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Journal

Oncolmunology, 5(2)

ISSN

2162-4011

Authors

Mall, Christine
Sckisel, Gail D
Proia, David A
[et al.](#)

Publication Date

2016-02-01

DOI

10.1080/2162402x.2015.1075114

Peer reviewed


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To cite this article: Christine Mall, Gail D. Sckisel, David A. Proia, Annie Mirsoian, Steven K. Grossenbacher, Chien-Chun Steven Pai, Mingyi Chen, Arta M. Monjazeb, Karen Kelly, Bruce R. Blazar & William J. Murphy (2016) Repeated PD-1/PD-L1 monoclonal antibody administration induces fatal xenogeneic hypersensitivity reactions in a murine model of breast cancer, *OncImmunity*, 5:2, e1075114, DOI: [10.1080/2162402X.2015.1075114](https://doi.org/10.1080/2162402X.2015.1075114)



To link to this article: <http://dx.doi.org/10.1080/2162402X.2015.1075114>

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 Accepted author version posted online: 12 Aug 2015.
Published online: 12 Aug 2015.

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ORIGINAL RESEARCH

Repeated PD-1/PD-L1 monoclonal antibody administration induces fatal xenogeneic hypersensitivity reactions in a murine model of breast cancer

Christine Mall^a, Gail D. Sckisel^a, David A. Proia^b, Annie Mirsoian^a, Steven K. Grossenbacher^a, Chien-Chun Steven Pai^a, Mingyi Chen^c, Arta M. Monjazeb^d, Karen Kelly^f, Bruce R. Blazar^e, and William J. Murphy^{a,f}

^aDepartment of Dermatology, School of Medicine, University of California, Davis, Sacramento, CA, USA; ^bSynta Pharmaceuticals Corp, Lexington, MA, USA; ^cDepartment of Pathology, School of Medicine, University of California, Davis, Sacramento, CA, USA; ^dDepartment of Radiation Oncology, School of Medicine, University of California, Davis, Sacramento, CA, USA; ^eDepartment of Pediatrics, Division of Blood and Marrow Transplantation, University of Minnesota Masonic Cancer Center, Minneapolis, MN, USA; ^fDepartment of Internal Medicine, School of Medicine, University of California, Davis, Sacramento, CA, USA

ABSTRACT

Monoclonal antibodies (mAbs) targeting coinhibitory molecules such as PD-1, PD-L1 and CTLA-4 are increasingly used as targets of therapeutic intervention against cancer. While these targets have led to a critical paradigm shift in treatments for cancer, these approaches are also plagued with limitations owing to cancer immune evasion mechanisms and adverse toxicities associated with continuous treatment. It has been difficult to reproduce and develop interventions to these limitations preclinically due to poor reagent efficacy and reagent xenogeneity not seen in human trials. In this study, we investigated adverse effects of repeated administration of PD-1 and PD-L1 mAbs in the murine 4T1 mammary carcinoma model. We observed rapid and fatal hypersensitivity reactions in tumor bearing mice within 30–60 min after 4–5 administrations of PD-L1 or PD-1 mAb but not CTLA-4 antibody treatment. These events occurred only in mice bearing the highly inflammatory 4T1 tumor and did not occur in mice bearing non-inflammatory tumors. We observed that mortality was associated with systemic accumulation of IgG1 antibodies, antibodies specific to the PD-1 mAb, and accumulation of Gr-1^{high} neutrophils in lungs which have been implicated in the IgG mediated pathway of anaphylaxis. Anti-PD-1 associated toxicities were alleviated when PD-1 blockade was combined with the therapeutic HSP90 inhibitor, ganetespib, which impaired immune responses toward the xenogeneic PD-1 mAb. This study highlights a previously uncharacterized fatal hypersensitivity exacerbated by the PD-1/PD-L1 axis in the broadly used 4T1 tumor model as well as an interesting relationship between this particular class of checkpoint blockade and tumor-dependent immunomodulation.

ARTICLE HISTORY

Received 14 May 2015
Revised 14 July 2015
Accepted 15 July 2015

KEYWORDS

Anaphylaxis; breast cancer; checkpoint blockade; hypersensitivity; PD-1; PD-L1; 4T1; neutrophils

Introduction

Cancer immunotherapy utilizing checkpoint inhibitors was recently recognized as breakthrough of the year for its role in the paradigm shift of cancer therapies.¹ While checkpoints play a role in maintaining self-tolerance, they are often utilized by tumors to modulate the host immune system and develop immune evasion and resistance.² The cytotoxic T lymphocyte protein-4 (CTLA-4), expressed on activated T cells, antagonizes their activation. Programmed cell death-1 (PD-1) is another checkpoint also expressed on activated T cells. PD-1 engages its ligand, programmed death ligand 1 (PD-L1), which is expressed on immune and non-immune cell types contributing to T cell anergy or apoptosis.² The therapeutic targeting of these inhibitory immune checkpoints is seen with successful antitumor efficacy in clinical trials across multiple cancer types.^{3–5} Due to their clinical success as monotherapies, many recent investigations have broadened to elucidate the mechanisms of immune checkpoints within different tumor

microenvironments as well as to utilize them as targets in combinational approaches with neoadjuvant and adjuvant therapies for enhanced therapeutic benefits.^{6–8}

Despite clinical successes, however, checkpoint blockade is often associated with toxicities referred to as immune related adverse events (irAEs). These can include colitis, dermatitis, pneumonitis and hepatitis. Approximately 25 to 30 percent of patients treated with anti-CTLA-4 and 10 to 20 percent of patients treated with anti-PD-1 or anti-PD-L1 have been reported to experience these toxicities limiting the application and efficacy of these treatments.^{3,5} To date, the etiology of these toxicities remains unclear. More importantly, there remains significant shortcomings in preclinical models used to investigate antitumor efficacy and associated toxicities of immune checkpoint blockade therapies, thus leaving irAEs as a poorly represented area in preclinical studies.^{9,10} Aside from differences in antitumor efficacy, preclinical tumor models utilizing PD-1/PD-L1 blockade have not reported irAEs as reported in

human trials.¹¹ Furthermore, preclinical investigations mostly utilize xenogeneic mAbs which can often lead to the generation of neutralizing mouse anti-xenogen antibodies able to provoke hypersensitivity reactions.¹² Reactions associated with these immunogenic mAb therapies can include anaphylaxis, serum sickness or cytokine release syndrome.¹³ In humans these are referred to as human anti monoclonal antibody (HAMA) responses.¹⁴ Due to these associated immunogenic responses, human trials have advanced to utilize fully human or humanized chimeric mAbs which is the case for all current FDA approved checkpoint inhibitors.¹⁵ In preclinical murine models, however, the continuous use of immunogenic xeno-mAbs may lead to immune responses causing limitations in the assessment of optimal dosing, timing and repeated administration of treatments.

In addition to the use of xenogeneic mAb treatments in preclinical settings, poor animal modeling relative to checkpoint blockade has also limited antitumor efficacy to combinational strategies rather the monotherapeutic efficacy seen clinically.^{16,17} PD-1 function and expression changes in mice have been shown to vary with age and strain. In C57BL/6 mice, PD-1 expression is increased in aged versus young mice.¹⁸ In Balb/c mice, deficiency in PD-1 is associated with autoantibodies leading to spontaneous cardiomyopathy.¹⁹ Furthermore, tumor models for checkpoint blockade also increase variability by inherent heterogeneous tumor expression of PD-L1 previously reported to correlate with clinical efficacy.¹¹ Tumor microenvironment constituents may also develop checkpoint blockade resistance as reported previously by others.^{8,20,21}

Here, we show that repeated intraperitoneal administration of mAbs targeting the PD-1/PD-L1 axis induces fatal hypersensitivity reactions specifically in the orthotopic 4T1 murine mammary carcinoma model which has previously been shown to express PD-L1 *in vivo*.²² We observed >85% mortality in tumor bearing mice receiving anti-PD-1 or anti-PD-L1 treatments upon repeated dosing. This mortality was not seen in non-tumor bearing mice, mice bearing tumors other than 4T1 and 4T1 tumor bearing mice treated with isotype controls or anti-CTLA-4. We show that fatal hypersensitivity reactions are associated with an accumulation of neutrophils within the lungs of anaphylactic mice as well as an increase in treatment specific-IgG1 antibodies in serum. Treatment of mice with anti-Gr-1 (neutrophil depletion) further corroborated the role of neutrophils by increasing survival of anti-PD-1 treated mice. Together, this suggests that cellular constituents of the highly myeloproliferative 4T1 model prime hypersensitivity reactions which may be exacerbated by antibodies targeting the PD-1/PD-L1 axis. Furthermore, we show that the accumulation of neutrophils and IgG1 antibodies by anti-PD-1 treatment was prevented by administration of therapeutic ganetespib, an inhibitor of the molecular chaperone heat shock protein 90 (HSP90), which was recently shown to suppress neutrophil migration into lungs.²³ Our findings caution the use xenogeneic antibodies in preclinical studies of checkpoint blockade immunotherapies and indicate a possible relationship between hypersensitivity reactions and the PD-1/PD-L1 axis, particularly in those cancers that have a strong myeloproliferative component.

