Antagonizing Integrin β3 Increases Immunosuppression in Cancer

Xinming Su, Alison K. Esser, Sarah R. Amend, Jingyu Xiang, Yalin Xu, Michael H. Ross, Gregory C. Fox, Takayuki Kobayashi, Veronica Steri, Kirsten Roomp, Francesca Fontana, Michelle A. Hurchla, Brett L. Knohlhoff, Melissa A. Meyer, Elizabeth A. Morgan, Julia C. Tomasson, Joshua S. Novack, Wei Zou, Roberta Faccio, Deborah V. Novack, Stephen D. Robinson, Steven L. Teitelbaum, David G. DeNardo, Jochen G. Schneider, and Katherine N. Weilbaecher

Abstract

Integrin β3 is critical for tumor invasion, neoangiogenesis, and inflammation, making it a promising cancer target. However, preclinical and clinical data of integrin β3 antagonists have demonstrated no benefit or worse outcomes. We hypothesized that integrin β3 could affect tumor immunity and evaluated tumors in mice with deletion of integrin β3 in macrophage lineage cells (β3KOM). β3KOM mice had increased melanoma and breast cancer growth with increased tumor-promoting M2 macrophages and decreased CD8+ T cells. Integrin β3 antagonist, cilengitide, also enhanced tumor growth and increased M2 function. We uncovered a negative feedback loop in M2 myeloid cells, wherein integrin β3 signaling favored STAT1 activation, an M1-polarizing signal, and suppressed M2-polarizing STAT6 activation. Finally, disruption of CD8+ T cells, macrophages, or macrophage integrin β3 signaling blocked the tumor-promoting effects of integrin β3 antagonism. These results suggest that effects of integrin β3 therapies on immune cells should be considered to improve outcomes.

Introduction

Integrins are heterodimeric cell surface receptors that directly bind components of the extracellular matrix (ECM) and participate in cell migration and other important cellular functions (1, 2). Integrin B3 can heterodimerize with integrin αv or αIIb and is critical for mature osteoclastic bone resorption, platelet aggregation, and angiogenic endothelial cell function (3, 4). Tumor cells expressing high levels of integrin β3 exhibit enhanced proliferation and metastasis (5–7). In vitro and in animal models, the integrin αvβ3 antagonist cilengitide was shown to decrease tumor cell proliferation, migration, and neoangiogenesis (8). Mice genetically deleted for integrin β3 (lgb3−/−) have decreased metastasis (9). Surprisingly, primary tumor growth is enhanced in lgb3−/− mice (10). However, cilengitide can enhance tumor growth and angiogenesis if applied suboptimally in preclinical models (11). In clinical trials, integrin β3 antagonists did not demonstrate improved outcomes in many cancer types (12–14). These findings suggest a need to better understand the role of integrin β3 in the tumor microenvironment before translating encouraging preclinical discoveries to patients.

We previously reported that integrin β3 deletion in platelets has no effect on primary tumor growth and angiogenesis, but specific deletion of integrin β3 in myeloid cells via lysozyme M promoter-driven Cre recombinase (LysM-Cre) results in enhanced tumor growth (15), consistent with both the phenotype observed in lgb3−/− mice (10) and results from bone marrow transplantation studies (16). Interestingly, in endothelial cells, acute depletion of integrin β3 transiently inhibits tumor growth and angiogenesis, but long-term deletion has no effect on primary tumor growth (17). These results suggest that integrin β3 signaling in myeloid cells is most likely to account for the enhanced tumor growth seen in genetic and pharmacologic studies.

Fibroblasts, stromal cells, myeloid cells/macrophages, and lymphocytes are very common tumor-infiltrating cells that play key roles in tumor progression and metastasis (18). Of these, macrophages are among the most abundant recruited host cells in tumors (19). For many solid tumor types, including breast, brain, and skin cancer, high densities of tumor-associated macrophages (TAM) are generally associated with poor clinical outcome.
(19, 20). Macrophage recruitment and polarization are dynamic processes in the tumor microenvironment because of the complex milieu of chemotactant and polarization signals in vivo (20). TAMs that express high levels of MHCI usually have a tumoricidal, antitumor, M1 phenotype, whereas TAMs expressing scavenger receptors, such as CD163, CD204, and CD206, are associated with a tumor-promoting and immunosuppressive M2 phenotype (19, 21). Lymphocyte-secreted factors, such as IL4, IL10, IL13, IFNγ, TNFα, and immunoglobulins, are very strong regulators of macrophage polarization and function (20, 22). M2 TAMs can suppress immune responses by decreasing intratumor CD8⁺ T-cell numbers and have potent proangiogenic activity through secretion of factors like VEGF (19, 23, 24). STAT1 is a dominant transcriptional factor that regulates M1 macrophage polarization. Lipopolysaccharide (LPS)-activated TLR4 signaling and IFNγ promote macrophage M1 polarization through activation of STAT1 (25, 26). STAT1 is required to drive M2 macrophage polarization in the presence of IL4 or IL13 (25, 26). STAT1 and STAT6 signaling events regulate and inhibit each other and control the polarization and function of macrophages (25, 26).

