Myeloid-derived suppressor cells (MDSCs) favor tumor promotion, mainly by suppressing antitumor T cell responses in many cancers. Although the mechanism of T cell inhibition is established, the pathways leading to MDSC accumulation in bone marrow and secondary lymphoid organs of tumor-bearing hosts remain unclear. We demonstrate that down-regulation of PLCγ2 signaling in MDSCs is responsible for their aberrant expansion during tumor progression. PLCγ2−/− MDSCs show stronger immune-suppressive activity against CD8+ T cells than WT MDSCs and potently promote tumor growth when adoptively transferred into WT mice. Mechanistically, PLCγ2−/− MDSCs display reduced β-catenin levels, and restoration of β-catenin expression decreases their expansion and tumor growth. Consistent with a negative role for β-catenin in MDSCs, its deletion in the myeloid population leads to MDSC accumulation and supports tumor progression, whereas expression of β-catenin constitutively active reduces MDSC numbers and protects from tumor growth. Further emphasizing the clinical relevance of these findings, MDSCs isolated from pancreatic cancer patients showed reduced p-PLCγ2 and β-catenin levels compared with healthy controls, similar to tumor-bearing mice. Thus, for the first time, we demonstrate that down-regulation of PLCγ2−β-catenin pathway occurs in mice and humans and leads to systemic β-catenin blockade as anti-cancer therapy.

Accumulating evidence indicates that myeloid-derived suppressor cells (MDSCs) are critically involved in tumor progression. Despite the ambiguity surrounding their origin, MDSCs are recognized for their ability to suppress antitumor immune responses. MDSCs exert their pro-neoplastic effects through the release of small soluble oxidizers, impairment of T cell/antigen recognition, and depletion of essential amino acids from the local extracellular environment, all ultimately leading to T cell suppression (Mazzoni et al., 2002; Kusmartsev and Gabrilovich, 2003). Thus, it is not surprising that presence of MDSCs in the blood and tumor biopsies of cancer patients is associated with poor prognosis (Almand et al., 2001; Lechner et al., 2011; Solito et al., 2011; Porembka et al., 2012).

MDSCs comprise a heterogeneous population of immature myeloid cells (Bronte et al., 2000; Gabrilovich et al., 2001; Liu et al., 2003; Kusmartsev et al., 2004; Kusmartsev et al., 2005; Zea et al., 2005; Gallina et al., 2006), identified by the co-expression of Gr-1 and αm.

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integrin (CD11b) in mice. More recently, MDSCs were subdivided into two different subsets based on the expression of Ly6C and Ly6G. One subset comprises monocytic and mononuclear CD11b+Ly6G−Ly6Chigh cells, called MO-MDSCs, which primarily release NO to suppress T cell activation (Movahedi et al., 2008; Youn et al., 2008). The second subset, termed PMN-MDSCs, includes CD11b+Ly6G+Ly6Clow cells with granulocytic and polymorphonuclear morphology and they exert T cell inhibition by producing mainly reactive oxygen species (ROS). The expansion of these two MDSC subsets has been shown to be tumor cell line dependent. Most tumors show increased PMN-MDSCs, whereas MO-MDSCs are expanded in only a few models (Youn et al., 2008). Nevertheless, both PMN- and MO-MDSC subsets exert comparable immune suppressive activity primarily against CD8+ T cells.

A large body of work has focused on the identification of the factors modulating MDSC activation and the signals mediating their recruitment to the tumor microenvironment or secondary lymphoid organs. Several tumor-derived growth factors, including macrophage colony-stimulating factor (M-CSF), IL-6, and granulocyte/macroage colony-stimulating factor (GM-CSF) promote the expansion of MDSCs through stimulation of myelopoiesis and inhibition of the differentiation of mature myeloid cells reviewed by (Gabrilovich and Nagarjuna, 2009). Other factors such as IFN-γ, ligands for TLRs, and TGFβ produced mainly by activated T cells and tumor stroma, are involved in MDSC activation. In most instances, phosphorylation of signal transducer and activator of transcription 3 (STAT3) and STAT1 are observed in MDSCs during tumor progression. Although STAT3 was shown to modulate MDSC differentiation and function, recent reports suggest that STAT3 primarily controls the ability of MDSCs to suppress antigen-dependent T cell activation (Kortylewski et al., 2005; Chalmin et al., 2010). Other transcription factors important for myeloid cell fate determination, such as PU.1 (Spi-1) or CAAT/enhancer binding protein-β (C/EBP-β), have been implicated in MDSC differentiation and immunosuppressive functions (Schroeder et al., 2003; Marigo et al., 2010). However, one of the major unresolved questions is the mechanism leading to MDSC expansion in the bone marrow, an event that occurs very early during tumor progression, even when the tumor itself is at a distant site. Thus, the focus of our work is to better understand the mechanisms involved in MDSC accumulation in response to the tumor.

Activation of PLCγ2, an enzyme converting phosphatidylinositol 4,5-bisphosphate (PIP2) into diacylglycerol (DAG) and inositol triphosphate (IP3), is implicated in proliferation and migration of several cancers (Smith et al., 1998; Feng et al., 2012). However, its deletion in the host also leads to increased tumor growth (Zhang et al., 2011). We have recently shown that PLCγ2−/− mice are more susceptible to tumor growth in bone despite a decrease in osteoclast number and function. Although PLCγ2 is not required for T cell activation, we observed impaired CD8+ T cell responses in PLCγ2−/− tumor-bearing mice (Zhang et al., 2011). Our current study shows that down-regulation of PLCγ2 signaling promotes MDSC expansion in the bone marrow of tumor-bearing mice and enhances MDSC immunosuppressive activities via modulation of β-catenin levels. Together these results highlight a novel molecular pathway regulating the expansion of MDSCs and their crucial role in tumor progression.

RESULTS

MDSCs are responsible for increased tumor growth in PLCγ2−/− mice

To understand the mechanism responsible for the impaired antitumor T cell response observed in PLCγ2−/− mice, we measured the percentage of MDSCs after s.c. inoculation of B16 melanoma and Lewis lung carcinoma (LLC) cell lines. Consistent with a significant increase in s.c. tumor growth in PLCγ2−/− mice, we also found a higher percentage of Gr-1+CD11b+ MDSCs in the bone marrow, spleen, and tumor site 14 d after inoculation of both tumor cell lines (Fig. 1, A and B). The increase in percentage of MDSCs observed in the null mice was not detected at baseline, reflecting a rather specific response to the tumor itself (not shown). Between the two cell lines, LLC induced stronger MDSC accumulation than B16 cells in both PLCγ2−/− and WT mice, and thus this cell line was used for all subsequent experiments. Next, to determine whether the expanded MDSC population observed in the null mice was a consequence of greater tumor growth, we injected WT and PLCγ2−/− mice with 106 LLC and sacrificed the animals 7 d after tumor challenge when no detectable differences in tumor weight were yet observed (Fig. 1 C). PLCγ2−/− mice already displayed greater MDSC accumulation in bone marrow and spleen, whereas only a small percentage of MDSCs was detected in the tumor at this time in both genotypes (Fig. 1 C).

To determine whether increased numbers of MDSCs are responsible for enhanced tumor growth in PLCγ2−/− mice, we adoptively transferred 3 × 106 MDSCs isolated from LLC tumor-bearing PLCγ2−/− or WT mice into WT animals. MDSCs were i.v. injected into the recipient mice 3 and 6 d after s.c. LLC inoculation. Tumor growth was monitored for 2 wk, and the percentage of MDSCs in the spleen was measured at time of sacrifice. We found that adoptive transfer of PLCγ2−/− MDSCs into WT recipients significantly enhanced tumor growth compared with animals receiving WT MDSCs, and it also further increased MDSC accumulation in the spleen (Fig. 2 A). To address whether the expansion of MDSCs caused by administration of PLCγ2-deficient MDSCs was caused by proliferation of the null cells or by increased expansion of endogenous MDSCs, we adoptively transferred CD45.2+ PLCγ2−/− or WT MDSCs into tumor-bearing CD45.1+ WT recipients. Mice injected with saline were used as controls. Tumor growth was monitored for 14 d and the percentage of endogenous CD45.1+ MDSCs and total Gr-1+CD11b+ MDSCs (CD45.1+ and CD45.2+) in the spleen was analyzed at time of sacrifice. As in Fig. 2 A, animals receiving PLCγ2−/− MDSCs showed increased tumor growth compared with animals receiving WT MDSCs, accompanied by greater MDSC expansion (Fig. 2 B). Interestingly, the
2.5 fold-increase in the percentage of MDSCs in the spleen (Fig. 3). All together these results indicate that PLC\(\gamma_2\) is a negative regulator of MDSC expansion and function in tumor-bearing hosts.

