Immune-Checkpoint Protein VISTA Regulates Antitumor Immunity by Controlling Myeloid Cell–Mediated Inflammation and Immunosuppression

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Abstract

Immune-checkpoint protein V-domain immunoglobulin suppressor of T-cell activation (VISTA) controls antitumor immunity and is a valuable target for cancer immunotherapy. This study identified a role of VISTA in regulating Toll-like receptor (TLR) signaling in myeloid cells and controlling myeloid cell–mediated inflammation and immunosuppression. VISTA modulated the polyubiquitination and protein expression of TRAF6. Consequently, VISTA dampened TLR-mediated activation of MAPK/AP-1 and IKK/NF-κB signaling cascades. At cellular levels, VISTA regulated the effector functions of myeloid-derived suppressor cells and tolerogenic dendritic cell (DC) subsets. Blocking VISTA augmented their ability to produce proinflammatory mediators and diminished their T-cell–suppressive functions. These myeloid cell–dependent effects resulted in a stimulatory tumor microenvironment that promoted T-cell infiltration and activation. We conclude that VISTA is a critical myeloid cell–intrinsic immune-checkpoint protein and that the reprogramming of tolerogenic myeloid cells following VISTA blockade promotes the development of T-cell–mediated antitumor immunity.

Introduction

The B7 family of immune-checkpoint receptors are valuable targets for cancer immunotherapy. Antibodies targeting two B7 family coinhibitory receptors, CTLA-4 and PD-1, have elicited durable clinical outcomes in previously refractory cancer types, and are considered a breakthrough therapy for cancer (1, 2). Despite this success, the response rate following CTLA-4, PD-L1, or PD-1–blocking monoclonal antibody (mAb) therapies is generally less than 30%, indicating that additional nonredundant immune-checkpoint pathways hamper antitumor immunity (2).

VISTA (gene Vsir, RIKEN cDNA 4632428N05, also known as GI24, Dies-1, PD-1H, and DD1et) stands for V-domain immunoglobulin suppressor of T-cell activation and is an inhibitory B7 family immune-checkpoint molecule (3, 4). VISTA is constitutively expressed on multiple immune cell types such as CD11b+ myeloid cells, naïve CD4+ and CD8+ T cells, Foxp3+ CD4+ regulatory T cells, and TCRγδ T cells. Studies from our group and others have shown that VISTA controls T cell–mediated autoimmunity and antitumor immunity (3, 5). Although other immune-checkpoint regulators such as CTLA-4 and PD-L1/PD-1 control T-cell activation by recruiting SHP1/2, which antagonizes proximal T-cell receptor (TCR) signaling (6, 7), VISTA plays a broader role in regulating both myeloid cell–mediated and T cell–mediated immune responses (4). Our previous studies in a murine model of psoriasisiform inflammation show that VISTA inhibits TLR7–induced IL23 production in myeloid dendritic cells (DC) and dampens the IL17 production in TCRγδ T cells (8). VISTA expressed on myeloid antigen-presenting cells (APC) engages an unidentified inhibitory receptor on CD4+ and CD8+ T cells and suppresses their proliferation and cytokine production (3). VISTA expressed on CD4+ T cells also limits T-cell activation in a T cell–intrinsic manner (9).

VISTA knockout (KO, Vsir−/−) mice demonstrate the loss of T cell–mediated peripheral tolerance and develop inflammatory phenotypes in multiple organs (10). When bred onto an autoimmune-prone background, VISTA deficiency accelerates disease development in the experimental autoimmune encephalomyelitis model of multiple sclerosis and the ske1−/−ske3 model of lupus (10, 11).

The critical role of VISTA in regulating antitumor immunity has been demonstrated by genetic deletion of VISTA gene (Vsir) or treatment with a VISTA-blocking mAb (4, 10, 12, 13). Blocking VISTA deters tumor growth in multiple preclinical...
models (10, 12), and use of a VISTA-blocking mAb further synergizes with either the PD-L1-blocking mAb or a Toll-like receptor (TLR) agonistic peptide vaccine and CD40-specific agonistic mAb to optimally control tumor growth and achieve long-term survival (12, 13). The mechanism of synergy has been attributed to the role of VISTA in directly inhibiting TCR signaling and T-cell activation (13). Whether or not VISTA regulates the activation of myeloid cells that indirectly control tumor-specific T-cell responses has not been elucidated.

This study uncovered a function of VISTA in regulating the TLR/TRAF6-mediated signaling axis and the effector function in myeloid cells. Blocking VISTA together with a TLR-agonistic vaccine abolished the suppressive functions of myeloid-derived suppressor cells (MDSC) and tumorigenic DCs and augmented the production of proinflammatory cytokines. These effects collectively led to a T cell–permissive tumor microenvironment (TME) that facilitated tumor rejection. The reprogramming of tumor-associated myeloid cells contributes to the protective antitumor immunity following VISTA inhibition.

Materials and Methods

Mice

C57BL/6 (wild-type, WT) mice were purchased from The Charles River Laboratories. Vsr/$^+$/- mice on a fully backcrossed C57BL/6 background were obtained from Mutant Mouse Regional Resource Centers (www.mmrc.org; stock # 031656-UCD; refs. 10, 13). Myd88$^/-$ mice were purchased from The Jackson Laboratory. OT-1 CD8$^{+}$ TCR transgenic mice were purchased from Jax (stock # 003831). Animals were maintained in a specific pathogen-free facility at the Medical College of Wisconsin, Milwaukee, WI. All animal protocols were approved by the institutional animal care and use committee of the Medical College of Wisconsin. All methods were performed in accordance with the relevant guidelines and regulations.