Results

Repeated dosing of anti-PD-1 and anti-PD-L1 results in fatal hypersensitivity reactions specifically in 4T1 tumor bearing mice

Given its clinical success in melanoma and non-small cell lung cancer and to pursue combinational regimens, we sought to determine whether checkpoint blockade had efficacy in a murine breast cancer model. We utilized the 4T1 murine mammary carcinoma model, a widely used non-transgenic breast cancer model, which metastasizes to lungs, liver, brain and bone similar to human breast cancer.²⁴⁻²⁷ We inoculated Balb/c mice orthotopically with 4T1 tumor cells and compared mice treated with anti-PD-1 (clone J43; hamster IgG) to other available checkpoint blockade antibodies such as anti-CTLA-4 (clone UC-10; hamster IgG) or anti-PD-L1 (clone 10F.9G2; Rat IgG). Polyclonal hamster IgG and monoclonal rat IgG were used as isotype controls for the hamster anti murine PD-1 and CTLA-4 antibodies and the rat anti murine PD-L1 antibody. Schemas depicting dosing and timing of antibody administration can be found in Fig. S1A-B. Expression of PD-L1 on 4T1 cells and *ex-vivo* tumor was confirmed by flow cytometry (Fig. S1C-D). Briefly, anti-PD-1, anti-CTLA-4 and hamster IgG were given intraperitoneally (IP) at a bolus of 500 μ g starting on day 14 post tumor inoculation followed by 250 μ g every other day. Anti-PD-L1 and rat IgG were given at a dose of 200 μ g IP every 3 d starting on day 13 post tumor inoculation. Overall, we observed that 90% (9/10) of mice treated with anti-PD-1 succumbed to mortality compared to 8% (1/12) of mice receiving hamster isotype control (Fig. 1A, Table 1). Affected mice were observed to reach fatality within 30 min of a fifth or sixth dose of anti-PD-1 (Fig. 1B). Remaining mice which did not reach fatality within an hour reached tumor endpoint-related survival. Mice treated with anti-CTLA-4, however, tolerated therapy at equivalent doses and reached tumor endpoint related survival. Surprisingly, 86% (12/14) of mice treated with anti-PD-L1 also suffered similar fatal hypersensitivity reactions within 30–60 min of a fourth dose (Fig. 1C and D, Table 1). We observed 21% (3/14) of mice treated with the rat isotype to also have fatal hypersensitivity reactions. Additionally, following the final dose of anti-PD-1 or anti-PD-L1, mice were noted to become immobile in a prostrate position. Further subjective analyses included footpad swelling, cutaneous cyanosis of tail and other extremities, mild piloerection, periorbital puffiness and dyspnea. These symptoms exhibited by mice closely resembled those well documented to be associated with hypersensitivity reactions.²⁸⁻³⁰ No treatment as a monotherapy had any significant effect on tumor growth or tumor endpoint-related survival (Fig. 1A and C, Fig. S2A-B). Taken together, these results suggested that these events were not specific to checkpoint blockade in general but possibly exacerbated by the PD-1/PD-L1 axis.

Although PD-1 and PD-L1 blockade have been administered extensively in preclinical settings of both tumor and infectious models of disease, there have been no reports showing monotherapy efficacy or hypersensitivity in the 4T1 model with either PD-1 or PD-L1 blockade.³¹ Furthermore, prior investigations have either used different clones with fewer repeated dosing regimens or different antibody clones.³² To

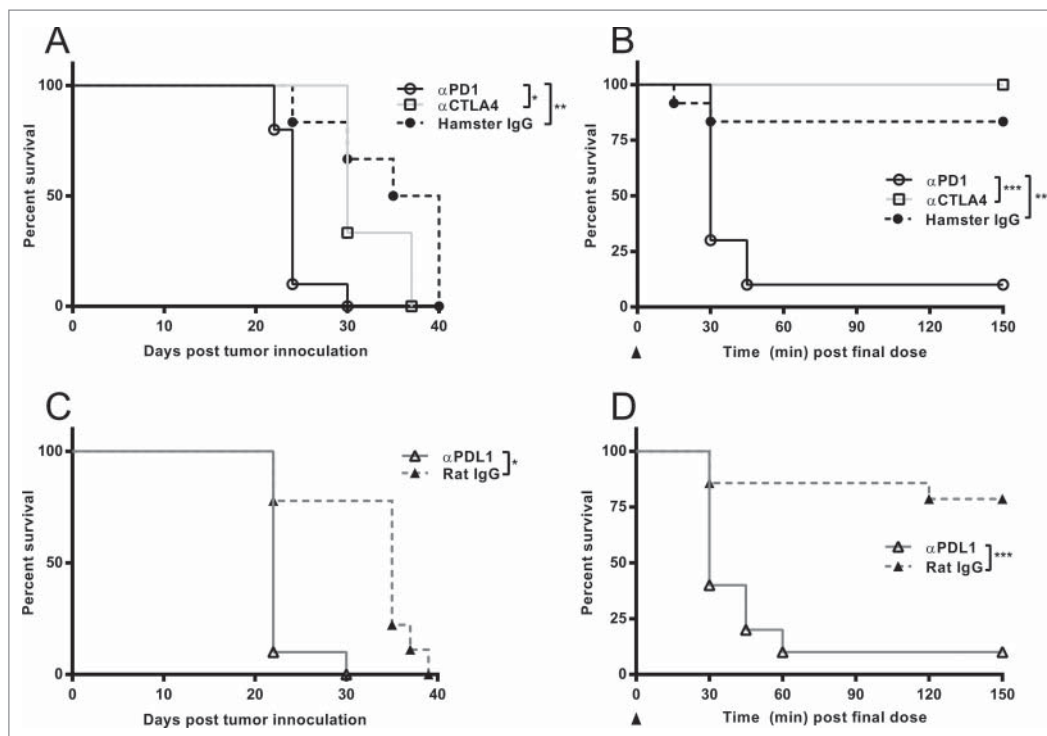


Figure 1. Repeated dosing of PD-1 and PD-L1 monoclonal antibodies induces fatal hypersensitivity reactions in 4T1 tumor bearing mice. (A) Survival of Balb/c mice bearing 4T1 tumors post repeated dosing with anti-PD-1 (5–6 doses) or anti-CTLA-4. (B) Anti-PD-1 and anti-CTLA-4 treated mice succumbed to fatal hypersensitivity within the first 30–60 min post the final 5th or 6th dose of anti-PD-1 ($n = 6–8$ mice per group). (C) Survival of Balb/c mice bearing 4T1 tumors post repeated dosing with anti-PD-L1 (4 doses). (D) Anti-PD-L1 treated mice reached mortality within an hour of the final 4th dose ($n = 6–10$ mice per group). Survival analysis was plotted according to the Kaplan–Meier method, and statistical differences were determined with the log-rank test. **** $P < 0.0001$, *** $P < 0.001$, ** $P < 0.01$ and * $P < 0.05$. Percent mortality of non-tumor bearing and Balb/c 4T1 breast, Balb/c Renca renal cell, and C57BL/6 B16 melanoma tumor bearing mice receiving repeat doses of anti-PD-1, anti-PDL1, anti-CTLA4 or isotype controls. ($n = 4$ or more mice per group. Hypersensitivity experiments were repeated and pooled).

determine whether the observed hypersensitivity reactions were 4T1 tumor specific or a general phenomenon associated with the PD-1/PD-L1 axis, we studied similar regimens of repeated dosing of anti-PD-1 or anti-PD-L1 in non-tumor bearing mice (Balb/c and C57BL/6 strains), Renca tumor bearing Balb/c mice and B16 tumor bearing C57BL/6 mice. Interestingly, we did not observe mortality or hypersensitivity in any of these models using the same dosing and timing regimen as depicted for 4T1 tumor bearing mice (Table 1, Fig. S2C–D). In some cases, extra repeated doses were given without any fatal hypersensitivity noted (data not shown). Taken together, these data suggest that hypersensitivity reactions exacerbated by PD-1/PD-L1 blockade were specific to the 4T1 mammary carcinoma model.

Fatal hypersensitivity reactions correlated with neutrophilic accumulation in lungs following PD-1/PD-L1 blockade

Due to the rapid onset of mortality within 30 to 60 min of respective final doses of antibody treatments and due to reactions seen in some mice treated with isotype controls, we suspected that mortality was likely due to a hypersensitivity reaction to the xenogeneic nature of the antibody treatments. Hypersensitivity reactions to immunogenic xeno-proteins are well characterized.³³ These reactions can lead to mortality by increased vascular permeability, respiratory arrest due to

smooth muscle contraction, multi organ failure or cardiac arrest due to decreased cardiac output.³⁴

To better understand the underlying mechanism of toxicity in mice treated with repeat anti-PD-1 treatment, heart, lungs, liver and kidney tissues were examined by a trained pathologist

Table 1. Anti-PD-1 and anti-PD-L1 induced fatal hypersensitivity reactions are specific to 4T1 tumor bearing mice. Percent mortality of non-tumor bearing and Balb/c 4T1 breast, Balb/c Renca renal cell, and C57BL/6 B16 melanoma tumor bearing mice receiving repeat doses of anti-PD-1, anti-PDL1, anti-CTLA4 or isotype controls. ($n = 4$ or more mice per group. Hypersensitivity experiments were repeated and pooled).

4T1 tumor	Treatment [‡]	Survival [†]	% Mortality
No tumor (Balb/c)	α PD1	8/8	0%
	α CTLA4	8/8	0%
	α PDL1	8/8	0%
4T1	α PD1	1/10	90%
	α CTLA4	8/8	0%
	Hamster IgG	11/12	8%
	α PDL1	2/14	86%
Renca	Rat IgG	11/14	21%
	α PDL1	5/5	0%
No tumor (C57BL/6)	Rat IgG	4/4	0%
	α PDL1	4/4	0%
B16	α PDL1	5/5	0%
	Rat IgG	5/5	0%

[†] Survival based on time of death (within one hour of last dose of mAb treatment) of one or more experiments combined.

[‡] PD1 (J43) and CTLA4 (UC-10) were given at a bolus of 500 μ g/mouse followed by 250 μ g/mouse and PDL1 (10F.9G2) was given at 200 μ g/mouse. Isotype control treatments were matched.

(M.C.) for evidence of hypersensitivity responses. In addition to the increased neutrophils previously shown to accumulate in lungs of 4T1 mice³⁵, the lungs of anti-PD-1 treated mice showed marked leukostasis with interstitial and alveolar neutrophilic accumulation and bronchiolitis pneumonia (Fig. 2A). Livers of anti-PD-1 treated mice showed mixed inflammatory infiltrates, granulomatous changes and geographic necrosis (Fig. 2B). These pathological changes were not seen in mice treated with isotype control or anti-CTLA-4. Interestingly, we observed similar lung and liver tissue pathology in anti-PD-L1 treated mice but not in rat IgG isotype control treated mice (Fig. 2C-D). It should be noted, however, that liver necrosis and granulomatous changes were much more significant in anti-PD-L1 treated groups than in anti-PD-1 treated group. Heart and kidney tissues showed no significant findings (data not shown). Since mAb treatments have been associated with cytokine storms or the release of proinflammatory chemokines which can lead to the recruitment of neutrophils, macrophages and lymphocytes we considered whether the observed neutrophil accumulation was due to an increase in systemic proinflammatory signals.³⁶ Thus, we analyzed sera of tumor bearing

mice. Briefly, levels of inflammatory cytokines IL-6 and TNF α in sera of 4T1 bearing mice treated with anti-PD-1 or isotype control were detected by cytokine bead array. Interestingly, we did not observe variations in levels of these inflammatory cytokines (Fig. S3A-B). Together, these results in addition to mice reaching fatality within 30–60 min. of a final dose further implicated a hypersensitivity or anaphylactic response in 4T1 tumor bearing mice exacerbated by the PD-1/PD-L1 axis.

Antibody mediated fatal hypersensitivity reactions correlated with systemic IgG1 accumulation

Anaphylaxis has been shown to occur through two distinct pathways in mice and humans. Both species include the traditional IgE pathway. In mice, a second pathway can induce systemic hypersensitivity through an IgG1 pathway recognizing antigen-IgG1 complexes.³⁷ This pathway is associated with the cross-linking of IgG1 with Fc γ RIII or Fc γ RIV, which are both Fc receptors expressed on neutrophils and basophils.³⁸ Due to the accumulation of neutrophils observed in the histology, we hypothesized that hypersensitivity reactions to the PD-1/PD-

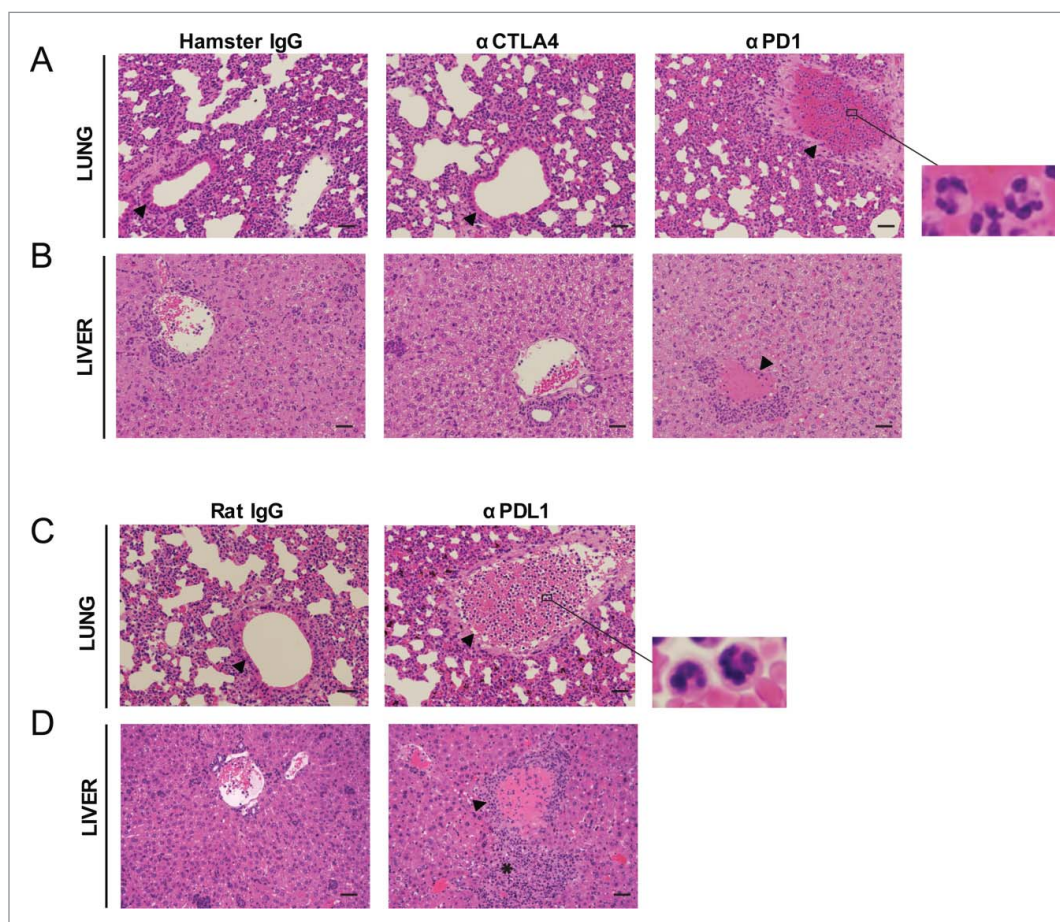


Figure 2. Fatal hypersensitivity correlated with leukostasis in lungs following blockade of PD-1/PD-L1 in 4T1 bearing mice (A) Representative H&E stains of lungs (A, C) and liver (B, D) of mice treated with anti-CTLA-4, anti-PD-1 (A, B) or anti-PD-L1 (C, D) antibodies. (A, C) Lung tissues show leukostasis with neutrophilic accumulation (arrowhead), alveolar filling with neutrophilic abscess and bronchiolitis pneumonia seen only in anti-PD-1 and anti-PD-L1 treated mice but not in anti-CTLA4 or isotype control treated mice receiving similar dosing (200X). (B) Liver tissues show tumor metastasis associated with mixed inflammatory infiltrate and granulomatous changes and geographic necrosis (arrowhead) in anti-PD-1 treated mice but not in anti-CTLA4 or isotype control mice receiving similar dosing (200X). (D) Representative H&E stain of liver tissues from anti-PD-L1 and isotype control treated mice show pathology consistent with anti-PD-1 treated mice. Asterisk indicates granuloma with histiocytic proliferation and eosinophilic infiltrates (200X). Bars represent 100 μ m.

L1 axis were mediated by the IgG1 pathway. Therefore, we collected sera from mice following the last injection and assayed for IgG1 titers by an enzyme-linked immunosorbent assay (ELISA). ELISA analysis of sera detected significantly increased levels of IgG1 in anti-PD-1 and anti-PD-L1 treated mice (Fig. 3A-B). Next, we sought to determine whether there was an increase in anti-PD-1 specific IgG1 antibodies. An ELISA for anti-PD-1 specific IgG1 showed that IgG1 specific to anti-PD-1 was detectable only in mice receiving anti-PD-1 therapy (Fig. 3C). In order to rule out an IgE response, we assayed sera for IgE titers. Interestingly, while there were no increases in IgE levels in affected mice treated with anti-PD-1 or anti-PD-L1, IgE levels in isotype control treated mice either trended toward an increase (hamster IgG) or significantly increased (rat IgG) (Fig. S4A-B). Together these findings suggest that while hypersensitivity reactions occurred in mice receiving PD-1/PD-L1 mAbs or isotype controls, the PD-1/PD-L1 axis specifically led to an IgG1 mediated anaphylaxis.

Increase in PMN cells in lungs of mice treated with anti-PD-1 and anti-PD-L1

As mentioned, IgG1 mediated anaphylaxis has been reported to correlate with an increase in neutrophils.³⁴ Additionally,

neutrophils have been identified as key players in mediating anaphylactic reactions in mice and humans.³⁸ To further evaluate the cause of death in mice treated with PD-1/PD-L1 blockade, we compared lungs and spleens of mice treated with anti-PD-1, anti-CTLA4 or isotype control antibody, both in non-tumor bearing and 4T1 bearing mice, for overall neutrophil and T cell content in the lungs. When observing CD11b+Gr-1+ dot plots, there was an overall 27% increase in Gr-1^{hi} cells noted in the lungs of affected mice (Fig. 4A). These granulocytic or polymorphonuclear (PMN) leukocytes are typically suggested as a more mature neutrophil phenotype as opposed to the less mature myeloid derived suppressor cell (MDSC) phenotype also associated with the 4T1 model.^{39,40} Interestingly, when quantified, there was an overall 2–3-fold increase in total PMN CD11b+Gr-1^{high} (Gr-1^{hi}) cell population in lungs of mice treated with anti-PD-1 antibody (Fig. 4B). We also observed a significant increase in CD4⁺ and CD8⁺ T cell counts in lungs of these mice (Fig. 4C-D). Differences in total Gr-1^{hi} cells, CD4⁺ or CD8⁺ T cells were not detected in the spleens of affected mice (data not shown). Similar changes in CD11b+Gr-1^{hi} cells were also noted in anti-PD-L1 treated mice (data not shown). All together, these data suggest an important role of neutrophil accumulation in the

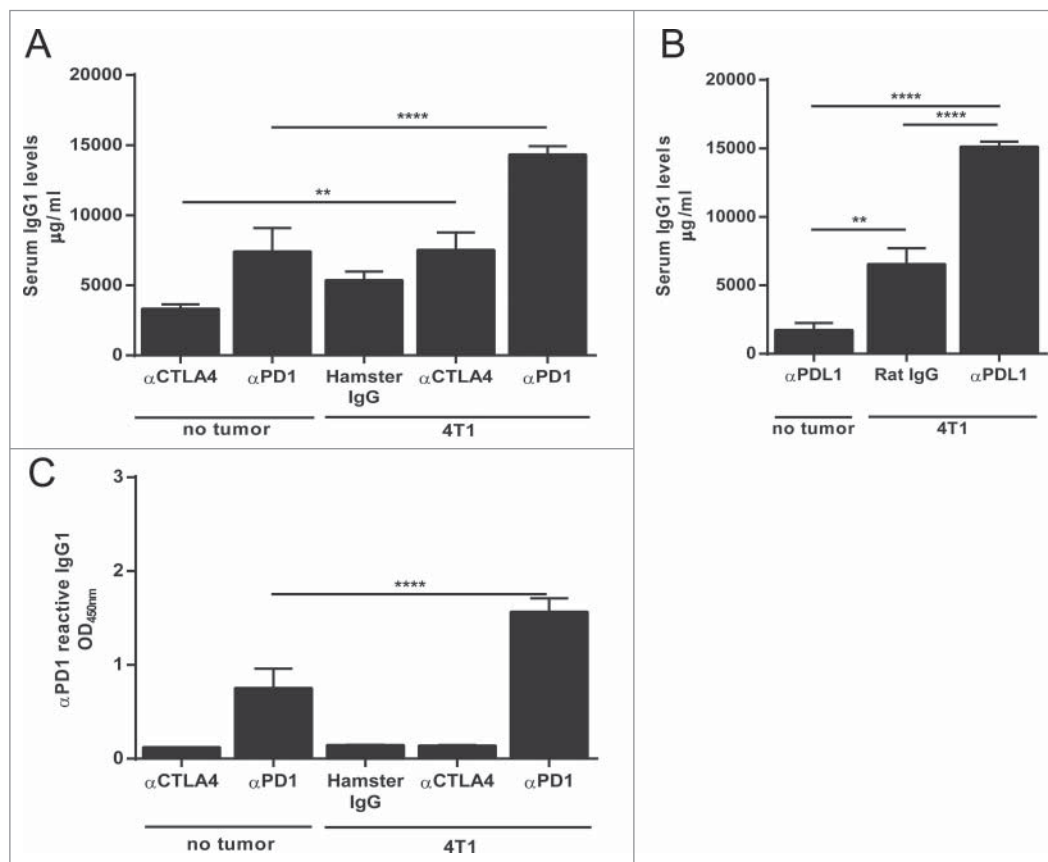


Figure 3. Systemic IgG1 accumulation following PD-1 and PD-L1 blockade in 4T1 bearing mice and increase in IgG1 antibodies toward anti-PD1. Sera from tumor bearing and non-tumor bearing mice treated with anti-PD-1, anti-CTLA4 or anti-PD-L1 was collected and analyzed for mouse IgG1 levels by ELISA. (A) Serum IgG1 levels as shown by $\mu\text{g}/\text{mL}$ in mice treated with anti-PD-1, anti-CTLA4 or isotype control; (B) Serum IgG1 levels are shown by $\mu\text{g}/\text{mL}$ in mice treated with anti-PD-L1 or isotype control; Sera from mice treated with anti-PD-1 or anti-CTLA4 were tested IgG1 antibody reactive to anti-PD1 antibody. (C) Anti-PD-1 reactive IgG1 antibodies were detected in serum of non-tumor and tumor bearing mice receiving repeated doses of anti-PD-1, CTLA4 or isotype control shown by OD (450nm). Results are representative of 2–4 mice per group and plate replicates of two. Mice were monitored post repeated dosing sacrificed when mice became moribund. Bar graph (Mean value \pm SEM) statistics were performed using One-way ANOVA with Fisher's LSD post tests. ** $P = 0.002$. **** $P = <0.0001$.

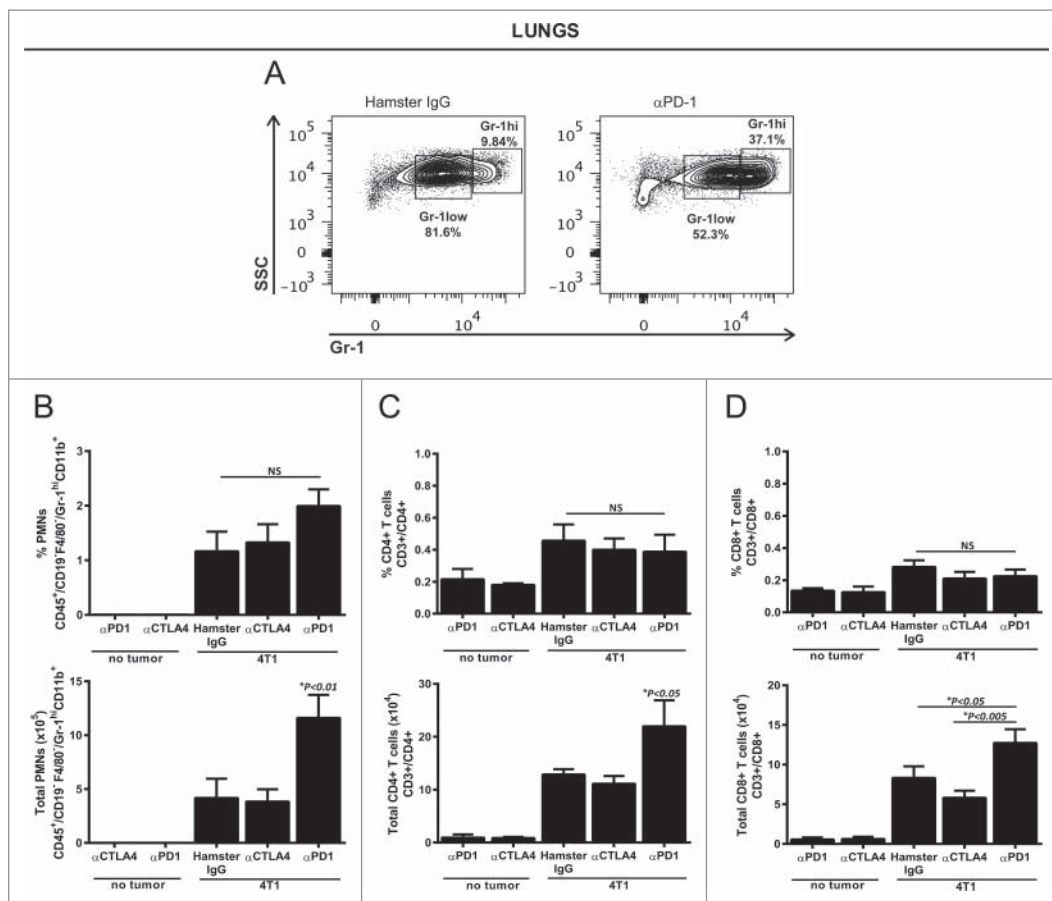


Figure 4. Increase in Gr-1^{hi} cells in lungs of mice treated with anti-PD-1 and anti-PD-L1. Lungs from tumor bearing and non-tumor bearing treated mice were harvested and analyzed for PMN cells (Gr-1^{hi}) (A) Representative dot plot showing a 27% increase in Gr-1^{hi} cells following treatment of 4T1 mice with anti-PD-1 treatment compared to isotype control. (B) Overall percent and counts of lung PMN cells (Gr-1^{hi}) by FACS analysis gating on CD45⁺/CD19⁻/F4/80⁺/CD11b⁺ cells; (C) Percent and total counts of CD4⁺ T cells by FACS analysis gating on CD3⁺/CD4⁺ T cells; (D) Percent and total counts of CD8⁺ T cells by FACS analysis gating on CD3⁺/CD8⁺ T cells; Results are representative of 2–3 mice per group for experiments repeated three times. Bar graph (Mean value ± SEM) statistics were performed using One-way ANOVA with Fisher's LSD post tests.

lungs and anti-PD-1 specific IgG1 antibodies in the serum of affected mice indicative of an IgG1 mediated anaphylaxis.

Depletion of neutrophils increases survival of anti-PD-1 treated mice

The use of granulocyte receptor-1 (Gr-1) mAb has previously been shown to deplete neutrophils in the 4T1 model.⁸ Given the significant increase of neutrophils in mice treated with PD-1 blockade and to investigate the role of these cells in our current model, we hypothesized that the depletion of neutrophils could rescue mice from mortality. We treated mice receiving repeated dosing of anti-PD-1 with a single dose of 200 μg of rat anti mouse Gr-1 (clone RB6–8C5; Rat IgG) monoclonal antibody one day prior to the sixth dose of anti-PD-1. Interestingly, aside from the 28% of mice that reached mortality by the fifth dose of anti-PD-1, there was no mortality by hypersensitivity of mice treated with anti-PD-1 in combination with anti-Gr-1 on the sixth dose (Fig. 5A). This was an approximately 60% increase in the overall survival of mice compared to the 90% mortality observed in prior experiments. In order to confirm depletion, we took sera from mice receiving anti-PD-1 in combination with anti-Gr-1 prior to the sixth anti-PD-1 dose

and observed an approximately 70% decrease in Ly6G⁺ cells compared to the isotype control (Fig. 5B). We saw no increase in survival of mice treated with anti-PD-L1 in combination with anti-Gr-1 (data not shown). These mice reached mortality within 30–60 min as observed in prior experiments. Together these results show an important role of neutrophils in this model but do not rule out other cells that may be involved.

Ganetespib decreases neutrophil accumulation in lung and suppresses anti-PD-1 reactive IgG1 antibody accumulation

The HSP90 inhibitor, ganetespib, plays a major role in the stability and activity of a diverse range of HSP90 client proteins and signaling pathways including JAK/STAT, RAF/MEK/ERK and PI3K/AKT.⁴¹ Ganetespib has recently been shown to suppress lung inflammation by suppressing neutrophil mobilization into lungs *in vivo* without myelosuppression and has also been implicated in the suppression of B cell accumulation, both of which were noted as dysregulated in our current model.²³ Additionally, it has previously been shown that treatment with ganetespib results in a significant decrease in lung metastasis in the 4T1 tumor model.⁴² Given its demonstrated efficacy in multiple cancer models including 4T1, its demonstrated immune modulating effects, and due to the lack of protection

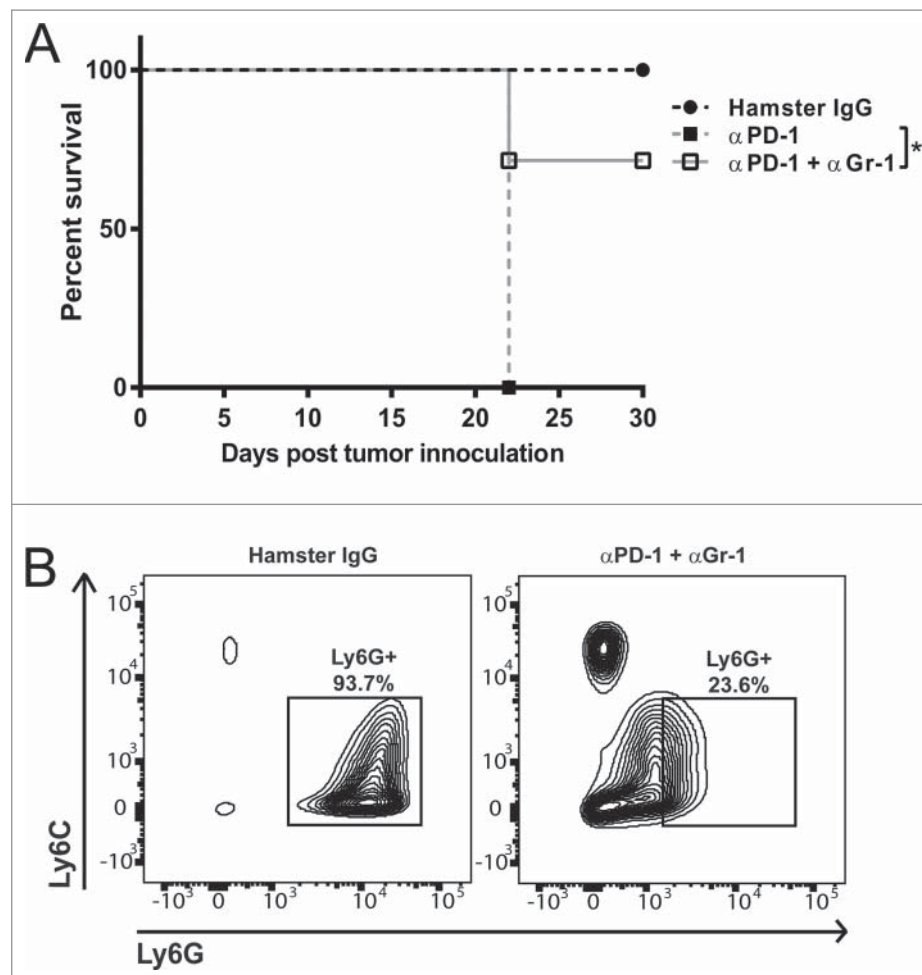


Figure 5. Depletion of neutrophils increases survival of anti-PD-1 treated mice. 4T1 tumor bearing mice were treated with anti-PD-1 as per earlier experiments. One day prior to receiving the sixth dose of anti-PD-1, mice received neutrophil depleting Gr-1 mAb (200 μ g IP). Immediately prior to receiving the sixth final dose of anti-PD-1, blood from mice was drawn and analyzed for PMN cells (Ly6G+) (A) Survival of Balb/c mice bearing 4T1 tumors post repeated dosing with anti-PD-1 (5–6 doses) in combination with anti-Gr-1. (B) Representative dot plot showing a 70% decrease in Ly6G+ cells following combination treatment of 4T1 mice with anti-PD-1 and anti-Gr-1 compared to isotype control or anti-Gr-1 alone.

seen by anti-Gr-1 mAb therapy, we hypothesized that treatment of mice receiving repeated dosing of anti-PD-1 in combination with ganetespib would protect mice from hypersensitivity reactions. We treated mice with a combined therapy of ganetespib and anti-PD-1 to determine if fatal hypersensitivity reactions persist (Fig. 6A).^{23,43} In contrast to mice receiving anti-PD-1 as a monotherapy, mice receiving anti-PD-1 in combination with ganetespib were able to survive the full course of the combination regimen before eventually succumbing to primary tumor burden (Fig. 6B). As expected, mice bearing the 4T1 tumor treated with ganetespib and anti-PD-1 showed a decrease in serum accumulation of IgG1 antibody compared to mice receiving anti-PD-1 alone (Fig. 6C). Accumulation of CD11b+Gr-1^{hi} cells in the lungs was also significantly decreased (Fig. 6D).

Discussion

In the current study, we show that repeated dosing with PD-1/PD-L1 blockade induces rapid fatal hypersensitivity in 4T1 tumor bearing mice. These adverse reactions were not reproducible by anti-CTLA-4 or isotype control treatments,

suggesting a unique role of the PD-1/PD-L1 axis in exacerbating hypersensitivity. Interestingly, hypersensitivity reactions were not observed in other tumor models or non-tumor-bearing mice highly suggestive of a tumor specific occurrence. The role of the PD-1/PD-L1 axis in hypersensitivity reactions was further supported by the observation of accumulating IgG1 antibodies in serum and neutrophils in lungs as both have previously been indicated in IgG1 mediated anaphylaxis.³⁸ Importantly, the use of ganetespib in combination with anti-PD-1 therapy protected mice from fatal hypersensitivity and resolved neutrophil and IgG1 accumulation associated with checkpoint blockade. Therefore, we suggest an adjuvant role of the PD-1/PD-L1 axis in xeno-mediated hypersensitivity reactions as well as an important priming role of a preexisting myeloproliferative tumor microenvironment.

Our observations that 8 to 21 percent of mice bearing 4T1 had fatal hypersensitivity reactions when treated with hamster and rat isotype controls as well as the rapid onset of these reactions suggest that a xeno-antibody mediated anaphylaxis may have been exacerbated by the PD-1/PD-L1 axis. This role is further suggested by the dramatic decrease in survival of mice upon treatment with PD-1/PD-L1 blockade but not by CTLA-4

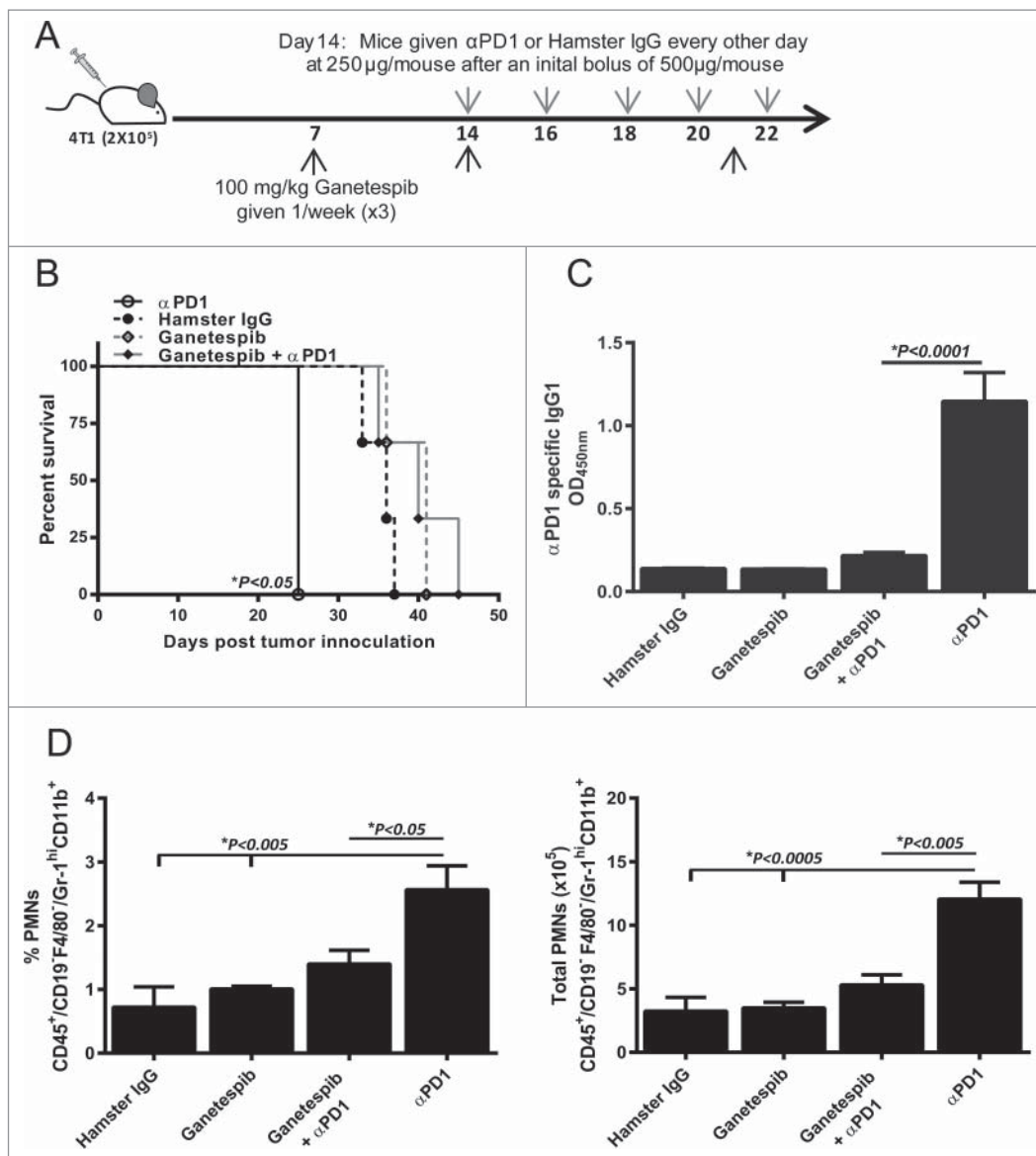


Figure 6. GanetespiB protects against fatal hypersensitivity, decreases neutrophil accumulation in lung and suppresses reactive IgG1 antibody accumulation. (A) Treatment schema. 4T1 tumor bearing mice were treated with ganetespiB (100mg/kg 1/week ×3) starting on day 7 post tumor inoculation as a monotherapy or combined with anti-PD-1 (B) Survival of tumor bearing mice treated with ganetespiB, anti-PD-1 or combined therapy. Mice receiving ganetespiB combined with anti-PD-1 treatment were rescued from fatal hypersensitivity (C) Anti-PD-1 specific anti-IgG1 antibodies in the serum of mice receiving ganetespiB combined with anti-PD-1. (D) Percent and total counts of PMN cells (Gr-1^{hi}) y FACS analysis gating on CD45⁺/CD19⁻F4/80⁺/Gr-1^{hi}CD11b⁺ cells. Mice treated with the combination regimen of ganetespiB and anti-PD-1 had reduced lung PMN percent and total counts. Survival analysis included n = 3 mice per group and was repeated 2–3 times. For tissue analysis, n = 3 mice per group were monitored until moribund and euthanized. Serum and lung tissue were collected and analyzed. Survival analysis was plotted according to the Kaplan–Meier method, and statistical differences were determined with the log-rank test. Bar graph (Mean value ± SEM) statistics were performed using One-Way ANOVA with Fisher’s LSD post tests.

checkpoint blockade. Recently, a similar report showed that repeated dosing of mice with anti-DTA-1 (GITR agonist antibody) was associated with IgG1 mediated anaphylaxis in a B16-F10 murine melanoma model.⁴⁴ In this report, anaphylaxis was not observed using isotype control antibodies however, did persist upon treatment with murinized antibody for the same target. Unlike observations seen by this study, our observations may have occurred due to the preexisting Th-2 skewed phenotype associated with the 4T1 tumor model.⁴⁵ This phenotype may have exacerbated the anaphylactic response to foreign antibodies. Additionally, it has been shown that both Th1 and Th2 responses can stimulate IgG1 production and that a switch from Th2 to Th1 can occur and suppress IgE responses; this

can also occur in an organ specific manner if mixed Th1/Th2 responses occur.^{46–48} Further studies will be important in addressing the role of PD-1/PD-L1 blockade in such skewed environments. It will also be important to discern whether these reactions persist with murine antibody treatments against checkpoint inhibitors as was seen with the GITR agonist mAb treatment.⁴⁴ However, the lack of hypersensitivity seen with anti-CTLA-4, the tumor specific reactions and observations that both anti-PD-1 and anti-PD-L1 induced fatal hypersensitivity does exclude the possibility of a clone specific response.

As mentioned, there was a lack of hypersensitivity reactions in non-tumor bearing mice or mice bearing Renca and B16 tumors, previously shown to have comparable immune

suppressor cell activity, suggesting a 4T1 tumor specific event in conjunction with PD-1/PD-L1 blockade.⁴⁹ The 4T1 tumor model is known for its high expression of G-CSF promoting a granulocytic rich phenotype.²⁴ 4T1 induced G-CSF has also been shown to dysregulate immunoglobulin production.⁵⁰ Both of these parameters were found dysregulated in our current study suggesting a primed tumor microenvironment for hypersensitivity reactions. Interestingly, neutrophils have previously been shown to accumulate in pre-metastatic lungs of 4T1 bearing mice in addition to their physiological nature to persist longer in lung tissue.^{35,51,52} They have also been implicated in both mouse and human anaphylaxis as well as in behaving as “B-cell helpers” inducing class switch and immunoglobulin production.^{38,53} Given these previous data on the preexisting 4T1 microenvironment and our current findings that Gr-1^{hi} cells increase in lungs with PD-1/PD-L1 blockade, our data suggests that preexisting neutrophils in 4T1 lungs may have played a significant role in airway hypersensitivity. Increase in the survival of mice treated with anti-Gr-1 further corroborated their role in hypersensitivity in anti-PD-1 treated mice. Of consideration, it should be noted that given CD4⁺ and CD8⁺ T cells were shown to have increased in the presence of increased levels of Gr-1^{hi} cells in lungs as well as previously reported data showing the lack of suppressive mechanism of these granular phenotypic cells, we suggest here that these are neutrophils.⁴⁰ However, other reports correlate this phenotype with the G-MDSC (granulocytic-MDSC) subset of MDSCs, also known as PMN-MDSCs, which will be important to resolve in future studies.^{39,54} Interestingly, with regards to an increase in Gr-1+ PMN-MDSCs, two recent investigations demonstrated that eradication or suppression of MDSC population enhances checkpoint blockade efficacy.^{8,21}

While the immune mediators of clinical irAEs remain unclear or noted as lymphocytic, this is the first report to show organ specific toxicities in the context of mice treated with anti-PD-1 and anti-PD-L1 and the role of neutrophil accumulation in lungs.⁵⁵ Interestingly, pneumonitis has previously been reported to occur in 3% of patients in an anti-PD-1 trial with 1% reaching mortality by treatment associated pneumonitis.⁵ This toxicity has not been significantly associated with anti-CTLA-4 treatment of melanoma.⁵⁶ This suggests that while anti-PD-1/anti-PD-L1 mediated hypersensitivity in 4T1 tumor bearing mice is an isolated event, dysregulated generation of neutrophilic and immunoglobulin responses may be of concern when using checkpoint blockade within certain tumor microenvironments primed for a hypersensitivity response. The 4T1 model may serve as a model for further elucidating such responses and perhaps the relationship between a myeloproliferative microenvironment and the PD-1-PD-L1 axis. However, we caution its use for preclinical studies utilizing this class of immunotherapies unless the monoclonal antibody is of mouse origin.

Despite the lack of antitumor efficacy with the combinational treatment of ganetespib and anti-PD-1 observed in our current model, there was a substantial decrease in aberrant immune responses mediated by anti-PD-1. Furthermore, it has been previously been shown that ganetespib can decrease lung metastasis in the 4T1.⁴² An important consideration of this observation is how this may be translatable to clinical use of checkpoint blockade. Currently, treatments for irAEs associated with checkpoint blockade have been narrowed to

discontinuation of treatment or management by corticosteroids or the addition of infliximab, a chimeric monoclonal antibody against the tumor necrosis factor alpha (TNF α).^{52,55} While these treatments are not known to obstruct antitumor responses of immunotherapy, they have no additional benefits to patients currently known. Additionally, immunosuppressive treatments alongside immunotherapy raise concern for opportunistic infections.⁵⁷ HSP90 inhibition, however, has recently been noted for its potential beyond antitumor efficacy for potential against inflammatory and infectious diseases.⁵⁸ Therefore, ganetespib may be a key combinational therapeutic against immunotherapy associated irAEs as well as opportunistic infections while possibly adding to antitumor efficacy. Further studies are required to optimize these possible combinational strategies and to isolate ideal tumor models.