PLC\(\gamma_2\) deficiency favors MDSC accumulation over myeloid cell differentiation in vitro

To better understand the role of PLC\(\gamma_2\) in MDSC accumulation, we isolated MDSCs from the spleen of tumor-bearing mice and analyzed the balance between anti- (Bcl-2, Bcl-xl) and proapoptotic (Bax) signals by quantitative RT-PCR. PLC\(\gamma_2\)\(^{-/-}\) MDSCs showed similar relative mRNA expression levels of Bcl-2, Bcl-xl, and Bax to WT MDSCs (not depicted). Next, we wondered whether PLC\(\gamma_2\) deficiency would affect the cell fate differentiation from myeloid progenitor cells in the bone marrow. We isolated lineage negative hematopoietic progenitor cells from PLC\(\gamma_2\)\(^{-/-}\) or WT bone marrow and cultured them in the presence of GM-CSF, IL-4, and tumor conditioned medium (TCM) (Bronte et al., 2000; Youn et al., 2008). After 5 d, we determined the percentage of cells expressing DCs (CD11c), mature DCs (B7.2), macrophages (F4/80), and granulocyte lineage (Gr-1) markers. We found a significant reduction in the percentage of F4/80-positive cells (PLC\(\gamma_2\)\(^{-/-}\): 31.6 ± 1.3% versus WT: 53.3 ± 1.4%; \(P = 0.00004\)), as well as CD11c (PLC\(\gamma_2\)\(^{-/-}\): 22.3 ± 2.3% versus WT: 35.7 ± 3.0%; \(P = 0.004\)) and CD11c/B7.2-positive cells (PLC\(\gamma_2\)\(^{-/-}\): 13.8 ± 1.7% versus WT: 24.5 ± 2.2%; accumulation of MDSCs after adoptive transfer of either WT or PLC\(\gamma_2\)\(^{-/-}\) cells was due to expansion of endogenous CD45.1\(^+\) MDSCs, as their percentage was similar to that of total MDSCs (Fig. 2 B and not depicted). This finding indicates that the transfer of exogenous PLC\(\gamma_2\)-deficient MDSCs into WT recipient animals causes the expansion of endogenous MDSCs, which might be the result of enhanced tumor growth. Next, to use a second approach to show that PLC\(\gamma_2\) deficiency in the myeloid population, including MDSCs, is responsible for the observed tumor phenotype, we turned to PLC\(\gamma_2\) conditional KO mice (PLC\(\gamma_2\)\(^{-/-}\)cKO), in which deletion of PLC\(\gamma_2\) is under control of LysM-Cre. Reduced PLC\(\gamma_2\) expression in MDSCs was confirmed by Western blot, and similarly to the global PLC\(\gamma_2\)\(^{-/-}\) mice, PLC\(\gamma_2\)\(^{-/-}\)cKO animals also displayed increased tumor growth and MDSC accumulation compared with LysM-Cre controls (not shown).

As MDSCs have also a recognized role in tolerance, we next analyzed whether PLC\(\gamma_2\)\(^{-/-}\) mice, which are on a C57BL/6 background, could allow growth of the allogeneic 4T1 breast cancer cell line derived from BALB/c mice. 5 × 10\(^6\) 4T1 tumor cells were inoculated s.c. in WT and PLC\(\gamma_2\)\(^{-/-}\) mice and tumor growth followed for 2 wk. As expected by the different background of the recipient C57BL/6 mice and the 4T1 tumor cell line isolated from BALB/c mice, the tumor engraftment was rejected in WT mice (Fig. 3). However, 4T1 tumor cells grew in PLC\(\gamma_2\)\(^{-/-}\) mice with a concomitant accumulation of MDSCs in the bone marrow and a
PLCγ2 deficiency expands PMN- and MO-MDSC subsets and potentiates their immunosuppressive functions

To gain more insights into the role of PLCγ2 in MDSC-induced tumor progression, we performed phenotypic and functional analyses comparing WT and PLCγ2−/− MDSCs during tumor progression. We measured the relative proportion of PMN-MDSCs (CD11b+Ly6G+Ly6Clow) and MO-MDSCs (CD11b+Ly6G+Ly6Chigh) by FACS in PLCγ2−/− and WT mice. We first confirmed that the percentage of both PMN- and MO-MDSC subsets in bone marrow and spleen was similar at baseline in the two genetic backgrounds (Fig. 4 A). PMN-MDSCs represented the dominant subpopulation before and after the tumor challenge in both WT and KO (Fig. 4, A and B). However, both MDSC subfractions were significantly increased in bone marrow and spleen of PLCγ2−/− mice compared with WT mice in response to LLC-tumor inoculation (Fig. 4 B). Thus, PLCγ2 does not seem to exert differential effects on PMN- and MO-MDSC subsets.

Next, to determine whether PLCγ2−/− MDSCs exhibited stronger immunosuppressive functions compared with WT, in addition to being more numerous, we examined the inhibitory effects of PMN- and MO-MDSCs on T cell proliferation in vitro. As MDSCs were shown to suppress both antigen-driven and mitogen-driven T cell proliferation, we examined the immune-suppressive activity of MDSCs under both conditions. Splenocytes from OT-1 transgenic mice were labeled with CFSE and incubated with MHC class I–restricted SIINFEKL (OT-1) peptide (10 pM) to induce CD8+ T cell antigen–specific proliferation or with anti-CD3 antibody (10 µg/ml) for mitogen-driven CD8+ T cell stimulation. The immune-suppressive effects of MDSCs were determined by culturing WT and PLCγ2−/− PMN- or MO-MDSCs with three different ratios of splenocytes (1:10; 1:5; 1:1). Proliferation of targeted CD8+ T cells was measured in terms of CFSE dilution by flow cytometric analysis 72 h later. We found that both PLCγ2−/− PMN- and MO-MDSCs had greater immune-suppressive effects on CD8+ T cell proliferation than WT MDSCs in both antigen- and mitogen-driven T cell stimulatory conditions (Fig. 5, A and B). It is established that PMN-MDSCs inhibit CD8+ T cell proliferation in vitro. As MDSCs were shown to suppress both antigen- and mitogen-driven T cell stimulatory conditions, we examined the immune-suppressive activity of MDSCs under both conditions. Splenocytes from OT-1 transgenic mice were labeled with CFSE and incubated with MHC class I–restricted SIINFEKL (OT-1) peptide (10 pM) to induce CD8+ T cell antigen–specific proliferation or with anti-CD3 antibody (10 µg/ml) for mitogen-driven CD8+ T cell stimulation. The immune-suppressive effects of MDSCs were determined by culturing WT and PLCγ2−/− PMN- or MO-MDSCs with three different ratios of splenocytes (1:10; 1:5; 1:1). Proliferation of targeted CD8+ T cells was measured in terms of CFSE dilution by flow cytometric analysis 72 h later. We found that both PLCγ2−/− PMN- and MO-MDSCs had greater immune-suppressive effects on CD8+ T cell proliferation than WT MDSCs in both antigen- and mitogen-driven T cell stimulatory conditions (Fig. 5, A and B). It is established that PMN-MDSCs inhibit CD8+ T cell proliferation mainly through ROS production and that MO-MDSCs induce T cell apoptosis mostly via NO release. To evaluate the mechanism by which PLCγ2 deficiency controls MDSC immune suppressive activity, we measured ROS and NO levels in PMN- and MO-MDSCs in response to PMA (300 nM) or LPS (1 µg/ml) for indicated time. As expected (Movahedi et al., 2008; Youn et al., 2008), WT PMN-MDSCs predominantly produced ROS, whereas WT MO-MDSCs mainly released nitrates (Fig. 5, C and D). In contrast, PMN-MDSCs...
and MO-MDSCs from PLCγ2−/− mice released both high levels of ROS and NO (Fig. 5, C and D). Thus, PLCγ2 deficiency in both MDSC subsets induces higher release of soluble oxidizers, making them more effective in suppressing both Ag-specific and nonspecific immune responses.

