VISTA Regulates the Development of Protective Antitumor Immunity

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Abstract

V-domain Ig suppressor of T-cell activation (VISTA) is a novel negative checkpoint ligand that is homologous to PD-L1 and suppresses T-cell activation. This study demonstrates the multiple mechanisms whereby VISTA relieves negative regulation by hematopoietic cells and enhances protective antitumor immunity. VISTA is highly expressed on myeloid cells and Foxp3+CD4+ regulatory cells, but not on tumor cells within the tumor microenvironment (TME). VISTA monoclonal antibody (mAb) treatment increased the number of tumor-specific T cells in the periphery and enhanced the infiltration, proliferation, and effector function of tumour-reactive T cells within the TME. VISTA blockade altered the suppressive feature of the TME by decreasing the presence of monocytic myeloid-derived suppressor cells and increasing the presence of activated dendritic cells within the tumor microenvironment. In addition, VISTA blockade impaired the suppressive function and reduced the emergence of tumor-specific Foxp3+CD4+ regulatory T cells. Consequently, VISTA mAb administration as a monotherapy significantly suppressed the growth of both transplantable and inducible melanoma. Initial studies explored a combinatorial regimen using VISTA blockade and a peptide-based cancer vaccine with TLR agonists as adjuvants. VISTA blockade synergized with the vaccine to effectively impair the growth of established tumors. Our study therefore establishes a foundation for designing VISTA-targeted approaches either as a monotherapy or in combination with additional immune-targeted strategies for cancer immunotherapy. Cancer Res; 74(7); 1933–44. ©2014 AACR.

Introduction

Immune responses against cancer are negatively regulated by multiple checkpoints, including CTLA-4, PD-L1/PD-1, and B7-H4 pathways. Targeting of these negative immune regulators has proved to be a clinically effective strategy to enhance tumor-specific immune responses (1, 2).

The critical role of T CTLA-4 in suppressing tumor-specific immunity was demonstrated when antibody-mediated CTLA-4 targeting in combination with a cellular vaccine induced regression of established, poorly immunogenic B16 melanoma (3). Ipilimumab, an anti-human CTLA-4 monoclonal antibody (mAb), as a monotherapy has proved to exert clinical benefit in late-stage melanoma in patients and has been approved for treating advanced melanoma, as well as undergoing early-phase trials for other cancers (4–6).

Programmed death-1 (PD-1) and its ligands PD-L1/PD-L2 represent another immune negative checkpoint axis (7, 8). The PD-1 pathway downregulates tumor-specific immunity by impairing T-cell responses and promoting the induction of Foxp3+ Tregs in the periphery (1, 9). Blocking the PD-L1/PD-1 pathway, in conjunction with other immune therapies inhibits tumor progression (10–15). MDX-1106/BMS-936558, the human αPD-1 mAb, as well as a human αPD-L1 mAb, have entered clinical trials, and early studies have shown resounding clinical results (16–18).

Given the success of immune-checkpoint regulator blockade in improving both endogenous and vaccine-elicited antitumor immune responses, identification of additional negative checkpoint regulator pathways would likely have important therapeutic implications. We have recently discovered a novel immunoglobulin (Ig) superfamly ligand, designated V-domain Ig suppressor of T-cell activation (VISTA; Genbank: JN602184; ref. 19). VISTA bears homology to PD-L1 but displays a distinct expression pattern. Within the hematopoietic compartment, VISTA is constitutively and highly expressed on CD11b+ myeloid cells, and expressed at lower levels on CD4+ and CD8+ T cells and Foxp3+ Tregs. The human and murine homologs share 90% homology, have indistinguishable functional properties, and are similar in their lineage restricted expression (20). VISTA expressed on APCs directly suppresses CD4+ and CD8+ T-cell proliferation and cytokine production (19).
We hypothesize that VISTA is a novel negative checkpoint regulator and a promising new target for cancer immunotherapy. This study has utilized a VISTA-specific blocking mAb to examine the role of VISTA in regulating antitumor immunity. Our data show that VISTA mAb treatment impaired the suppressive character of the tumor microenvironment (TME) and enhanced protective antitumor immunity. Furthermore, initial studies exploring a combination regimen of VISTA blockade together with a peptide vaccine show synergistic efficacy. As such, our study establishes a foundation for designing optimal therapeutic approaches that incorporate VISTA blockade either as a monotherapy or in combination with additional immune-targeted therapies.

