Immune Inhibitory Molecules LAG-3 and PD-1 Synergistically Regulate T-cell Function to Promote Tumoral Immune Escape

Seng-Ryong Woo1, Meghan E. Turnis1, Monica V. Goldberg4, Jaishree Bankoti1, Mark Selby8, Christopher J. Nirschl4, Matthew L. Bettini1, David M. Gravano1, Peter Vogel2, Chih Long Liu9, Paul J. Utz9, Creg J. Workman1, Drew M. Pardoll5, Alan J. Korman8, Charles G. Drake4, and Dario A.A. Vignali1

Abstract

Inhibitory receptors on immune cells are pivotal regulators of immune escape in cancer. Among these inhibitory receptors, CTLA-4 (targeted clinically by ipilimumab) serves as a dominant off-switch while other receptors such as PD-1 and LAG-3 seem to serve more subtle rheostat functions. However, the extent of synergy and cooperative interactions between inhibitory pathways in cancer remain largely unexplored. Here, we reveal extensive coexpression of PD-1 and LAG-3 on tumor-infiltrating CD4+ and CD8+ T cells in three distinct transplantable tumors. Dual anti-LAG-3/anti-PD-1 antibody treatment cured most mice of established tumors that were largely resistant to single antibody treatment. Despite minimal immunopathologic sequelae in PD-1 and LAG-3 single knockout mice, dual knockout mice abrogated self-tolerance with resultant autoimmune infiltrates in multiple organs, leading to eventual lethality. However, Lag3−/−/Pdcd1−/− mice showed markedly increased survival from and clearance of multiple transplantable tumors. Together, these results define a strong synergy between the PD-1 and LAG-3 inhibitory pathways in tolerance to both self and tumor antigens. In addition, they argue strongly that dual blockade of these molecules represents a promising combinatorial strategy for cancer.

Cancer Res; 72(4); 917–27. ©2011 AACR.

Introduction

T-cell–mediated antitumor immune responses are essential for effective deletion of primary tumor lesions and for protection against metastases (1). Although the immune system can detect and destroy malignant cells, tumors escape surveillance by a variety of cell intrinsic and extrinsic mechanisms (1–3). As with chronic viral infection (4), tumor antigen-specific CD4+ and CD8+ T cells display impaired effector function and an exhausted phenotype characterized by decreased production of proinflammatory cytokines and hyporesponsiveness to antigenic restimulation (5). This is mediated by cell extrinsic mechanisms, such as regulatory T cells (Treg), and cell intrinsic mechanisms, such as inhibitory molecules that are upregulated on exhausted, tumor-infiltrating lymphocytes (TIL). In combination, these inhibitory mechanisms represent a formidable barrier to effective antitumor immunity (6–10).

Inhibitory receptors such as cytotoxic T-lymphocyte–associated protein 4 (CTLA-4, CD152), lymphocyte-activation gene 3 (LAG-3, CD223), and programmed cell death 1 (PD-1, CD279) function at multiple levels to ensure appropriate T-cell homeostasis, activation, and differentiation (7, 11–17). Furthermore, all 3 inhibitory molecules also contribute to cell extrinsic regulation by controlling Treg homeostasis and function, mediating induced Treg development, and mitigating dendritic cell differentiation and function (13–16, 18, 19). Data from genetically manipulated mice indicate that CTLA-4 represents a basic and indispensable “off switch,” whereas PD-1 and LAG-3 play more subtle roles in immune regulation. Whereas Clta4−/− mice develop a severe lymphoproliferative disease and are usually moribund by 3 to 4 weeks of age (20), Pdcd1−/−
(which encodes PD-1) mice live beyond 1 year while developing subtle and variable immune-based disease manifestations depending on genetic background; Pdcd1−/− BALB/c mice develop dilated cardiomyopathy 5 to 30 weeks of age, whereas Pdcd1−/− C57BL/6 mice develop a protracted lupus-like condition that takes over 6 months to develop (21, 22). Unmanipulated Lag3−/− C57BL/6 mice do not develop any disease manifestations within the first year of life (23).