Cell lines

B16-BL6 murine melanoma cells were cultured in RPMI-1640 supplemented with 10% FBS, 0.1% β-mercaptoethanol, and penicillin (100 U/mL)/streptomycin (100 μg/mL). B16-BL6 (female origin) was originally obtained from I. Fidler (The University of Texas MD Anderson Cancer Center, Houston, TX) and were verified by in vivo growth in syngeneic mice and their expression of melanoma antigens TRP1, TRP2, and gp100 but have not been authenticated further by other methods. Murine thymoma EG7 (female origin), human THP-1 (male origin) monocytes, and human HEK293T cells were authenticated and obtained from ATCC and were cultured in the same RPMI-1640 media as above. For HEK293T cells that were transfected with HA-tagged mutant ubiquitin and VISTA-expressing or control vectors, cells were lyzed in RIPA buffer (50 mmol/L Tris pH 7.4, 150 mmol/L NaCl, 1% NP-40, 0.5% deoxycholic acid, 0.1% SDS, 1 mmol/L EDTA, 1 mmol/L EGTA, 5 mmol/L sodium pyrophosphate, 50 mmol/L NaF, 10 mmol/L β-glycerophosphate) supplemented with 1 x Halt Protease Inhibitor Cocktail (cat. # 78442, Thermo Fisher Scientific) at indicated time points. Phosphorylated and total levels of proteins including Jnk1, Jnk2, Erk1, Erk2, p38, IκB, and 30 minutes. When indicated, cells were pretreated with MG132 (10 μg/mL; Sigma-Aldrich) or DMSO vehicle control for 30 minutes before TLR stimulation. Culture supernatants were harvested and examined by ELISA.

For denaturing immunoprecipitation (IP) of TRAF6, WT or Vsr/$^+$/- macrophages were lysed in lysis buffer (1% NP-40, 50 mmol/L Tris-HCl pH 7.5, 150 mmol/L NaCl, 5 mmol/L EDTA) containing 1% SDS and denatured for heating for 5 minutes. Lysates (200 μg) were diluted with SDS-free lysis buffer until the concentration of SDS reached 0.1%. TRAF6 was immunoprecipitated using a mouse mAb (4 μg for 200 μg lysates; sc-8409) from Santa Cruz Biotechnology and 20 μL protein G agarose beads (MilliporeSigma). Bound proteins were eluted using 1 x SDS sample buffer and analyzed by Western blotting as described below. For HEK293T cells that were transfected with HA-tagged wild-type ubiquitin and VISTA-expressing or control vectors, cells were stimulated with R848 (10 μg/mL) for 15 minutes before lysed in the denaturing IP buffer, and TRAF6 was immunoprecipitated as described above.

Electrophoretic mobility shift assays (EMSA) were used to measure the DNA binding activity of the transcription factors AP-1 and NF-κB, using radiolabeled DNA probes as previously described (14). Briefly, WT or Vsr/$^+$/- macrophages (2 x 10$^5$ to 3 x 10$^6$ cells) were lysed in 50 μL buffer containing 20 mmol/L HEPES pH 7.9, 350 mmol/L NaCl, 1 mmol/L MgCl$_2$, 0.5 mmol/L EDTA, 0.5 mmol/L DTT, 20% glycerol, and 1% NP-40. Cell lysates (3 μL) were incubated with [32P]labeled NF-κB probe (cat. # SC-2505) or AP-1 probe (cat. # SC-2501; 25,000 cpm per reaction; Santa Cruz Biotechnology) for 15 minutes at room temperature and then resolved on a 4% polyacrylamide gel.

Real-time quantitative RT-PCR

Single-cell suspensions of tumor-draining lymph nodes (LN) were obtained by passing cells through 70-μm cell strainers. For
peritoneal macrophages, cells (1–2 million) were stimulated with Cpg (1 μg/mL) or R848 (5 μg/mL) for 3 hours before RNA isolation. Total RNA of LN cells or macrophages was prepared using the RNeasy Kit (QIAGEN). One microgram of total RNA was reverse transcribed in 50 μL of TaqMan reverse transcription reagents (Applied Biosystems) using random hexamer primers and according to the manufacturer’s instructions. Ten nanogram cDNA and 1 μmol/L primers were used in a 20 μL PCR reaction with SYBR Green Supermix (Bio-Rad). PCR reaction was performed in the Bio-Rad iQ5 Real-Time PCR System (Bio-Rad). Each sample has triplicate replication, and the 2^-ΔΔCt method was used to determine gene expression. Expression of the target genes was normalized to 18s and displayed as fold change relative to the WT sample. Primers were purchased from Integrated DNA Technologies.


Western blotting

For Western blotting, antibodies specific for K63-linked and K48-linked polyubiquitin chains, p-Erk1/2 (Thr202/Tyr204), Erk1, p-IκB (β), IκKα/β, p-NF-κBp65, NF-κBp65, IkBα, TRAF6, pERK1 (Thr185/187), and TAK1 were purchased from Cell Signaling Technology. Antibodies specific for actin were purchased from Santa Cruz Biotechnology. IFN-γ, TNF-α, and TNFα were harvested after 24 hours. Secreted cytokines (IL12p40, IL6, and MIP-1β) were measured by ELISA as described above. Cytokines and chemokines in the supernatant were detected by ELISA as described above. For detecting cytokines in blood, heparinized blood samples were harvested via cardiac puncture and centrifuged at 2,500 rpm for 10 minutes. Plasma was collected and also examined by ELISA.

