PI3Kγ activates integrin α4 and promotes immune suppressive myeloid cell polarization during tumor progression

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ABSTRACT

Immunosuppressive myeloid-derived suppressor cells (MDSCs) and tumor-associated macrophages (TAMs) accumulate in tumors where they inhibit T cell–mediated antitumor immune responses and promote tumor progression. Myeloid cell PI3Kγ plays a role in regulating tumor immune suppression by promoting integrin α4-dependent MDSC recruitment to tumors and by stimulating the immunosuppressive polarization of MDSCs and TAMs. Here we show that integrin α4 promotes immunosuppressive polarization of MDSCs and TAMs downstream of PI3Kγ, thereby inhibiting antitumor immunity. Genetic or pharmacological suppression of either PI3Kγ or integrin α4 blocked MDSC recruitment to tumors and also inhibited immune suppressive myeloid cell polarization, thereby reducing expression of IL10 and increasing expression of IL12 and IFNγ within tumors. Inhibition of PI3Kγ or integrin α4 within tumors stimulated dendritic cell and CD8+ T-cell recruitment and maturation, as well as tumor cell cytotoxicity in vivo, thereby inhibiting tumor growth. As blockade of PI3Kγ or integrin α4 prevents accumulation of MDSC and reduces myeloid cell expression of immunosuppressive factors that stimulate tumor immune escape, these results highlight PI3Kγ and integrin α4 as targets for the design of cancer therapeutics.
INTRODUCTION

Chronic inflammation promotes tumor progression by stimulating angiogenesis and suppressing the antitumor immune response (1-3). CD11b^+Gr1^+ immunosuppressive myeloid cells [myeloid-derived suppressor cells (MDSC)] accumulate in the blood, spleen, lymph nodes, bone marrow and tumor microenvironments in tumor-bearing animals and cancer patients (4-8). These cells, as well as CD11b^+Gr1^- tissue resident macrophages, inhibit innate and adaptive immunity, thereby promoting tumor immune escape (1-8). These immunosuppressive cells inhibit maturation of antigen-presenting dendritic cells (DC), suppress T-cell activation and NK-cell cytotoxicity and induce regulatory T-cell (T_{reg}) development, leading to dysfunctional cell-mediated antitumor immunity through the release of immunosuppressive factors such as IL10, TGFβ1, IL6, VEGF, and ROS (9-10).

Experimental evidence suggests that therapeutic strategies targeting MDSCs may be beneficial in cancer therapy (11-15). Studies show that modulation of recruitment, differentiation, and expansion of myeloid cells could promote antitumor immunity and block tumor progression (11-15). Strategies targeting the mCSF1 receptor (CSF1R), the CCL2 receptor (CCR2), or PI3Kγ reduce tumor growth and inflammation in animal models and are currently undergoing testing in cancer patients (11-14). We previously found that CD11b^+Gr1^+ monocyte and granulocyte trafficking to tumors from circulation depends on PI3Kγ-mediated activation of integrin α4β1, a receptor for endothelial cell VCAM (14-18). As the most prevalent PI3K isoform in normal myeloid cells, PI3Kγ is also required for neutrophil motility and trafficking during inflammatory...
disease (19-20). We found that numerous cytokines and chemokines activate PI3Kγ, including SDF-1, VEGF-A, IL4, IL6, and CSF1 (17). Once activated, PI3Kγ stimulates BTK, PLCγ, RAPGEF, Rap1a, RIAM, and paxillin-dependent integrin α4 activation (14-18). Inhibition of PI3Kγ, BTK, PLCγ, RAPGEF, Rap1a, RIAM, or integrin α4β1 suppresses monocyte and granulocyte accumulation in tumors and inhibits tumor growth (14-18).

PI3Kγ not only controls myeloid cell trafficking during tumor growth and inflammation, but also controls myeloid cell polarization by regulating immune suppressive gene expression in tumor associated macrophages, monocytes and granulocytes (21-22). Our studies showed that PI3Kγ stimulates mTor, S6Kα and C/EBPβ-mediated anti-inflammatory gene expression and inhibits NFκB-mediated pro-inflammatory gene expression, thereby promoting expression of immune suppressive factors, such as IL10, TGFβ, and Arginase, and inhibiting expression of IL12, IFNγ and Nos2 (21-22). PI3Kγ activity thereby suppresses T-cell immunity and stimulates tumor growth (21). Since our prior studies showed that PI3Kγ signaling activates integrin α4β1 to promote myeloid cell adhesion and invasion, we speculated that integrin α4β1 might also regulate myeloid cell polarization downstream of PI3Kγ and thereby affect the antitumor immune response.

We show here that integrin α4β1 promotes immune suppressive myeloid cell polarization, inhibits antitumor immunity and stimulates tumor growth. Genetic or pharmacologic blockade of integrin α4β1 or its activator PI3Kγ inhibits immunosuppressive myeloid cell polarization, stimulates dendritic cell maturation and restores antitumor T cell–mediated immunity in mouse
models of cancer. These studies indicate that blockade of the PI3Kγ–integrin α4 pathway can suppress tumor growth.
MATERIALS AND METHODS

Tumor cell lines

Lewis Lung Carcinoma cells (LLC) were obtained from the American Type Culture collection (ATCC) and cultured in Dulbecco’s Modified Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS), 2 mM glutamine, 100 U/ml Penicillin and 100 μg/ml Streptomycin.