Materials and Methods

Mice

C57BL/6 mice were from NCI. TRP1 and OTII CD4 transgenic mice were from the Jackson Laboratory. FoxP3-GFP reporter mice were as described (21) and were generously provided by Dr. Alexander Rudensky (University of Washington School of Medicine, Seattle, WA). The triple transgenic mouse strain B6.Cg-BrafCA/+; Ptenlox5/lox5 Tg(Tyr::Cre/ERT2) was obtained from Dr. Bosenberg (Yale School of Medicine, New Haven, CT). All animals were maintained in a pathogen-free facility at Dartmouth Medical School. All animal protocols were Institutional Animal Care and Use Committee approved at the Dartmouth College.

Tumor models, tumor vaccine, and treatment

MB49 (300,000), B16OVA (120,000), and B16BL6 (18,000) tumor cells were inoculated on the right back. Tumor vaccine containing tumor cells were inoculated on the right side of the mice with additional immune-targeted therapies. VISTA blockade either as a monotherapy or in combination with other therapies was examined in the B16OVA model. Despite the apparent immunogenicity and short half-life of 13F3 in vivo, 13F3 treatment significantly suppressed tumor growth in the B16OVA model (Fig. 1A). Increased number of IFN-γ–producing cells in the tumor-draining lymph node was detected by ELISPOT assay in response to irradiated tumor cells, indicating that VISTA mAb treatment enhanced tumor-specific T-cell responses. Even though we cannot detect any expression of VISTA on nonhematopoietic cells, we examined in vitro cultured tumor cells to exclude the possibility that VISTA mAb directly affected tumor cell proliferation and apoptosis (Supplementary Fig. S2).

Flow cytometry and analysis

Flow cytometry analysis was performed either on FACSCAN using CellQuest software (BD Biosciences) or on MACSQuant 7 color analyzer (Miltenyi). Data analysis was performed using FlowJo software (Treestar).

Graphs and statistical analysis

All graphs and statistical analysis were generated using Prism 4 (GraphPad Software, Inc.). A Student t test (two-tailed) was used for the data analyses.

Materials and Methods are described in detail in the Supplementary Data.

Results

VISTA mAb treatment impairs tumor growth in immunogenic transplantable tumor models

We have previously generated a hamster monoclonal antibody (clone 13F3) that neutralizes the suppressive activity of VISTA (19). We hypothesized that VISTA mAb-mediated blockade would enhance antitumor immune responses. This hypothesis was tested in murine tumor models. We first examined the immunogenicity and in vivo clearance of 13F3 (Supplementary Fig. S1). Our data show that mice developed strong immune response against 13F3 and accumulated high levels neutralizing antibodies, which presumably leads to fast clearance of 13F3. In fact, after a week of continuous treatment, we can no longer detect any 13F3 in the serum when blood was analyzed 24 hours after each injection. Our data indicates that the most effective window of 13F3-mediated VISTA blockade in vivo might be within the first week of treatment.

Next, we examined the impact of VISTA mAb treatment in mice bearing melanoma B16OVA, which expresses the chicken ovalbumin as a neo tumor antigen. Despite the apparent immunogenicity and short half-life of 13F3 in vivo, 13F3 treatment significantly suppressed tumor growth in the B16OVA model (Fig. 1A). Increased number of IFN-γ–producing cells in the tumor-draining lymph node was detected by ELISPOT assay in response to irradiated tumor cells, indicating that VISTA mAb treatment enhanced tumor-specific T-cell responses. Even though we cannot detect any expression of VISTA on nonhematopoietic cells, we examined in vitro cultured tumor cells to exclude the possibility that VISTA mAb directly affected tumor cell proliferation and apoptosis (Supplementary Fig. S2).
T-cell populations (Fig. 1F–H). VISTA mAb administration enhanced the proliferation and the effector function of tumor-infiltrating CD8⁺ T cells, as evidenced by increased number of Ki67⁺ cells, enhanced effector molecule production (i.e., IFN-γ and granzyme B), and CD107 mobilization (Fig. 1F–H and Supplementary Fig. S1). There are no significant alterations of myeloid cells and T-cell lineages in the spleen or tumor-draining lymph nodes upon VISTA mAb treatment (data not shown), indicating that VISTA mAb did not induce antibody-mediated deletion of VISTA⁺ cells. VISTA blockade therefore appears to act predominantly on altering the TME by enhancing the frequency of tumor-infiltrating effector T cells as well as their effector functions, resulting in enhanced control of tumor growth.