Examination of tumor-associated myeloid cells

Thirty to 40 B16-BL6 tumors (7–8 mm in diameter) were harvested from untreated tumor-bearing C57BL6 WT mice. Tumors were pooled, cut into smaller pieces, and digested for 20 minutes at 37° C in RPMI buffer containing Liberase TL (150 μg/mL) and Dnase I (120 μg/mL; Sigma-Aldrich). Single-cell suspensions were generated by passing cells through 70-μm cell strainers. Myeloid cells, including polymorphonuclear MDSCs (PMN-MDSC; CD11b^+CD11c^-Ly6C^hiLy6G^-), monocytic MDSCs (MDSC-M; CD11b^+CD11c^-Ly6C^hiLy6G^-), myeloid conventional DCs (cDC; CD11b^-CD11c^-Ly6C^-Ly6G^+), inflammatory DCs (Inf-DC; CD11b^-CD11c^-Ly6C^-Ly6G^-FcγRi^+), and CD103^- DCs (CD103^-CD11c^-Ly6C^-Ly6G^-F4/80^-) were purified from dissociated tumor tissues by FACS using BD FACSaria II with 99% purity. To test TLR-mediated cytokine production, purified myeloid cell subsets (10,000 cells) were stimulated with TLR agonists Cpg (1 μg/mL) in the presence of VISTA-blocking mAb (20 μg/mL) or isotype-control IgG (20 μg/mL) as indicated. Culture supernatants were harvested after 24 hours. Secreted cytokines (IL12p40, IL6, and TNFα) were quantified by ELISA as described above. For testing the T cell–suppressive function of MDSCs, sorted myeloid cDCs and Inf-DCs (10,000) were cocultured with 30,000 culture supernatants from MDSC suppression assays were diluted 50-fold. All dilutions were using PBS with 2% BSA as diluent.

ELISAs

For ELISA analyses, culture supernatants from peritoneal macrophages were diluted 5-fold, homogenized tumor tissues were diluted 5-fold, plasma samples were diluted 5-fold, and
whole splenocytes from OT-I CD8\(^+\) TCR transgenic mice together with ovalbumin peptide 257–264 (1 \(\mu\)g/mL, GenScript). Cpg (1 \(\mu\)g/mL), and VISTA-blocking mAb or control IgG (20 \(\mu\)g/mL) as indicated. Culture supernatants were harvested after 48 hours, and secreted IFN\(\gamma\) was quantified by ELISA. To test the T cell–stimulatory function of CD103\(^+\) DCs, sorted CD103\(^+\) DCs (10,000) were cocultured with 30,000 purified OT-I CD8\(^+\) T cells, ovalbumin peptide 257–264 (1 \(\mu\)g/mL), Cpg (1 \(\mu\)g/mL), and VISTA-blocking mAb or control IgG (20 \(\mu\)g/mL) as indicated. Culture supernatants were harvested after 48 hours, and secreted IFN\(\gamma\) was quantified by ELISA.

Flow cytometry and data analysis
For flow cytometry, antibodies specific for CD4 (GK1.5), CD8 (53-6.7), CD16/CD32, and IFN\(\gamma\) (XMG1.2) were purchased from BioLegend. Single-cell suspension tumor tissues and spleens were stained with lineage-specific antibodies (CD4, CD8, CD11b, CD11c, Ly6C, Ly6G, MHCIIF, F4/80, and FcRlI) to identify CD4\(^+\) and CD8\(^+\) T cells and myeloid cell populations. To detect intracellular cytokines in T cells, single-cell suspensions of tumors tissues (2–3 million) were stimulated for 5 hours in RPMI medium containing phorbol 12-myristate 13-acetate (PMA; 50 \(\mu\)g/mL), ionomycin (1 \(\mu\)g/mL), 10% FBS, 2 mM L-glutamine, 50 \(\mu\)mol/L 2-mercaptoethanol, 1% penicillin-streptavidin, 1 \(\times\) monensin, and 1 \(\times\) Brefeldin A (BioLegend). Cells were stained with the LIVE/DEAD Fixable Violet Dead Cell Stain Kit first (Thermo Fisher Scientific), then stained with T-cell–lineage markers in the presence of pan-CD16/CD32 blocking antibody before fixation with 1% paraformaldehyde, and then permeabilized with 0.5% saponin and stained for intracellular IFN\(\gamma\). Cells were analyzed on an LSR II flow cytometer (BD Biosciences). Data were analyzed with FlowJo version 9.9.6 analysis software (Tree Star).

Graphs and statistical analysis
All graphs and statistical analysis were generated using Prism 7 (GraphPad Software). Unpaired two-tailed t tests were used for comparing WT and Vsir\(^{-/-}\) cells or mice, or comparing treatments using VISTA-blocking mAb or control IgG. Survival differences of tumor-bearing mice were assessed using Kaplan–Meier curves and analyzed by log-rank testing. A P value less than 0.05 was considered as statistically significant. *, P < 0.05; **, P < 0.025; ***, P < 0.005; ****, P < 0.0001.