β3 integrins play key roles in myeloid lineage osteoclast differentiation and function and in preclinical models of pathologic inflammation (3, 6). Because loss of integrin β3 in myeloid cells results in enhanced tumor growth (15), we hypothesized that integrin β3 may be involved in macrophage polarization and function in the tumor microenvironment. Here, we present data uncovering an antitumoral role for integrin β3 signaling in this context. Integrin β3 signaling controls the balance between antitumor and protumor immune cells through effects on STAT1/STAT6 signaling, which in part explains the mixed results of integrin antagonists in the clinic. Taken together, our findings highlight the important role of TAMs when designing clinical trials with integrin β3-targeted treatments in cancer.

Materials and Methods

Mice

All animal studies were performed according to the guidelines established by the Animal Studies Committee at the Washington University in St. Louis (St. Louis, MO). Wild-type (WT), LysM-Cre knockin mice and Stat6ox/ox mice are from The Jackson Laboratory (all C57BL/6J background). Igbgαfl/fl (15) and Igbg3−/− mice (27) on a pure C57BL/6J background were used. All mice are housed under pathogen-free conditions according to the guidelines of the Division of Comparative Medicine, Washington University School of Medicine. Unless noted, all mice were used at 6 to 10 weeks of age.

Cell lines

In 2013, the parental MMTV-PyMT cells (PyMT-B6; kindly provided by D.G. DeNardo) were isolated from a fully invasive mammary tumor that spontaneously arose at day 120 in a C57BL/6 background MMTV-PyMT mouse, a mouse model that represents an antiestrogen-sensitive, luminal B breast cancer. The tumor was collagenase treated, grown in single-cell suspension on a collagen-coated plate, and cloned to establish the parent PyMT-B6 cell line. Parent PyMT-B6 cells were injected into the mammary fat pad (MFP) tissue of a female C57BL/6J mouse, and after reaching a tumor size approaching 1 cm, tumor cells were collagenase treated and cultured in a cell culture dish. The cultured tumor cells were intracardially injected into a 6-week-old female C57BL/6J mouse to establish bone metastases. Twelve days after intracardiac inoculation, the bone tumor was harvested and cultured in a cell culture dish with DMEM media plus 10% FBS to establish the PyMT-BO1 subline, which when compared with the parent PyMT-B6 cells, had a higher incidence of inducing bone metastases after either orthotopic MFP or intracardiac injection. The PyMT-BO1 cells were infected with lentivirus containing the GFP-firefly luciferase genes as described previously (28). GFP-expressing PyMT-BO1 cells were FACS sorted, cultured, and validated for luciferase expression; this cell line was named PyMT-BO1-GFP-Luc. PyMT-B6, PyMT-BO1, and PyMT-BO1-GFP-Luc cells were evaluated by qPCR, and all express the PyMT, Est1, Est2, and Igbg3 genes. These cell lines were tested as CD45 negative and integrin β3 positive by FACS.

Mice tumor models

For MFP injection, 1 × 10⁵ PyMT-BO1-GFP-Luc cells mixed with BD Matrigel (BD Biosciences) were injected into MFP tissue of 8-week-old female mice. For subcutaneous injections, 1 × 10⁶ B16F10-Luc cells in 200 µL PBS were injected into the flank of WT and B3KOM mice. Tumor growth was measured at each indicated time point. Tumor size (mm³) was calculated by measuring the longest (L) and shortest (S) distance of tumor tissue, with this formula: tumor size = 0.5 × L × S².