Pancreatic adenocarcinoma cells (Panc02) were obtained from the David Cheresh laboratory, University of California, San Diego and were cultured in RPMI (GIBCO) supplemented with L-glutamine, 100 U/ml Penicillin and 100 μg/ml Streptomycin and 10% fetal bovine serum.

Cultures were maintained in a humidified incubator at 37°C in an atmosphere containing 5% CO₂. Both cell lines were verified by RT-PCR or RNA sequencing, in vitro morphological and biochemical criteria and in vivo tumor assays in syngeneic mice.

Murine macrophage differentiation and culture

Bone marrow derived cells (BMDC) were aseptically harvested from 6-8 week-old female mice by flushing leg bones of euthanized mice with phosphate buffered saline (PBS), 0.5% BSA, 2 mM EDTA, incubating in red cell lysis buffer (155 mM NH4Cl, 1 mM NaHCO3 and 0.1 mM EDTA) and centrifuging over Histopaque 1083. Purified mononuclear cells were cultured in RPMI + 20% serum + 50 ng/ml M-CSF (PeproTech). Bone marrow derived macrophages were polarized with either IFNγ (20 ng/ml, Peprotech) plus LPS (100 ng/ml, Sigma) or LPS alone for 24 h or IL4 (20 ng/ml, Peprotech) for 24-48h. Total RNA was harvested from macrophages using the RNeasy Mini Kit (Qiagen) according to the manufacturer’s instructions.
**In vivo tumor studies**

Animal studies were approved by the Institutional Animal Care and Use Committee (IACUC), University of California, San Diego.: 5 x 10^5 LLC cells were injected subcutaneously into syngeneic (C57Bl/6J) 6- to 8- week old wild-type (WT), integrin α4Y991A, or PI3Kγ−/− (p110γ−/−) mice (n = 8-10). Tumors dimensions were recorded and excised at 14-21 days. Tumors were cryopreserved in O.C.T., solubilized for RNA purification or collagenase-digested for flow cytometric analysis of immune cell infiltration as detailed below. Alternatively, orthotopic Panc02 pancreatic tumor were initiated by implanting 1 x 10^6 Panc02 pancreatic carcinoma cells into the pancreas of syngeneic mice (n = 8-10). The abdominal cavities of immunocompetent C57Bl/6J mice, integrin α4Y991A mutant and PI3Kγ−/− mice were opened and the tails of the pancreas were exteriorized. One million Panc02 cells were injected into the pancreatic tail, the pancreas was placed back into the abdominal cavity, and the incision was closed. Pancreas were excised and cryopreserved after 5 weeks. Tumor weight and immune cell infiltration were quantified as described.

**Drug treatment of tumors**

Studies with blocking mAb to integrin α4;C57Bl/6J mice were subcutaneously implanted on day 1 with 5 X 10^5 LLC cells. Mice were treated every third day with intraperitoneally (i.p.) injections of mAb PS/2, an α4 blocker, or isotype-matched control rat IgG2b, at a dose of 200 μg/mouse (10 mg/kg) in a 100 μl volume (n = 8 per group). Tumors were harvested at 14-21 days, weighed and further analyzed by quantitative RT-PCR, flow cytometry and immunohistochemistry.
IL10 blocking studies: C57Bl/6J mice were subcutaneously implanted on day 1 with $5 \times 10^5$ LLC cells. Mice were treated on day 7 and day 11 with i.p. injections of function-blocking anti-IL10 (JES052A5, R&D Systems) or isotype-matched (rat IgG1) control antibodies at doses of 200 μg/mouse ($n = 6$ per group). Tumors were harvested at 14 days, weighed and further analyzed by quantitative RT-PCR, flow cytometry and immunohistochemistry. PI3Kγ inhibitor studies: C57Bl/6J mice were subcutaneously implanted on day 1 with $5 \times 10^5$ LLC. Mice were treated by i.p. injection with 2.5 mg/kg of PI3Kγ inhibitor (TG100-115) or with a chemically similar inert control ($n = 10$) twice daily for fourteen days for a total daily dose of 5 mg/kg. Tumor volumes and weights, as well as myeloid cell densities were measured.

**Isolation of bone marrow derived cells for bone marrow transplantation**

Bone marrow cells were aseptically harvested from 6-8 week-old female mice by flushing leg bones of euthanized mice with phosphate buffered saline (PBS) containing 0.5% BSA and 2mM EDTA, incubating cells in red cell lysis buffer and centrifuging over Histopaque 1083. Approximately $5 \times 10^7$ bone marrow cells were purified by gradient centrifugation from the femurs and tibias of a single mouse. Two million cells were intravenously injected into tail veins of each lethally irradiated (1000 rad) 6-week-old syngeneic recipient mouse. After 4 weeks of recovery, tumor cells were injected in BM transplanted animals. LLC ($n = 8$, 3 experiments) tumor growth in C57BL/6 and α4Y991A mice transplanted with BM from α4Y991A or WT were compared as described above.

**Isolation of tumor-infiltrating immune cells**
Tumors were isolated, minced and digested to single cell suspension for 1 h at 37°C in 5ml of Hanks Balanced Salt Solution (HBSS, Invitrogen) containing 1 mg/ml collagenase type IV (Sigma), 0.1 mg/ml hyaluronidase (Sigma) and 20 U/ml DNase type IV (Sigma). Cell suspensions were filtered through a 70 μm cell strainer and then incubated with different antibodies to perform flow cytometry.