Results
Absence of VISTA augments TLR-mediated proinflammatory cytokine production
TLR stimulation initiates MyD88- or TRIF-dependent signaling pathways to induce the expression of proinflammatory cytokines, chemokines, and type I interferons (17). Owing to the high expression of multiple TLRs on peritoneal macrophages, we chose this cellular system as a model to dissect the role of VISTA in regulating TLR-mediated signaling pathways. Peritoneal macrophages were isolated from WT and Vsir\(^{-/-}\) mice following thioglycollate treatment and stimulated ex vivo with TLR agonists Cpg (TLR9) and R848 (TLR7; Fig. 1A–C). The expression of cytokines IL12 and IL6 was examined by real-time quantitative RT-PCR (qRT-PCR) and ELISA. Cpg and R848 significantly upregulated gene expression and protein production of IL12 and IL6 in Vsir\(^{-/-}\) macrophages (Fig. 1A and B). The expression of multiple cytokines and chemokines, including GM-CSF, IFN\(\gamma\), MIP-1\(\alpha\), and MIP-1\(\beta\), was also significantly increased in Vsir\(^{-/-}\) macrophages (Fig. 1C). In addition to Cpg and R848, Vsir\(^{-/-}\) macrophages were hyperresponsive toward additional TLR agonists, including Pam3CSK4 (TLR2), LPS (TLR4), and poly (I:C) (TLR3; Fig. 1D).

Although the results above indicated that VISTA directly regulated TLR-mediated signaling, we wanted to assess if cell-extrinsic factors such as TNF\(\alpha\) or additional soluble factors may have contributed to the hyperresponses of Vsir\(^{-/-}\) cells in an autocrine manner. We first tested a TNFR-specific neutralizing mAb and found minimal effects (Supplementary Fig. S1A). Next, we harvested supernatants from Cpg-treated WT and Vsir\(^{-/-}\) cell cultures (designated as WT and KO sups, respectively) and tested their effects on freshly isolated peritoneal macrophages (Supplementary Fig. S1B). The residual Cpg in the supernatants directly stimulated cells, and adding Cpg to the culture further boosted cytokine production. Under all tested conditions, WT and KO supernatants stimulated cells to similar levels. These data support the conclusion that the hyperresponsiveness in Vsir\(^{-/-}\) macrophages was due to cell-intrinsic TLR signaling rather than secondary responses to soluble factors.

VISTA inhibits the activation of MAP kinases and NF-\(\kappa\)B signaling cascades
Transcription factors AP-1 and NF-\(\kappa\)B are critical for the gene expression of many inflammatory cytokines and chemokines (18). Previous studies have established the role of MAP kinases (MAPK Erk1/2, Jnk1/2, and p38) in the activation of AP-1 (19, 20). The activation of NF-\(\kappa\)B requires the phosphorylation of IkB\(\kappa\)B and degradation of the inhibitor IkB\(\kappa\)B (21). We, therefore, examined the activation of MAPKs/AP-1 and IKK/NF-\(\kappa\)B signaling cascades following TLR stimulation. Cell lysates from WT and Vsir\(^{-/-}\) macrophages were stimulated with Cpg or R848. Phosphorylation of MAPKs and IKK\(\beta\) was examined by Western blotting. Both Cpg (Fig. 2A) and R848 (Fig. 2B) induced greater phosphorylation of Erk1/2, Jnk1/2, and p38 in Vsir\(^{-/-}\) macrophages. Phosphorylation of IKK\(\beta\) and degradation of IkB\(\kappa\) in Vsir\(^{-/-}\) macrophages following Cpg stimulation but were not significantly increased following R848 stimulation.

Next, we carried out EMSAs to measure the DNA binding activity of AP-1 and NF-\(\kappa\)B. As shown in Fig. 2C, Cpg stimulation induced higher activation of AP-1 and NF-\(\kappa\)B in Vsir\(^{-/-}\) macrophages than in WT cells. These results indicate that VISTA inhibited TLR-mediated activation of MAPKs/AP-1 and IKK/NF-\(\kappa\)B signaling cascades.

Previous studies show that Vsir\(^{-/-}\) mice develop systemic tissue inflammation (10). It is possible that Vsir\(^{-/-}\) macrophages were partially activated due to exposure to the existing tissue inflammation. To rule out this possibility and validate the cell-intrinsic role of VISTA, we examined the effects of VISTA overexpression in THP-1 human monocyte cells. VISTA\(^+\) and control THP-1 cells were stimulated with TLR2 agonist Pam3CSK4 (10 \(\mu\)g/mL) for the indicated time, and total cell lysates were harvested and analyzed by Western blotting (Fig. 3). VISTA expression in THP-1 cells suppressed the phosphorylation of MAPKs (Jnk1/2, Erk1/2, and p38; Fig. 3A and C), the phosphorylation of IKK\(\beta\) and NF-\(\kappa\)B p65, and the degradation of IkB\(\kappa\) (Fig. 3B and C) following TLR2 stimulation.
VISTA controls the activation of MAPKs and NF-κB by regulating TRAF6

TLRs utilize either MyD88-dependent (TLR2/4/5/7/8/9) or TRIF-dependent (TLR3/4) signaling pathways [22]. Following TLR2/4/5/7/8/9 engagement, the MyD88/IRAK1/4 complex recruits and activates the RING-domain E3 ubiquitin ligase TRAF6, which is required for the activation of MAPKs and NF-κB downstream of TLR3 by binding to TRIF [23]. Because Vsr⁻/⁻ macrophages are hyperresponsive to all TLR agonists (Figs. 1A–C), we hypothesized that VISTA targeted a shared signaling adaptor such as TRAF6 (24). We first examined the total protein level of TRAF6 and observed increased TRAF6 expression in freshly isolated Vsr⁻/⁻ macrophages compared with WT cells (Fig. 4A). Because K48-linked polyubiquitination could target proteins to proteasomes for degradation and K63-linked polyubiquitination activates TRAF6 (25), we analyzed the ubiquitination status of TRAF6 after immunoprecipitating TRAF6 under denaturing conditions (Fig. 4B). Western blots showed that VISTA deletion resulted in lower K48-linked polyubiquitination and higher K63-linked polyubiquitination of TRAF6 following CpG stimulation (Fig. 4B).