Recent studies have revealed that Lag-3 and PD-1 are coexpressed on tolerized TILs suggesting that they may contribute to tumor-mediated immune suppression (5, 24). Preclinical models using antibody treatment to block Lag-3 for cancer treatment show enhanced activation of antigen-specific T cells at the tumor site and disruption of tumor growth (25). Ablation of PDI signaling in mice leads to enhanced CTL killing, cytokine production, and tumor-bearing animal survival over several different tumor models (26). On the basis of their roles in T-cell inhibition and antitumor immune regulation, individual antibody blockade of both CTLA-4 and PD-1 have been reported to show clinical utility (27, 28). Given this information, Lag3−/− Pdcd1−/− mice were provided by Lieping Chen (Johns Hopkins University) with permission from C. Benoist and D. Mathis (N418), CD25 (PC61), CD44 (IM7), CD45R/B220 (RA3-6B2), CD69 (H1.2F3), PD-1 (RMP1-30), TCR-β (H57-597), Thy1.1 (HS51), IFN-γ (XMG1.2), TNF-α (MP6-XT22), IL-17 (TC11-18H10), Foxp3 (150D), and Lag-3 (4–10-C9; ref. 31). For intracellular cytokine staining, cells were activated with phorbol 12-myristate 13-acetate (PMA; 100 ng/mL) plus ionomycin (500 ng/mL) for 4 hours in the presence of GolgiPlug (32), processed with a Cytofix/Cytoperm kit (32), and stained as indicated. Measurement of IFN-γ, TNF-α, and MCP-1 in serum was determined by IFN-γ or TNF-α–specific ELISA kits (eBioscience) and a MCP-1–specific bead based kit (Millipore).

Materials and Methods

Mouse strains and cell lines

C57BL/6 mice were purchased from The Jackson Laboratory. Lag3−/− mice were provided by Y.H. Chien (Stanford University) with permission from C. Benoist and D. Mathis (Joslin Diabetes Center; refs. 23, 29). Pdcd1−/− mice were provided by Lieping Chen (Johns Hopkins University) with permission from T. Honjo (Kyoto University; ref. 30). At St. Jude Children’s Research Hospital, the Lag3−/−, Pdcd1−/− and Lag3−/−/Pdcd1−/− mice were backcrossed onto a C57BL/6 background an additional 5, 9, and 5 generations respectively, and a genome-wide single-nucleotide polymorphism analysis indicated that 100% of the markers were C57BL/6 for Lag3−/− and Pdcd1−/− mice and 90% for the Lag3−/− Pdcd1−/− mice. At Johns Hopkins, the Lag3−/−/Pdcd1−/− mice were backcrossed 5 generations onto a B10.D2 background and crossed with Clone 4 (CL4) TCR transgenic mice. Animal experiments were carried out in specific pathogen-free facilities accredited by the American Association for the Accreditation of Laboratory Animal Care (AAALAC) at St. Jude Children’s Research Hospital and Johns Hopkins Kimmel Cancer Center and approved by the respective Animal Care and Use Committees. The mice at St. Jude Children’s Research Hospital are also Helicobacter- and MNV free. B16 melanoma cells were obtained from MJ Turk (Dartmouth College, Hanover, NH). This line has been authenticated by the RADIL at the University of Missouri (September 18, 2008) and maintained in continuous culture for no more than 6 months posttesting. It was also tested by IMPACT I PCR Profile at the RADIL at the University of Missouri (October 10, 2008). MC38 cells were obtained from J.P. Allison (Memorial Sloan-Kettering Cancer Center, NY), authenticated by the RADIL (March 10, 2003), and tested by IMPACT I at the RADIL at the University of Missouri (March 18, 2010). SaIN cells were originally obtained from J.P. Allison (Memorial Sloan-Kettering Cancer Center, NY). Although these cells have not been authenticated, they perform as described in the literature in syngeneic A/J mice (14) and tested by IMPACT I at the RADIL at the University of Missouri (January 29, 2011).

Flow cytometry, intracellular cytokine staining, and cytokine analysis

Single-cell suspensions were prepared from spleens, inguinal, brachial, and axillary lymph nodes, and tumors. Cells were stained with fluorescent-labeled antibodies (BioLegend, BD-Bioscience Pharmingen, or eBiosciences) and analyzed by either FACSCalibur or LSR II flow cytometer (BD). The following clones were used: CD4 (GK1.5), CD8α (53–6.7), CD11c (N418), CD25 (PC61), CD44 (IM7), CD45R/B220 (RA3–6B2), CD69 (H1.2F3), PD-1 (RMP1–30), TCR-β (H57–597), Thy1.1 (HS51), IFN-γ (XMG1.2), TNF-α (MP6-XT22), IL-17 (TC11–18H10), Foxp3 (150D), and Lag3 (4–10-C9; ref. 31). For intracellular cytokine staining, cells were activated with phorbol 12-myristate 13-acetate (PMA: 100 ng/mL) plus ionomycin (500 ng/mL) for 4 hours in the presence of GolgiPlug (32), processed with a Cytofix/Cytoperm kit (32), and stained as indicated. Measurement of IFN-γ, TNF-α, and MCP-1 in serum was determined by IFN-γ or TNF-α–specific ELISA kits (eBioscience) and a MCP-1–specific bead based kit (Millipore).

