

PI3-Kinase γ Promotes Rap1a-Mediated Activation of Myeloid Cell Integrin $\alpha 4\beta 1$, Leading to Tumor Inflammation and Growth

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Abstract

Tumor inflammation, the recruitment of myeloid lineage cells into the tumor microenvironment, promotes angiogenesis, immunosuppression and metastasis. CD11b+Gr1lo monocytic lineage cells and CD11b+Gr1hi granulocytic lineage cells are recruited from the circulation by tumor-derived chemoattractants, which stimulate PI3-kinase γ (PI3K γ)-mediated integrin $\alpha 4$ activation and extravasation. We show here that PI3K γ activates PLC γ , leading to RasGrp/CalDAG-GEF-I&II mediated, Rap1a-dependent activation of integrin $\alpha 4\beta 1$, extravasation of monocytes and granulocytes, and inflammation-associated tumor progression. Genetic depletion of PLC γ , CalDAG-GEFI or II, Rap1a, or the Rap1 effector RIAM was sufficient to prevent integrin $\alpha 4$ activation by chemoattractants or activated PI3K γ (p110 γ CAAX), while activated Rap (RapV12) promoted constitutive integrin activation and cell adhesion that could only be blocked by inhibition of RIAM or integrin $\alpha 4\beta 1$. Similar to blockade of PI3K γ or integrin $\alpha 4\beta 1$, blockade of Rap1a suppressed both the recruitment of monocytes and granulocytes to tumors and tumor progression. These results demonstrate critical roles for a PI3K γ -Rap1a-dependent pathway in integrin activation during tumor inflammation and suggest novel avenues for cancer therapy.

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Introduction

The link between cancer and inflammation is increasingly recognized; at least fifteen percent of cancer cases arise in association with chronic inflammation, such as those caused by infectious agents (helicobacter pylori/gastric cancer, hepatitis C/liver cancer, and papilloma virus/cervical cancer), environmental toxins (asbestos, coal dust, and tobacco smoke), autoimmune disorders (Crohn's disease) and potentially obesity (1–3). Extensive infiltration of tissues by immunosuppressive macrophages is a common element of inflammatory diseases and tumors (4–6). In chronically inflamed tissues and tumors, among the most populous inflammatory cells are macrophages (TAMs), which express numerous factors that can stimulate angiogenesis, metastasis, inflammation and immunosuppression, as well as relapse after therapy (4–16). Targeting the causes and consequences of chronic inflammation is likely to provide significant benefit in the treatment and prevention of a wide variety of cancers. Thus, identification of the common mechanisms controlling inflammatory cell recruitment to tumors is a promising approach to suppress tumor growth and progression.

Diverse chemoattractants recruit innate immune cells to chronically inflamed tissues and tumors; these can activate G

protein coupled receptors (GPCR), receptor tyrosine kinases (RTK) or Toll-like/interleukin-1 receptors (TLR/IL1R) to initiate myeloid cell recruitment (9–10, 12–15). We recently showed that each of these receptors stimulate myeloid cell recruitment by activating PI3-kinase γ , but not α , β or δ , in circulating myeloid cells (9). It is well-accepted that Class IA PI3K isoforms p110 α , β and δ can be activated downstream of RTKs through the engagement of the regulatory p85 subunit by receptor phosphotyrosines. In contrast, the Class IB isoform p110 γ can be activated by GPCRs via the β - γ subunits of heterotrimeric G proteins. We recently demonstrated that whereas GPCRs activate p110 γ in a Ras/p101-dependent manner, RTKs and TLR/IL1Rs directly activated p110 γ in a Ras/p87-dependent manner. Once activated, p110 γ promoted inside-out activation of integrin, $\alpha 4\beta 1$, causing granulocytic and monocytic cell adhesion to endothelium and invasion into tumors (Figure 1A–B). Pharmacological or genetic blockade of p110 γ suppressed recruitment of both monocytes and granulocytes, and suppressed angiogenesis, tumor growth, progression and metastasis of implanted and spontaneous tumors, revealing an important therapeutic target in oncology (10). These findings indicated that targeting the molecular mechanisms promoting the recruitment of myeloid cells during inflammation