For intracardiac injections, the left ventricular chamber of 6-week-old mice was injected with 1 × 10⁵ B16F10-Luc cells or 1 × 10⁵ PyMT-BO1-GFP-Luc cells in 50 µL PBS as described previously (30). Bioluminescence imaging (BLI) was used to quantify tumor growth after injection. For the cingitide treatment, mice were given cingitide (5 mg/kg, Selleckchem) by intraperitoneal injection for the indicated time point. For anti-CSF1 antibody (clone 5A1) treatment, mice were given three doses of antibody (1, 0.5, and 0.5 mg per mouse) by intraperitoneal injection. For CD8⁺ T-cell depletion, mice were given an intraperitoneal injection of 100 µg anti-CD8α (53-6.7; BioLegend) or rat IgG2b κ-chain isotype-matched control antibody (RTK4530; BioLegend) on the appropriate days.

Flow cytometric analysis

Tumor tissues were prepared in a single-cell suspension for FACS analysis (31), with minor modifications (Supplementary Information).

Primary macrophage culture

Primary bone marrow macrophages (BMM) were generated from the femurs and tibias of mice as described previously (30) (Supplementary Information).

Microarray analysis

Microarray was performed with the Genome Technology Access Center at Washington University School of Medicine (Supplementary Information). The microarray data in this article are available on the GEO database with accession number GSE75882.
qPCR
qPCR was performed using SYBR Advantage Mix (Bio-Rad) with mouse-specific primers as specified in Supplementary Table S4 (Supplementary Information).

BLI
Live BLI analysis was described previously (30), with minor modifications (Supplementary Information).

Western blot analysis
Antibodies and protocol were used from Cell Signaling Technology (Supplementary Information).

Statistical analysis
Data are shown as mean ± SD unless noted otherwise. All experiments were analyzed using two-tailed unpaired Student t test for two groups or one-way ANOVA with a Bonferroni post hoc test for three or more groups by Prism (GraphPad Software). P < 0.05 was considered significant.

Results
Mice with myeloid-specific–targeted disruption of integrin β3 have enhanced primary tumor growth and increased M2/M1 macrophage ratios
Previous studies have shown that Itgb3−/− mice have enhanced primary tumor growth (10), but the mechanism is largely unknown. Here, we evaluated tumor growth in β3KOM mice lacking integrin β3 specifically in myeloid cells (LysM-Cre+/−, Itgb3fl/fl−) and in WT (LysM-Cre+/−, Itgb3fl/fl+) mice using immunocompetent allograft orthotopic tumor models. We found that in both orthotopic melanoma subcutaneous tumor model and a breast cancer MFP model, tumor growth was enhanced in β3KOM mice (Fig. 1A–D). There was no difference in the number of blood vessels between WT and β3KOM mice as measured by endomucin staining in size-matched tumor tissue (Supplementary Fig. S1).

TAMs, osteoclasts, and myeloid-derived suppressor cells are Ly6M+ myeloid cells that have been demonstrated to support tumor growth through immunosuppression, promotion of bone resorption, and stimulation of neoangiogenesis (19, 32, 33). We asked whether loss of integrin β3 in myeloid cells altered immune cell infiltration of primary tumor tissue. Tumor-infiltrating immune cells from WT and β3KOM mice were analyzed by flow cytometry. To avoid effects from varying tumor size, we analyzed similar size tumors before statistically significant changes in tumor growth were observed (day 11 for B16F10 and PyMT-B01). Myeloid cell populations were defined by FACS as described previously (31) and by the FACS gating strategy detailed in Supplementary Fig. S2. In both tumor types, although the overall number of tumor-infiltrating CD45+ cells and TAMs (CD45+CD11b+Ly6C−Ly6G−F4/80−) was not significantly different between WT and β3KOM mice (Fig. 1E and F), β3KOM tumors displayed a significant increase in the number of tumor-promoting M2 TAMs (CD206hi) and a significant decrease in M1 TAMs (MHCIIhi; Fig. 1E and F). Gene expression of FACS-sorted CD11b+ cells from tumors established in β3KOM mice and WT mice revealed upregulation of the M2 TAM markers CD163, Fizz1, Arg1, and Ym1 in β3KOM CD11b+ cells as compared with WT CD11b+ cells (Fig. 1G). Furthermore, there was a significant decrease in CD8T-cell number in β3KOM tumors as compared with WT tumors (Supplementary Table S1). These results suggest that genetic deletion of integrin β3 in myeloid cells increases the M2/M1 TAM ratio in both orthotopic melanoma and breast cancer models.