Similar analysis was performed in an immunogenic bladder tumor model MB49 (27). Consistent with that observed in the melanoma model, VISTA mAb treatment significantly suppressed MB49 tumor growth (Fig. 2A). Similar to the B16OVA model, VISTA is highly expressed on tumor-infiltrating myeloid cell populations (Fig. 2B). Unlike the B16OVA tumors, VISTA mAb treatment in MB49 tumors did not reduce the relative percentage of MDCS within the CD45⁺ TILs. Further analysis indicated that more than 90% of the MDCS populations infiltrating MB49 tumors were of the granulocytic phenotype (CD11b⁺Gr1⁺Ly6G⁻Ly6C⁻), which is in contrast to the predominant monocytic MDCS populations (CD11b⁺Gr1⁻Ly6G⁻Ly6C⁺) infiltrating B16 melanoma (Supplementary Fig. S3). Despite the unaltered presence of MDCSs, VISTA mAb enhanced the activation status of tumor-associated CD11c⁺ DCs, which showed higher expression of MCHII and CD80, and higher production of interleukin (IL)-12 and TNF-α (Fig. 2D). In αVISTA-treated mice, tumor-specific T-cell responses were significantly enhanced, both in the tumor-draining lymph node and within the tumor tissue (Fig. 2).

Because both B16OVA and MB49 tumors express neontigenic markers that elicit strong immune responses, we sought to evaluate whether VISTA blockade might impact on the growth of poorly immunogenic tumors, such as the B16B6L6 melanoma. VISTA mAb in this model treatment significantly delayed tumor growth (Fig. 3A). Similar to the B16OVA model, reduction of tumor-infiltrating monocyteic MDCSs and increase of tumor-infiltrating CD4⁺ and CD8⁺ T cells were observed upon VISTA mAb treatment (Fig. 3B). To determine whether VISTA mAb could directly promote T-cell infiltration to tumor tissues, we tracked the tumor-infiltration of TRP1 TCR transgenic CD4⁺ T cells that are specific for the melanoma antigen tyrosinase-related protein-1 (TRP1), in B16 tumor-bearing mice. Our data show that VISTA mAb treatment significantly enhanced tumor infiltration of TRP1 transgenic CD4⁺ T cells (Fig. 3C). Phenotyped analysis of tumor-infiltrating polyclonal populations of CD4⁺ and CD8⁺ T cells demonstrated heightened activation status upon VISTA blockade, which was evidenced by enhanced Ki67⁺ cells and heightened frequency of cells bearing a CD44⁺CD62L⁻ surface phenotype (Fig. 3D). When restimulated with BMDCs pulsed with tumor antigens in vitro, TIL CD4⁺ and CD8⁺ T cells produced enhanced levels of effector molecules (IFN-γ and/or granzyme B; Fig. 3E). Consistent with the MB49 model, tumor-infiltrating CD11c⁺ DCs expressed higher level of MCHII and CD80, as well as inflammatory cytokines IL-12 and TNF-α (Fig. 3F). Taken together, these results demonstrated the ability of VISTA mAb to alter the suppressive signature of the TME and promote tumor-specific effector T-cell function, which likely contributed to reduced tumor growth.

