Blockade of PD-L1, an immune checkpoint molecule, in a murine model of melanoma

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Highlights

Injection of anti-PD-L1 antibody in a mouse melanoma model:

• Increases levels of activated CD4+ splenic T cells.
• Stimulates production of Th cytokines and suppresses pro-inflammatory cytokines.
• Increases antigen-specific T cells and reduces tumor growth.
Summary

Immune checkpoint molecules help to regulate and limit the immune response. As tumor cells are able to exploit these mechanisms, new immunotherapy treatments target immune checkpoints. We used a monoclonal antibody to block PD-L1 binding in a murine model of melanoma and characterized T cell subsets and cytokine profiles. We found that treatment with an anti-PD-L1 antibody resulted in activation of T cells, increased production of Th cytokines and chemokines, increased levels of tumor-specific T cells and caused a reduction in tumor growth.

Introduction

Cancer treatment is classically composed of a combination of surgery, radiation, and chemotherapy. More recently, immunotherapy-based strategies have emerged as another treatment option. Immunotherapy relies on using the body's own immune system to recognize and mount an attack against cancer cells. Immunotherapy can be broadly categorized into cell-based therapies in which cells are engineered \textit{ex vivo} before being reintroduced into the patient and soluble factor-based therapies in which monoclonal antibodies or other proteins are introduced into the patient to mount an anti-tumor response.

One approach to developing immunotherapy-based treatments is through the inhibition of immune checkpoint molecules (reviewed in 1). Immune checkpoints maintain immune homeostasis and limit the immune response, in part, to prevent autoimmunity. Tumor cells are able to exploit this system to prevent an anti-tumor response by expressing checkpoint markers typically found on antigen presenting cells (APCs). Because immune checkpoint molecules are often composed of receptors and ligands, they are able to be targeted using monoclonal antibodies. Antibodies that can block ligand-receptor binding allow T cells to remain in an activated state to mount an immune response against the tumor cells.

One such immune checkpoint molecule that has been studied for use in immunotherapies is the PD-1 receptor and one of its ligands, PD-L1 \textsuperscript{2}. Engagement of PD-L1 and PD-1 initiates downstream signaling pathways ultimately leading to T cell death\textsuperscript{3}. As melanoma cells show elevated expression of PD-L1, the PD-L1 blockade may be particularly useful in the treatment of melanoma\textsuperscript{4,5}. In this report, we use a GoInVivo\textsuperscript{\textregistered} anti-mouse PD-L1 antibody in a murine model of melanoma and characterize the T cells in the tumor microenvironment and draining lymph nodes, study the antigen-specific T cell response, and phenotype the cytokine profile in the serum.
Materials and Methods

In vivo injection of PD-L1 Antibody

BALB/cJ or C57BL/6 mice were injected with \(10^6\) B16F10 melanoma cells in the flank. Mice were subsequently treated with either 100 or 200 µg of GoInVivo™ anti-PD-L1 antibody clone 10F.9G2 (Cat. No. 124328) or Isotype control (Cat. No. 400666) every 3 days for a total of 3 doses. Dosing schedule was performed as indicated in the figure legend. Tissue samples and serum were collected 14 or 24 days following initial dose. Spleen samples were collected and made into a single cell suspension for flow staining.

Flow cytometry staining

Cells were stained with anti-CD25 (Cat. No. 102033), CD69 (Cat. No. 135209), CD152 (CTLA-4) (Cat. No. 106305), CD279 (PD-1) (Cat. No. 135209), CD278 (ICOS) (Cat. No. 313531), I-A/I-E (MHC II) (Cat. No. 107605), CD11c (Cat. No. 117317), CD80 (Cat. No. 104729), CD86 (Cat. No. 105011), MHC pentamer (ProImmune), and 7-AAD (Cat. No. 420403). Cells were acquired with an LSR Fortessa™ (BD Biosciences) and analyzed using FlowJo software.

Microscopy

Draining lymph nodes were isolated. Tissues were sectioned in a microtome cryostat at 7 micrometers after freezing in OCT media. Sections were stained with anti-CD4 (Cat. No. 100425), CD8 (Cat. No. 100727), CD11c (Cat. No. 117346), and B220 (Cat. No. 103251), without fixation or antigen retrieval. Images were taken on an Olympus IX83 inverted microscope.

Cytokine and chemokine measurement

Cytokine and chemokine profiles were characterized using BioLegend’s LEGENDplex™ system (Cat. Nos. 740740, 740134, 740007). A detailed protocol has been published. For more information regarding LEGENDplex™, please visit: biolegend.com/legendplex

All reagents are from BioLegend, unless otherwise noted.
Results

Effective blocking of PD-L1 using a monoclonal antibody in a melanoma model

To examine the efficacy of a PD-L1 blocking antibody in the treatment of a mouse cancer model, melanoma, we injected mice with $10^6$ B16F10 melanoma cells in the flank. Mice were subsequently treated with a GoInVivo™ anti-PD-L1 blocking antibody every three days (or as indicated). Following treatment, tissues and serum were collected for immunophenotyping (Figure 1).

Anti-PD-L1 treatment increases T cell activation status

Splenic CD4$^+$ cells were harvested 14 days after treatment with anti-PD-L1 antibody or the corresponding isotype control and stained for common T cell activation markers. Mice that had been treated with anti-PD-L1 showed an activated phenotype as measured by increased levels of CD25, CD69, ICOS, and CD279 (PD-1) as compared to mice treated with an isotype control (Figure 2). Importantly, CTLA-4, another immune checkpoint molecule which negatively regulates the immune response, was expressed at low levels and remained unchanged in mice that had been injected with anti-PD-L1.