Integrin β3 knockout M2 macrophages have enhanced tumor-promoting function
To determine whether the increased number of M2 macrophages alone can explain the increased tumor growth seen in β3KOM mice, or whether these β3KOM M2 TAMs also display enhanced function, we adoptively transferred ex vivo–polarized WT and Itgb3−/− M2 macrophages directly into B16F10 tumors. We found that the Itgb3−/− M2 macrophages were more potent in promoting tumor growth than WT M2 macrophages (Fig. 2A). To account for the effects of integrin loss on M2 cell migration and homing, we adoptively transferred BMMs through intracardiac injection into mice bearing PyMT-B01 MFP tumors. Again, we observed that breast cancer bearing mice that received Itgb3−/− BMMs had larger tumors (Fig. 2B). Together, these data suggest that integrin β3 is a negative regulator of tumor-promoting function in M2-polarized macrophages.

Despite comparable numbers of Foxp3+ Tregs, we also found that CD8T-cell numbers were significantly decreased in β3KOM tumors compared with WT tumors (Supplementary Table S1). To determine whether CD8+ T cells contributed to the enhanced tumor growth in β3KOM mice, we depleted CD8+ T cells in WT and β3KOM mice by anti-CD8α antibody injection and monitored tumor growth. As expected, depletion of CD8+ T cells enhanced tumor growth in WT mice; however, there was no additional enhancement of tumor growth in β3KOM mice (Fig. 2C and D). These data show that CD8+ T cells in the tumor microenvironment contributed to the enhanced tumor growth phenotype observed in β3KOM mice.

Pharmacologic blockade of integrin αβ3–enhanced tumor growth and increased M2 TAM infiltration
Recent clinical trials with cilengitide have failed to improve survival in glioblastoma, non–small cell lung cancer (NSCLC), metastatic melanoma, prostate cancer, and advanced resectable pancreatic cancer (12–14, 34–36). As the β3KOM mice exhibit enhanced tumor growth and increased M2 macrophage numbers (Fig. 1), we evaluated the effect of pharmacologic blockade of integrin β3 on TAMs by establishing PyMT-B01 MFP tumors in WT immunocompetent mice. Treatment with cilengitide, initiated when the tumors were fully established (day 10, ~200 mm3), significantly enhanced tumor growth (Fig. 3A–C). Analysis of the tumor-infiltrating myeloid cell population by FACS revealed that cilengitide treatment increased M2 TAMs in the tumor tissue (Fig. 3D). Interestingly, cilengitide administration to early-stage tumors (less than 100 mm3) did not increase tumor burden; however, it did not decrease tumor burden either (Supplementary Fig. S3). Day 10 tumors had almost double the number of myeloid cells and exhibited 50% decreases in T-cell numbers compared with day 6 tumors (Supplementary Fig. S3), making them potentially more susceptible to the myeloid cell effects of cilengitide. These results show that cilengitide treatment can adversely affect breast cancer tumor growth in immunocompetent mice and results in increased tumor-infiltrating M2 TAMs.
Tumor cells and M2 macrophages from β3KOM mice have diminished immune response signatures

To better understand the impact of integrin β3 signaling in myeloid cells on the tumor microenvironment, we compared the gene expression profiles of FACS-isolated GFP⁺ PyMT-BO1 MFP tumor cells and M2 TAMs (CD11b⁺ Gr1⁻ F4/80⁺ CD206⁺) from WT and β3KOM tumor tissue. For the microarray experiment data analysis, an unadjusted P value cutoff of less than 0.01 was selected to generate lists of differentially expressed genes (DEG; accession number GSE75882). For the CD206⁺ TAMs WT versus β3KOM comparison, 122 unique annotated genes were identified (Supplementary Table S2). Term enrichment (produced by the PANTHER classification system) for genes upregulated in M2 TAMs from the β3KOM tumor identified many immune-related pathways.

Figure 1. Mice with specific deletion of integrin β3 in myeloid cells promoted tumor growth with increased M2 TAM infiltration. A, growth curve of subcutaneously injected B16F10-Luc cells (1 × 10⁵) in 8-week-old male WT (LysM-Cre⁺⁻, Itgb3f/-) and β3KOM (LysM-Cre⁻⁻, Itgb3f/-) mice (n = 8). B, tumor weight. C, growth curve of MFP-injected PyMT-BO1-GFP-Luc cells (1 × 10⁵) in 8-week-old female WT and β3KOM mice (n = 6). D, tumor weight. E and F, FACS staining was performed on cells from day 11 tumor tissue. TAMs were gated on CD45⁺ cells, and M1 TAMs were gated as MHCII⁺/CD206⁻ cells, and M2 TAMs were gated as MHCII⁻/CD206⁺ cells. G, M2 TAM markers CD163, Fizz1, Arg1, and Ym1 mRNA expression in CD11b⁺ cells isolated from day 11 tumor tissue (n = 3). Data, mean ± SEM. *P < 0.05; **P < 0.01; ***P < 0.001.
biologic processes to be significantly overrepresented (Fig. 4A). A more diverse list of biologic processes was identified from the downregulated genes. Among them, IFNβ response genes were found to be significantly downregulated in B3KOM M2 TAMs (Fig. 4A).