**Reduced β-catenin levels are observed in PLCγ2−/− MDSCs**

Dysregulated β-catenin signaling has been noted in many cancers. Furthermore, β-catenin pathway is also known to modulate the self-renewal and maintenance of hematopoietic stem cells and myeloid progenitor cells (Scheller et al., 2006). Because MDSCs are immature myeloid progenitor cells, we hypothesized that β-catenin could be an important player in their development. First, we confirmed deletion of PLCγ2 and reduced phosphorylation levels of the PLCγ2 effector PKC (p-PKC; Fig. 6 A). Next, we measured β-catenin and phosphorylated-GSK3β (p-GSK3β) levels in WT and PLCγ2−/− MDSCs isolated from tumor-bearing mice by Western blot. Surprisingly, we observed a striking decrease in MDSCs isolated from tumor-bearing mice by Western blot.

To further understand the importance of β-catenin downregulation in MDSCs in vivo, we turned to the Cre-loxP recombination system to conditionally delete β-catenin in myeloid cells. β-catenin−floxed mice were bred with animals expressing the Cre recombinase under the control of LysM-Cre (LysM-Cre/β-cateninflx/flx; herein defined as β-cat.cKO), and deletion of β-catenin in MDSCs was confirmed by Western blot (not shown). We used animals expressing either the Cre recombinase (LysM-Cre/β-cateninflx/flx) or the floxed allele (β-cateninflx/flx) as a control (CTR). As no differences in tumor growth and MDSC expansion in the two control genotypes were observed, we used either one or the other as control group. LLC tumor cell line was inoculated s.c. into β-cat.cKO or control mice, and tumor growth was followed by caliper measurements for 14 d. The percentage of MDSCs in the bone marrow, spleen, and tumor site was analyzed by flow cytometry at time of sacrifice. Similarly to PLCγ2−/− mice, β-cat.cKO animals displayed greater tumor growth and over twofold increase in the percentage of MDSCs in spleen and tumor site compared with CTR (Fig. 6 C). A significant increase in the percentage of MDSCs in the bone marrow was also observed. To further determine the ability of β-catenin-deficient MDSCs to support tumor growth, we adoptively transferred MDSCs isolated from tumor-bearing β-cat.cKO or CTR mice into tumor-bearing WT mice. Mice receiving saline were used as additional controls. We found that mice transfected with β-cat.cKO MDSCs displayed enhanced tumor volume and twofold increase in splenic MDSCs compared with animals receiving CTR MDSCs or saline (Fig. 6 D).

**β-catenin stabilization in myeloid cells protects from tumor growth by reducing MDSC accumulation and function**

To further demonstrate that β-catenin is a critical modulator of MDSC expansion in response to tumor, we turned to a similar LysM-Cre-loxP genetic mouse model to express a constitutively active mutant of β-catenin (LysM-Cre/β-cateninCA; Harada et al., 1999) in myeloid cells, including MDSCs (not shown). LLC cells were inoculated s.c. into β-cat.CA and LysM-Cre CTR mice. Tumor growth was monitored every 2 d, and MDSC accumulation in bone marrow, spleen, and tumor site was evaluated 14 d after tumor challenge. In contrast to β-cat.cKO mice, expression of a stable form of β-catenin in MDSCs reduced tumor growth compared with...
PLC-2/2 catenin pathway regulates MDSCs in cancer | Capietto et al.

MDSCs were more potent inhibitors than β-cat.CA MDSCs at 1:1 ratio (Fig. 7 B). Collectively, these results indicate that modulation of β-catenin levels in MDSCs is critical for their accumulation and function and for modulation of tumor progression.

β-catenin stabilization in PLC-2/2 MDSCs decreases their accumulation and immune suppressive activity

Next, to determine if PLCγ2 controls MDSC expansion in response to tumor via β-catenin, we bred LysM-Cre PLCγ2 conditional KO mice (PLCγ2cKO) with LysM-Cre/β-catenin<sup>−/−</sup>/floxed<sup>−/−</sup> mice (β-cat.CA) to obtain the double-mutant PLCγ2cKO/β-cat.CA animals. Because the double

CTR mice (Fig. 7 A). Although the percentage of MDSCs was not different in the bone marrow, a significant decrease in MDSC accumulation was observed in the spleen and tumor site of β-cat.CA mice (Fig. 7 A). To determine whether stabilization of β-catenin levels in MDSCs would affect their immune suppressive activity in addition to their expansion, we isolated MDSCs from the spleen of tumor-bearing CTR and β-cat.CA mice and co-cultured these cells with WT CFSE-labeled splenocytes at 1:5 and 1:1 ratios. CD8+ T cell proliferation was induced by incubation with anti-CD3 antibody for 72 h and analyzed by FACS. Whereas the T cell proliferation rate was similar in CTR- and β-cat.CA-MDSC cultures at 1:5 ratio, we found that WT

Figure 5. PLCγ2<sup>−/−</sup> MDSCs are more potent immune suppressors than WT MDSCs in vitro. (A and B) T cell proliferation assay. PMN- or MO-MDSCs isolated from WT and PLCγ2<sup>−/−</sup> mice were co-cultured for 3 d with CFSE-labeled splenocytes from OT-1 transgenic mice (1:10, 1:5, and 1:1 ratios) and stimulated with SIINFEKL peptide (10 pM) in antigen-driven (A) or with anti-CD3 (10 µg/ml) in mitogen-driven (B) experiments. Bar graphs show mean ± SD of three independent experiments. *, P < 0.05; **, P < 0.01. Representative flow cytometric analysis of CD8+ T cell proliferation (shown as CFSE dilution) in the presence of WT (dashed line) and PLCγ2<sup>−/−</sup> (solid line) MDSCs is also shown. (C and D) Suppressive mechanisms of PMN- and MO-MDSCs isolated from WT and PLCγ2<sup>−/−</sup> mice. Level of ROS (mean fluorescence intensity, MFI) in PMN and MO subsets in response to PMA stimulation (300 nM for 30 min) was measured using DCFDA staining and FACS. NO<sub>2</sub> release in supernatant of 10<sup>5</sup> MDSC subfractions was assayed by a standard Greiss reaction after LPS stimulation (1 µg/ml for 24 h; D). Mean ± SD from three independent experiments are shown. *, P < 0.05; **, P < 0.01; ***, P < 0.001.
mice with 105 LLC and sacrificed the animals 5 d after tumor challenge when no detectable differences in tumor weight were yet observed (Fig. 7 D). Similar to the global PLC\(\gamma\)2-deficient mice, we observed greater accumulation of PLC\(\gamma\)2\(cKO\) MDSCs compared with CTR in mice with similar tumor size (Fig. 7 D). In contrast, PLC\(\gamma\)2\(cKO/\beta\)-cat.CA mice showed significantly lower MDSC expansion than PLC\(\gamma\)2\(cKO\) mice (Fig. 7 D). Consistent with this observation, the percentage of CD8\(^+\) T cells infiltrating the tumor was significantly increased in PLC\(\gamma\)2\(cKO/\beta\)-cat.CA compared with PLC\(\gamma\)2\(cKO\) (Fig. 7 E). As these findings suggested that stabilization of \(\beta\)-catenin levels in PLC\(\gamma\)2\(cKO\) MDSCs impairs cell immune suppressive activity, we performed functional T cell proliferation assays. CTR-, PLC\(\gamma\)2\(cKO\)-, and PLC\(\gamma\)2\(cKO/\beta\)-cat.CA MDSCs were cultured with CFSE-labeled WT splenocytes stimulated with anti-CD3 antibody (10 µg/ml). After 3 d, the proliferation of targeted CD8\(^+\) T cells was analyzed in terms of CFSE dilution by flow cytometry. As expected from our previous result (Fig. 5 B), CD8\(^+\) T cell proliferation is strongly diminished by PLC\(\gamma\)2\(cKO\) MDSCs compared with control. In contrast, PLC\(\gamma\)2\(cKO/\beta\)-cat.CA MDSCs have significantly lower MDSC expansion than PLC\(\gamma\)2\(cKO\) mice (Fig. 7 D). Similar to the global PLC\(\gamma\)2-deficient mice, we observed greater accumulation of PLC\(\gamma\)2\(cKO\) MDSCs compared with CTR in mice with similar tumor size (Fig. 7 D). In contrast, PLC\(\gamma\)2\(cKO/\beta\)-cat.CA mice showed significantly lower MDSC expansion than PLC\(\gamma\)2\(cKO\) mice (Fig. 7 D). Consistent with this observation, the percentage of CD8\(^+\) T cells infiltrating the tumor was significantly increased in PLC\(\gamma\)2\(cKO/\beta\)-cat.CA compared with PLC\(\gamma\)2\(cKO\) (Fig. 7 E). As these findings suggested that stabilization of \(\beta\)-catenin levels in PLC\(\gamma\)2\(cKO\) MDSCs impairs cell immune suppressive activity, we performed functional T cell proliferation assays. CTR-, PLC\(\gamma\)2\(cKO\)-, and PLC\(\gamma\)2\(cKO/\beta\)-cat.CA MDSCs were cultured with CFSE-labeled WT splenocytes stimulated with anti-CD3 antibody (10 µg/ml). After 3 d, the proliferation of targeted CD8\(^+\) T cells was analyzed in terms of CFSE dilution by flow cytometry. As expected from our previous result (Fig. 5 B), CD8\(^+\) T cell proliferation is strongly diminished by PLC\(\gamma\)2\(cKO\) MDSCs compared with control. In contrast, PLC\(\gamma\)2\(cKO/\beta\)-cat.CA MDSCs have significantly lower MDSC expansion than PLC\(\gamma\)2\(cKO\) mice (Fig. 7 D).
Figure 7. Increased β-catenin expression inhibits MDSC expansion, activity, and tumor growth. (A) $10^5$ LLC cells were s.c. injected in β-cat.CA or control mice (CTR), and tumor growth was followed for 14 d. Bone marrow, spleen, and tumors were then analyzed by FACS using anti-Gr-1 and CD11b staining to measure the percentage of MDSCs. Mean ± SD ($n = 4$) are shown. One representative out of two independent experiments is shown. *, $P < 0.05$; **, $P < 0.01$. (B) T cell proliferation assay. MDSCs were isolated from CTR and β-cat.CA mice, co-cultured with CFSE-labeled splenocytes from WT mice (1:5 and 1:1 ratios) and stimulated with anti-CD3 (10 µg/ml) for 3 d. Bar graphs show mean ± SD of three independent experiments. *, $P < 0.05$. Representative flow cytometric analysis of gated CD8$^+$ T cell proliferation in the presence of CTR- (dashed line) and β.cat.CA-MDSCs (solid line) is also
less ability to suppress T cell proliferation compared with PLCy2cKO MDSCs (Fig. 7 F). All together these findings reveal that stabilization of β-catenin in PLCy2−/− MDSCs modulates MDSC expansion and their suppressive activity.