**VISTA regulates the induction of Foxp3⁺ iTregs from naïve CD4⁺ T cells as well as the suppressive activity of nTregs**

Our previous study showed that VISTA-Ig fusion protein directly suppressed T-cell activation by inhibiting T-cell proliferative responses and cytokine production (19). Our current data show that VISTA-Ig promoted the induction of Foxp3⁺ CD4⁺ regulatory T cells (iTregs) in the presence of TGF-β in vitro (Fig. 4C). This effect is also seen on human CD4⁺ T cells treated with human-VISTA-Ig fusion protein in vitro (manuscript submitted). Tregs induced by VISTA-Ig obtained similar suppressive activity in vitro when compared with control iTregs (Fig. 4C). To validate the role of VISTA on promoting the differentiation of iTregs, we examined the effect of VISTA mAb treatment on the induction of tumor-specific iTregs, as previously described (9). Naïve OVA-specific OTII CD4⁺ T cells (purified from Foxp3GFP reporter mice as CD25⁺CD62L⁺ Foxp3GFP⁺) were adoptively transferred into sublethally irradiated host bearing the B16OVA tumor. Mice were treated with either control-Ig or VISTA mAb. The induction of Foxp3GFP⁺ OTII iTreg cells in the tumor-draining lymph node and within the tumor tissue was examined when tumors reached >8 mm diameter (approximately day 20). As shown in Fig. 4D, VISTA blockade significantly diminished the percentage of Foxp3GFP⁺ iTregs within the tumor-infiltrating OTII transgenic CD4⁺ T-cell population (from 61.51% ± 5.71% to 35.75% ± 7.09%). Similar reduction was seen in the tumor-draining lymph node (from 3.51% ± 0.49% to 1.75% ± 0.25%).

VISTA expression level on tumor-infiltrating Tregs is higher than Tregs from peripheral lymph nodes (Fig. 4B), indicating that VISTA expressed on Tregs within the TME might play a role in suppressing tumor-specific immunity. Studies were developed to functionally assess whether VISTA mAb-mediated blockade impaired the suppressive function of Tregs. Thymus-derived natural Tregs (nTregs) contain different subsets that are distinguished by surface markers. Our analysis show that VISTA is more highly expressed on CD62L⁻ and ICOS⁻ Treg subsets, when compared with CD62L⁺ and ICOS⁺ Treg subsets (Fig. 4A; refs. 28, 29). No difference of VISTA expression was observed on other Treg subsets based on surface markers such as CD25, GITR, CD73, Folate receptor 4, and IL-7 receptor (data not shown). Treg subsets were sorted from naïve mice based on markers ICOS and CD62L, and tested for their suppressive activity in vitro. VISTA mAb enhanced naïve target T-cell proliferation in the presence of all the Treg subsets tested. This data indicates that VISTA blockade might directly impair the suppressive activity of Tregs. Notably, VISTA mAb also enhanced T-cell proliferation in the absence of Tregs, indicating an alternative possibility that VISTA
Figure 1. VISTA mAb treatment suppressed tumor growth and altered the tumor microenvironment in B16OVA melanoma model. A, B16OVA tumor-bearing mice (n = 8 for control group; n = 10 for 13F3 group) were treated with anti-VISTA mab (13F3) or control-Ig starting from day 0, every 2 days throughout the duration of the experiment. Tumor size was measured with a caliper. IFN-γ ELISPOT was performed using tumor-draining lymph node cells on day 14. Cells were restimulated in vitro with irradiated tumor cells for 20 hours. IFN-γ-producing cells were visualized and counted. B and C, cells were harvested from peripheral lymph node, tumor-draining lymph node, and tumor tissues around day 20 when tumors reached approximately 8–10 mm diameter. VISTA expression on myeloid-derived suppressor cells (MDSC, CD11bhi CD11c−/Gr1+ ) and myeloid DCs (CD11b+ CD11c+ ), and a comparison of VISTA and PD-L1 expression on tumor cells and tumor-infiltrating leukocytes (TIL) was shown by flow cytometry. D, VISTA expression within B16 melanoma tumor tissue was examined by immunofluorescence. 4′, 6-diamidino-2-phenylindole (DAPI), blue; Cd11b, green; VISTA or control-IgG, red. (Continued on the following page.)
blockade might enhance the resistance of naïve T cells to Treg-mediated suppression. Either mechanism might ultimately contribute to the therapeutic efficacy of VISTA mAb-mediated blockade in tumor models.