**Flow cytometry**

Tumor-infiltrating immune cells were incubated with Fc-blocking reagent (anti CD16/CD32, BD Biosciences), followed by CD11b-APC (M1/70, BD Biosciences), Gr1-FITC (RB6-8C5, BD Biosciences), CD11c-APC (HL3, BD Biosciences), MHC II-FITC (I-Ab, AF6-120.1, BD Biosciences), CD3-APC (145-2C11, eBioscience), CD4-FITC (GK1.5, eBioscience), CD8a-APC (53-6.7, eBioscience) along with isotype-matched control. In vitro cultures dendritic cells (DCs) were stained with CD11b-APC, Gr1-FITC, CD11c-APC, MHC II-FITC, and CD80-FITC (16-10A1, eBioscience) and CD86-FITC (GL1, eBioscience).

**Analysis of gene expression**

Total tumor RNA was prepared with TRIzol® Reagent (Invitrogen) according to the manufacturer’s instructions. In some experiments, total isolated CD11b+ or CD90.2+ cells RNA was isolated using RNeasy Mini Kit (Qiagen). cDNA synthesis was performed from 0.5-1 μg RNA with SuperScript III First-Strand Synthesis Kit (Invitrogen). Quantitative PCR was performed using primers for Gapdh, Arg1, Il10, Il6, Vegfa, Tgfb, Il12, or Ifng (QuantiTect Primer Assay). qPCR analysis was performed using a Smart Cycler System (Cepheid).
**Immunohistochemistry**

Tumor samples were collected and progressively frozen in cold 2-methylbutane solution. Sections (6 μm) were fixed in 100% cold acetone, blocked with 8% normal goat serum for 2 hours, and incubated with the appropriate primary antibodies (rat anti-mouse CD11b, Hamster anti-mouse CD11c, rat anti-mouse CD8a or rat anti-mouse CD11b, BD Biosciences) for 2 hours at room temperature. Sections were washed 3 times with PBS and incubated with goat anti-rat secondary antibodies coupled to Alexa488 or 597 for CD11b, CD8 and CD4 studies or with Cy5-goat anti-Hamster for CD11c studies. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI). The slides were washed and mounted in DAKO fluorescent mounting medium. The detection of apoptotic cells was performed using a TUNEL-assay (ApoTag Fluorescein In Situ Apoptosis Detection Kit, Promega) in accordance with the manufacturer’s instructions. Immunofluorescence images were collected on a Nikon microscope (Eclipse TE2000-U) and pixels of fluorescence or area of staining was quantified using MetaMorph Software.

**Isolation of tumor-infiltrating myeloid cells and T cells**

Myeloid CD11b$^+$ cells and CD90.2$^+$ (Thy1.2) T cells were isolated from tumor cell suspensions using CD11b or CD90.2 microbeads (Miltenyi Biotech), respectively. The purity of the sorted cells was typically >80% as assessed by FACS analysis. In some experiments, tumor-infiltrating lymphocytes were stained with CD4-FITC (GK1.5, eBioscience) and CD8-APC (53-6.7, eBioscience).
In vitro cytotoxicity assay

Thy1.2+ T cells (effector cells) in tumor were purified from LLC tumor-bearing WT and α4Y991A mice and then co-incubated with LLC tumor cells (target cells) at 2.5:1, 5:1 and 10:1 ratios (2x10^3 LLC tumor cells per well) for 6 hours. Target cell killing was assayed by collecting the supernatants from each well for measurement of the lactate dehydrogenase release (Cytotox96 NonRadioactive Cytotoxicity Assay kit, Promega).

Effect of T cells from mice bearing tumors on tumor cell killing in vivo

CD90.2+ T cells were first isolated from the spleens of WT, α4Y991A and PI3Kγ−/− mice bearing tumors using the MACS system and then mixed with viable LLC tumor cells at ratio 1:2. The mixture was injected into the flanks of naïve mice. Tumor growth, intratumoral apoptosis and necrosis were investigated after 10 days.

IL10 Elisa

Tumors were collected and homogenized in RIPA lysis buffer (137mM NaCl, 20mM Tris-HCL pH8, 10% Glycerol, 1% Triton X-100, 0.1% SDS and protease inhibitor cocktail). Protein extracts were clarified by centrifugation at 12,000 g for 10 minutes at 4°C, and clarified protein samples were used for IL10 ELISA quantification (R&D Systems).

Statistical Analysis

Results were expressed as mean ± SEM. Data comparing differences between two groups were assessed using unpaired Student’s t-test. One-way ANOVA with post hoc testing was used for
multiple comparisons. A value of $P < 0.05$ was considered significant.
RESULTS

Inhibition of PI3Kγ or integrin α4 reduced MDSC accumulation and tumor progression

Tumor inflammation promotes the accumulation of CD11b^+Gr1^+ MDSCs and CD11b^+Gr1^- TAMs that inhibit antitumor immunity, thereby favoring tumor progression (4-8). Recruitment of circulating Gr1^+ myeloid cells into tumors is regulated by PI3Kγ-mediated integrin α4β1 activation (14-18). PI3Kγ also promotes TAM polarization by activating an mTor, S6Kα and C/EBPβ-dependent transcriptional pathway, leading to expression of immune suppressive factors, such as Arginase, IL10 and TGFβ that promote tumor immune suppression (21-22). As integrin α4β1 is downstream of PI3Kγ, we speculated that integrin α4β1 might also regulate immune suppressive transcription in myeloid cells.