Tumor growth experiments and TIL preparation

B16 melanoma and MC38 colon adenocarcinoma models were carried out as previously described with some modifications (33, 34). Briefly, on day 0 mice were injected with 1.25 × 105 to 5.0 × 105 B16 cells i.d. in the back or 2.0 × 106 to 5.0 × 106 MC38 cells subcutaneously (s.c.) in the right flank. Lag3−/− Pdcd1−/− (and appropriate controls) were used at approximately 5 weeks of age. Tumor diameter was measured every 2 to 3 days with an electronic caliper and reported as volume using the formula m1/2 × m2 × π/6 (35). To isolate TILs, solid tumors were excised after 12 to 14 days, single-cell suspensions prepared by mechanical dissociation, followed by density gradient centrifugation on an 80%/40% Percoll (GE Healthcare) gradient. For CD4+ and CD8+ T-cell depletion experiments, anti-mouse CD4 (GK1.5) and anti-mouse CD8 (2.43) ascites were administered i.p. on days 1, 2, 5, 8, and 11 (pretreated for maximal deletion).

Dual antibody blocking experiments

SaIN fibrosarcoma cells or MC38 cells (2 × 106) were implanted s.c. into A/J mice (Harlan) or C57BL/6 mice (Charles River), respectively. Tumor volumes were measured with an electronic caliper (l × w × h/2) and randomized by size (10 mice per group). Mice with palpable tumors (SaIN ~60 mm3/2; MC38 ~40 mm3/2) were injected i.p. at a dosage of 10 mg/kg for chimeric mouse anti-PD-1 (4H2, IgG1; ref. 36) and/or rat
anti-mouse LAG-3 (C987W, IgG1; ref. 37). Control murine IgG1 (MOPC 21; BioXCell) was dosed at 20 mg/kg or added to individual anti–PD-1 or anti–LAG-3 antibody treatments at 10 mg/kg. Tumor growth inhibition (TGI) was calculated when all mice within a group were available for tumor measurement.

Adoptive transfer into Rag-1−/− mice

Splenocytes and lymph node cells from female mice (5–7 weeks old) were pooled, and 10⁷ cells injected i.v. into age-matched female Rag-1−/− (5–6 weeks old) mice. CD4⁺ or CD8⁺ cells were detected from splenocytes and lymph node cells with biotinylated anti-CD4 or anti-CD8 by macrophage separation using streptavidin-coupled beads (Milteny Biotec) to achieve purity above 95%.

Histopathology

Full necropsies were completed independently at St Jude Children’s Research Hospital and Johns Hopkins. Tissues from knockout and control mice were collected and fixed in 10% neutral buffered formalin, except eyes, which were fixed for 24 hours in Davidson’s fixative, embedded in paraffin, sectioned at 4 μm and stained with hematoxylin and eosin for histopathologic examination. Collagen deposition was detected by a Masson’s Trichrome stain. Macrophages and T cells were detected using rat anti-mouse MAC2 (CL8942AP; Accurate) and stained with hematoxylin and eosin for histopathologic examination. Collagen deposition was detected by a Masson’s Trichrome stain. Macrophages and T cells were detected using rat anti-mouse MAC2 (CL8942AP; Accurate Chemical) and goat anti-human CD3 (sc-1127; Santa Cruz Biotechnology) antibodies, respectively. Heat-induced epitope retrieval was carried out by heating slides in ER2 (AR9640; Leica) for 30 minutes and the Refine system (DS9800; Leica Microsystems) used for detection. Treg cells were detected with a rat anti-mouse FoxP3 antibody (14–5773–82; eBioscience) with heat-induced retrieval, pH 6.0, target retrieval buffer (S699; Dako) followed by a horseradish peroxidase–labeled streptavidin detection system (TS-125-HR; Thermo Shandon). In all immunohistochemical assays, 3,3′-diaminobenzidine was used as the chromogenic substrate with a light hematoxylin counterstain.

Autoantibody analysis

Mouse sera were analyzed by indirect ELISA, alongside a positive control (serum from a MRL/lpr mouse, a SLE disease model), using 96-well Nunc MaxiSorp plates (Nalgene Nunc). Multiple antigen blot assay (MABA) was conducted as described (38).

Clone 4 TCR transgenic T-cell experiments

CL4 adoptive transfer and in vivo CTL studies were carried out as previously described (25, 39).

Statistical analyses

Summary statistics are presented as mean ± SEM. Group means were compared with 2-sample t tests. Event-free survival (moribund) estimates were calculated with the Kaplan–Meier method; mouse groups were compared by log-rank test. The proportions of tumor-free mice were evaluated with the binomial distribution; synergy hypotheses were tested based on the maximum likelihood method. Trends in weight over time and tumor growth over time among different mice groups were analyzed using mixed models. All P values are 2-sided, and statistical significance was assessed at the 0.05 level. Analysis was conducted by SAS (version 9.2).