Next, we utilized HA-tagged mutant ubiquitin containing only K48 (HA-Ub-K48) or K63 (HA-Ub-K63) to determine the effect of transient VISTA expression on TRAF6 ubiquitination. HEK293T cells were transfected with HA-Ub-K48 or HA-Ub-K63, and the effect of VISTA expression on TRAF6 ubiquitination was assessed by Western blotting.

Figure 1.
Genetic deletion of VISTA augments the expression of inflammatory cytokines in macrophages in response to TLR stimulation. A–C, WT and Vsr⁻/⁻ peritoneal macrophages were isolated and pooled from 3 mice of each genotype. Cells were stimulated ex vivo with PBS, R848 (5 μg/mL, TLR7/8), and CpG (1 μg/mL; TLR9). RNA was extracted and supernatants were collected after 3 and 6 hours of culture, respectively. A, Gene expression of Ifi202/p35, Il12p40, and Il6 was examined by real-time quantitative RT-PCR. B, Secreted IL12p40 and IL6 were examined by ELISA. C, Additional cytokines (GM-CSF and IFNγ) and chemokines (MIP-1α and MIP-1β) were also examined by ELISA. D, WT and Vsr⁻/⁻ macrophages were stimulated with TLR agonists LPS (1 μg/mL; TLR4), Pam3Cys4 (Pam3, 10 μg/mL; TLR2), and poly (I:C)(25 μg/mL; TLR3) for 6 hours. Secreted IL6 and IL12p40 were quantified by ELISA. Statistical significance was determined by unpaired two-tailed t-test. Error bars, SEM. *, P < 0.05; **, P < 0.01; †††, P < 0.001; ††††, P < 0.0001. Shown are representative results from at least three independent repeats.
cells were cotransfected with mutant ubiquitin together with a VISTA-expressing vector or empty control vector and analyzed at 24 hours after transfection (Fig. 4C). Transient VISTA expression significantly augmented K48-linked polyubiquitination of TRAF6 and simultaneously decreased K63-linked polyubiquitination levels (Fig. 4C).
The TAK1/TAB1/TAB2 complex is recruited to the MyD88/IRAK/TRAF6 complex by binding to the K63-polyubiquitin chains of TRAF6 and mediates the activation of the Jnk1/2/AP-1 and IKKα/β/NF-κB signaling pathways (25). Consistent with the higher protein level and K63 polyubiquitination of TRAF6, phosphorylation of TAK1 at Thr184/187 in Vsir/C0/C0 macrophages was significantly enhanced compared with WT cells following CpG stimulation (Fig. 4D). Reduced protein expression of TRAF6 and diminished TAK1 activation were also seen in VISTA-expressing THP-1 cells compared with control THP-1 cells following Pam3Cys4 treatment (Fig. 4E and F).

These results prompted us to test whether proteasomal inhibition reversed the inhibitory effects of VISTA. As shown in Fig. 5A, pretreatment with proteasome inhibitor MG132 effectively reversed the inhibitory effects of VISTA in THP-1 cells by rescuing the phosphorylation of Jnk1/2, Erk1/2, p38, and IKKα/β. MG132 pretreatment also enhanced TLR signaling in WT but not VISTA-deficient macrophages (Fig. 5B). These results, therefore, support the conclusion that VISTA downregulates TLR-mediated signaling by promoting TRAF6 protein degradation and restricting the activation of MAPKs/AP-1 and IKKα/β/NF-κB signaling pathways.

Blocking VISTA augments the TLR/MyD88-mediated proinflammatory responses

We have previously shown that treatment with a VISTA-blocking mAb deters tumor growth in preclinical models (12). Our current study indicated that the proinflammatory responses induced by VISTA blockade promote the development of antitumor immunity. Supporting this hypothesis, treatment with the VISTA-blocking mAb led to a significant accumulation of IL12p40, IL12p70, TNFα, and IL27 in the serum of mice (Fig. 6A). This cytokine response was absent in tumor-bearing MyD88 KO hosts (Fig. 6B). Consistent with the lack of cytokine induction, the therapeutic efficacy of the VISTA-blocking mAb was diminished in MyD88 KO hosts (Fig. 6C). These results indicated that blocking VISTA elicited an endogenous TLR/MyD88-mediated proinflammatory response that contributed to the development of antitumor immune responses.