The list of DEGs generated from the comparison of CD206hi TAMs in WT versus B3KOM mice was used for pathway analyses with IPA Ingenuity. An upstream analysis was performed, and four genes (Tgfβ1, Hgf, Tnfa, and Vegf) were predicted to be upregulated in B3KOM with an activation Z-score greater than 2.0 (Fig. 4B). TGFβ1, HGF, TNFα, and VEGF may generally promote tumor growth and are related to TAM function. All genes connected directly to these four upstream genes were placed in a network, which also included mir-10 (also called mir-99a). Mir-10 was the most downregulated gene/miRNA in B3KOM and has been shown to inhibit TNF (37). Cytotoxic T lymphocyte antigen-2α and -2β (Cita2a, Cita2b) were also upregulated in B3KOM CD206hi TAMs; of the two, Cita2a was shown to inhibit effector T-cell function (38). Together, these data suggest that loss of integrin β3 results in a tumor-promoting, immunosuppressive gene expression signature in TAMs.

Gene expression analysis of isolated tumor cells established in WT versus B3KOM mice identified 130 unique annotated genes (Supplementary Table S2). Changes in tumor cell gene expression reflected myeloid integrin β3-dependent changes to the tumor microenvironment. An interaction network consisting of 53 nodes was subsequently generated from the DEGs in tumor cells from WT versus B3KOM mice (Fig. 4C). In this network, Stat1 (downregulated in B3KOM) and Il1b (upregulated in B3KOM) are two highly connected nodes. Among the downregulated genes in tumor cells isolated from B3KOM mice, Stat1 and Cxcl10 belong to the IFNγ pathway. Taken together, these data indicate that IFN-related pathways are downregulated in the B3KOM
tumor microenvironment. STAT1 is an important regulator in IFN-related pathways (39), suggesting that integrin β3 may be involved in the regulation of STAT1 pathway genes in the tumor microenvironment.

Integrin β3 favors STAT1 activation and suppresses STAT6 signaling in macrophages

We found that macrophages lacking integrin β3 had down-regulation of IFN (STAT1) pathway genes in vivo (Fig. 4). We evaluated the role of integrin β3 signaling when macrophages were polarized in M1 (LPS-STAT1) or M2 (IL4-STAT6) conditions. After LPS treatment, *Itgb3*−/− BMMs had decreased phosphorylation of STAT1 compared with WT (Fig. 5A and B). The mRNA expression of the STAT1 downstream gene *Ccl5* was also decreased in *Itgb3*−/− BMMs after LPS treatment (Supplementary Fig. S4A). Spleen tyrosine kinase (SYK) phosphorylation is usually associated with integrin activation (40). Genetic or pharmacologic (cilengitide) disruption of integrin β3 significantly decreased SYK phosphorylation in macrophages (Fig. 5A and C). We evaluated the effect of LPS on integrin β3 expression and activation in BMMs and found that in a short time course, LPS had little effect on integrin β3 expression (Supplementary Fig. S5A). However, LPS had a potent effect on integrin β3 activation as measured by binding of IntegrinSense-680, a ligand for activated integrin αvβ3 (Supplementary Fig. S5C). To determine whether decreased STAT1 signaling was caused by the loss of integrin β3, we performed a rescue experiment. Integrin β3 was transduced into *Itgb3*−/− BMMs and rescued the defect in STAT1 phosphorylation after LPS treatment (Fig. 5D). To determine whether the expression of other integrins was altered by genetic disruption of *Itgb3*, we compared the mRNA expression of other integrin genes between polarized and nonpolarized WT and *Itgb3*−/− BMMs (Supplementary Fig. S6). Interestingly, the mRNA expression of some integrins was indeed affected, suggesting that integrin β3 disruption can modulate expression of other integrins. That said, ITGAV and ITGB5, which together bind the same RGD motif in integrin αvβ3, were not significantly changed after disruption of ITGB3 and were thus unlikely to have compensatory effects in *Itgb3*−/− BMMs. Together, these results demonstrate that disruption of integrin β3 caused decreased phosphorylation of both the integrin β3 signaling molecule SYK and the M1-polarizing molecule STAT1 in macrophages.