Results

Combinatorial anti–LAG-3/anti–PD-1 immunotherapy inhibits tumor growth

PD-1 monoclonal antibody treatment has shown clinical efficacy against multiple malignancies including melanoma, prostate, renal cell, and lung cancer (27). LAG-3 has been suggested to directly modulate the activity of PD-1⁺ cells (5); furthermore, coexpression of LAG-3 and PD-1 has been shown in malignant mouse and human tissue (5, 24). Given these data, we hypothesized that LAG-3 and PD-1 act synergistically to control immune homeostasis and mediate tumor-induced tolerance. Consistent with previous reports, a significant percentage of CD4⁺ and CD8⁺ TILs from transplanted B16 melanoma, MC38 colorectal adenocarcinoma, and Sa1N fibrosarcoma expressed high levels of LAG-3 and PD-1 (32, 34), whereas similar upregulation was not observed on peripheral T-cell populations (Fig. 1). Next, we asked if antibody-mediated dual blockade of these pathways would reduce tumor growth by assessing the potential efficacy of combined anti–LAG-3 and anti–PD-1 blockade in mice with established tumors. Reduced growth of Sa1N fibrosarcoma and MC38 colorectal adenocarcinoma (32, 40–42) was observed in some but not all mice treated with the anti–LAG-3 or anti–PD-1 monotherapy (Fig. 2); only a few mice were tumor free after 50 days (0%–40%). For anti–LAG-3, this is the first demonstration of TGI with anti–LAG-3 as a monotherapy. In striking contrast, 70% and 80% of the Sa1N-and MC38-inoculated mice, respectively, were tumor free after 50 days following combinatorial anti–LAG-3/anti–PD-1 immunotherapy (Fig. 2). However, this regimen had no effect against established B16 tumors. Using the maximum likelihood method, there seemed to be a synergistic benefit of anti–LAG-3/anti–PD-1 combinatorial immunotherapy that is superior to either the additive effect of anti–LAG-3 and anti–PD-1 or monotherapy. Dual treatment with anti–LAG-3/anti–PD-1 did not result in immunopathologic manifestations such as lymphocytic infiltration in the Sa1N fibrosarcoma model as determined by detailed histologic analysis of multiple tissues. Despite efficient tumor clearance, no evidence of systemic or organ-specific autoimmunity was observed.

To investigate the mechanism underlying decreased tumor growth in antibody-treated mice, MC38 tumor-bearing mice were treated with the antibody combinations used above and draining lymph node (DLN) T cells, non-DLN (NDLN) T cells, and TILs analyzed using mixed models. All P values are 2-sided, and statistical significance was assessed at the 0.05 level. Analysis was conducted by SAS (version 9.2).
higher percentage of IFN-γ⁺CD4⁺ and IFN-γ⁺CD8⁺ TILs, and to a lesser extent TNF-α⁺CD4⁺ and CD8⁺ TILs, were observed in anti-LAG-3/anti–PD-1–treated mice than in control groups (Fig. 3B). Taken together, these data suggest that anti-LAG-3/anti–PD-1 combinatorial immunotherapy may act synergistically to reduce tumor growth by increasing the proportion of effector T cells in the tumor and DLNs.

To further investigate the synergy between these 2 inhibitory molecules, we next assessed whether LAG-3 and PD-1 cooperate to control immune homeostasis and mediate tumor-induced tolerance with a genetic approach. LAG3⁻/⁻ Pdcd1⁻/⁻ mice develop lethal systemic autoimmunity

To further investigate the synergy between these 2 inhibitory molecules, we next assessed whether LAG-3 and PD-1 cooperate to control immune homeostasis and mediate tumor-induced tolerance with a genetic approach. LAG3⁻/⁻ Pdcd1⁻/⁻ mice were generated at 2 independent
locations (see Materials and Methods), and disease manifestation and immune pathology analyzed over time. Lag3\(^{-/-}\)/Pdcd1\(^{-/-}\) mice developed an early onset (~4 weeks of age), lethal autoimmune condition that resulted in approximately 80% of the mice moribund by approximately 10 weeks (Fig. 4A and Supplementary Fig. S2). The major histopathologic manifestations included diffuse fibrosing lymphohistiocytic endocarditis, myocarditis, and pancreatitis (Fig. 4B, Supplementary Table S1 and Fig. S3). Extensive infiltration by CD3\(^+\) T cells, Foxp3\(^+\) Treg cells, and Mac2\(^-\)F4/80\(^+\) macrophages was observed, in conjunction with substantial collagen deposition but limited B-cell and neutrophil infiltration; however, negligible autoantibody reactivity was seen in serum from Lag3\(^{-/-}\)/Pdcd1\(^{-/-}\) mice but not single knockout or wild-type mice (Supplementary Fig. S4). Lag3\(^{-/-}\) and Pdcd1\(^{-/-}\) single KO mice lacked any disease manifestations or histopathology over this period of observation. These results show that the PD-1 and LAG-3 pathways synergistically regulate self-reactivity.