TLR agonists are promising vaccine adjuvants for cancer therapy (26, 27). We hypothesized that VISTA inhibitors would synergize with TLR-agonistic vaccines owing to an augmented proinflammatory response. We tested this hypothesis in the B16-BL6 melanoma model (Fig. 6C). Mice bearing 3-day established B16-BL6 tumors were treated with either VISTA-blocking mAb, or a vaccine containing TLR7/8/9 agonists (CpG and R848)
Figure 4.
VISTA modulates the K48-linked and K63-linked polyubiquitination of TRAF6 and inhibits the activation of TAK1 following TLR stimulation. **A**, WT and Vviar−/− peritoneal macrophages (pooled from 2–3 mice of each genotype) were pretreated with MG132 (10 μg/mL) or vehicle control DMSO for 30 minutes before being stimulated with CpG (1 μg/mL) for 0, 15, and 30 minutes. TRAF6 and actin in total cell lysates were determined by Western blotting. **B**, To examine the ubiquitination status, WT and Vviar−/− peritoneal macrophages (pooled from 2–3 mice of each genotype) were stimulated with CpG (1 μg/mL) for 15 minutes. TRAF6 protein was immunoprecipitated from total lysates under denaturing conditions. Both K63-linked and K48-linked polyubiquitination (pUb) were examined by Western blotting using antibodies specific for K48-linked or K63-linked polyubiquitin chains. **C**, TLR7-expressing HEK293T cells were transfected with HA-tagged mutant ubiquitin HA-Ub-K48 or HA-Ub-K63, together with a VISTA-expressing plasmid or vector control (Ctrl) as indicated. Cells were stimulated with R848 at 24 hours after transfection. TRAF6 was immunoprecipitated as in B and examined by Western blotting (WB) using anti-HA. **D**, WT and Vviar−/− peritoneal macrophages were stimulated with CpG (1 μg/mL) for indicated amount of time. Phosphorylated (p-) and total TAK1 in cell lysates were examined by Western blotting and quantified using ImageJ software. The ratio of pTAK1/TAK1 is shown. **E** and **F**, VISTA-expressing THP-1 cells (THP-1VISTA) and control cells (THP-1Ctrl) were stimulated with Pam3Cys4 (10 μg/mL) for indicated amount of time. TRAF6, actin, pTAK1, and total TAK1 in total cell lysates were examined by Western blotting and quantified using ImageJ. Ratios of TRAF6/actin and pTAK1/TAK1 are shown. Shown are representative results from at least three independent repeats. min, minutes.
and peptides derived from melanoma antigens TRP1 and TRP2, or both. Combining VISTA-blocking mAb with the TLR vaccine resulted in tumor-free long-term survival in ~50% mice, whereas either monotherapy transiently delayed tumor growth without long-lasting effects (Fig. 6C). This stronger therapeutic effect correlated with significantly increased numbers of IFNγ-expressing tumor-infiltrating CD8⁺ and CD4⁺ T cells (Fig. 6D and E).

To dissect the inflammatory TME, we examined the expression of inflammatory mediators within tumor tissues following CpG/R848 treatment (Fig. 6F and H). The VISTA-blocking mAb significantly enhanced the expression of cytokines (IL12p40, IL12p70, IL23p19, IL6, IFNγ, and TNFα) and chemokines (CCL4, CCL5, CXCL9, and CXCL10). To exclude the possibility that this inflammatory response may be due to additional effects of the antibody (i.e., Fc receptor-mediated activation of myeloid cells), we examined WT and Vsir−/− mice proinflammatory responses in the periphery and within the TME, which are critical for the development of antitumor T-cell responses.

### VISTA regulates the effector functions of tumor-associated myeloid cells

Tumor tissues are enriched with immunosuppressive MDSCs and dysfunctional DCs (28, 29). Importantly, VISTA is expressed on tumor-associated myeloid cDCs (CD11b⁺CD11c⁺ MHCII⁺Ly6C⁻), Inf-DCs (CD11b⁺CD11c⁺ MHCII⁺Ly6C⁺Ly6C⁻FceRI⁺), PMN-MDSCs (CD11b⁺CD11c⁺Ly6C⁺Ly6C⁻), and M-MDSCs (CD11b⁺CD11c⁺Ly6C⁺Ly6C⁺), as well as on CD103⁺ DCs (Fig. 7A). This result led us to examine how VISTA directly regulates the effector function of these myeloid cell types in tumor-bearing hosts.

We first analyzed the accumulation of MDSCs and DC subsets in tumor-bearing mice treated with CpG/R848 and VISTA-blocking mAb (Fig. 7B). VISTA inhibition reduced the accumulation of M-MDSCs and PMN-MDSCs and concurrently expanded Inf-DCs within tumor tissues (Fig. 7B). A similar reduction of PMN-MDSCs and expansion of Inf-DCs was observed in the spleen, whereas the number of M-MDSCs in the spleen remained unaltered (Supplementary Fig. S3). The number of CD103⁺ DCs and myeloid cDCs within tumor tissues and spleen was not significantly altered.