Reduced PLCy2/β-catenin in MDSCs occurs during tumor progression in mice and cancer patients

We then wondered if down-regulation of PLCy2−β-catenin pathway is an important regulatory mechanism involved in the aberrant MDSC expansion and/or activity during tumor progression. To test the hypothesis that down-regulation of PLCy2−β-catenin pathway occurs in WT MDSCs, we compared protein expression levels of phosphorylated-PLCy2 (p-PLCy2) and β-catenin in MDSCs isolated from tumor-free and tumor-bearing WT mice. Strikingly, we found down-regulation of p-PLCy2 and β-catenin levels in MDSCs isolated from WT tumor-bearing mice compared with tumor-free controls. Both LLC and B16 tumor cell lines induced a similar phenomenon (Fig. 8 A).

Because various studies implicated MDSCs in the metastatic process (Li et al., 2013; Sawant et al., 2013; Yu et al., 2013), we wondered whether down-regulation of PLCy2−β-catenin signaling in MDSCs would also occur at late stages of tumor dissemination. To answer this question, we injected firefly-conjugated B16 melanoma (B16-Fl) cells into the left ventricle of WT animals, a model widely used to study tumor dissemination to bone, visceral organs, and lungs. Interestingly, we found down-regulation of p-PLCy2 and β-catenin levels in MDSCs isolated from WT tumor-bearing mice compared with tumor-free controls. Both LLC and B16 tumor cell lines induced a similar phenomenon (Fig. 8 A).

Increased percentage of MDSCs in peripheral blood correlates with disease progression and stage in many human cancers, including pancreatic cancer (Porembka et al., 2012), we isolated MDSCs from PBMCs of pancreatic cancer patients and healthy donors to compare p-PLCy2 and β-catenin expression levels. Similarly to mice, we isolated the whole human MDSC population, characterized by the co-expression of CD11b and the common myeloid marker CD33 (Greten et al., 2011; Filipazzi et al., 2012). All samples were collected before surgical or medical therapy. 5 patients (49–74 yr of age), 2 with resectable T2A or T3 stages and 3 with unresectable T3 or T4 stages of pancreatic ductal adenocarcinoma were tested by Western blot. Considering the variability between individuals, we normalized protein levels with total amount of protein loaded visualized by β-actin. We found significant reduction of p-PLCy2 and its downstream effector p-PKC in MDSCs from pancreatic cancer patients compared with healthy donors. β-catenin and p-GSK3β levels were also decreased (Fig. 8 C). In conclusion these results demonstrate that the down-regulation of PLCy2–β-catenin signaling is a critical step in MDSC expansion in humans and mice.

DISCUSSION

The mechanism behind MDSC differentiation and the signals that control their commitment and biological function in tumor-bearing hosts are not well understood. MDSCs have been detected in bone marrow, secondary lymphoid organs, and tumor site in many murine tumor models and in patients with advanced malignancies (Young et al., 1988; Gabitass et al., 2011; Porembka et al., 2012). The variability in the percentage of MDSCs and their efficiency to suppress antitumor T cell responses depend on the type and stage of tumor (Youn et al., 2008; Dolcetti et al., 2010; Younos et al., 2011). Several studies focused on identifying tumor-derived factors involved in the accumulation of MDSCs (Bronte et al., 2000; Barreda et al., 2004; Serafini et al., 2006; Pan et al., 2008; Roland et al., 2009; Xiang et al., 2009). Fewer studies, however, analyzed the signaling pathways involved in MDSC expansion in the tumor host. We now provide new evidence demonstrating that down-regulation of PLCy2 and β-catenin signaling promote MDSC accumulation in the bone marrow and subsequent recruitment to secondary lymphoid organs and tumor site, where they favor tumor escape from immune control. We found that PLCy2−/− MDSCs, with reduced β-catenin levels, are increased in number and strongly suppress CD8+ T cell activity via production of ROS and NO species. This increase in MDSC number and immune suppressive effects is likely responsible for the greater s.c. growth of LLC and B16 tumor cell lines and for the allogeneic tumor progression.
of 4T1 breast carcinoma cells observed in PLCγ2−/− mice. Although a tumor/MDSCs vicious cycle is expected to take place, in which larger tumors cause greater MDSC expansion to promote tumor escape from immune control, we found increased PLCγ2−/− MDSC numbers at early time points after tumor inoculation, when differences in tumor size between WT and KO mice were not present. Although DC and NK cells from PLCγ2−/− mice also have functional defects and could enhance tumor growth in PLCγ2−/− mice, the MDSCs adoptive transfer studies demonstrate that PLCγ2−/− MDSCs alone can greatly increase tumor progression in WT recipient mice. This finding indicates that PLCγ2 controls MDSC development and/or proliferation in response to the tumor.

Enhanced accumulation of MDSCs is also likely to be responsible for the unexpected bone tumor phenotype recently observed in PLCγ2−/− animals (Zhang et al., 2011). In the bone metastasis field, it has been established that bone resorbing osteoclasts are required to sustain tumor growth in bone. Interestingly, PLCγ2−/− mice display enhanced tumor growth in bone despite decreased osteoclast numbers, due to impaired CD8+ T cell activation (Zhang et al., 2011). Thus, expansion of PLCγ2−/− MDSCs in this tumor model seems to overcome the requirement for active osteoclasts to sustain tumor growth in bone. Importantly, the down-regulation of PLCγ2 and β-catenin can also occur in MDSCs from WT mice during tumor dissemination to bone, indicating that PLCγ2/β-catenin pathway modulates MDSC expansion in primary tumors as well as in a metastatic setting.