Consistent with the histopathology observed, substantially increased numbers of CD4\(^+\) and CD8\(^+\) T cells were observed in the regional LNs, but not the spleens, of Lag3\(^{-/-}\)/Pdcd1\(^{-/-}\) mice (Fig. 4C, Supplementary Fig. S5). These cells possessed a predominately activated/memory phenotype as indicated by CD69/CD44 staining. Nevertheless, there seemed to be minimal difference in the extent of division in vivo based on ex vivo Ki67 staining, even though Lag3\(^{-/-}\)/Pdcd1\(^{-/-}\) T cells proliferate more in vitro following anti-CD3 stimulation (data not shown). The number of CD4\(^+\) Foxp3\(^+\) Treg cells, B cells, and CD11c\(^+\) dendritic cells were also increased in Lag3\(^{-/-}\)/Pdcd1\(^{-/-}\) mice (Fig. 4C, Supplementary Fig. S5). Given that Lag3\(^{-/-}\) Treg cells exhibit reduced suppressive activity (13, 14) and PD-L1 (PD-1 ligand)
contributes to iTreg development (15), it is possible that the combined loss of LAG-3 and PD-1 alters Treg cell homeostasis. To further probe the cellular defects in Lag3−/−/Pdcd1−/− mice, we adoptively transferred splenocytes into lymphopenic Rag-1−/− mice. In contrast to healthy wild-type and single knockout controls, Lag3−/−/Pdcd1−/− splenocyte recipients started to lose body weight approximately 6 days posttransfer with 100% morbidity by day 20 (Supplementary Fig. S6A and S6B). Adoptive transfer experiments T-cell–depleted Lag3−/−/Pdcd1−/− splenocytes clearly showed that both CD4+ or CD8+ T-cell populations contributed to the disease observed, with a dominant role for the former (Supplementary Fig. S6A and S6B). Adaptive transfer experiments T-cell–depleted Lag3−/−/Pdcd1−/− splenocytes clearly showed that both CD4+ or CD8+ T-cell populations contributed to the disease observed, with a dominant role for the former (Supplementary Fig. S6A and S6B). Consistent with these survival and weight loss data, histologic analysis of CD4+ T-cell–depleted Lag3−/−/Pdcd1−/− splenocyte recipients revealed relatively normal bone marrow cellularity and density, whereas Lag3−/−/Pdcd1−/− splenocyte recipients exhibited a near total absence of hematopoietic cell precursors in bone marrow and severe lymphoid depletion in the spleen, LNs, and Peyer’s patches (Supplementary Table S2, Fig. S6E and S7). These data indicate that CD4+ T cells are primarily responsible for the pathology observed. Cytokine analysis revealed high levels of IFN-γ, TNF-α, and MCP-1 in the serum of Lag3−/−/Pdcd1−/− recipients but not single knockout or wild-type control recipients (Supplementary Fig. S6F–S6H). Taken together, these data suggest that Lag3−/−/Pdcd1−/− splenocyte recipients, in contrast with their single knockout and wild-type controls, develop an autoimmune GvHD-like syndrome with evidence of aplastic anemia and bone marrow failure as a cause of death.

The data thus far suggested that while a reasonable level of tolerance is maintained in single knockout Lag3−/− or Pdcd1−/− mice, dual loss of LAG-3 and PD-1 expression results...
in a loss of peripheral self-tolerance of CD4+ and CD8+ T cells. To test this in an antigen-specific system, we asked whether hemagglutinin-specific tolerance induced in transgenic mice expressing hemagglutinin as a self-antigen in multiple epithelial tissues (C3-HAlo mice; refs. 13, 43), could also be broken if adoptively transferred hemagglutinin-specific T cells [from

Clone 4 (CL4) TCR transgenics] lacked both inhibitory molecules. Compared with wild-type CL4 T cells, significant expansion of Lag3-/- Pdcd1-/- clonotypic CD8+ T cells was observed 5 days posttransfer (Supplementary Fig. S6). Although this was not substantially greater than that seen with Pdcd1-/- T cells, the Lag3-/- Pdcd1-/- T cells exhibited a significantly enhanced effector phenotype, as determined by intracellular expression of IFN-γ and IL-17, compared with their single knockout and wild-type controls (Supplementary Fig. S6 and S6K). Similarly, when tolerance was broken in C3-HAlo transgenic mice (39), adoptive transfer of antigen-specific Lag3-/- Pdcd1-/- CD8+ T cells expanded significantly more than their single knockout and wild-type controls, although enhanced in vivo CTL activity was comparable in the Pdcd1-/- and Lag3-/- Pdcd1-/- CD8+ T cell recipients (Supplementary Fig. S8). Collectively, these data suggest that the loss of Lag-3 and PD-1 also results in loss of tolerance to a model self-antigen.