Next, we assessed whether VISTA blockade augmented the ability of tumor-associated myeloid cells to produce immunostimulatory cytokines, such as IL12 (30). We purified MDSCs and DC subsets from tumor tissues and examined their cytokine expression following CpG/TLR9 stimulation. Our results showed...
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Figure 6.
TLR/MyD88-mediated inflammation promotes the therapeutic efficacy of VISTA blockade. A, WT or MyD88 KO mice were inoculated with B16-BL6 melanoma cells (200,000) on the flank on day 0. On day +7, mice bearing established tumors (~5 mm in diameter) were treated with VISTA-blocking mAb or isotype control IgG (n = 10/group). Three hours later, serum was harvested and examined by ELISA for cytokines. B, WT and MyD88 KO mice (n = 6–7/group) were inoculated with EG7 thymoma cells (200,000) on the flank on day 0. On day +7, mice bearing established tumors (~5 mm in diameter) were treated with VISTA-blocking mAb or control IgG. Tumor size was measured by a caliper. Shown are representative results from three independent repeats. C-E, Naïve WT mice (n = 10) were inoculated with B16-BL6 melanoma cells (30,000) on day 0. On day +3, tumor-bearing mice were treated with a peptide vaccine containing CpG, R848, and peptides (details in Materials and Methods). Mice were also treated with VISTA-blocking mAb or control IgG every 2 to 3 days. Tumor size was recorded. In a separate group of mice (n = 10), tumor tissues (~6–8 mm in diameter) were harvested. Tumor-infiltrating CD4^+ and CD8^+ T cells were stimulated with anti-CD3. Percentage and number of IFNγ-expressing CD4^+ T cells (D) and CD8^+ T cells (E) were examined by flow cytometry. F and H, To examine the inflammatory TME, we treated B16-BL6 tumor-bearing mice (n = 5–7/group) with the peptide vaccine and VISTA-blocking mAb or control IgG. Tumor tissues were harvested after 3 hours and homogenized. Cytokines (IL12p70, IL12p40, IL23p19, IL6, TNFα, and IFNγ) and chemokines (CCL4, CCL5, CXCL9, and CXCL10) in the homogenates were quantified by ELISA. G and I, B16-BL6 tumors were harvested from WT and VISTA^−/− mice (n = 5–7/group) following treatment with the peptide vaccine as described above. Cytokines and chemokines in tumor tissue homogenates were examined by ELISA. Ctrl, control. Statistical significance of all results was determined by an unpaired two-tailed t test. Error bars, SEM. *, P < 0.05; **, P < 0.01; ††, P < 0.001; †††, P < 0.0001; ns, not significant. Shown are representative results from three independent repeats.
Figure 7.
VISTA regulates the accumulation and effector functions of tumor-associated MDSCs and DC subsets. A, Tumor tissues were harvested from 5 B16-BL6 tumor-bearing mice and pooled. VISTA expression on tumor-infiltrating MDSCs and DC subsets were examined by flow cytometry. B, Mice bearing established B16-BL6 tumors (4–5 mm in diameter) were treated with TLR agonists CpG and R848 together with the VISTA-blocking mAb or isotype control IgG. Numbers of tumor-infiltrating M-MDSCs, PMN-MDSCs, and Inf-DCs were examined by flow cytometry 48 hours after treatment. P values are shown. C, Tumor-associated PMN-MDSCs, M-MDSCs, myeloid cDCs, Inf-DCs, and CD103+ DCs were sorted from pooled B16-BL6 tumor tissues (7–8 mm in diameter) harvested from 20 to 30 tumor-bearing mice. Each type of purified myeloid cell (10,000 cells each) was stimulated ex vivo with CpG (1 μg/mL) in the presence of VISTA-blocking mAb or control IgG. Culture supernatants were collected after 24 hours, and IL12p40 was quantified by ELISA. M-MDSCs (WT and MyD88 KO; D), PMN-MDSCs (E), Inf-DCs (F), and myeloid cDCs (G) were sorted from B16-BL6 tumor tissues (pooled from 20–30 tumor-bearing mice) and cocultured with whole splenocytes from the OT-I transgenic mice in the presence of OVA peptide. CpG and VISTA-blocking mAb or control IgG were added as indicated. Culture supernatants were harvested after 48 hours, and IFNγ was examined by ELISA. H, Tumor-associated CD103+ DCs were sorted from B16-BL6 tumor tissues and incubated with purified naive OT-I CD8+ T cells in the presence of OVA peptide and VISTA-blocking mAb or control IgG. After 48 hours, secreted IFNγ was examined by ELISA. Ctrl, control. Statistical significance of all results was determined by an unpaired two-tailed t test. Error bars represent SEM. *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001; ns, not significant. Results from three independent experiments are shown.
that, with the exception of PMN-MDSCs, VISTA blockade significantly augmented the expression of IL12p40 in M-MDSCs, Inf-DCs, myeloid cDCs, and CD103+ DCs (Fig. 7C). In addition to cytokine production, we sought to determine whether VISTA regulated the immunosuppressive functions of tumor-associated myeloid cells. Tumor-associated MDSCs (Fig. 7D and E), Inf-DCs (Fig. 7F), and myeloid cDCs (Fig. 7G) inhibited IFNγ production by OT-1 CD8+ T cells stimulated with splenic APCs. The VISTA-blocking mAb or TLR stimulation partially reversed the suppressive functions of M-MDSCs, myeloid cDCs, and Inf-DCs, whereas combined treatments maximally abolished suppression (Fig. 7D–F). MyD88 KO M-MDSCs failed to respond to the VISTA-blocking mAb, thus validating the role of MyD88 in mediating the myeloid-intrinsic function of VISTA (Fig. 7D). On the other hand, PMN-MDSCs were not responsive to VISTA inhibition or TLR stimulation (Fig. 7E).

In contrast to the tolerogenic tumor-infiltrating myeloid cDCs and Inf-DCs, Batf3-dependent CD103+ DCs are the most potent cross-presenting DCs that prime CTLs at the tumor site (29,31,32). We showed that VISTA was expressed on CD103+ DCs (Fig. 7A), and treatment with VISTA-blocking mAb significantly augmented IFNγ production from purified OT-1 CD8+ T cells cocultured with CD103+ DCs, whereas CpG treatment had only minimal impact (Fig. 7H). Combining VISTA blockade with CpG treatment led to optimal T-cell activation and IFNγ production (Fig. 7H). Together, these results indicated that blocking VISTA promotes the development of antitumor immunity by reprogramming tumor-associated tolerogenic MDSCs and DCs.

Discussion

Previous studies have shown that VISTA is expressed on myeloid cells at steady state and in tumor-bearing hosts, but the myeloid-intrinsic functions of VISTA have not been elucidated (3,12,33,34). This study demonstrated that VISTA downregulated the TLR/TRAF6/TAK1 signaling pathway by modulating the polyubiquitination and protein expression of TRAF6. At cellular levels, VISTA regulated the effector functions of MDSCs and DC subsets. Targeting VISTA in tumor-associated myeloid cells induced inflammatory cytokines and promoted antitumor T-cell responses.