Similarly to PLCγ2-deficient animals, mice with deletion of β-catenin in the myeloid compartment are more susceptible to tumor growth due to increased MDSC numbers, whereas expression of a constitutively active form of β-catenin decreases MDSC accumulation and tumor growth. Importantly, expression of β-catenin constitutively active in PLCγ2-deficient MDSCs is also sufficient to reduce the expansion and the immune suppression of tumor growth in bone.
suppressive activity of PLCγ2−/− MDSCs, allowing greater infiltration of CD8+ T cells at tumor site. Consequently, tumor growth is reduced in PLCγ2cKO/β-cat.CA mice compared with PLCγ2cKO animals. The importance of down-regulation of PLCγ2–β-catenin axis in MDSC accumulation and activity is not limited to our KO mouse models but is a general mechanism involved in the modulation of WT MDSC responses occurring in the presence of tumor, including in cancer patients. Our data show that pancreatic cancer patients at advanced stage have reduced p-PLCγ2 and β-catenin levels compared with MDSCs isolated from healthy controls. These results demonstrate that PLCγ2 is a negative regulator of MDSC expansion and immune suppressive effects in tumor-bearing hosts via the β-catenin pathway in humans and mice. Several questions arise from this finding.

How is PLCγ2 signaling regulated in MDSCs? Activation of PLCγ2, via phosphorylation of its tyrosine residues, is often observed in hematopoietic cells, including myeloid cells, as well as in cancer cells downstream of ITAM-containing immune receptors, growth factor receptors, and G protein coupled receptors. Considering that the tumor microenvironment is enriched with tumor-derived factors that can potentially signal via PLCγ2, it was very surprising to observe reduction of PLCγ2 phosphorylation in MDSCs from tumor-bearing mice and cancer patients compared with healthy controls. In myeloid cells PLCγ2 activation downstream of ITAM containing receptors can be counterbalanced by negative regulatory signals emanated by receptors containing ITIM domains. A recent study indicated that the ITIM-containing paired immunoglobulin-like receptor (PIR) B is highly expressed in MDSCs (Ma et al., 2011). Deletion of PIR-B switches the MDSC phenotype from immune-suppressive to proinflammatory, thus leading to reduced tumor growth. Therefore, one possible mechanism for down modulation of PLCγ2 activation in WT MDSCs would be through suppression of ITAM signaling by PIR-B. Because β-catenin activation has also been reported to occur downstream of ITAM receptors (Otero et al., 2009), reduced β-catenin levels in MDSCs isolated from tumor-bearing mice or cancer patients could also be caused by suppression of ITAM signaling by PIR-B.

How does PLCγ2 modulate β-catenin? Regardless of which ITAM-containing receptor may activate PLCγ2 and β-catenin, we propose that these molecules lie on the same pathway. This assumption is supported by the observation that β-catenin levels are reduced in PLCγ2−/− MDSCs and that expression of constitutively active β-catenin in PLCγ2−/− marrow cells limits MDSC expansion and immune suppressive effects and reduces tumor growth. Although PLCγ2 has never been shown to directly modulate β-catenin levels, activation of PKC can induce β-catenin stabilization in T cells (Lovatt and Bijlmakers, 2010). It is likely that PKC, a downstream effector of PLCγ2, phosphorylates and thus inactivates GSK3β, a kinase that targets β-catenin for ubiquitination and subsequent proteasomal degradation. In support of this hypothesis, we observed impaired PKC phosphorylation and reduced p-GSK3β levels in PLCγ2−/− MDSCs compared with WT, whereas the rescue of PKC activation by PDBu in PLCγ2−/− MDSCs increases β-catenin protein levels. Nevertheless, we cannot exclude additional PLCγ2-independent down-regulation of β-catenin in MDSCs, possibly via modulation of Wnt ligands or Wnt inhibitors by the tumor cells.

How does β-catenin control MDSC expansion? Elevated levels of β-catenin are often associated with increased cell proliferation. This is especially true for cancer cells. Therefore, it was puzzling to observe that MDSC expansion was associated with reduced β-catenin levels. β-catenin has been extensively analyzed in hematopoiesis, at times leading to contradictory findings. Conditional expression of a stabilized, active form of β-catenin in hematopoietic stem cells (HSC) resulted in hematopoietic failure because of a reduction in cell cycle quiescence, HSC exhaustion, and blocked differentiation. Consistent with our hypothesis that β-catenin limits MDSC expansion, constitutive activation of β-catenin in early hematopoietic precursors significantly reduces the Gr-1+CD11b+ myeloid cell population (Scheller et al., 2006). Thus, it is possible that down-regulation of β-catenin, rather than controlling MDSC proliferation, allows MDSC accumulation by preventing their differentiation into mature myeloid cells. In support of this hypothesis, we find that in vitro cultures of hematopoietic progenitor cells from PLCγ2-deficient mice give rise to less mature myeloid populations but more Gr-1+ cells than WT cultures. Another plausible mechanism that could lead to accumulation of MDSCs in tumor-bearing PLCγ2- and β-catenin–null animals would be through increased differentiation from myeloid progenitors in the bone marrow. Several transcription factors involved in myelopoiesis such as interferon–regulatory factor 8 (IRF-8), C/EBP-β, and PU.1 have been demonstrated to regulate MDSC development (Scheller et al., 1999; Schroeder et al., 2003; Kistetter et al., 2006; Marigo et al., 2010). However IRF-8, PU.1, and C/EBP-β are also involved in monocyte/macrophage maturation, neutrophil differentiation, and/or DC development (Tamura et al., 2000; Hamdorf et al., 2011; Batliner et al., 2012; Pham et al., 2012). Therefore, further studies are required to determine whether down-regulation of PLCγ2–β-catenin pathway may affect MDSC differentiation via transcriptional regulation.

In conclusion, our results identify the PLCγ2–β-catenin pathway as a negative modulator of MDSC accumulation and activation in response to tumors. This finding is clinically relevant because we confirmed down-regulation of PLCγ2 and β-catenin in human MDSCs isolated from pancreatic cancer patients. This observation is particularly important, as β-catenin targeting is currently in clinical trials because of the positive role of β-catenin on cell growth in many cancers. However, based on our finding this approach could be undermined by tumor escape from immune control through expansion of MDSCs.

**MATERIALS AND METHODS**

**Animals and tumor models.** Animals were housed in a pathogen-free animal facility at Washington University. 6–8-wk-old littermate mice were used in all experiments according to protocols approved by the Institutional

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Animal Care and Use Committee. PLCy2<sup>-/-</sup> mice were on a C57BL/6 background and have been previously described (Wang et al., 2000). WT and PLCy2<sup>-/-</sup> littermates were used throughout the study. LysM-Cre/β-catenin<sup>flx/flx</sup> (conditional KO, β-cat.KO) and LysM-Cre/β-catenin<sup>flx/flx/LysM-Cre</sup> (constitutively active, β-cat.CA) mice on C57BL/6 background were provided by F. Long (Washington University, St. Louis, MO) and have been previously described (Clausen et al., 1999; Harada et al., 1999; Brautl et al., 2001). In brief, β-catenin<sup>flx/flx</sup> mice bear two LoxP sites flanking exon 2-6, which lead to a loss-of-function deletion upon Cre-mediated excision. In β-catenin<sup>flx/flx/LysM-Cre</sup> mice, excision of exon 3 leads to a stabilized, non-degradable form of β-catenin. C57BL/6 PLCy2<sup>2cKO</sup> mice were obtained from T. Kurosaki (Kansai Medical University, Moriguchi, Japan; Hashimoto et al., 2000, and the mating strategy to obtain the conditional KO under the LysM-Cre promoter (PLCy2<sup>2KO</sup>) was performed in our laboratory. LysM-Cre and floxed littermates were used as controls (CTR). All in vivo figures are shown as representative experiments. Importantly, significant differences are shown when all CTR groups are pooled together (Fig. 6 C and F and Fig. 7 A).

CD45.1 C57BL/6 WT mice used for the MDSC transfer experiments were purchased from The Jackson Laboratory. C57BL/6 OT-1 mice were obtained from M. Colonna’s laboratory (Washington University, St. Louis, MO).