Reduced tumor growth and enhanced survival in Lag3-/- Pdcd1-/- mice

To continue our analysis of Lag-3/PD-1 synergy in the regulation of antitumor immunity, we assessed tumor growth in Lag3-/- Pdcd1-/- and controls over time. Of the 3 transplantable tumor models examined in this study, B16 is regarded as the least immunogenic and thus the hardest to eliminate by immunologic intervention (32, 34). A low dose of B16 cells (1.25 × 10⁵) progressively grew in wild-type and Lag3-/- mice inoculated intradermally at day 0, whereas limited growth was observed in Pdcd1-/- and Lag3-/- Pdcd1-/- mice (Supplementary Fig. S9A). Although previous studies suggested that PD-1 deletion did not affect subcutaneously injected tumor growth (44), our experiments revealed reduced tumor growth in Pdcd1-/- mice compared with wild-type mice. Paradoxically, Lag3-/- mice developed slightly larger tumors. Whether this is due to reported defects in natural killer cell cytolyis (23), or an unexpected role of pDCs, which highly express Lag-3 (45), remains to be determined. Statistical analysis with the maximum likelihood method for synergy found that the lack of tumor growth in the Lag3-/- Pdcd1-/- mice was greater than the additive effects of tumor growth in Lag3-/- mice and Pdcd1-/- mice at day 11 (P < 0.05) and day 13 (P < 0.0005) suggesting that Lag-3 and PD-1 synergize to mediate tumor-induced tolerance. Depletion of CD4+ and CD8+ T cells restored normal B16 tumor growth in compound-deficient mice, indicating the necessity of adaptive immunity to the antitumor response (Supplementary Fig. S9B).

As the difference in resistance to B16 growth between Pdcd1-/- and Lag3-/- Pdcd1-/- mice seemed small, we evaluated tumor B16 and MC38 growth at different doses. At the higher B16 dose (5 × 10⁵ cells per mouse), wild-type and Lag3-/- mice show uncontrolled tumor growth and lethality with an average survival time of less than 20 days (Fig. 5A). Although limited tumor growth was observed in Pdcd1-/- mice (Supplementary Fig. S10), Lag3-/- Pdcd1-/- mice (80%) eliminated tumors compared with only 40% of Pdcd1-/- mice; however, B16 survivors did not display autoimmune vitiligo as is often seen with this model (46). We also investigated growth of subcutaneously implanted MC38 adenocarcinoma cells at 2 different doses. Whereas MC38 growth and survival were
Lag3

type cells. Taken together, these data clearly show that
less susceptible to tumor-induced tolerance than wild-type
CD8
increased killing efficacy in comparison with the wild type, and slightly
high-dose (5 \times 10^5/C2
Lag3
and
Pdcd1
comparable in Lag3
/Pdcd1
and
Pdcd1
mice when a low
dose (2 \times 10^5 cells per mouse) was used (70% vs.
71%, respectively; Fig. 5B and Supplementary Fig. S10).
Lag3
/Pdcd1
mice were clearly more effective at preventing
high-dose (5 \times 10^5 cells per mouse) MC38 tumor growth and
ensuring survival (83% vs. 38%, respectively; Fig. 5C and
Supplementary Fig. S10). Phenotypic analysis revealed
enhanced IFN-\gamma expression by
Lag3
/Pdcd1
mice compared with wild-type
and
Lag3
mice, and NDLNs from all groups (Fig. 6). These
data suggest that combined loss of LAG-3 and PD-1 limits
tumor-mediated tolerization and enhances tumor-specific
immunity and resistance to tumor growth.

To further investigate the killing efficacy of
Lag3/Pdcd1
T cells in vivo in the presence of an established
tumor, clonotypic CL4 CD8
T cells were transferred into
ProTRAMP male mice, which develop prostate cancer driven
by the probasin promoter and express hemagglutinin. After
vaccination with hemagglutinin-expressing Vaccinia virus,
Lag3/Pdcd1
recipients showed significantly increased
killing ability in comparison with the wild type, and slightly
increased killing efficiency in comparison with
Pdcd1
single knockouts (Supplementary Fig. S11). These data sup-
port the conclusion that
Lag3/Pdcd1
CD8
T cells are less susceptible to tumor-induced tolerance than
wild-type cells. Taken together, these data clearly show that
Lag3/Pdcd1
mice are more capable of resisting high-
dose tumor growth than
Pdcd1
and wild-type mice.

