for subsequent analysis. Preprocessing and normalization of data sets were carried out by Affymetrix Expression Console using gene level Signal Space Transformation–Robust Multiarray Average (SST-RMA) normalization. All downstream analyses were conducted using R and Bioconductor (28). The “limma” package was used for differential gene expression (29). GSEA was performed using Metacore (https://portal.genego.com) and MSigDB (Molecular Signatures Database v6).

Detection of antigen-specific T cells. In order to detect SOX2-specific T cells, PBMCs were stimulated for 48 hours with SOX2 peptide library as described (10). Following stimulation, the presence of T cell activation was determined based on the detection of IP-10 in the culture supernatant by Luminex.

Detection of plasma cytokines. Plasma samples were used for the detection of a panel of 38 cytokines/chemokines using the Milliplex MAP Human Cytokine/Chemokine Magnetic Bead Panel kit.
Generation of Mo-DCs. Purified CD14+ monocytes were cultured in 1% plasma in the presence of IL-4 (25 ng/mL; R&D Systems) and GM-CSF (20 ng/mL sargramostim [Leukine]; Genzyme) to yield Mo-DCs. Immature Mo-DCs were used to study the effects of anti–PD-L1 or anti–PD-1 antibodies. DCs were cultured with anti–PD-L1 (clone 29E.2A3; BioLegend), anti–PD-1 (clone EH12.2H7; BioLegend) or their respective isotype control antibodies (IgG2b and IgG1; BioLegend) (200 μg/mL). For some experiments, immature Mo-DCs were cultured with CD40L (250 ng/mL; R&D Systems).

Effects of PD-L1 blockade on BM myeloid cells. Bone marrow mononuclear cells (BMMNCs) were treated with atezolizumab (200 μg/mL) every 24 hours for 48 hours or left untreated. Following incubation, samples were stained with antibodies for CD14 (MoP9), CD19 (SJ25C1), and CD11c (B-ly6) (BD Biosciences); BDCA3 (AD5-14H12, Miltenyi Biotec); and PD-L1 (29E.2A3), CD40 (5C3), CD16 (3G8), and HLA-DR (L243) (BioLegend).

Antigen-specific T cell stimulation. For some experiments, immature Mo-DCs differentiated from HLA A2.1+ donors (n = 4) were stimulated with CD40L (250 ng/mL) in the presence of anti–PD-L1 (200 μg/mL). After overnight culture, DCs were loaded with HLA A2.1–restricted Flu-MP (sequence GILGFVFTL) at 0.1 μg/mL for 2 hours. Flu-MP–loaded DCs were then used to stimulate autologous T cells at a DC/T cell ratio 1:30 in the presence of IL-2 (10 U/mL). Flow cytometry analysis was performed to detect the presence of Flu-MP–specific CD8+ T cells using MHC tetramers (MBL International).

Detection of caspase-1 activation. Immature Mo-DCs were treated with anti–PD-L1 (200 μg/mL), anti–PD-1 (200 μg/mL), or left untreated for 4 hours. Activation of caspase-1 was assayed with the FAM-YVAD-FMK Caspase-1 Detection Kit (Cell Technology Inc). FAM-YVAD-FMK was added to the culture 1 hour before the end of culture period, following manufacturer protocol; washed twice with Caspase-1 kit wash buffer; and detected using flow cytometry.

Measurement of oxygen consumption and spare respiratory capacity. Basal, maximal, and coupled oxygen consumption rates were measured in a Mito stress assay using a Seahorse extracellular flux (XFe96) analyzer. Immature Mo-DCs were treated with anti–PD-L1 (200 μg/mL) or left untreated. After 3 hours, DCs were harvested, washed 1× with PBS, and plated at 200,000 cells per well in 5–8 replicates on Cell-Tak– precoated (Becton Dickinson) 96-well plates custom designed for XFe96 analysis. Oxygen consumption rate was evaluated over time with sequential injection of oligomycin (MilliporeSigma, catalog 495455; final concentration 2.5 μM), carbonyl cyanide p-trifluoro-methoxy-phenyl hydrazone (FCCP; Enzo Life Sciences, BML-CM120-0010; final concentration 0.5 μM), and antimycin (Atr; MilliporeSigma, A8674) or rotenone (Rot; MilliporeSigma, R8875; final concentration 2 μM each). Spare respiratory capacity was calculated as the difference between maximal and basal respiration.

Statistics. Data from individual cohorts were compared using GraphPad analysis software. Paired 2-tailed t tests and nonparametric tests were used to analyze the data with significance set to P < 0.05 and Bonferroni’s to correct for multiple comparisons.

Study approval. The clinical trial was approved by the Yale University IRB and monitored by data safety monitoring committee at Yale Cancer Center. All specimens were collected following informed consent under institutional IRB guidelines at Emory and Yale universities.

Author contributions
NB supervised the clinical trial. FC, RD, and AD designed and performed experiments. M. Samur and SM performed bioinformatic analysis. SNG, NN, TLP, MLX, TA, NG, AN, and HJC performed clinical research. JKB, KP, RB, LZ, AR, and M. Shanmugam performed some experiments. MVD and KMD designed and performed oversight to entire project. All authors performed data analysis and approved the final manuscript.

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