B16 (C57BL/6 murine melanoma cells), LLC (C57BL/6 murine LLC cells), and 4T1 (BALB/c murine mammary tumor cells) were cultured at 37°C in complete media (DMEM supplemented with 2 mM l-glutamine, 100 µg/ml streptomycin, 100 IU/ml penicillin, and 1 mM sodium pyruvate) containing 10% FBS. To establish tumors, B16 (10<sup>6</sup>, LLC (10<sup>6</sup>), or BALB/c/4T1 (5 × 10<sup>5</sup>) tumor cells were suspended in PBS and inoculated s.c. in the flank of mice. Tumor measurements were performed every 2 or 3 d with a caliper, and volumes were calculated using the following formula: V = 0.5 (length [mm] × width [mm]<sup>2</sup>). For adoptive transfer experiments, appropriate control mice and indicated KO (PLCy2<sup>-/-</sup> or β-cat.KO) animals were s.c. injected with 10<sup>6</sup> LLC. 14 d after tumor xenograft, MDSCs were isolated from the spleen as described below, and 3 × 10<sup>6</sup> MDSCs were i.v. injected into WT tumor-bearing mice on days 3 and 6 after tumor challenge. WT mice receiving saline injection on days 3 or 6 were used as additional controls.

10<sup>6</sup> firefly-conjugated B16 melanoma cells (B16-Fl) suspended in 50 µl of saline solution were injected into the left cardiac ventricle (i.v.) of 6-wk-old female mice as previously described (Arguello et al., 1988; Kang et al., 2003). Recruitment of tumor cells to bone was monitored on days 9, 12, and 14-wk-old female mice as previously described (Arguello et al., 1988; Kang et al., 2003). In brief, appropriate control mice and indicated KO (PLCy2<sup>-/-</sup> or β-cat.KO) animals were s.c. injected with 10<sup>6</sup> LLC. 14 d after tumor xenograft, MDSCs were isolated from the spleen as described below, and 3 × 10<sup>6</sup> MDSCs were i.v. injected into WT tumor-bearing mice on days 3 and 6 after tumor challenge. WT mice receiving saline injection on days 3 or 6 were used as additional controls.

Bone marrow transplantation. 4-wk-old female C57BL/6 mice were lethally irradiated using a 137Cs source with 1,000 rad to generate recipient mice. Bone marrow was harvested from 6-wk-old female PLCy2<sup>2KO</sup>, PLCy2<sup>2KO/β-cat.CA</sup>, or LysM-Cre control mice. Cells were then suspended in PBS and 10<sup>6</sup> cells/200 µl were injected into the lateral tail vein of recipient mice. 4 wk after bone marrow transplantation, these mice were inoculated with tumor cells as described above.

Flow cytometric analysis. Immediately upon sacrifice, single-cell suspensions were prepared from bone marrow, spleen, and tumor. In brief, bone marrow cells were harvested from tibias and femurs by centrifugation, whereas spleens and tumors were mechanically dissociated and individual cell suspensions obtained through 70-µm cell strainer. Red blood cells were then removed with lys buffer and cells counted. Cell suspensions were then washed and stained in PBS with 0.5% FBS with the following anti-mouse antibodies: allophycocyanin (APC)-conjugated anti-Gr-1 or -Ly6C, FITC-conjugated anti-F4/80 (eBioscience); and phycoerythrin (PE)-conjugated antibodies: allophycocyanin (APC)-conjugated anti–Gr-1 or -Ly6C, FITC–conjugated anti-Ly6G, -CD11c, -CD86 (B7.2), or -CD45.1, APC-conjugated anti-CD8<sup>α</sup> (BD). The respective isotype-matche conjugated controls were purchased from eBioscience and BD, respectively. Cell surface staining on isolated cells from human PBMCs were performed using monoclonal (PerCP-Cy5.5)-conjugated anti-human CD33 antibody (BiolLegend) and (PE)-conjugated anti-human CD11b antibody (BD). Corresponding isotype controls yielded no significant staining. Acquisition was performed on a FACScalibur and the dedicated software CellQuest (BD). Data were analyzed with FlowJo 7.5.5 software (Tree Star).

MDSC isolation. In vivo experiments were assessed using freshly isolated MDSCs from spleens of tumor-bearing mice. Cells were purified by immunomagnetic separation using biotinylated anti-Gr-1 antibody and streptavidin-conjugated MicroBeads with MiniMACS columns according to the manufacturer’s protocol (Miltenyi Biotec). In vitro functional and mechanistic assays were done either with whole MDSC population or with PMN- and MO-MDSC subsets. Cells were isolated from spleens of tumor-bearing mice using the Myleoid-Derived Suppressor Cell Isolation kit from Miltenyi Biotec. Cell purity was checked by flow cytometric analysis using anti-CD11b and Gr-1 antibodies (>95%), and viability was checked by Trypan blue dye exclusion.

Human PBMCs were obtained from D.C. Linehan’s laboratory (Washington University, St. Louis, MO) as previously described (Porembka et al., 2012). In brief, informed consent was prospectively obtained from all patients before obtaining human blood according to the institutionally approved Human Studies Committee Protocol. Peripheral blood samples were collected in vacuum tubes containing EDTA (BD). PBMCs were isolated by Ficol-densi centrifugation and frozen in DMSO with 10% FBS. Cells were then thawed, washed, and processed for cell isolation using CD33 and CD11b MicroBeads with MiniMACS columns according to the manufacturer’s protocol (Miltenyi Biotec). Purity was confirmed by flow cytometric (>95%) and Western blot analyses, which were immediately performed.

Real-time PCR. MDSCs from spleens were isolated 14 d after tumor challenge as previously described. Total RNA was extracted with TRIzol (Invitrogen) and quantified on ND-1000 spectrophotometer (NanoDrop Technologies). The cDNA was synthesized with 1 µg RNA using cDNA EcoDry Premix (oligo dT) RT-PCR kit from Takara. The amount of Bcl-2, Bcl-xl, or Bad was determined using Power SYBR Green mix on 7300 Real-Time PCR System (Applied Biosystems). Cyclophilin mRNA was used as internal control. Specific primers were as follows: Bcl-2, 5′-TGATGACCTGCAGGCACCATC-3′ and 5′-GCATCCATGCGGTTCGTTAT-3′; Bcl-xl, 5′-ACAGAGGAGACCCAGTTAAGT-3′ and 5′-ACCGGATCTAAGCAACT-3′; Bad, 5′-AGATATTGAAGAGG-3′ and 5′-CAGAGTAAAGGAGCCAC-3′; cyclophilin, 5′-AGCATAAGGCCTGCCAGAT-3′ and 5′-TTTCCCTTCAAAAGACCAC-3′. Relative quantification of transcription was calculated as the power of the difference between amplification of the target gene and amplification of cyclophilin i.e. 2<sup>ΔΔCT</sup> (where Ct represents threshold cycle).

Generation of cells from bone marrow progenitors. Hematopoietic progenitor cells (HPCs) were isolated from WT and PLCy2<sup>-/-</sup> bone marrow using the Lineage Cell Depletion kit (Miltenyi Biotec). 5 × 10<sup>6</sup> HPCs were cultured in 24-well plates containing 2 ml of RPMI 1640 supplemented with 10% FBS, 20 ng/ml GM-CSF, 10 ng/ml IL-4, and 20% vol/vol tumor conditioned medium (TCM; Youn et al., 2008). The TCM was generated from primary EL-4 tumor cells injected into WT C57BL/6 for 2 wk, collected as single-cell suspension with collagenase and cultured for 2 d in RPMI supplemented with 10% FBS. TCM were frozen at −80°C until further use. After 5 d of HPC cultures, percentages of CD11c<sup>+</sup>, CD11c<sup>+</sup>/B7.2<sup>+</sup>, F4/80<sup>+</sup>, and Gr-1<sup>+</sup> cells in total WT and PLCy2<sup>-/-</sup> cell cultures were analyzed by flow cytometry.

T cell suppression assay. Freshly isolated splenocytes (5 × 10<sup>6</sup> cells/ml) from OT-1/TCR transgenic mice were depleted of red cells and labeled with CFSE (1 µM; Molecular Probe, Carlsbad, CA) for 10 min at 37°C and washed with fresh culture media, according to the manufacturer’s instructions. OT-1
Bone marrow cell suspensions were harvested as pre-PKC stimulation. The oxidation-sensitive dye DCFDA (Invitrogen) was used to measure ROS production by purified PMN- or MO-MDSCs. Cells were incubated at 37°C in RPMI in the presence of DCFDA (3 µM) and stimulated or not with PMA (300 nM; Sigma-Aldrich) for 30 min. Cells were then washed twice with cold PBS and labeled with anti-Gr-1 and CD11b antibodies before flow cytometric analysis.