To investigate whether integrin α4 regulates immune suppressive myeloid cell polarization in tumors, we first compared tumor growth and inflammation in two different models of tumor growth, the syngeneic subcutaneous Lewis Lung Carcinoma (LLC) and orthotopic Panc02 pancreatic adenocarcinoma (PDAC) models in WT, PI3Kγ^-/- or α4Y991A knock-in mice and in mice treated with inhibitors of integrin α4 or PI3Kγ^-/- (Fig. 1). Integrin α4Y991A mice express a knock-in point mutation in the α4 cytoplasmic tail that inhibits integrin activation, which is controlled by PI3Kγ signaling in myeloid cells (17). LLC and Panc02 tumors implanted in integrin α4Y991A and PI3Kγ^-/- mice were both significantly smaller than tumors implanted in control treated mice (P < 0.001), whether analyzed at endpoint or over time (Fig. 1A-C). Growth of lung
LLC tumors was similarly inhibited in animals treated with inhibitors of α₄ or PI3Kγ: PS2, a function-blocking antibody (anti-α₄) or TG100-115, a PI3Kγ inhibitor (Fig. 1A, Supplementary Fig. S1). Flow cytometric analyses of LLC or Panc02 tumors implanted in WT, integrin α4Y991A and PI3Kγ⁻/⁻ mice revealed significant (P < 0.01) reductions in infiltration of total CD11b⁺Gr1⁺ myeloid cells (Fig. 1D-F), as well as in CD11b⁺Gr1⁻ and CD11b⁺Gr1⁺ (P < 0.05) myeloid cell subpopulations but not of CD11bGr1⁻ cells (Fig. 1G) in PI3Kγ⁻/⁻ and α4Y991A animals compared with WT animals. These populations were further defined by marker analysis as CD11b⁺Gr1⁻Ly6G⁻F4/80⁻CD206⁻CD11c⁻ monocytes, CD11b⁺Gr1⁺Ly6G⁺F4/80⁻CD206⁻CD11c⁻ granulocytes and CD11b⁺Gr1⁻Ly6G⁻F4/80⁻CD206⁻CD11c⁺ macrophages (Fig. 1H). Similar results were observed in tumor-bearing mice treated with the anti-α4 or with the PI3Kγ inhibitor TG100-115 (Fig. 1D-G). These data show that loss of PI3Kγ or integrin α₄ activity affects trafficking of Gr1⁺ but not Gr1⁻ myeloid cells.

Tumors from PI3Kγ⁻/⁻ and α4Y991A animals not only exhibited decreased CD11b⁺ cell infiltration but also increased CD11c⁺ dendritic cell infiltration in tumors and lymph nodes compared to control animals (Fig. 2A-C). Similar results were observed in α4Y991A mice transplanted with α4Y991A bone marrow but not in mice transplanted with WT bone marrow (Fig. 2D-F). The percentages of mature MHCII-expressing dendritic cells (CD11c⁺MHCII⁺) increased in α4Y991A and PI3Kγ⁻/⁻ Panc02 and LLC tumors compared to WT tumors (P < 0.01) and in tumors from animals that were treated with anti-α4 or the PI3Kγ inhibitor TG100-115 (Fig. 2G-I). The ratio of MHCII⁺ mature DC to MHCII⁻ immature DC was 2-fold higher in tumors from these mice compared to control mice (P < 0.001) (Fig. 2J-K). In addition, levels of the co-
stimulatory protein CD80 were significantly ($P < 0.05$) higher in dendritic cells in tumors from PI3Kγ--/- and α4Y991A animals (Fig. 2L). Together, these results indicate that PI3Kγ and α4 integrin inhibit dendritic cell recruitment and maturation in association with tumor inflammation and growth and that inhibitory targeting of these molecules can stimulate dendritic cell recruitment and activation.

**Integrin α4 regulates myeloid cell polarization**

PI3Kγ promotes immune suppressive transcription in TAMs and MDSCs, thereby contributing to tumor immune escape (18, 21-22). Therefore, as integrin α4 is downstream of PI3Kγ, we investigated the role of integrin α4 in myeloid cell polarization. We used RT-PCR to assess the expression of pro- and anti-inflammatory factors in LLC and Panc02 tumors from WT, α4Y991A and PI3Kγ--/- mutant mice and in LLC tumors from anti-α4 and TG100-115 treated mice. We observed increased expression of mRNAs encoding immunostimulatory cytokines, such as IL12b and IFNγ and decreased expression of mRNAs encoding immune suppressive and pro-angiogenic factors, such as IL10, IL6, Arg1, and VEGFA in LLC and Panc02 tumors from α4Y991A and PI3Kγ--/- mice (Fig. 3A-B). Treatment of LLC tumors with anti-α4 or the PI3Kγ inhibitor (TG100-115) had similar effects on inflammatory factor expression (Fig. 3C-D). These gene expression changes were also observed in tumor associated CD11b+ myeloid cells purified from α4Y991A and PI3Kγ--/- mice (Fig. 3E).

To determine whether integrin α4β1 like PI3Kγ, controls macrophage polarization, we also
performed gene expression analyses on in vitro cultured, IL4-stimulated macrophages derived from bone marrow of WT and α4Y991A mice. We found that loss of integrin activity in α4Y991A macrophages suppressed expression of Il10, Arg1, and Tgfb1 and stimulated expression of mRNAs encoding immune stimulatory factors such as IL12 and IFNγ in vitro (Fig. 3F). As IL4-stimulated macrophages exhibit gene expression patterns similar to those of tumor derived macrophages (21), our results indicate that integrin α4β2 plays a role in the PI3Kγ signaling cascade that regulates myeloid cell polarization. Taken together, these results suggest that integrin α4 regulates immunosuppressive myeloid cell transcription downstream of PI3Kγ during tumor inflammation.