Discussion

The data presented here illustrate clear synergy between the
inhibitory receptors LAG-3 and PD-1 in controlling immune
homeostasis, preventing autoimmunity, and enforcing tumor-
induced tolerance. First, we show coexpression of LAG-3 and
PD-1 on tumor-infiltrating lymphocytes. Second, we show that
dual blockade of these receptors leads to decreased tumor
growth and enhanced antitumor immunity. Importantly, dual
antibody–treated mice show more robust immune responses
than either single-treated group. Third, analysis of mutant
mice revealed a cooperative requirement for LAG-3 and PD-1
in maintaining immune homeostasis. Consistent with our
observations following antibody-mediated blockade of LAG-3
and PD-1,
Lag3/Pdcd1
mice prevented growth of high-
dose B16 and MC38 tumors and ensured survival while single
knockout controls and wild-type mice succumbed to disease.
Taken together, these data reveal an unprecedented synergistic
cooperation between LAG-3 and PD-1 in limiting tumor
growth.

Although anti–LAG-3/anti–PD-1 combinatorial immuno-
therapy effectively cleared established Sa1N and MC38 tumors,
this therapy was not effective against established B16 tumors.
In contrast, B16 tumors were more difficult to establish in
Lag3/Pdcd1
mice. B16 is a more difficult tumor to
eradicate than MC38 and Sa1N and thus there could be several
possible explanations for this apparent discrepancy. First,
expression of LAG-3/PD-1 on TILs from B16 is lower than for

Figure 5. Reduced tumor growth in tumor-bearing Lag3
/Pdcd1
mice. Wild type, Lag3
, Pdcd1
, and Lag3
/Pdcd1
mice were inoculated on day 0 with
5 \times 10^5 B16 cells i.d. (A), 2 \times 10^6
MC38 cells s.c. (B), or 5 \times 10^6
MC38 cells s.c. (C). Tumors were
measured with an electronic caliper and reported as volume (see
Materials and Methods). Data are combined from 2 to 3 repeated
experiments, 3 to 5 animals per animals per group. Data were
analyzed by the Maximum
likelihood method to determine
synergy p values: 0.0253 (A),
0.94 (B), and 0.0273 (C) for the
Lag3
/Pdcd1
mice compared with the additive effect of the 2
single knockouts. Animals were euthanized when tumors became
large, ulcerated, and/or necrotic.
discussed above, these observations could be due to the different tumors analyzed or temporal differences between these experiments, as IFN-γ expression was determined 1 week after mAb treatment compared with 2 weeks after tumor inoculation into Lag3−/−Pdcd1−/− mice. Finally, we cannot rule out the possibility that there is a phenotypic difference in the immune cells in the Lag3−/−Pdcd1−/− mice. However, there does not seem to be an active systemic defect at the time of the experiment as high IFN-γ expression is not observed in the NDLN.

Although Lag3−/−Pdcd1−/− mice develop a lethal autoimmune condition, the disease is slower (~10 weeks vs. 3–4 weeks) and less penetrant (80% vs. 100%) than the phenotype observed in Cita4−/− mice (20). Recently, analogous observations were reported by Honjo and colleagues in which BALB/c mice harboring a loss-of-function mutation in Lag3 combined with genetic deletion of Pdcd1 develop lethal myocarditis (47). Heart-infiltrating T cells from these compound-deficient mice were shown to produce high amounts of IFNγ compared with distal lymphoid organs such as the spleen. Our results are consistent with their data, as we also observed enhanced production of proinflammatory cytokines by T cells infiltrating sites of inflammation, such as the heart and pancreas in Lag3−/−Pdcd1−/− mice and in tumors and DLNs in knockout and antibody-treated mice. Furthermore, the Honjo group also observed accelerated autoimmune diabetes in NOD mice expressing a loss-of-function Lag-3 mutant, consistent with our recent observations in Lag3−/− NOD mice (48). However, the mice used by Honjo and colleagues that lacked functional Lag3−/−Pdcd1−/− expression were on a BALB/c background, whereas our data were derived from mice on a C57BL/6 or B10.D2 background. Strain-specific differences between these mice have been well documented (49, 50) and may have contributed to subtle differences in phenotypic and mechanistic observations reported. For instance, loss of Lag3−/−Pdcd1−/− on a B10.D2 background can lead to an increase in IL-17+ cells which was not seen in mice on a Balb/c background.