Nitrre quantification was assayed by a standard Greiss reaction, as previously described (Lee et al., 2003). In brief, purified 10⁵ PMN- or MO-MDSCs were stimulated with LPS (1 µg/ml) for 24 h in RPMI 1640 media with 10% FBS and 10% G-CSF. The absorbance at 540 nm was measured using a microplate reader (Bio-Tek). Nitrre concentrations were determined by comparing the absorbance values for the test samples to a standard curve generated by serial dilution of 0.25 mM sodium nitrite.

PKC stimulation. Bone marrow cell suspensions were harvested as previously described and plated in 6-well plates (3 × 10⁶ cells/ml) with RPMI supplemented with 2 mM L-glutamine, 100 µg/ml streptomycin, 100 IU/ml penicillin, 1 mM sodium pyruvate, and 10% FBS for 18 h in the presence or not of PDBu (10 ng/ml). MDSCs were then isolated as previously described and prepared for Western blot analysis, as described below.

Western blotting. MDSCs were isolated as previously described and immediately lysed with RIPA buffer in the presence of protease and phosphatase inhibitors. An equal amount of total protein lysates were subjected to immediate lysis with RIPA buffer in the presence of protease and phosphatase inhibitors. An equal amount of total protein lysates were subjected to 8% SDS-PAGE gel and transferred to PVDF membranes. Membranes were blocked in 5% BSA in PBS/Tween-20 for 1 h, and then probed with appropriate primary antibodies overnight at 4°C. Membranes were washed and incubated for 2 h at room temperature with secondary antibody-conjugated with peroxidase. Results were visualized by chemiluminescence detection using a SuperSignal West Dura Extended Duration Substrate (Thermo Fisher Scientific).

Antibodies against p-PLC (pan; Castagna, M., Y. Takai, K. Kaibuchi, K. Sano, U. Kikkawa, and Y. Nishizuka. 1982. Direct activation of calcium-activated, phospholipid-dependent protein kinase by tumor-promoting phorbol esters. J. Biol. Chem. 257: 7847–7851.).

The authors declare no conflict of interest.

REFERENCES

Almand, B., J.J. Clark, E. Nikitina, J. van Beynen, N.R. English, S.C. Knight, D.P. Carbone, and D.J. Gabrilovich. 2001. Increased production of immature myeloid cells in cancer patients: a mechanism of immunosuppression in cancer. J. Immunol. 166:678–689.

Arguello, F., R.B. Baggs, and C.N. Frantz. 1988. A murine model of experimental metastasis to bone and bone marrow. Cancer Res. 48:6876–6881.

Barreda, D.R., P.C. Hannington, and M. Belosevic. 2004. Regulation of myeloid development and function by colony stimulating factors. Dev. Dyn. 228:509–554.

Batliner, J., E. Buehrer, E.A. Federzoni, M. Jenal, A. Tobler, B.E. Torbett, M.F. Fey, and M.P. Tschan. 2012. Transcriptional regulation of MIR29B by PU.1 (SPI1) and MYC during neutrophil differentiation of acute promyelocytic leukemia cells. Br. J. Haematol. 157:270–274. http://dx.doi.org/10.1111/j.1365-2141.2011.08964.x.

Braith, V., R. Moore, S. Kutsch, M. Inhibishi, D.H. Rowitch, A.P. McMahon, L. Sommer, O. Boussadia, and R. Kemler. 2001. Inactivation of the beta-catenin gene by Wnt1-Cre-mediated deletion results in dramatic brain malformation and failure ofcraniofacial development. Development. 128:1253–1264.

Bronte, V., E. Apolloni, A. Cabrellle, R. Ronca, P. Serafini, P. Zamboni, N.P. Restifo, and P. Zanovello. 2000. Identification of a CD11b(+)Gr-1(+)CD3(+) myeloid progenitor capable of activating or suppressing CD8(+) T cells. Blood. 96:3838–3846.

Castagna, M., Y. Takai, K. Kaibuchi, K. Sano, U. Kikkawa, and Y. Nishizuka. 1982. Direct activation of calcium-activated, phospholipid-dependent protein kinase by tumor-promoting phorbol esters. J. Biol. Chem. 257: 7847–7851.

Chalmun, F., S. Ladoire, G. Magnot, J. Vincent, M. Bruchard, P. Zanovello, et al. 2006. Tumors induce immunosuppression of mouse and human myeloid-derived suppressor cells. J. Immunol. 176:678–689.

Dolcetti, L., E. Peranzoni, S. Ugel, I. Marigo, A. Fernandez Gomez, C. Mesa, M. Geilich, G. Winkels, E. Traggiai, A. Casati, et al. 2010. Hierarchy of immunosuppressive strength among myeloid-derived suppressor cell subsets is determined by GM-CSF.

Dolcetti, L., E. Peranzoni, S. Ugel, I. Marigo, A. Fernandez Gomez, C. Mesa. 2010. Hierarchy of immunosuppressive strength among myeloid-derived suppressor cell subsets is determined by GM-CSF Eur J. Immunol. 40:22–35. http://dx.doi.org/10.1002/eji.201039903

Feng, L., I. Reynisdóttir, and J. Reynisson. 2012. The effect of PLC-β(II) Ser660, and p-GSK3β (Ser9) were obtained using GeneTools software (Syngene).

Fey, and M.P. Tschan. 2012. Transcriptional regulation of MIR29B by PU.1 (SPI1) and MYC during neutrophil differentiation of acute promyelocytic leukemia cells. Br. J. Haematol. 157:270–274. http://dx.doi.org/10.1111/j.1365-2141.2011.08964.x.

Gabitass, R.F., N.E. Annels, D.D. Stocken, H.A. Pandha, and G.W. Middleton. 2010. Conditional gene targeting in macrophages and granulocytes using LysMcre mice. Transgenic Res. 19:285–293.

Gabiass, R.F., N.E. Annels, D.D. Stocken, H.A. Pandha, and G.W. Middleton. 2010. Conditional gene targeting in macrophages and granulocytes using LysMcre mice. Transgenic Res. 19:285–293.

Galina, G., L. Dolcetti, P. Serafini, C. De Santo, I. Marigo, M.P. Colombo, G. Basso, F. Brumbacher, I. Borrello, P. Zanovello, et al. 2006. Tumors induce immunosuppression of mouse and human myeloid-derived suppressor cells. J. Immunol. 176:678–689.

Gabiass, R.F., N.E. Annels, D.D. Stocken, H.A. Pandha, and G.W. Middleton. 2010. Conditional gene targeting in macrophages and granulocytes using LysMcre mice. Transgenic Res. 19:285–293.

Gabiass, R.F., N.E. Annels, D.D. Stocken, H.A. Pandha, and G.W. Middleton. 2010. Conditional gene targeting in macrophages and granulocytes using LysMcre mice. Transgenic Res. 19:285–293.

Gabiass, R.F., N.E. Annels, D.D. Stocken, H.A. Pandha, and G.W. Middleton. 2010. Conditional gene targeting in macrophages and granulocytes using LysMcre mice. Transgenic Res. 19:285–293.
a subset of inflammatory monocytes with immunosuppressive activity on CD8+ T cells. J. Clin. Invest. 116:2777–2790. http://dx.doi.org/10.1172/JCI82828

Greten, T.F., M.P. Manns, and F. Korangy. 2011. Myeloid derived suppressor cells in human diseases. Int. Immunopharmacol. 11:802–807.

Harada, N., Y. Tama, T. Ishikawa, B. Sauer, K. Takaku, M. Oshima, and M.M. Taketo. 1999. Intestinal polyposis in mice with a dominant stable mutation of the beta-catenin gene. EMBO J. 18:5931–5942

Hamdor, M., A. Berger, S. Schüle, J. Reinhardt, and E. Flory. 2011. PKCδ-induced PI3k phosphorylation promotes hematopoietic stem cell differentiation to dendritic cells. Stem Cells. 29:297–306. http://dx.doi.org/10.1002/stem.564

Hashimoto, A., K. Takeda, M. Inaba, M. Sekimata, T. Kasino, S. Ikehara, Y. Homma, S. Akira, and T. Kurosko. 2000. Cutting edge: essential role of phospholipase C-γ2 in B cell development and function. J. Immunol. 165:1738–1742.