IL10, an immune suppressive factor expressed by Tams, MDSCs and other immune cells, impairs DC maturation and effector T-cell function (10). We observed that CD11b+ myeloid cells are the principal source of Il10 expression in LLC tumors (Fig. 4A). IL10 protein and mRNA expression was suppressed in α4Y991A MDSCs and in tumors from α4Y991A BM transplanted animals (Fig. 4B-C). As integrin α4 activity promotes IL10 expression, we further elucidated the role of IL10 in the control of DC maturation during tumor growth. Mice bearing LLC tumors were treated over the course of 10 days with anti-IL10. Although this treatment did not affect tumor growth, it did increase the total percentage of intratumoral mature MHCI+ DC compared to treatment with control isotype-matched antibody (Fig. 4D-F). Mice treated with blocking mAbs to IL10 exhibited increased expression of Il12b, Ifng, and Cd8a mRNA (Fig. 4G-I) as well as increased infiltration of CD8+ T cells as detected by IHC (Fig. 4J-K). Together, these data suggest that the
integrin α4 regulated production of IL10 by CD11b+ myeloid cells prevents DC maturation and inhibits antitumor T-cell responses.

**PI3Kγ–integrin α4 pathway inhibited CD8+ TIL recruitment and activation**

To promote an effective antitumor immune response, tumor-specific T cells must be present in sufficient numbers to kill their targets. A direct correlation exists between the number of tumor-infiltrating lymphocytes (TILs) and a favorable clinical outcome (23-26). Furthermore, the functional status of TILs has been correlated with a favorable prognosis in human malignancies (25-26). To further understand the mechanisms underlying the immune suppression mediated by inhibition of the PI3Kγ–integrin α4 pathway, we evaluated whether blockade of this pathway alters both the number and the activation state of the tumor-infiltrating lymphocytes.

Histological examination of tumors revealed reduced numbers of CD4+ T cells and increased numbers of CD8+ T cells in tumors from both α4Y991A and PI3Kγ−/− mice when compared to WT mice (Fig. 5A-B). Additionally, flow cytometric analysis of tumor-infiltrating lymphocytes isolated from tumors showed that numbers of CD4+ T cells were reduced, whereas numbers of CD8+ T cells were increased, in α4Y991A and PI3Kγ−/− mice compared to WT mice (Fig. 5C-D). These observations were supported by increases in total Cd8a mRNA in tumors from both α4Y991A and PI3Kγ−/− mice when compared to WT mice (Fig. 5E). TILs from α4Y991A and PI3Kγ−/− mice expressed more Ifng and less Tgfb1 and Il10 mRNAs than WT TILs, suggesting that inhibition of myeloid cell PI3Kγ or integrin α4β1 resulted in increased recruitment and priming of CD8+ T cells in tumors (Fig. 5F). To determine whether myeloid cell PI3Kγ or integrin α4β1
promote T cell–mediated tumor cell killing, TILs isolated from WT, α4Y991A or PI3Kγ−/− mice were co-cultured with LLC cells in a direct ex vivo CTL assay. TILs from α4Y991A or PI3Kγ−/− mutant mice exhibited a 2-3 fold increase in LLC tumor cell cytotoxicity over TILs from WT mice (Fig. 5G).

To determine whether PI3Kγ and α4 integrin also suppress T cell–mediated cytotoxicity in vivo, we mixed tumor-infiltrating T lymphocytes isolated from tumors of WT, α4Y991A or PI3Kγ−/− mice with LLC tumor cells and injected the mixture into the flanks of C57Bl/6J naïve mice. When LLC tumor cells were mixed with α4Y991A and PI3Kγ−/− T cells, tumor dimensions and weights were reduced compared to tumors derived from LLC cells mixed with WT T cells (P < 0.001) (Fig. 6A-B). Tumors derived from LLC cells mixed α4Y991A and PI3Kγ−/− T cells exhibited increased intratumoral cell death, as detected by in situ TUNEL assay (Fig. 6C-D) and H&E staining (Fig. 6E-F) in mice injected with α4Y991A and PI3Kγ−/− T cells compared to mice receiving WT T cells. Together, these studies show that integrin α4β1, like PI3Kγ, suppresses T cell–mediated tumor cell cytotoxicity and that inhibiting the PI3Kγ–integrin α4 pathway can stimulate T-cell cytotoxicity.

These studies indicate that myeloid cell PI3Kγ and integrin α4 regulate myeloid cell polarization to control antitumor T-cell activation. In summary, these data show that blocking the PI3Kγ–integrin α4 pathway in myeloid cells can restore antitumor immunity by modulating the tumor microenvironment and promoting tumor cell killing by T cells. We show here that integrin α4β1 regulates myeloid cell polarization and antitumor immunity and that inhibition of this pathway...
could be a useful therapeutic approach to stimulate antitumor immunity.
Discussion