Although CTLA-4, PD-1, and Lag-3 are all negative regulatory molecules expressed during T-cell activation, high level, dual Lag-3/PD-1 expression has been shown to promote tumor-specific responses relative to nonspecific or self-antigen-specific immune responses and thus may be less toxic than CTLA-4 blockade. Given the recent phase 3 results with anti-CTLA-4 treatment of patients with metastatic melanoma, showing a clear survival benefit (albeit with notable immune toxicity; ref. 28), our results suggest that combined blockade of PD-1 and Lag-3 is a highly promising combinatorial strategy for the immune-based therapy of cancer.

Disclosure of Potential Conflict of Interest

The authors declare competing financial interests. D.M. Pardoll, D.A.A. Vignali, C.G. Drake, and C.J. Workman have submitted patents that are pending and are entitled to a share in net income generated from licensing of these patent rights for commercial development. A.J. Korman, M. Selby, and F.J. Grosso are employees of Bristol-Myers Squibb. C.G. Drake has an ownership interest in Amplimmune and has served as a consultant to Dendreon, Bristol-Myers Squibb, and Pfizer.

Figure 6. Tumor-draining (DLN; right) and nondraining (NDLN; left) LN T cells were isolated on day 14 post-B16 inoculation and were activated with PMA + ionomycin for 4 hours in the presence of brefeldin A. After intracellular cytokine staining, CD8+ IFN-γ+ (A) and CD4+ IFN-γ+ (B) cells were analyzed by flow cytometry. Data are representative of 10 to 15 animals per group. Nonparametric 1-way ANOVA with Kruskal-Wallis test (P < 0.0001) was used; *P < 0.05; **P < 0.01; and ***P < 0.001 vs. Lag3−/−Pdcd1−/−.
Acknowledgments

The authors thank Many Jo Turk and Jim Allison for cell lines. At St. Jude, the authors thank Karen Forbes, Amy Krause, and Ashley Castellaw for maintenance and breeding of mouse colonies. Clifford Guy for help with cytokine analysis, Paul Thomas for anti-CD4 and anti-CD8 depleting Abs, Richard Cross, Greg Lennon and Stephanie Morgan for FACS, Song Wu and Hui Zhang for help with statistical analysis, the staff of the Shared Animal Resource Center at St Jude for the animal husbandry, the Veterinary Pathology Core Laboratory at St. Jude for histology and immunohistochemistry support, and the Hartwell Center for Biotechnology and Bioinformatics at St Jude for real-time PCR primer/probe synthesis and qPCR synthesis and purification. At Johns Hopkins, the authors thank Dib-Dib Huang for the maintenance and breeding of the mouse colonies, and technical support. At Bristol-Myers-Squibb, the authors thank David Klytzing and the staff of the Milpitas animal facility for carrying out the tumor experiments and Rangan Vanganipuram, Brian Lee, and Shilpa Mankikar for provision of antibodies.

Grant Support

This work was supported by the NIH (R01 AI39480 to D.A.A. Vignali and R01 CA127153 and P50 CA85266–15 to C.G. Drake), a Hartwell Postdoctoral Fellowship (to M.L. Bettini), NCI Comprehensive Cancer Center Support CORE grant (CA33572 to D.A.A. Vignali), the American Lebanese Syrian Associated Charities (ALSAC to D.A.A. Vignali), the Patrick C. Walsh Fund (to C.G. Drake), the Koch Fund (to C.G. Drake), NHLBI contract - HHSN-26620199904C and CHIR - 26820141 to P.J. Utz, and NIAID F32 AI080086 to C.L. Liu. C.G. Drake is a Damion Runyon Lilly Clinical Investigator. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received May 11, 2011; revised December 7, 2011; accepted December 10, 2011; published OnlineFirst December 20, 2011.

References

Immune Inhibitory Molecules LAG-3 and PD-1 Synergistically Regulate T-cell Function to Promote Tumoral Immune Escape

Seng-Ryong Woo, Meghan E. Turnis, Monica V. Goldberg, et al.

Cancer Res 2012;72:917-927. Published OnlineFirst December 20, 2011.

Updated version
Access the most recent version of this article at:
doi:10.1158/0008-5472.CAN-11-1620

Supplementary Material
Access the most recent supplemental material at:
http://cancerres.aacrjournals.org/content/suppl/2011/12/19/0008-5472.CAN-11-1620.DC1

Cited articles
This article cites 50 articles, 23 of which you can access for free at:
http://cancerres.aacrjournals.org/content/72/4/917.full#ref-list-1

Citing articles
This article has been cited by 100 HighWire-hosted articles. Access the articles at:
http://cancerres.aacrjournals.org/content/72/4/917.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, use this link
http://cancerres.aacrjournals.org/content/72/4/917.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.