VISTA mAb treatment suppresses tumor progression in a genetic model of melanoma

Encouraged by results seen in the transplantable tumor models, we tested the therapeutic impact of VISTA mAb

(Continued.) E-H, VISTA mAb treatment altered the TME and improved the effector function of tumor-infiltrating CD8+ T cells. B16OVA-bearing mice were treated with 13F3 or control Ig from day 0 until tumors in the control group reached 9–10 mm diameter (day 19). Tumors were harvested and CD45+ TILs were quantified. Tumor-infiltrating DCs were analyzed by flow cytometry for their surface expression of MHCII and CD80 and cytokine production (i.e., IL12p40 and TNF-α). Tumor-infiltrating CD4+ and CD8+ T cells were stimulated with irradiated tumor cells for 20 hours. Cytokine production (i.e., IFN-γ and TNF-α) was analyzed by flow cytometry and quantified. Data are representative of 2–3 independent experiments.
administration in an inducible melanoma model. This model relies on the interbreeding of three transgenic mouse lines (30): (i) BrafCΔ, which carries a conditional BrafV600E allele. (ii) PtencΔ, which carries a conditional allele of Pten, permitting Cre-mediated deletion. (iii) Tyr::CreER2, which carries a 4-hydroxytamoxifen (4-OHT)-inducible CreER2 allele, allowing melanocyte-specific inducible expression of Cre. The triple mutant mice, B6.Cg-BrafCA/CD11C Δ0 CreER2 Tg (Tyr::Cre/ERT2), develop pigmented skin lesions upon topical treatment of 4-OHT within 21 days, which quickly progress to malignant melanoma (Fig. 5A). Analysis of tumor-infiltrating leukocytes reveals high levels of VISTA expression on tumor-associated myeloid cells (Fig. 5B). No VISTA expression was observed on tumor cells, whereas some level of PD-L1 expression was detected (Fig. 5C). Importantly, VISTA mAb treatment significantly delayed tumor progression (Fig. 5A). Further analysis of TIL populations demonstrated similar alterations of TME as shown in the transplantable B16 tumor models, including increased tumor infiltration of T cells, decreased tumor-infiltration of myeloid cells, and enhanced IFN-γ production of tumor-infiltrating CD8 T lymphocytes (Fig. 5D–F).

**VISTA mAb synergizes with a tumor vaccine to achieve optimal therapeutic outcome**

Although VISTA mAb as a single-agent therapy delayed tumor progression, it was not curative under the conditions tested. In an attempt to increase the magnitude of the tumor-specific immune response, a peptide-based cancer vaccine was used in combination with VISTA blockade. Based on previous melanoma vaccine studies from our lab (R.J. Noelle) and others (31–34), we applied a modified vaccine platform containing the...
agonistic CD40 antibody FGK, TLR agonists, and tumor antigen peptides. Both a MHCII-restricted mutant TRP1 peptide and a MHCI-restricted TRP2 peptide were incorporated (22–24). Early therapeutic intervention on 2-day tumors using a combinatorial therapy of αVISTA mAb and a single dose of peptide vaccine effectively eradicated tumors in a majority of mice, whereas either reagent alone only transiently impaired tumor growth without significant survival benefit (Fig. 6A). Combinatorial treatment of 7-day established tumors (with average tumor diameter ~3mm) showed significant suppression of tumor growth and led to approximately 30% long-term survival, whereas monotherapy had little effect (Fig. 6B). A prime-boost vaccine regimen was applied on day +7 and +14 for treating 7-day established tumors, and showed better long-term survival (from 10% to 30%) than a single vaccine dose in this setting (Fig. 6C). Tumor-infiltrating T cells showed synergistically enhanced responses against immunizing peptide, indicating that the development of T-cell–mediated immune responses might be critical for the efficacy of the combination therapy (Fig. 6C). Additional evidence supporting this hypothesis is provided by the loss of therapeutic efficacy upon predepletion of CD8+ T cells, or when treating immune-deficient tumor-bearing Rag-/- hosts (Supplementary Fig. S7). This data is consistent with the mechanisms of action associated with CD40/TLR-based vaccines (31–34). A second tumor challenge of survivors at day +60 post-treatment showed delayed tumor growth (8/12) or no tumor growth (4/12), indicating that certain levels of T-cell memory was developed during the combination therapy (Fig. 6D). The protection rate for the rechallenge did not improve regardless whether a second boost vaccine was introduced during the primary tumor challenge (data not shown). These studies set
the stage for extensive efforts in the future to define successful combination therapies with αVISTA mAb as a platform.