β3KOM mice had increased M2 macrophages with enhanced tumor-promoting function (Fig. 1), so we evaluated the M2-polarizing STAT6 signaling pathway in *Itgb3*−/− BMMs. In contrast to WT, *Itgb3*−/− BMMs treated with the M2 polarization factor IL4 showed enhanced STAT6 signaling and increased expression of STAT6 downstream gene *Ym1* (Fig. 5E and F and Supplementary Fig. S4B). Enhanced STAT6 signaling was also observed in WT BMMs pretreated with cilengitide (Supplementary Fig. S7). IL4 has been shown to increase integrin β3 mRNA expression in BMMs (41). We found that while IL-4 induced integrin β3 gene and protein expression in WT BMMs, this was abrogated in STAT6−/− BMMs (Fig. 5G and H). Taken together, we find that integrin β3 signaling regulates the balance of

Figure 3.

Cilengitide treatment enhanced tumor growth and increased M2 TAM population. A, experimental schema. PyMT-BO1-GFP-Luc cells (1 × 10⁶) were injected into MFP of 8-week-old female WT mice (*n* = 5). Starting at day 10 after tumor cell injection, mice were treated daily with cilengitide for 5 days. At day 14, all mice were sacrificed. i.p., intraperitoneal. B, tumor growth was measured at the indicated time points by BLI. C, tumor weight at day 14. D, TAM populations in tumor tissue were analyzed by FACS. Data, mean ± SEM. *, *P* < 0.05; **, *P* < 0.01. ns, nonsignificant.
Figure 4.
Gene expression profiling of tumor cells and M2 macrophages from B3KOM mice reveals diminished immune response signatures. Pathway-based analysis of microarray data. A, biologic process, term enrichment results produced by PANTHER classification system (GO) for both up- and downregulated genes in B3KOM tumors. B, upstream analysis of WT CD206hi macrophages versus B3KOM CD206hi macrophages, in which genes upregulated in B3KOM are shown in red, and genes downregulated in B3KOM are shown in green (the darker the more extreme the increase/decrease). For the edges, orange indicates predicted activation, blue indicates predicted inhibition, yellow indicates findings inconsistent with the state of the downstream molecule, and gray indicates that the effect is unknown. Solid edges, direct interactions; dashed edges, indirect interactions. C, interaction network from the DEGs of WT tumor cells versus B3KOM tumor cells. Solid gray edges, direct interactions; dashed gray edges, indirect interactions.
p-STAT1 and p-STAT6. These results show an integrin β3-negative feedback loop within M2 macrophages, in which integrin β3 expression is induced by STAT6 signaling, but that integrin β3 signaling favors STAT1 activation and suppression of STAT6 signaling (Fig. 6).

Myeloid cells are required for increased tumor growth after cilengitide treatment

Because there were increased numbers of M2 tumor-promoting macrophages in cilengitide-treated tumors, we asked whether the direct effects of cilengitide on myeloid cells were required for
enhanced tumor growth. We administered cilengitide to tumor-bearing β3KOM mice, where only LysM+ myeloid cells would be unresponsive to cilengitide. In contrast to the larger MFP tumors observed when cilengitide was administered to WT mice, we found that cilengitide treatment significantly reduced tumor burden in β3KOM mice (Fig. 7A). These results show that integrin β3 expression on TAMs is required for some of the cilengitide-induced tumor-promoting effects.

Next, we used anti-CSF1 antibody to decrease the number of macrophages and myeloid cells in tumor-bearing mice and evaluated the effect of cilengitide treatment. Coadministration of cilengitide with anti-CSF1 resulted in no enhancement of tumor growth compared with cilengitide alone (Fig. 7B and C). Analysis of tumor-infiltrating cells demonstrated that anti-CSF1 antibody treatment significantly decreased myeloid cell numbers in the tumor, with an expected concomitant increase in CD4+ and CD8+ T-cell numbers in WT mice (Fig. 7D–G). Anti-CSF1 antibody also decreased tumor-infiltrating myeloid cells and increased CD4+ and CD8+ T cells in tumor-bearing β3KOM mice (Supplementary Fig. S8). Taken together, these data show that macrophage/myeloid lineage cells are required for the tumor-enhancing effects of cilengitide and that coadministration of cilengitide and antimacrophage therapy reverses the tumor-enhancing effects of integrin β3 blockade.