TLRs expressed on DCs and other APCs maintain antitumor immunosurveillance by recognizing endogenous danger-associated molecular patterns derived from commensal/invasive microbes or necrotic tumors (35,36). Our study showed that in the absence of a TLR-agonistic vaccine, the efficacy of the VISTA-blocking mAb relied upon endogenous TLR/MyD88-mediated signaling and correlated with the production of immune-stimulatory cytokines such as IL12, IL27, and TNFα. Thus, VISTA inhibited antitumor immunosurveillance at the steady state.

Synthetic TLR agonists such as imiquimod (a TLR7 agonist) and PF-3512676 (Cpg ODN for TLR9) are promising vaccine adjuvants (27,37). We showed that in conjunction with the TLR7/8/9 agonistic vaccine, VISTA blockade augmented the expression of chemokines (CXCL9/10 and CCL4/5) and cytokines (INFγ, IL12, IL27, IL23, IL6, and TNFα) within tumor tissues. Intratumor CXCL9/10 and CCL4/5 are critical for recruiting Th1 CD4+ T cells and CD8+ cytotoxic T cells and are associated with better clinical outcomes in various cancer types (38–40). Although IL12, IL27, TNFα, and INFγ play well-established roles in promoting antitumor T-cell responses (30,41,42), IL6 impairs antitumor immunity by inducing STAT3 activation in tumor cells and MDSCs (43). IL23 can both promote and inhibit tumor growth, depending upon the stage of tumor development (44). It remains to be determined whether neutralizing IL6 and IL23 will improve the antitumor efficacy of VISTA inhibitors and reduce immune-related adverse events (irAE).

Tumor tissues are enriched with dysfunctional and immunosuppressive MDSCs and myeloid DCs (29,45). MDSCs utilize diverse mechanisms such as the production of reactive oxygen species, nitric oxide (NO), and the secretion of arginase I, IL10, and TGFβ to inhibit T-cell responses (45). Tumor-associated myeloid DCs impair T-cell activation by expressing PD-L1 or secreting soluble mediators such as galectin-1 (46,47), and the frequencies of circulating MDSCs have been correlated with resistance to ipilimumab therapy in melanoma patients (45,48). Myeloid-inflamed TME profile associates with poor clinical outcomes in pancreatic cancer and head and neck cancer following GVAX vaccine therapy (49).

Therefore, effective strategies that reduce the accumulation and suppressive functions of tumorigenic myeloid cells are urgently needed.

Our study showed that VISTA was an immune-checkpoint protein that regulated the immunosuppressive functions of tumor-associated myeloid cells. Blocking VISTA together with TLR stimulation ablated the suppressive functions of M-MDSCs and myeloid DCs and induced IL12 production. These myeloid cell–dependent effects converted the immunosuppressive TME into a T cell–stimulatory one that may sensitize tumors to additional immunotherapies. Supporting this notion, we have previously shown that blocking VISTA synergizes with PD-L1 inhibitors in murine tumor models (13).

In contrast to M-MDSCs, the suppressive function of PMN-MDSCs was not reversed by the VISTA-blocking mAb despite high VISTA expression on these cells. This result indicated established that tumors enriched with PMN-MDSCs may be resistant to VISTA-targeted inhibitors. Previous studies have identified approaches to reduce the accumulation and suppressive functions of PMN-MDSCs, including treatment with sildenafl (50), gemcitabine (51), inhibitors of DNA methyltransferase or histone deacetylase (52), and PI3Kγ/δ inhibitor (53). However, it remains to be determined if combining VISTA inhibitors with these reagents will maximally block both PMN-MDSCs, M-MDSCs, and other tumorigenic myeloid DCs, thereby achieving optimal therapeutic outcomes.

In conclusion, this study established VISTA as an immune-checkpoint protein that regulates the functions of myeloid cells and established that myeloid cell activation contributes to the overall antitumor mechanisms of VISTA inhibitors. Unlike VISTA, immune-checkpoint proteins CTLA-4 and PD-1 do not directly regulate the function of myeloid cells (28). Targeting VISTA may be a valuable approach for patients resistant to the existing immune-checkpoint inhibitors. Results from this study provide insights for monitoring myeloid cell–dependent inflammatory responses that precede T cell–mediated antitumor responses following VISTA-targeted therapies.
Disclosure of Potential Conflicts of Interest

D. Wang is Adjunct Professor at Medical College of Wisconsin and Fujian Normal University. L. Wang has ownership interest (including stock, patents, etc.) in ImmuNext Inc. No potential conflicts of interest were disclosed by the other authors.

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Immune-Checkpoint Protein VISTA Regulates Myeloid Cells

Acknowledgments

This study was supported by research funding from NCI R01 CA164225 and NCI R01 1R01CA223804 (L. Wang), Advancing A Healthier Wisconsin Research and Education Program (AHW REP) fund (L. Wang), the Office of the Assistant Secretary of Defense for Health Affairs through the Peer Reviewed Cancer Research Program under Award No. W81XWH-14-1-0587 (L. Wang), Worldwide Cancer Research Foundation (UK) research grant 16-1161 (L. Wang), and a Research Scholar Grant, RSG-18-045-01-LIB, from the American Cancer Society (L. Wang). This work is also supported by NIH R01 AI02893 (S. Malarkannan), NCI R01 CA179363 (S. Malarkannan), Nicholas Family Foundation (S. Malarkannan), Gardetto Family (S. Malarkannan and D. Wang), P01 CA 06950 and P30 CA 016056 (M.S. Ernstoff), and NIH grants Al079087 and HL130724 (D. Wang).

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Received July 19, 2018; revised January 4, 2019; accepted July 19, 2019; published first August 1, 2019.
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