![Figure 1: Experimental layout of treatment of anti-PD-L1 antibody treatment in a melanoma model.](image1.png)

![Figure 2: Splenic CD4$^+$ T cells treated with anti-PD-L1 (200 µg by I.V. injection on days 0, 3, 6) demonstrated an activated phenotype. Cells were collected 14 days following implantation.](image2.png)
Splenic Dendritic Cells Downregulate CD80 following PD-L1 treatment

Next, we examined splenic dendritic cells to determine whether the increase in activated T cells correlated with alterations in the population of APCs. For these experiments, we first gated on classical dendritic cells that are MHC II high and CD11c high. Dendritic cells (DCs) from animals that had been treated with the anti-PD-L1 antibody showed lower expression of CD80 (Figure 3). We hypothesize that downregulation of CD80 may occur to allow for CD86-CD28 interaction and less binding to CTLA-4.

Anti-PD-L1 treatment results in elevated numbers of CD8⁺ DCs in draining lymph node

To further characterize the APCs, we examined the draining lymph nodes from animals that had been injected with anti-PD-L1 and isotype controls. In animals that received the anti-PD-L1 treatment, there is an increased level of CD8a⁺ dendritic cells in draining lymph nodes. CD8a⁺ dendritic cells are APCs that have the ability to cross-present antigens to CD8⁺ T cells, which is a common phenomenon during tumor elimination.⁷

Figure 3: Splenic DCs show decreased levels of CD80 (top). Cells were first gated on classical DCs (MHC II high, CD11c high). Increased levels of CD8⁺ DCs in the draining lymph node of animals treated with anti-PD-L1 (bottom). Tissue was stained with CD4 (green), CD8 (red), CD11c (cyan), and B220 (blue).
Anti-PD-L1 treatment in melanoma models alters the cytokine and chemokine profile in serum

To more completely analyze the immune response following anti-PD-L1 treatment, we used our LEGENDplex™ multiplex platform to analyze the chemokine and cytokine profile in serum (Figure 4). Notably, we found that animals treated with anti-PD-L1 had increased content of cytokines typically associated with a Th1 response, including IFN-γ and TNF-α. Additionally, there was an increase in cytokines associated with Th9 and Th22 phenotypes, IL-9 and IL-22, respectively. However, this did not correspond with an overall increase in soluble mediators, as they simultaneously limited the production of other pro-inflammatory cytokines including IL-12p70, IL-27, IL-33, and GM-CSF, indicating that the cytokine response in these animals was specific. Lastly, anti-PD-L1 treatment resulted in the elevated production of important chemokines including MIP-3α, Eotaxin, MIG, and IP-10. These chemokines may play a role in anti-tumor immunity by mobilizing other cells.

Figure 4: Cytokine profile in the serum from treated mice shows elevation of Th cytokines and chemokines. Animals treated with PD-L1 also showed a simultaneous decrease of other pro-inflammatory cytokines.
Increase in antigen-specific CD8+ tumor infiltrating lymphocytes (TIL) following anti-PD-L1 treatment

To examine antigen-specific T cells during tumor development, we implanted a B16 melanoma cell line that expresses the peptide SIYRYYGL, which is derived from the Nucleoprotein of Vesicular stomatitis virus (498-505) and examined the tumor 24 days after implantation and antibody treatment. Using an MHC pentamer loaded with the SIY peptide, we were able to track CD8+ TILs. As expected, animals that received the anti-PD-L1 treatment had expanded pools of antigen-specific TILs as compared to those that received the isotype control (Figure 5). Finally, as a confirmation of other published models, treatment with an anti-PD-L1 antibody significantly reduced the tumor growth, as measured by tumor area, in this melanoma model.

Figure 5: Animals treated with anti-PD-L1 (100 µg I.P. injection on days 7, 13, 16 following implantation of melanoma cell line) show increased antigen-specific TILs and a reduction in total tumor area.
Conclusions

Immune checkpoint molecules make an attractive target for anti-cancer therapy, as inhibition of these pathways results in an elevated immune response, and they often rely on a ligand-receptor interaction. Blockade of these pathways can often be easily performed using a bioactive antibody, like our GoInVivo™ functional antibodies. As melanoma cells express PD-L1 to help evade an anti-tumor response, we sought to understand the specific cellular phenotype of the PD-L1 blockade in a mouse melanoma model. As expected, the blockade of PD-L1 resulted in elevated activation of T cells as measured by expression of common T cell activation markers, including CD25 and CD69. Interestingly, this activation phenotype corresponds with a downregulation of CD80, another T cell co-stimulatory molecule, on APCs. We hypothesize that this downregulation may occur to alter the interaction of other immune checkpoint molecules, including reducing binding to CTLA-4 and to allow for more interaction between CD86 and CD28. Using our LEGENDplex™ multiplex platform, we surveyed the cytokine and chemokine response in the serum following anti-PD-L1 treatment. Interestingly, anti-PD-L1 treatment increased certain cytokines associated with a Th response; however, there was not an overall increase in all soluble mediators, as the treatment also caused simultaneous downregulation of other pro-inflammatory cytokines. Importantly, treatment with anti-PD-L1 resulted in increased levels of CD8+ tumor-specific T cells which is also correlated with a reduction in tumor size. Collectively, these results describe the cellular response to the blockade of PD-L1 in a mouse melanoma model and suggest that anti-PD-L1 monoclonal antibodies may provide an effective treatment for melanoma.

References: