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Research Article

Modulating T-Independent and T-Dependent Antibody Responses via Checkpoint Blockade and CD40 Agonist Insights for Vaccination Strategies During Immunotherapy

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Abstract

Background: Patients with cancer often do not receive vaccines to preventable infectious diseases such as influenza and pneumococcal pneumonia because of a lack of knowledge about the optimal timing of vaccination relative to their underlying disease or their current cancer treatments. Cancer immunotherapies, which rely on the ability to promote immune responsiveness to tumors, are a promising therapeutic modality, but their impact on vaccination is largely unexplored.

Methods: We used a pre-clinical mouse model to evaluate the antibody response to a T-dependent (TD) or a T-independent (TI) antigen immunization with concomitant administration of either checkpoint inhibitors such as antibodies to CTLA-4 or PD-L1 or an antibody to CD40 that has adjuvant properties.

Results: We found that checkpoint blockade with anti-CTLA-4 or anti-PD-L1 antibodies provided reduction in IgM, IgG, and most IgG subclasses when immunized with either TI or TD antigens. On the other hand, a CD40 agonist antibody provoked modest reductions in all immunoglobulins in response to TD antigen but provided marked increases in most immunoglobulins and IgG subclasses in response to TI antigen.

Conclusions: These data suggest that the timing of vaccinations relative to immunotherapies might be an important factor in determining the efficacy of vaccination. If these findings are shown to extend to humans, the antibody response to vaccination might be attenuated and patients might be at increased risk for infection. This pilot study provides potential mechanistic insights into an important consideration in patients receiving immunotherapies.

Keywords: Vaccination; Immunotherapy; Checkpoint blockade

Abbreviations

APC: Antigen Presenting Cell; BSA: Bovine Serum Albumin; CFA: Complete Freund's Adjuvant; CTLA-4: Cytotoxic Lymphocyte Antigen-4; ELISA: Enzyme-Linked Immunosorbent Assay; IFA: Incomplete Freund's Adjuvant; KLH: Keyhole Limpet Hemocyanin; PD-L1: Programmed Death Ligand-1; TD: T-Dependent antigen; TI: T-Independent antigen; TNP: Tri-NitroPhenol.

Background

Patients with cancer are more susceptible to infections, either due to the malignancy itself or immunosuppressive treatments [1]. In many cases, infections in cancer patients are due to organisms to which there are available vaccines such as influenza and pneumococcal pneumonia [2]. Uus, the coordination of optimal timing of vaccination with cancer treatment is a key to achieving better protection against infection. Many novel cancer immunotherapies have emerged over the past several years, including those that rely on augmentation of the immune responses that recognize solid tumors or hematologic malignancies [3-5]. Two types of immune augmentation include checkpoint blockade agents or co-stimulation agonists that can act as adjuvants.

Ue most widely characterized checkpoint blockade agents include those that block the CTLA-4 pathway or the PD-L1/PDL pathway. Briefly, the CTLA-4 receptor present on T-cells functions as an immune checkpoint and the therapeutic antibodies that block CTLA-4 allow B7 ligands to interact with the co-stimulatory CD28 molecule. Uus, these antibodies promote stimulatory signals to T-cells that are reactive to antigens expressed on tumor cells [6,7]. Likewise, the antibodies that interfere with PD-L1 binding to its receptor PD-1 interfere with T-cell exhaustion thereby enhancing T-cell reactivity to their cognate tumor antigens [8,9]. On the other hand, antibodies with agonist activity to co-stimulatory pathways such as the CD40/CD40L pathway can act as immunologic adjuvants that can enhance antigen presenting cells (APCs) such as dendritic cells, B cells, and cells of the monocyte-macrophage series [10]. Enhancement of antigen presentation to T and B lymphocytes leads in turn to enhanced and more durable immune responses to tumor antigens [11-13].

Although the effects of agents with co-stimulatory or checkpoint blockade activities on the cellular immune response to tumors are

relatively well characterized, there is much less known about their effect on antibody response to tumors or other antigens. Ue pathways of antibody responses to antigens differ by the requirements for co-stimulation of B cells. Antigens are processed by the immune system either with or without the need for T-cell co-stimulation. In general, antigens can be classified as either T-lymphocyte dependent (TD) or as T-lymphocyte independent (TI) [14,15]. During the immune response to TD antigens, T-lymphocytes provide "help" in the form of cytokines and/or ligands to co-stimulatory receptors. Uese signals are essential for driving B-lymphocyte proliferation, production of immunoglobulins, immunoglobulin class switching, rescue of B-lymphocytes from apoptotic death, and generation of memory B cells [16]. Ue TD antigens include protein antigens that are processed and presented by professional APCs of the monocyte/macrophage/ dendritic cells system, as well as, in some cases, mature B-cells.

In contrast to TD antigens, TI antigens induce antibody production without the help of T-lymphocytes. TI antigens commonly consist of repetitive structures such as polymeric proteins or polysaccharides [17]. Ue most commonly used TI antigens in pre-clinical models are haptens such as di- or tri-nitrophenol conjugated to ficoll, a sucrose-epichlorohydrin co-polymer. Ue capsular polysaccharides of bacteria are a clinically important group of TI antigens [18]. Ue TI antibody response to the capsular polysaccharides of *Streptococcus pneumoniae, Haemophilus influenzae* and *Neisseria meningitidis* provide immunity to invasive infections with these bacteria [19]. Other examples of TD and TI antigens (vaccines and infectious agents) are listed in Table 1.

Antibody response	Vaccine	Infectious examples
TI	PPV23	*S. pneumonia* *H. influenza* *N. meningitidis*
TD	PCV13	*S. pneumonia*
TD	FV	Influenza, all other viruses

Table 1: TD and TI antigens.

Our clinical oncology program has recently developed an interest in vaccination patterns in our large population of myeloma patients, specifically with a focus on influenza and pneumococcal vaccination rates and patient outcomes [20-22]. As part of these studies, we hypothesized that the simultaneous administration of checkpoint blockade agents or CD40 agonists would have measurable effects on both TI and TD antibody responses. To test this in a pre-clinical model, we immunized mice with TI or TD antigens with simultaneous administration of CD40 agonist antibody or antibodies that block immune checkpoint molecules and measured total immunoglobulin (IgG and IgM) responses as well as IgG subclasses to the immunizing antigen. In this brief report, we show that immunization delivered simultaneously with checkpoint blockade or immunostimulatory agonist antibody can have profound effects on the magnitude of the antibody response to both T-dependent as well as T-independent antigens. Ue data suggest that the timing of vaccination of patients who receive immunotherapies should be considered to achieve and sustain maximum protections against certain infections.

Methods

Mice

Female C57BL/6 mice (5 per group) were from Envigo (Madison, WI), and were immunized between 6-10 weeks of age. Mice were housed in laminar flow cage systems and fed standard rodent chow and tap water ad libitum. All experiments were reviewed and approved by the Institutional Animal Care and Use Committee (protocol #257).

Antigens and antibodies

TNP-ficoll, TNP-KLH, and TNP-BSA) were purchased from BioSearch Technologies (Petaluma, CA). *In vivo* grade antibodies anti-PD-L1 (PDL1 BP101), anti-CTLA-4, (a pool of BP0164 and BP0131), and anti-CD40 (BP0016-2) were purchased from BioXCell (West Lebanon, NH).

Immunizations and *in vivo* antibody treatments

For TI responses, mice were immunized i.p. with 150 µg TNP-ficoll and bled on day 12. For T-dependent responses, mice were immunized s.c. with 100 µg TNP-KLH in delivered in 50uL complete Freund's adjuvant (CFA) on day 0, and the same dose in incomplete Freund's adjuvant (IFA) on day 14 and bled on day 21. Phosphate buffered saline was used as vehicle for both antigens. CFA and IFA were purchased from Sigma Chemical (St. Louis, MO). *In vivo* antibody treatments were given at 200 ug/animal and administered i.p. simultaneously with injection of antigens.

Enzyme immunoassay

Blood plasma samples were tested for IgG levels from hapten-immunized mice by ELISA. Briefly, microplate wells (Nunc Medisorp) were coated with 200 ng/well of TNP-BSA overnight at 4C. Ue wells were blocked with the addition of SuperBlock (#37515 Uermo-Fisher, Rockford, IL) and samples were diluted serially in 5% bovine serum albumin in PBS. Total IgG was detected with the addition of goat-anti-mouse IgG-HRP (# 115-035-166, Jackson ImmunoResearch, West Grove, PA), and IgM was detected with goat-anti-mouse IgM-HRP (# 115-035-020, Jackson ImmunoResearch). IgG subclasses were detected with subclass-specific HRP-conjugated antibodies (all from Southern Biotech, Birmingham, AL). Reactions were developed with two-part Turbo TMB substrate (UermoScientific, Rockford, IL), and read at 450 nm on an ELISA reader. Titers were determined at endpoint as defined by the highest dilution that was at least three times the background optical density of wells that received no serum.

Statistics

One-way ANOVA with Duncan's Comparison was used to determine statistical relationships between animals without antibody treatments to those with checkpoint blockade or CD40 agonist. Statistics were run on SIGMASTAT soIware (Systat SoIware Inc., San Jose, CA) for Windows Version 11.0. Except for IgG1 and IgG2a responses to the TNP-Ficoll antigen, all groups passed the Shaprio-Wilk test for normality. Accordingly, the data from these groups were log-transformed to conform to normality before testing by ANOVA. Ue statistical power with and alpha of 0.05 ranged from 0.60 to 0.96 for the different groups tested.

Results

Antibody responses to TI antigen

Control (no antibody treatment) mice immunized with the TI antigen TNP-ficoll, showed robust responses of both IgG and IgM immunoglobulins (Figures 1a and 1b), and the responses were predominated by the IgG3 subclass. Ue IgG3 response to the TI antigen (Figure 1f) was greater in magnitude than the IgG3 response to the TD antigen (Figure 2f), as would be expected for a TI antigen. Ue antibody responses to TNP-ficoll were differentially affected by checkpoint blockade compared to CD40 agonist (Figure 1). Specifically, the CD40 agonist mediated a highly increased IgM, total IgG, and most IgG subclasses compared to controls; whereas CTLA-4 or PD-L1 blockade resulted in moderate reduction of most immunoglobulins.

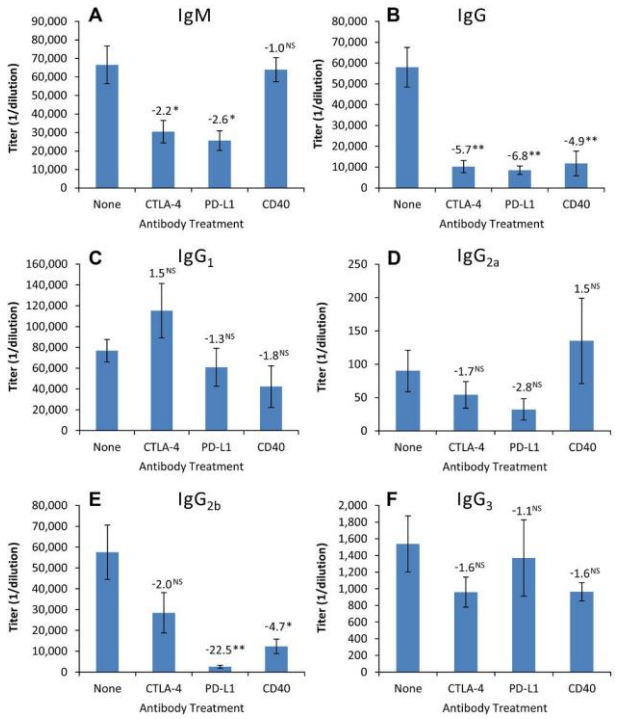

Figure 1: Antibody Responses to T-independent Antigens. Mice (n=5/group) were immunized with TNP-ficoll and tested for IgM and IgG titers to TNP-BSA. Values above bars represent + or – fold changes compared to control groups that received TNP-ficoll immunization alone. P values *=p<0.05; **=p<0.005; NS=not significant when compared to control group. P values where determined by One-way ANOVA. Non-immunized mice (n=5) showed titers of <1/100 for each Ig and IgG subclass (data not shown).

For example, CD40 agonist monoclonal antibody increased total IgM and IgG responses when compared to control by approximately 3-fold and 9-fold, respectively. Ue most pronounced increase among the IgG subclasses was for IgG1 (nearly 16-fold); whereas the IgG3 response was not significantly different from the control group. IgG2a and IgG2b responses were also significantly increased compared to control groups. On the other hand, total IgM and IgG responses to the TI antigen delivered with either CTLA-4 or PD-L1 blockade were mildly decreased by around 2-fold to 3-fold, respectively, and while most IgG subclasses showed small reductions, only the IgG2a response under PD-L1 blockade (Figure 1d) and the IgG3 response under CTLA-4 blockade (Figure 1f) reached statistical significance. Similar data were obtained when checkpoint blockade or CD40 agonist antibodies were given 48 hours before or 48 hours aler immunization (data not shown).

Antibody responses to TD antigen

Robust IgM and IgG responses were observed in control mice immunized with the TD antigen TNP-KLH (Figures 2a and 2b). Uese responses were predominantly of the IgG1 and IgG2b subclasses (Figures 2c and 2e) when compared to the TI antigen (Figures 1c and 1e), as expected for a TD antigen. Checkpoint blockade and CD40 agonistic antibodies showed variable effects on TD antibody responses. IgM responses to TNP-KLH were modestly reduced by the influence of CD40 agonistic antibody as well as to blockade with either anti-CTLA-4 or and PD-L1, and these reductions were statistically different from control responses (Figure 2a).

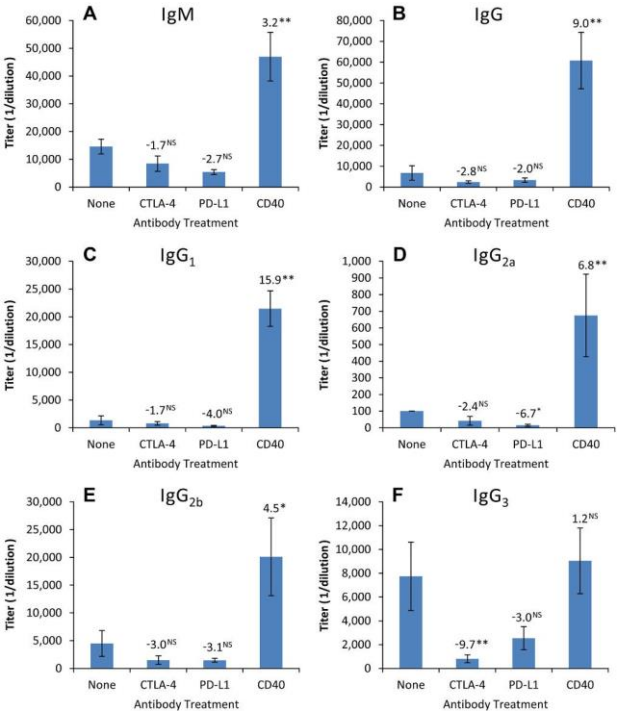

Figure 2: Antibody Responses to T-dependent Antigens. Mice (n=5/group) were immunized with TNP-KLH and tested for IgM and IgG titers to TNP-BSA. Values above bars represent + or – fold changes compared to control groups that received TNP-KLH immunization alone. P values *=p<0.05; **=p<0.005; NS=not significant when compared to control group. P values where determined by One-way ANOVA. Non-immunized mice (n=5) showed titers of <1/100 for each Ig and IgG subclass (data not shown).

On the other hand, total IgG responses were markedly reduced, ranging from 5-fold to 7–fold (Figure 2b). Much of the reduction of total IgG was likely due to suppression of the IgG2b subclass (Figure 2e), with reductions ranging from 2-fold to over 20-fold in the case of PD-L1 blockade. Only modest reductions of IgG3 were observed with either checkpoint blockade or CD40 agonist (Figure 2f), but these were not statistically significant. Similar data were obtained when checkpoint blockade or CD40 agonist antibodies were given 48 hours before or 48 hours aIer immunization (data not shown).

Discussion

Ue antibody response to vaccination under the cover of immune checkpoint inhibitors and co-stimulatory agonist antibodies is largely undefined. We describe two interesting findings in this brief report. Firstly, checkpoint blockade with either CTLA-4 or PD-L1 systems produced reduction in IgM, IgG, and most IgG subclasses when immunized with either TI or TD antigens. Secondly, a CD40 agonist showed modest reductions in all immunoglobulins in response to TD antigen, but provided marked increases in most immunoglobulins and IgG subclasses in response to TI antigen. Because vaccination to infectious diseases is performed in patients who receive immunotherapies, our findings may have implications on the timing of vaccination relative to treatment with these agents.

Our finding that checkpoint blockade with anti-CTLA4 or anti-PD-L1 reduced total IgM and IgG and many IgG subclasses was somewhat unexpected, as both of these antibodies are known to augment T-cell mediated immune responses [3-6,9]. We reasoned that enhanced co-stimulation afforded by increasing T-cell longevity (via PD-L1 inhibition) or inhibition of CTLA-4 would have a positive effect on antibody production. Ue mechanism(s) for the effects observed in these studies is unclear. It is possible that the similar effects of CTLA-4 and PD-L1 inhibition on antibody production is due to their sharing a common pathway of interaction with B-cells that express the CD80 (B7.1) molecule. It has been established that CD80 is part of the B-cell receptor complex (BCR), but its precise function in B-cell signaling is not well defined. CD80 is a known co-receptor for both CTLA-4 and PD-L1 [23], so it is feasible that inhibition of CD80 signaling on B-cells is due to the lack of availability of these ligands to B-cells. Anti-CD80 antibodies have been shown to block the proliferation and production of IgG and restrict the growth of CD80 expressing lymphomas. In addition, anti-CD80 provided an up-regulation of pro-apoptotic molecules and down-regulated the anti-apoptotic molecule Bcl-x(L) [24]. Also, a CTLA-4-Ig fusion protein was reported to inhibit antibody responses to influenza in a mouse model, further implicating the role of B7 family members in the down-regulation of antibody responses [25]. It is not clear, however, whether the anti-CD80 antibodies and fusion proteins used in these studies acted in a manner consistent with blockade of B7.1 ligands or were acting as agonists of the CD80 co-receptor. In either case, CD80 function on B cells could be responsible for the similar inhibition of IgG responses by CTLA-4 and PD-L1 blockade. Although it is possible that PDL-1 and CTLA-4 blockade effects immunoglobulin production via enhanced production of interferon-gamma, this mechanism is unlikely because while interferon-gamma inhibits production of IgG1, IgG2b, and IgG3 isotypes, it enhances IgG2a production [26].

IgG3 was the predominant subclass induced by the TI TNP-ficoll antigen among control mice, which is consistent with observations that IgG3 is the predominant subclass developed in response to polysaccharide antigens [27-30]. Mouse IgG3, (which is not a homologue of human IgG3), is the primary subclass of IgG that is induced in response to carbohydrates and repeating epitope antigens and, by nature of its self-associating properties, can elicit powerful effector function early in immune responses [31-33]. It is interesting that while IgG3 responses are relatively unaffected by CD40-costimulation, other IgG subclasses demonstrate marked increases ranging from 4-fold to nearly 16-fold. Uus, it would appear that CD40 co-stimulation contributes to the generation of a diverse pattern of IgG subclasses through class switching but does not influence the magnitude of the response to the IgG3 subclass. By contrast, CD40 agonist does not contribute to a rise in IgG subclasses in response to the TD antigen TNP-KLH. Uis may reflect a differential requirement for CD40-CD40L interactions on TI vs. TD antibody responses.

Although the role of CD40L–CD40 interactions is well established in the immune response to TD antigens, its role in the response to TI antigens is less clear [34,35]. Both CD40 and CD40L knockout mice mount immune responses the TI antigens DNP-ficoll and TNP-ficoll, at levels similar to those of wild-type mice, suggesting that the immune response to TI is independent of the CD40–CD40L interaction [36-38]. In addition, mice immunized with a capsular polysaccharide antigen mounted vigorous antibody responses when treated with a CD40L blocking antibody [39,40]. On the other hand, capsular polysaccharide antigens up-regulated the expression of CD40L on T lymphocytes [41,42]. Furthermore, Dullforce et al. demonstrated that administration of anti-CD40 antibody to mice immunized with pneumococcal polysaccharide provided a substitute for T-cell help that resulted in the generation of strong, isotype-switched antibody responses that afforded protection from subsequent challenged from infection [43]. Ue data obtained from our studies is mostly in line with those of Dullforce et al. support the concept that CD40 agonists provide enhanced immunoglobulin responses to TI antigens. It is interesting that CD40 agonist appears to act as an adjuvant to TI antigens despite findings that alum or Freund's adjuvants do not have a major effect on the immunogenicity of TI antigens [44]. Uis likely supports a direct effect of CD40 agonist activity on B-cells, as contrasted to the indirect effects of other adjuvants.

We acknowledge that this study has several limitations. Firstly, because this was designed to be a feasibility study, we only performed the experiments with checkpoint blockade or CD40 agonist antibodies given immediately around the timing of immunization. Future experiments that would help define the longevity of the duration of suppression or enhancement of the antibody responses to each type of antigen challenge would be important. Secondly, because these studies involved quantitation of antibody levels at a single time-point (12 days for TI and 21 days for TD antigens) we have not established whether these effects represent fixed changes in antibody responsiveness. It is possible, for example, that the suppression of antibody responses observed with checkpoint blockade represents delayed antibody production, rather than long-term suppression. It would be interesting to perform larger studies in which animals were bled at various time-points post-immunization. Uirdly, in the case of the augmentation of antibody production in CD40 treated animals give CD40 agonist, it also will be important to determine whether these responses are durable over longer periods on time. Finally, future pre-clinical studies might include other antigens comprised by a wider range of T and B cell epitopes rather than simple haptens as described here. In addition, it will be important to test these effects in tumor-bearing hosts as the immune system in these animals will adequately reflect the immune system in the setting of established cancers.

In addition to checkpoint inhibition and adjuvants described here, other cancer therapeutics can affect the immune system through direct or indirect mechanisms. Uese would include standard chemotherapies that involve tumor cell toxicity via their ability to directly damage DNA, immunomodulatory drugs such as lenalindomide, as well as other antibodies such as elotuzumab which can directly activate NK cells via the signaling lymphocytic activation molecule F7 (SLAMF7) [45]. We might improve the use and effectiveness of vaccination if we could better define the optimum timing of vaccinations relative to delivery of these various anti-cancer agents.

Uis exploratory animal study provides several interesting findings that might be considered in the optimization of vaccination strategies in humans who receive cancer immunotherapy. We designed this study to include both TI and TD antigens because both types of antigens are used in humans. For example, the Pneumovax vaccine consists of polysaccharide antigens from a variety of bacterial strains (23-valent) and is analogous to our TI TNP-Ficoll antigen. On the other hand, the pneumococcal-conjugate vaccines are bacterial polysaccharides that are chemically coupled to the tetanus toxoid protein which presents the antigen to APC in a manner analogous to the TD pathway. Influenza antigens are also in the class of TD antigens.

Although retrospective data [21] and prospective international registries [46] of vaccination in the setting of cancer have been described, there are little available data regarding endpoints such as antibody measurements, clinical outcomes, hospitalization, and infection in immunotherapy clinical trials. A recent study by Branagan and colleagues described improved duration of serological immunity to a two-series dose of influenza vaccines in patients with plasma cell disorders [47]. Practical application of data from these types of studies might help decrease the infectious morbidity and mortality in cancer. Such human studies might include evaluating antibody titers sequentially with vaccinations before or aler immunomodulatory drugs. To date, only a few studies have evaluated checkpoint inhibitor use and vaccination. Laubli and colleagues showed in 22 cancer patients (n=16 NSCLC, n=3 RCC, n=3 melanoma) that there was an adequate antibody response to influenza but an increased number of adverse events [48] while a larger study (n=108 total, n=71 melanoma, n=23 NSCLC) showed no differences in adverse events, but did not study antibody responses to influenza vaccination [49].

A fuller understanding of the potential for checkpoint inhibitors to influence the immune system globally will be increasingly important as clinical use of these drugs increases. Currently available PD-1 inhibitors include pemobrolizumab (Keytruda, Merck) and nivolumab (Opdivo, BMS). PD-L1 inhibitors include atezolizumab (Tecentriq, Genentech), avelumab (Bavencio, Pfizer), and Durvalumab (Imfinzi, Medimmune/AstraZeneca). Uese drugs may all behave similarly based on mechanism-of-action, or there may be differences that would be important to characterize. In addition, CD40 agonists that are currently in clinical trials such as dacetuzumab (SGN-40, Seattle Genetics) and lucatumumab (HCD122, CHIR-12.12, Novartis) may also be of interest to elucidate their influence on vaccination response.

Conclusions

A fuller understanding of the potential for checkpoint inhibitors and adjuvants to influence the immune response to vaccines will be increasingly important as the clinical use of these drugs increases. Currently available PD-1 inhibitors may all behave similarly based on mechanism-of-action, or there may be differences that would be important to characterize including in vaccination response and potential adverse events. Beyond checkpoint inhibitors and adjuvants, the interaction between environmental, therapeutic, or vaccination antigen exposure may help us better understand the immune response to these agents.

Declarations

Ethics approval

Uis study was performed under the oversite of the Aurora Health Care Institution for Animal Care and Use Committee (protocol # 257).

Competing interests

Ue authors declare that they have no competing interests.

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Authors' contributions

MO designed experiments, analyzed data, wrote manuscript; MAT analyzed data, wrote manuscript; KMM performed experiments, prepared figures; AG performed experiments.

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