Kang, Y.P., M. Siegel, W. Shi, M. Drobnjak, S.M. Kakonen, C. Cordón-Cardo, C. Chiang, J. Massague. 2011. Paired immunoglobin-like receptor-B regulates the suppression of the beta-catenin gene. Nat. Immunol. 12:734–743. http://dx.doi.org/10.1038/ni.1984

Kortylewski, M., M. Kujawski, T. Wang, S. Wei, S. Zhang, S. Pilon-Thomas, G. Lechner, M.G., C. Megiel, S.M. Russell, B. Bingham, N. Arger, T. Woo, and A.L. Roland, C.L., K.D. Lynn, J.E. Toombs, S.P. Dineen, D.G. Udagama, and R.A. Brekken. 2009. Cytokine levels correlate with immune cell infiltration after anti-VEGF therapy in preclinical mouse models of breast cancer. PLoS ONE. 4:e7669. http://dx.doi.org/10.1371/journal.pone.0007669

Liu, Y., J.A. Van Gelderachter, L. Lyons, P. De Baetselier, G. Raes, and A.B. Geldhof. 2003. Nitric oxide-independent CTL suppression during tumor progression: association with arginase-producing (M2) myeloid cells. J. Immunol. 170:5064–5074.

Ma, G., P.Y. Pan, S. Eisenstein, C.M. Divino, C.A. Lowell, T. Takai, and S.H. Chen. 2013. Paired immunoglobulin-like receptor-B regulates the suppression function and fate of myeloid-derived suppressor cells. Immunity. 38:385–395.

Maier, I., E. Bosso, S. Solito, C. Mesa, A. Fernandez, L. Dolcetti, S. Ugil, N. Sonda, S. Bicciato, E. Falisi, et al. 2010. Tumor-induced tolerance and immune suppression depend on the C/EBPbeta transcription factor. J. Immunol. 183:32790–802. http://dx.doi.org/10.4049/jimmunol.2010.05.010

Mazzoni, A., V. Bronte, A. Visintin, J.H. Spitzer, E. Apolloni, P. Serafini, P. Seruffino, D.A. van der Voort, and U. June. 2003. notch signaling induces multilineage myeloid differentiation and up-regulates PU.1 expression. J. Immunol. 170:5538–5548.

Otero, K., J.R. Turnbull, P.L. Poliani, W. Vermi, E. Cerutti, T. Aoshi, I. Tassi, T. Takai, S.L. Stanley, M. Müller, et al. 2009. Macrophage colony-stimulating factor induces the proliferation and survival of macrophages via a pathway involving DAP12 and beta-catenin. Nat. Immunol. 10:734–743. http://dx.doi.org/10.1038/ni.1744

Pham, T.H., C. Benner, M. Lichtinger, L. Schwartzfischer, Y. Hu, R. Andreassen, W. Chen, and M. Rehli. 2012. Dynamic epigenetic enhancer signatures reveal key transcription factors associated with monocytic differentiation states. Blood. 119:e161–e171. http://dx.doi.org/10.1182/blood-2012-01-402453

Plowman, G.P., B. Leung, J.R. George, R. Brange, and Y. Chen. 1996. Adenosinosea induces bone marrow mobilization of myeloid-derived suppressor cells which promote primary tumor growth. Cancer Immunol. Immunother. 41:1373–1385. http://dx.doi.org/10.1007/s00262-011-1178-0

Roland, C.L., K.D. Lynn, J.E. Toombs, S.P. Dineen, D.G. Udagama, and R.A. Brekken. 2013. Myeloid-derived suppressor cells function as novel osteoclast progenitors enhancing bone loss in breast cancer. Cancer Res. 73:672–682. http://dx.doi.org/10.1158/0008-5472.CAN-12-2202

Scheller, M., J. Foerster, C.M. Heyworth, J. Waring, J. Löhler, G.L. Gilmore, R. Shadduck, T.M. Dexter, and I. Horak. 1999. Altered development and cytokine responses of myeloid progenitors in the absence of transcription factor, interferon consensus sequence binding protein. Blood. 94:3764–3771.

Serafin, P., I. Borrello, and V. Bronte. 2006. Myeloid suppressor cells in cancer: recruitment, phenotype, properties, and mechanisms of immune suppression. Semin. Cancer Biol. 16:53–65.

Smith, M.R., D.W. Court, H.K. Kim, J.B. Park, S.G. Rhee, J.S. Rhim, and H.F. Kung. 1998. Overexpression of phosphoinositide-specific phospholipase C gamma in NIH 3T3 cells promotes transformation and tumorigenicity. Cancer Res. 58:1977–1985. http://dx.doi.org/10.1158/0008-5472.1998-32:7-323

Solito, S., E. Falisi, C.M. Diaz-Montero, A. Doni, L. Pinton, A. Rosato, S. Francescato, G. Basso, P. Zanovello, G. Onicescu, et al. 2011. A human promyelocytic-like population is responsible for the immune suppression mediated by myeloid-derived suppressor cells. Blood. 118:2254–2265. http://dx.doi.org/10.1182/blood-2010-12-325753

Tamura, T., T. Nagamuro-Inoue, Z. Shmeltzer, T. Kuwata, and K. Ozato. 2001. ICOSBP directs bipotential myeloid progenitor cells to differentiate into macrophages. Immunity. 13:155–165. http://dx.doi.org/10.1016/S1074-7613(00)00016-9

Wang, D., J. Feng, R. Wen, J.C. Marine, M.Y. Sangster, E. Parganas, A. Hoffmeyer, C.W. Jackson, J.L. Cleveland, P.J. Murray, and J.N. Ihle. 2000. Phospholipase Cgamm2a is essential in the functions of B cell and several Fc receptors. Immunity. 13:25–35. http://dx.doi.org/10.1016/S1074-7613(00)00005-4

Xiang, X., A. Poliakov, C. Liu, Y. Liu, Z.B. Deng, J. Wang, Z. Cheng, S.V. Shah, G.J. Wang, L. Zhang, et al. 2009. Induction of myeloid-derived suppressor
cells by tumor exosomes. *Int. J. Cancer.* 124:2621–2633. http://dx.doi.org/10.1002/ijc.24249

Youn, J.I., S. Nagaraj, M. Collazo, and D.I. Gabrilovich. 2008. Subsets of myeloid-derived suppressor cells in tumor-bearing mice. *J. Immunol.* 181:5791–5802.

Young, M.R., M.E. Young, and K. Kim. 1988. Regulation of tumor-induced myelopoiesis and the associated immune suppressor cells in mice bearing metastatic Lewis lung carcinoma by prostaglandin E2. *Cancer Res.* 48:6826–6831.

Younos, I., M. Donkor, T. Hoke, A. Dafferner, H. Samson, S. Westphal, and J. Talmadge. 2011. Tumor- and organ-dependent infiltration by myeloid-derived suppressor cells. *Int. Immunopharmacol.* 11:816–826. http://dx.doi.org/10.1016/j.intimp.2011.02.021

Yu, J., W. Du, F. Yan, Y. Wang, H. Li, S. Cao, W. Yu, C. Shen, J. Liu, and X. Ren. 2013. Myeloid-derived suppressor cells suppress antitumor immune responses through IDO expression and correlate with lymph node metastasis in patients with breast cancer. *J. Immunol.* 190:3783–3797. http://dx.doi.org/10.4049/jimmunol.1201449

Zea, A.H., P.C. Rodriguez, M.B. Atkins, C. Hernandez, S. Signoretti, J. Zabaleta, D. McDermott, D. Quiceno, A. Youmans, A. O’Neill, et al. 2005. Arginase-producing myeloid suppressor cells in renal cell carcinoma patients: a mechanism of tumor evasion. *Cancer Res.* 65:3044–3048.

Zhang, K., S. Kim, V. Cremasco, A.C. Hirbe, L. Collins, D. Pwnica-Worms, D.V. Nowack, K. Weilbaecher, and R. Faccio. 2011. CD8+ T cells regulate bone tumor burden independent of osteoclast resorption. *Cancer Res.* 71:4799–4808. http://dx.doi.org/10.1158/0008-5472.CAN-10-3922