Discussion

Our study introduces a new negative immune-checkpoint regulator, VISTA, whose expression within the TME plays a critical role in regulating protective immunity to cancer. Our data show that αVISTA monotherapy impairs tumor growth in multiple tumor models. We have dissected the multiple mechanisms whereby VISTA mAb-mediated blockade enhances antitumor immune responses.

First, VISTA mAb enhanced tumor-specific T-cell response, both in the periphery and within the TME. VISTA is constitutively highly expressed on myeloid cells, with even higher densities observed within the TME. This data suggests that VISTA may be abundantly present within the TME of any solid tumor types that are infiltrated with myeloid cells and T cells, indicating a broad clinical applicability for VISTA-blockade therapy. The high expression of VISTA on myeloid cells
within the TME suggests that VISTA might directly suppress tumor-infiltrating effector T cells. This hypothesis is supported by enhanced proliferation, activation, and effector function of tumor-infiltrating T cells in VISTA mAb-treated mice (Figs. 1–3). We have not detected VISTA expression on tumor cells investigated. As such, we predict that VISTA targeted immunotherapy will be effective independent of its expression on tumors. Similarly restricted hematopoietic expression of human VISTA has been observed in human melanoma, colorectal cancer, and lung cancer (data not shown). In contrast, PD-L1 is known to be expressed at high levels on nonhematopoietic tumor cells (Fig. 1; refs. 2, 35). It is therefore clinically relevant that VISTA mAb demonstrated efficacy despite the high levels of PD-L1 within the TME. Our future studies will compare the efficacy of VISTA-blockade with PD1 blockade and define synergistic treatment regimens for the remission of established tumors.

Second, VISTA blockade impaired the suppressive effect of natural Tregs and the differentiation of tumor-specific iTregs (Fig. 4). It remains to be determined whether VISTA expression on Tregs directly contributes to their suppressive function, even though VISTA neutralization in vitro failed to show any direct effect on Treg proliferation, apoptosis, and Foxp3 stability on in vitro cultured Tregs (Supplementary Fig. S4). Alternatively, it is possible that VISTA neutralization might enhance naïve T-cell proliferation, making them more resistant to Treg-mediated suppression. Future studies will utilize VISTA KO mice to tease out the underlying mechanisms whereby VISTA regulates the suppressive function of Tregs. In this context, both CTLA-4 and PD-1 impact on either the

Figure 6. VISTA mAb synergized with tumor vaccine to impair the growth of established B16-BL6 tumors. Naïve mice (n = 13 per group for A and n = 8–10 per group for B) were inoculated with B16-BL6 tumor cells (18,000) on the right flank. On day + 2 (A) or day + 7 (B) postinoculations, mice were treated with peptide vaccine complex (1 dose) or anti-VISTA mAb 13F3 (continuous treatment for the entire duration of the experiment) or the combination as indicated. C, to determine whether the combination therapy induced optimal T-cell responses when compared with single treatment, tumor-bearing mice were treated on day + 7 with vaccine or 13F3 or the combination as indicated. Tumor-infiltrating lymphocytes were harvested ~day -21, restimulated with the immunizing peptides TRP1/deltaV-TRP2, and analyzed for their cytokine production (i.e., IFN-γ) by flow cytometry as described in Materials and Methods. D, mice that survived the first B16BL6 tumor challenge (n = 12) after combination treatment were rechallenged with B16BL6 cells (18,000) on the same flank on day + 60 post primary tumor rejection. Naïve mice were used as the control group. Tumor size was measured every 2–3 days with a caliper. Data are representative of 2–3 independent experiments.
suppressive function and/or the peripheral induction of Foxp3\(^+\) Tregs (9, 36–38).

Third, in the B16 melanoma model, VISTA blockade altered the suppressive cellular signature of the TME, by reducing the tumor-infiltrating monocytic MDSCs while increasing the frequency of infiltrating effector T cells. Because there is no apparent deletion of both myeloid lineages and T-cell lineages in the periphery upon VISTA mAb treatment (data not shown), such alterations within the tumor tissue likely reflect either the impaired migration of monocytic MDSCs into tumor tissues, or impaired infiltration of immature myeloid progenitor cells, which might differentiate into MDSCs at tumor site (39, 40). In contrast to melanoma, VISTA blockade did not significantly alter the infiltration of the granulocytic MDSC population within the MB49 bladder tumors. This intriguing result might indicate different mechanisms whereby VISTA regulates monocytic and granulocytic myelopoiesis during tumor development.

In addition to affecting MDSCs, VISTA blockade directly enhanced the migration of tumor-specific effector T cells (i.e., TRP1 Tg CD4\(^+\) T cells) into tumor tissue (Fig. 3). Furthermore, VISTA blockade enhanced the immune-stimulatory phenotype of TIL DCs, which showed higher expression levels of MHCII and CD80, and cytokine production (i.e., IL-12 and TNF-\(\alpha\); Figs. 2 and 3). Collectively, these multiple effects of VISTA mAb treatment facilitate the establishment of an immune-stimulatory TME, which leads to enhanced antitumor immunity.

It is clear that combination immunotherapy for cancer using multiple biologics will be critical for improving the therapeutic outcome. In addition to the monotherapeutic targeting of negative checkpoint regulators, combining multiple checkpoint blockades, or combining checkpoint blockade with chemotherapy or cancer vaccines is moving into clinical trials. Some examples of such combinatorial approaches include CTLA4 blockade combined with GVax or Flt3vax (3, 41), CTLA4 blockade combined with PD-L1/PD1 blockade (42, 43), PD1 blockade combined with vaccine (44, 45). Our initial studies explore a combination regimen using VISTA blockade together with a peptide vaccine (22–24, 33). Our data show promising synergistic effects for treating both 2-day and 7-day established tumors. Future studies will focus on elucidating the cellular and molecular mechanisms of such synergy, as well as determining the limitations of this combination platform and strategies for improvement. For example, the use of defined peptides might lead to loss-of-antigen escape variants (46). The delivery of vaccine component might be optimized through the use of nanoparticles or other particulate formulations or TLR-peptide conjugates (47–49). Additional immune-suppressive pathways (i.e., other immune checkpoint pathways such as PD-1, and immune suppressive cytokines) might be blocked to achieve better long-term tumor-specific memory T-cell responses. Furthermore, the immunogenicity and the FcR binding activity of the VISTA mAb might be critical limiting factors for achieving optimal target neutralization and therapeutic efficacy, thus warrant future studies (50, 51).

Recent breakthroughs using CTLA-4 and PD-1 checkpoint blockade have reinvigorated the field of cancer immunotherapy (2, 35). Multiple checkpoints control the development of antitumor immunity, each with their own unique signature and mechanisms. Our studies provide compelling evidence supporting the role of VISTA as a negative immune checkpoint regulator that controls immunity against cancer. Taken together with the findings that VISTA expression and function on human leukocytes and within the human tumors have recapitulated the murine data (20), we present VISTA as a promising new target for cancer immunotherapy, either as a single target or in combination with other immunotherapeutic strategies.

**Disclosure of Potential Conflicts of Interest**

L. Wang is a consultant/advisory board member of Immunext. J.L. Lines is a consultant/advisory board member of Immunext. M. Day is a research associate and has ownership interest (including patents) in ImmuNext. R.J. Noelle is CSO, has commercial research grant, other commercial research support, ownership interest (including patents), and is a consultant/advisory board member of ImmuNext. No potential conflicts of interest were disclosed by the other authors.

**Authors’ Contributions**

Conception and design: L. Wang, R.J. Noelle

Development of methodology: L. Wang, W. Chen

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): L. Wang, L. LeMercier, J.L. Lines, P. Sergent

Analysis and interpretation of data (e.g., statistical analysis, biospecimens, computational analysis): L. Wang, L. LeMercier, W. Chen, J. Li, R.J. Noelle

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Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): L. Wang, M. Day, J. Li, P. Sergent

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