Discussion

In this study, we provide new evidence for the role of integrin β3 in TAM polarization and function. We found that modulating integrin β3 levels either genetically or pharmacologically resulted in enhanced tumor growth and increased M2 macrophage numbers and tumor-promoting function in the tumor microenvironment. We found that integrin β3 signaling favored M1-polarizing STAT1 signaling and suppressed M2-polarizing STAT6 signaling. However, integrin β3 gene expression was suppressed by STAT1 and induced by STAT6 signaling, creating a negative feedback loop for M2 polarization. Thus, loss of integrin β3 signaling promoted an immunosuppressive tumor environment through increased M2 TAM polarization and function and decreased CD8+ T-cell numbers. Finally, disruption of macrophages blocked the tumor-promoting effects of cilengitide, demonstrating an immediate way to improve the efficacy of integrin β3 therapies in cancer.

M1 TAMs are essential participants in Th1 responses and have potent immunostimulatory capacity (25). On the other hand, M2 TAMs help maintain an immunosuppressive environment and promote tumor growth by facilitating angiogenesis, tumor cell invasion, metastasis, and chemotherapeutic resistance (19, 25, 33, 42). We found that integrin β3 knockout TAMs had elevated expression of M2 macrophage markers and enhanced tumor-promoting function in vivo when compared with WT TAMs (Figs. 1 and 2). We also noticed a significant decrease in CD8+ T-cell number in the tumor tissue when integrin β3 was disrupted in myeloid cells (Supplementary Table S1). In the tumor microenvironment, CD8+ T cells play a central role in antitumor cellular immune responses. High CD8+ T cells, low CD4+ T cells, and low CD68+ TAMs in the tumor microenvironment correlated with better survival for breast cancer patients (42). In vivo, β3KOM
TAMs have elevated TGFβ signaling downstream gene expression, suggesting increased TGFβ in the β3KOM tumor microenvironment (Fig. 4B). In vitro, Itgb3−/− BMMs have higher levels of IL10 mRNA expression after LPS treatment when compared with WT BMMs (data not shown). Finally, although CD8+ T-cell depletion in WT mice enhanced tumor growth, the same treatment in β3KOM mice resulted in no significant difference in tumor size (Fig. 2C and D), indicating that the enhanced tumor-promoting function of integrin β3–null TAMs is mediated by suppressive effects on the CD8+ T-cell response. Therefore, our data describe a functional role for integrin β3 in the macrophage-dependent regulation of immunosuppression within tumors.

Several integrin β3 antagonists have been developed for clinical use in cancer, including peptide antagonists, such as cilengitide, and neutralizing antibodies, such as Vitaxin and c7E3 (43). Treating integrin αvβ3–expressing tumor cells with cilengitide in vitro reduces tumor cell proliferation and invasion (8, 43). On the basis of these data, integrin β3 inhibition initially appeared to be an exciting cancer therapeutic target, particularly after cilengitide treatment displayed positive results in some animal tumor models (8, 43). However, clinical trials with cilengitide on glioblastoma, NSCLC, metastatic melanoma, prostate cancer, and advanced pancreatic cancer showed no improvement in outcomes, even while cilengitide was well tolerated in patients (12–14, 34–36). We noticed that in WT mice with early-stage mammary tumors (day 6, less than 100 mm3), cilengitide treatment did not increase or decrease tumor growth (Supplementary Fig. S3). However, in established mammary tumors (day 10, ~200 mm3), cilengitide treatment significantly enhanced tumor growth (Fig. 3). We compared the number of tumor-infiltrating T cells and myeloid cells in both early and established mammary tumors; established tumors had almost double the number of infiltrating myeloid cells and a 50% decrease in infiltrating T-cell numbers compared with early-stage tumors (Supplementary Fig. S3). This result suggests that late-stage tumors with an increased number of TAMs may be more susceptible to the protumor effect of integrin β3 blockade. Importantly, cilengitide treatment decreased tumor burden in mice genetically lacking integrin β3 on myeloid cells (Fig. 7A). Cilengitide has been shown to inhibit tumor cell proliferation (8). We subsequently confirmed that cilengitide treatment inhibits PyMT-BO1 cell proliferation in vitro (Supplementary Fig. S9A), a direct inhibitory role that may explain the decreased tumor burden we observed in cilengitide-treated β3KOM mice. Taken together, tumor-promoting M2 macrophages played an important role in integrin β3 disruption–induced enhancement of tumor growth (cilengitide treatment and β3KOM). Moreover, when mice were pretreated with anti-CSF1 antibody to decrease myeloid cell numbers, this cilengitide-induced tumor progression was significantly abrogated (Fig. 7C). Thus, anti-integrin β3 (cilengitide) treatment has
several roles in the tumor microenvironment, directly inhibiting tumor proliferation and neoangiogenesis while indirectly promoting tumor progression through stimulation of macrophage M2 polarization and tumor-promoting function.

We found that LPS-induced phosphorylation of SYK and STAT1 was strongly reduced in Itgb3−/− BMMs. SYK is an important signaling molecule to transduce integrin β3 outside-in signaling (40). In addition, it has been shown that p-SYK can activate STAT1 (44). A similar decrease in SYK and STAT1 activation was observed if WT BMMs were pretreated with integrin αvβ3 inhibitor ciliengitide (Fig. 5A–C). Furthermore, Itgb3−/− BMMs and WT BMMs pretreated with ciliengitide displayed an increase in STAT6 activation upon IL4 treatment (Fig. 5E and Supplementary Fig. S7). These data demonstrate that integrin β3 downstream signaling enhances STAT1 activation, the key M1-polarizing signal, and negatively regulates STAT6 activation, the key M2-polarizing signal. Thus, integrin β3 activation and ligand binding induce SYK phosphorylation and then STAT1 activation in macrophages. LPS (TLR4 signaling) administration to macrophages induced integrin β3 activation and enhanced STAT1 signaling; disruption of integrin β3 (such as β3Kom) diminished this enhancement of STAT1 signaling. Defining the precise signaling pathway through which integrin β3, LPS, and STAT1 interact in macrophages is underway.

It is important to understand effects on M2 TAMs and other myeloid cells when administrating integrin β3-targeted therapy or antitumor immune therapy, particularly in patients whose tumors express high numbers of infiltrating macrophages. Infiltrating TAMs in cancer are more likely to polarize towards an M2 phenotype with tumor progression (21, 24). TAM burden correlates with poor survival in many cancer types (19, 20). TAM-targeted therapy is now being considered as an important facet of successful cancer treatment. We found that anti-CSF1 therapy prevented the increase in tumor growth associated with ciliengitide treatment in breast cancer. Single-agent anti-CSF1 antibody was effective to decrease TAMs by 30% to 50% and increase T cells in established tumors. However, it did not significantly decrease tumor burden. Most breast cancer cells, including PyMT-B1 cells, secrete macrophage colony-stimulating factor (M-CSF) 1 (CSF1). It is possible that anti-CSF1 antibody as dosed in our experiments did not completely block the M-CSF receptor (M-CSFR or CSF1R) signaling on tumor-infiltrating macrophages. Also, anti-CSF1 treatment targets both tumor-promoting M2 TAMs and tumor-suppressive M1 TAMs, thus diminishing the antitumor properties of the latter. Accordingly, longer treatment times or blockade of M-CSFR signaling pathways may have stronger effects on the tumor microenvironment in terms of rebalancing the T-cell population and reducing tumor burden. Taken together, we propose that M2 TAMs are a key player in the decreased efficacy of integrin αvβ3-targeted therapies and that concurrent treatment with macrophage antagonists represents a possible strategy to improve their clinical effectiveness. Our results suggest that when giving integrin β3-targeted therapy, myeloid cell function should be considered.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: X. Su, S.R. Amend, S.L. Teitelbaum, D.G. DeNardo, J.G. Schneider, K.N. Weilbaecher


Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): X. Su, S.R. Amend, Y. Xu, M.H. Ross, F. Fontana, M.A. Hurchla, B.L. Knolhoff, M.A. Meyer, E.A. Morgan, S.D. Robinson, D.G. DeNardo, J.G. Schneider, K.N. Weilbaecher


Writing, review, and/or revision of the manuscript: X. Su, A.K. Esser, Y. Xu, M.H. Ross, G.C. Fox, T. Kobayashi, K. Roomp, M.A. Hurchla, D.V. Novack, S.D. Robinson, J.G. Schneider, K.N. Weilbaecher

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): X. Su, I. Xiang, Y. Xu, J.G. Schneider, K.N. Weilbaecher

Study supervision: X. Su

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References


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