Trafficking of myeloid cells to sites of tumor inflammation depends on integrin $\alpha_4$ and PI3K$\gamma$ (14-18; 21-22). Here, we show that integrin $\alpha_4\beta_1$ controls myeloid cell polarization during tumor progression. Our studies demonstrate that integrin $\alpha_4$, like PI3K$\gamma$, not only regulates myeloid cell trafficking to tumors but also promotes immune suppressive myeloid cell polarization, inhibition of antitumor immunity and stimulation of tumor growth. PI3K$\gamma$ stimulates integrin $\alpha_4$ activation in a manner dependent on BTK, PLC$\gamma$, RAPGEF, Rap1a, RIAM, and paxillin (14-18). PI3K$\gamma$ activates BTK to promote immune suppressive myeloid cell polarization by inducing expression of IL10, TGF$\beta$, and Arginase, which are dependent on mTor, S6K$\alpha$, or C/EBP$\beta$, and inhibiting expression of IL12, IFN$\gamma$ and Nos2 (18, 21-22). C/EBP$\beta$ controls immune suppression in tumor-infiltrating myeloid cells (27). Our present studies show that a PI3K$\gamma$–integrin $\alpha_4$ pathway inhibits antitumor immunity through stimulation of CD11b$^+$Gr1$^+$ MDSC cell recruitment and immunosuppressive myeloid cell polarization. Although it is not clear whether integrin $\alpha_4$ directly participates in PI3K$\gamma$-mediated activation of mTor, S6K$\alpha$, or C/EBP$\beta$, our results indicate that the PI3K$\gamma$–integrin $\alpha_4$ pathway modulates tumor inflammation and immunosuppression.

Several lines of evidence presented here indicate that blockade of this pathway regulates antitumor immune responses. First, inhibition of the PI3K$\gamma$–integrin $\alpha_4$ pathway is associated with reduction of CD11b$^+$Gr1$^+$ MDSC infiltration and immunosuppressive factors (IL10, IL6,
TGFβ, or Arginase) in the tumor microenvironment and lymph nodes of tumor-bearing animals. Blocking this pathway reduced expression of immunosuppressive factors in tumor-infiltrating CD11b+ myeloid cells.

Second, disruption of the PI3Kγ–integrin α4 pathway increased the recruitment of mature DCs into the tumor microenvironment and into the draining lymph nodes. Defects in dendritic cells caused by the immunosuppressive tumor microenvironment result in the accumulation of immature dendritic cells. These immature dendritic cells can promote tumor angiogenesis (28-36) and suppress T-cell responses (10), thus favoring tumor progression. We observed that blockade of a PI3Kγ–integrin α4 pathway could facilitate CD11c+ dendritic cell infiltration and maturation. Dendritic cell differentiation and maturation depends on factors produced by the microenvironment, such as IL10. Our results showed that PI3Kγ and integrin α4 regulate IL10 production by myeloid cells and thereby regulate DC differentiation and maturation.

Third, blockade of the PI3Kγ–integrin α4 pathway increased expression of immunostimulatory cytokines (IL12β, IFNγ), reduced CD4+ T-cell infiltration and promoted CD8+ T-cell accumulation into the tumor microenvironment. Furthermore, inhibition of this pathway promoted T cell–mediated tumor cytotoxicity and tumor rejection. We did not observe fewer T cells, but rather more T cells, in tumors from α4Y991A and PI3Kγ−/− mice. Our studies show that integrin α4 does not control T-cell trafficking to tumors. Indeed, PI3Kγ deficiency increases CD8+ cell differentiation and Th1 cytokine production (IL12, IFNγ) but does not directly affect Th1/Th2 T-cell polarization (22).
Finally, the PI3Kγ–integrin α4 pathway also affects tumor progression by promoting angiogenesis (17). Myeloid cells can support tumor angiogenesis and growth through the production of proangiogenic factors (28-32). We found that blockade of the PI3Kγ–integrin α4 pathway reduced expression of proangiogenic factors and tumor angiogenesis. Inhibition of this pathway increased myeloid cell expression of IL12, an anti-angiogenic Th1 cytokine (33-34). Therefore, inhibition of PI3Kγ and integrin α4 controls both tumor angiogenesis and immunity.

Inhibition of the PI3Kγ-α4 integrin pathway could also influence development of regulatory T cells (Treg), which maintain tumor immune tolerance (34). In fact, we found that TGFβ, a cytokine that induces the generation of Treg (35), was down-regulated in T cells from PI3Kγ and α4 integrin mutant mice, suggesting that disruption of this pathway reduces the generation of Treg in the tumor microenvironment.

The tumor microenvironment is a barrier to immune cell function. Our results indicate that blocking the PI3Kγ-α4 integrin pathway can inhibit the immunosuppressive tumor microenvironment and promote antitumor immunity. Our results support the development of strategies based on the inhibition of PI3Kγ- integrin α4 pathway to control tumor immune suppression.
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FIGURE LEGENDS

Figure 1: PI3Kγ and integrin α4 similarly impact tumor progression and CD11b+Gr1+ MDSC infiltration.

(A) Weights of LLC tumors implanted in WT versus α4Y991A or PI3Kγ−/− animals and of tumors that were treated with anti-α4 blocking or isotype antibodies (10mg/kg every 3 days) or with the PI3Kγ inhibitor TG100-115 or vehicle (****P < 0.0001) (n = 10). (B) Time course of LLC tumor growth in WT versus α4Y991A or PI3Kγ−/− animals (n = 10). (C) Weights of orthotopic Panc02 pancreatic adenocarcinoma tumors from WT, α4Y991A or PI3Kγ−/− animals (***P < 0.001) (n = 10). (D) Representative FACs profiles of CD11b+ myeloid cell populations in LLC tumors from WT, α4Y991A, and PI3Kγ−/− mice from A (**P < 0.01). (E) Quantification of CD11b+Gr1+ myeloid cells as a percent of live cells in tumors from A (n = 4). (F) Quantification of CD11b+Gr1+ myeloid cells as a percent of live cells from C (**P < 0.01) (n = 4). (G) Quantification of CD11b+Gr1hi neutrophils, CD11b+Gr1lo monocytes and CD11b+Gr1− macrophages as a percent of live cells in tumors from A (n = 4) (*P < 0.05; **P < 0.01). (H) FACs profiles of Ly6G, F4/80, CD206 and CD11c in the three myeloid cell populations observed in D and quantified in G. Similar results were obtained in 3 replicate experiments. Error bars indicate standard error of the mean (SEM).