In conclusion, checkpoint blockade targeting the PD-1/PD-L1 axis causes fatal xenogeneic hypersensitivity with an associated increase in lung neutrophils and serum IgG1 levels in 4T1 tumor bearing mice. Our model emphasizes caution with regards to the use of foreign antibodies in preclinical models since it is yet unclear that these are reliable therapeutic models for translational approaches with regards to toxicities and efficacy. In future studies, the 4T1 tumor model may possibly be used for further understanding of the relationship between hypersensitivity and the PD-1/PD-L1 axis in a highly myeloproliferative microenvironment but caution should be applied in utilizing this model for further checkpoint blockade studies. Additionally, it is possible that checkpoint blockade using mAbs, even human, may result in increased hypersensitivity reactions to other antigens with repeated clinical administration.

Materials and methods

Animals and tumor models

Female Balb/c or C57BL/6 mice of 6–8 weeks of age were purchased from the National Cancer Institute (NCI-APA, Frederick, MD) or Charles River Laboratories (Wilmington, MA). For the 4T1 primary tumor model, 2×10^5 cells in 0.1 mL PBS were injected orthotopically into the mammary fat pad. Renca cells were injected at 1×10^5 cells in 0.2 mL PBS intravenously. B16 melanoma cells were injected at 2×10^5 cells in 0.2 mL PBS intravenously. Primary tumor volumes were determined using calipers to measure dimensions and calculated using the formula: Volume (mm³) = ((Width (mm))² \times Length (mm))/2. Mice were euthanized when moribund with hypersensitivity determined based on mice becoming immobile (prostrate position) and cyanotic with labored breathing within 30–60 min of the final dose of monoclonal antibody. All mouse procedures and care were completed in accordance with the protocols approved by the Institutional Animal Care and Use Committee (IACUC) of the University of California, Davis (UCD).

Tumor cell lines

The murine metastatic mammary carcinoma cell line, 4T1, was obtained from Dr. Sally Dupre (University of Nevada, Reno, NV). The murine renal cell carcinoma cell line, Renca, was obtained from Dr. Robert Wiltout (National Cancer Institute, Frederick, MD). The B16 murine melanoma cell line was

obtained from the National Cancer Institute, Frederick, MD. Cryopreserved cell lines were thawed and maintained in RF10 complete media growth conditions and used at 50–80% confluency for *in vivo* experiments.

Treatments

The hamster anti-murine PD-1 (clone J43) and hamster anti-murine CTLA-4 (clone UC10-4F10-1) mAbs have been described.^{59,60} The rat anti-murine PD-L1 (clone 10F.9G2) mAb, rat anti-murine Gr-1 (clone RB6-8C5) and Rat IgG isotype control (clone LTF-2) were all purchased from BioXcell (West Lebanon, NH). Hamster IgG was purchased from Jackson ImmunoResearch (West Grove, PA). The HSP90 inhibitor, ganetespib [3-(2,4-dihydroxy-5-isopropylphenyl)-4-(1-methyl-1H-indol-5-yl)-1H-1,2,4-triazon-5(4H)-one] was synthesized by Synta Pharmaceuticals Corp. (Lexington, MA). Stock solution was prepared in DMSO then diluted in 20% Cremophor RH40 (Sigma; 07076) in dextrose 5% in water (D5W). Final dose of 100 mg/kg was used once a week for three weeks starting at day seven of tumor inoculation. Schemas depicting dosing and timing of antibody administration can be found in Fig. S1. In the case of anti-Gr-1, mice received a single dose of 200 μg IP 24 h prior to the sixth dose of anti-PD-1 or the fourth dose of anti-PD-L1.

Histology

Formalin fixed paraffin embedded tissues were sectioned (5 μm) and stained with hematoxylin and eosin (H&E) stains. Pathological findings were summarized and reported by an experienced pathologist.

Isolation of lung cells

To obtain single cell suspension of lung tissues for immune phenotyping, lungs were digested in 2mg/mL collagenase-IV and 0.1mg/mL DNase I at 37°C for 1 h. Following incubation, tissue was mechanically disassociated, filtered and stained for flow cytometry as stated below.

Antibodies and flow cytometry

Spleen and lung tissues were processed, brought to single cell suspensions and stained for 20 mins at 4°C. Cells were washed with staining buffer (PBS + 1% FBS + 1mM EDTA + 0.02% NaN₃) and acquired on a Becton Dickinson Fortessa Flow Cytometer (BD, San Jose, CA). Data was analyzed using FlowJo software (TreeStar, Ashland, OR). Pacific Blue-CD44 (clone IM7), Pacific Blue-CD45 (clone 20-F11), Bright Violet 785-CD3 (clone 17A2), Bright Violet 785-CD19 (clone 6D5), Bright Violet 711-CD4⁺ (clone RM4-5), Alexa Fluor 700-CD8⁺ (Clone 53-6.7), PE-Cy7-Ly6G/C (Gr-1) (clone RB6-8C5), APC-F4/80 (clone BM8) and APC-Cy7-CD11b (clone M1/70) were purchased from Biolegend (San Diego, CA). Fc block and 7-AAD was purchased from BD Biosciences (San Jose, CA). Gr-1⁺ cells were gated on CD45⁺/F4/80-/CD19-/CD11b⁺ cells.

Elisa

For analysis of serum IgG1 levels, 96-well polysorb surface plates (Thermo Scientific Nunc, Waltham, MA) were coated with goat-α-mIgG1 (Southern Biotech, Birmingham, AL) and incubated at 4°C overnight. Plates were then washed with buffer (PBS + 0.05% Tween20) and 100 μL of diluted serum samples or standard were added to plate and incubated for 90 mins at room temperature (RT). After washing plate again with buffer, 100mL of diluted secondary HRP-conjugated α-mIgG1 (Southern Biotech, Birmingham, AL) was added and incubated for 90 mins at RT. Plate was washed again with buffer and 100 μL of TMB substrate solution (Cell Signaling Technology, Inc., Beverly, MA) was added to wells and incubated for up to 30 mins at RT. A stop solution (Fisher Scientific, Hanover Park, IL) was used to stop reactions and absorbance was read at 450nm on the VersaMax™ Tunable microplate reader (VWR, Radnor, PA) using the SoftMax Pro Data Acquisition and Analysis Software (Molecular Devices Corp., Sunnyvale, CA).

Statistics

Survival data was plotted by the Kaplan–Meier method and analyzed by the log-rank test. One-way or 2-way ANOVA or *Student's t* tests were performed to determine if mean values were significantly different followed by Bonferroni multiple-group comparison or Fisher's LSD post-test. Statistical analyses were performed in Graph-Pad Prism 6 Software (GraphPad Software, Inc., La Jolla, CA). Unless otherwise identified significance is as follows: *****P* < 0.0001, ****P* < 0.001, ***P* < 0.01 and **P* < 0.05

Disclosure of potential conflicts of interest

Author D.A.P is an employee and shareholder of Synta Pharmaceuticals Corp. Author K.K is an advisory to Synta Pharmaceuticals Corp. and a principle investigator of their phase III trial GALAXY 2.

Acknowledgments

The authors would like to acknowledge Weihong Ma, Stephanie Mac and Monja Metcalf for technical support. We would like to thank Anthony Zamora for his advice and technical support with the flow cytometry assays and analysis. We would also like to thank Ethan Aguilar for helpful discussions.

Funding

This work has been supported by the National Institutes of Health (NIH) grant R01CA095572 and R01 CA72669. We would also like to acknowledge the UC Davis Comprehensive Cancer Center Shared Resources Flow Cytometry Core which was used for all flow cytometry data acquisition and is funded by the UC Davis Comprehensive Cancer Center Support Grant (CCSG, NCI P30CA093373).

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