Figure 2. Blockade of PI3Kγ–integrin α4 pathway promotes infiltration and maturation of CD11c+ dendritic cell in the tumor microenvironment.
Representative images of CD11c<sup>+</sup> dendritic cells (green, arrowhead) and CD11b<sup>+</sup> myeloid cells (red, arrow) in cryosections from LLC and Panc02 tumors and LLC tumor draining lymph nodes from WT and α4Y991A mice. Scale bar: 100μm. (B) Quantitative analysis of CD11b<sup>+</sup> and CD11c<sup>+</sup> cells in LLC and Panc02 tumors in WT and α4Y991A mice from A, expressed as pixels per field (n = 5). **P < 0.01 vs WT. (C) Quantitative analysis of CD11b<sup>+</sup> and CD11c<sup>+</sup> cells in LLC tumor draining lymph nodes in WT and α4Y991A mice from A, expressed as pixels per field (n = 5). **P < 0.01 vs WT. Scale bar: 100μm. (D) Representative images of CD11c<sup>+</sup> dendritic cells and CD11b<sup>+</sup> myeloid cells in LLC tumors implanted in integrin α4Y991A mice transplanted with WT or α4Y991A BM and in WT mice transplanted with α4Y991A or WT BM. Scale bar: 100μm. (E-F) Quantitative analysis of (E) CD11b<sup>+</sup> and (F) CD11c<sup>+</sup> pixels in tumors from D (n = 5). (G) Quantitative analysis of CD11c<sup>+</sup>MHCII<sup>+</sup> mature dendritic cells in Panc02 tumors assessed by flow cytometry in WT, α4Y991A, and PI3Ky<sup>−/−</sup> mice (n = 5). **P < 0.01 vs WT. (H) Representative FACs plots of MHCII expression in CD11c<sup>+</sup> cells from G. (I) Quantification of CD11c<sup>+</sup>MHCII<sup>+</sup> mature DC infiltration in LLC tumors assessed by flow cytometry in WT, α4Y991A, and PI3Ky<sup>−/−</sup> mice and mice treated with anti-α<sub>4</sub> or PI3Ky inhibitor TG100-115 (n = 5), **P < 0.01. (J-K) Quantification of the ratio between mature DC (CD11c<sup>+</sup>/MHCII<sup>+</sup>) and immature DC (CD11c<sup>+</sup>/MHCII<sup>−</sup>) in Panc02 (J) and LLC (K) tumors from (G and I) (n = 5) *P < 0.05, **P < 0.01. (L) Mean fluorescence intensity of CD11c, CD80, and CD86 in CD11b<sup>+</sup> cells from d14 LLC tumors from WT, PI3Ky<sup>−/−</sup>, and α4Y991A animals (n=4). *indicates P < 0.05 for differences between WT and mutants. Error bars indicate standard error of the mean (SEM).

Figure 3. Blockade of PI3Ky–integrin α<sub>4</sub> pathway repolarizes tumor associated myeloid cells
(A-B) mRNA expression of immune related cytokines in (A) LLC and (B) Panc02 tumors from WT, α4Y991A, and PI3Kγ−/− mice (n = 4) *P < 0.05. (C-D) mRNA expression of immune related cytokines in LLC tumors from mice that were treated with (C) anti-α4 versus isotype matched control antibody or (D) PI3Kγ inhibitor TG100-115 versus control (n = 4) **P < 0.01. (E) mRNA expression of immune related cytokines in LLC tumor associated myeloid cells from WT, α4Y991A, and PI3Kγ−/− mice (n = 4) **P < 0.05. (F) mRNA expression of immune related cytokines in in vitro IL4 stimulated WT and α4Y991A macrophages (n = 4) *P < 0.05. Error bars indicate standard error of the mean (SEM).

Figure 4: Integrin α4 activation controls IL10 expression

(A) Il10 mRNA levels ± SEM in CD11b− and CD11b+ cell populations from LLC tumors (n = 3). (B) IL10 protein concentration (pg/mg total protein) ± SEM in LLC tumors from WT and α4Y991A animals (n = 3) *P < 0.05. (C) Il10 mRNA levels ± SEM in tumors from WT and α4Y991A bone marrow transplanted animals (n = 3) *P < 0.05. (D) Mean LLC tumor volumes ± SEM from mice that were treated with two doses of anti-IL10 or cIgG (n = 6) *P < 0.05. (E) Percent CD11c+MHC+ DCs in tumors from D (n = 6) *P < 0.05. (F) Quantification of the ratio of mature DC (CD11c+/MHCII+) and immature DC (CD11c+/MHCII−) in mice from D (n = 6) **P < 0.01. (G) Il12b, Ifng, and Cd8a mRNA expression in tumors from D (n = 3) *P < 0.05 **P < 0.01. (J) Representative images of CD8+ T cells in LLC tumors from D. Scale bar: 100μm. (K) Mean CD8+ T cells in tumors ± SEM (pixels/field) (n = 3) **P < 0.01. Error bars indicate standard error of the mean (SEM).
Figure 5. Blockade of PI3Kγ–integrin α4 pathway promotes CD8⁺ T-cell infiltration and T-cell cytotoxicity

(A) Representative images of CD4⁺ and CD8⁺ T-cell infiltration in LLC tumors in cryosections from WT, α4Y991A and PI3Kγ⁻/⁻. Scale bar: 100μm. (B) Quantitative analysis CD4⁺ and CD8⁺ positive T cells in tumors from WT, α4Y991A and PI3Kγ⁻/⁻ mice (n = 5), **P < 0.01 vs WT. (C) Flow cytometry analysis of CD4⁺ and CD8⁺ tumor-infiltrating lymphocytes from LLC tumor-bearing mice from WT, α4Y991A, and PI3Kγ⁻/⁻ animals. (D) The ratio of CD8⁺ to CD4⁺ T cells in tumors from WT, α4Y991A, and PI3Kγ⁻/⁻ animals (n = 3), *P < 0.05 vs WT. (E) Relative expression of Cd8a mRNA in TILs from LLC tumors from WT, α4Y991A, and PI3Kγ⁻/⁻ animals (n = 4), *P < 0.05. (F) mRNA expression of Ifng, Il10, and Tgfb mRNA in TILs from LLC tumors from WT, α4Y991A, and PI3Kγ⁻/⁻ animals (n = 4). (G) Tumor-infiltrating lymphocytes from LLC tumors from WT, α4Y991A, and PI3Kγ⁻/⁻ animals were isolated and mixed with LLC tumor cells. T cell-cytotoxic activity against parental LLC tumor cells was assessed in an ex vivo CTL assay (n = 6) *P < 0.05 vs WT. Error bars indicate standard error of the mean (SEM).

Figure 6. Blockade of PI3Kγ–integrin α4 pathway stimulates T cell–mediated cytotoxicity in vivo.

(A) LLC tumor cells were mixed with T cells isolated from LLC tumors grown in WT, α4Y991 or PI3Kγ⁻/⁻ mice. The mixture was injected in the flank of naïve mice. Representative images of LLC tumors are shown. Scale bar: 1cm. (B) Tumor weights analyzed after 10 days (n = 9-10), ***P < 0.001 vs WT. (C) Representative images of apoptotic cells in LLC tumor by TUNEL immunostaining. (D) Quantification of TUNEL⁺ cells from C (n = 9-10), ***P < 0.001 vs WT. (E)
Representative images of necrosis in LLC tumors by H&E staining. (F) Quantification of necrotic areas in LLC tumors (n = 9-10). ***$P < 0.001$ vs WT. Error bars indicate standard error of the mean (SEM).
Figure 3

A. LLC tumor

Relative mRNA expression

WT  | α4Y991A | PI3Kγ−/−
--- | --- | ---
Il10 | * | *
Arg1 | * | *
Tgfb | * | *
Il6 | * | *
Il1b | * | *
Vegfa | * | *
Il12b | * | *
lfng | * | *

B. Panc02 tumor

Relative cytokine expression

WT  | α4Y991A | PI3Kγ−/−
--- | --- | ---
Il10 | * | *
Il6  | * | *
Vegfa | * | *
Tgfb | * | *
Il12b | * | *
lfng | * | *

C. LLC tumor

Relative cytokine expression

IgG2b | anti-α4
--- | ---
Il10 | *
Il6  | *
Vegfa | *
Tgfb | *
Il12b | *
lfng | *

D. LLC tumor

Relative cytokine expression

Placebo | TG100-115
--- | ---
Il10 | *
Il6  | *
Vegfa | *
Tgfb | *
Il12b | *
lfng | *

E. TAMs

Relative mRNA expression

WT  | α4Y991A | PI3Kγ−/−
--- | --- | ---
Il10 | * | *
Arg1 | * | *
Tgfb | * | *
Il6  | * | *
Il1b | * | *
Vegfa | * | *
Il12b | * | *
lfng | * | *
iNos | * | *

F. BMM

Relative mRNA expression

WT  | α4Y991A
--- | ---
Il10 | *
Arg1 | *
Il6  | *
Tnfa | *
Il1b | *
Nos2 | *
Il12b | *
Figure 4

A) Relative If/10 mRNA expression

B) Concentration IL-10 (pg/ml)

C) Relative Ilf10 mRNA expression

D) Tumor volume (mm3)

E) % CD11c+ MHCII+ (mature DCs)

F) Ratio mDC/imDC

G) Relative If/2b mRNA expression

H) Relative Ifng mRNA expression

I) Relative C08 mRNA expression

J) CD8/DAPI

K) CD8+ pixel / field (x10^3)
Figure 6

A

Ctrl
WT T cells
α4Y991A T cells
PI3Kγ−/− T cells

B

LLC tumor weight (mg)

Ctrl
WT T cells
α4Y991A T cells
PI3Kγ−/− T cells

C

Ctrl
WT T cells
α4Y991A T cells
PI3Kγ−/− T cells

D

TUNEL-positive area

Ctrl
WT α4Y991A
PI3Kγ−/−

T cells + LLC

E

Ctrl
WT T cells
α4Y991A T cells
PI3Kγ−/− T cells

F

Intratumoral necrosis (mm²)

Ctrl
WT α4Y991A
PI3Kγ−/−

T cells + LLC

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PI3Kgamma activates integrin alpha4 and promotes immune suppressive myeloid cell polarization during tumor progression

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