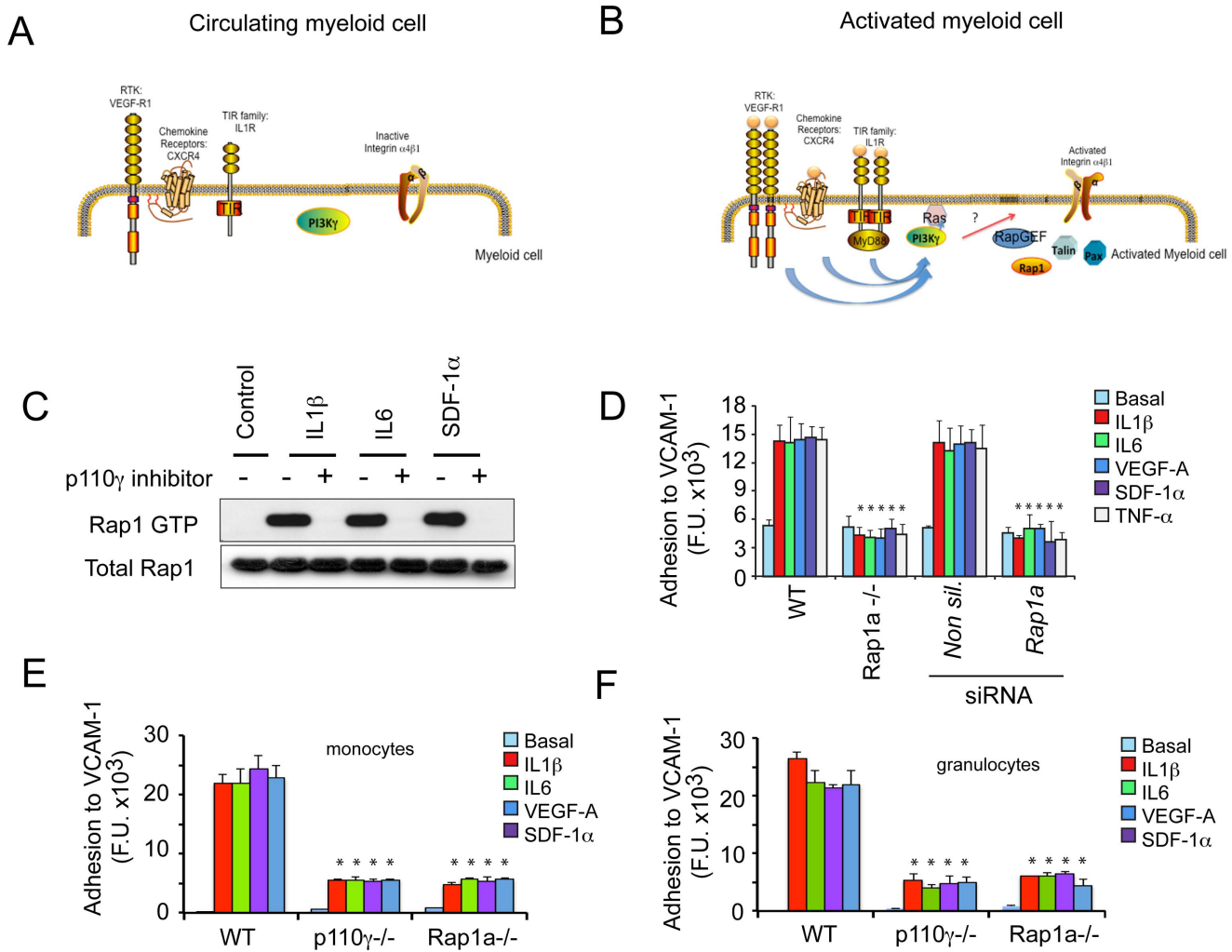


Figure 1. Rap1a and PI3K γ each promote integrin $\alpha 4\beta 1$ -dependent myeloid cell adhesion. (A) Schematic of circulating, unstimulated myeloid cells, indicating inactive receptor tyrosine kinases, chemokine receptors and Toll-like Receptor/IL-1 Receptor family members (TIR family) and inactive downstream PI3K γ and integrin $\alpha 4\beta 1$. (B) Schematic of stimulated myeloid cells showing all three receptor types that activate PI3K γ , thereby promoting integrin $\alpha 4\beta 1$ conformational changes, leading to integrin activation. Co-factors that may play roles in activating integrin $\alpha 4\beta 1$ include PKC, Rap1, RIAM, talin and paxillin. (C) Representative immunoblot of GTP-Rap and total Rap in myeloid cells that were stimulated with or without the chemoattractants IL-1 β , IL6 or SDF1 α and treated with the PI3K γ inhibitor, TG100-115 (+) or the chemically similar, inert control (-). (D) Adhesion to the integrin $\alpha 4\beta 1$ ligand VCAM-1 (expressed as fluorescence units, F.U.) of chemoattractant-stimulated WT and Rap1a $^{-/-}$ CD11b $^{+}$ cells (n=3), *P<0.01, Rap1a $^{-/-}$ vs WT and Rap1a vs non-silencing siRNA. (E) Adhesion to VCAM-1 of CD11bGr1lo monocytes sorted from WT, p110 $\gamma^{-/-}$ and Rap1a $^{-/-}$ animals (n=3), *P<0.01, p110 $\gamma^{-/-}$ and Rap1a $^{-/-}$ vs WT. (F) Adhesion to VCAM-1 of CD11bGr1hi granulocytes sorted from WT, p110 $\gamma^{-/-}$ and Rap1a $^{-/-}$ animals (n=3), *P<0.01, p110 $\gamma^{-/-}$ and Rap1a $^{-/-}$ vs WT. doi:10.1371/journal.pone.0060226.g001

could provide significant benefit in the treatment of a wide variety of cancers.

The integrin family of adhesion proteins plays a significant role in regulating inflammation (17–19). Activation of integrin $\alpha 4\beta 1$ by inside-out signaling induced by chemoattractants is required for lymphocyte and myeloid cell extravasation from the vasculature (9–10, 18–20). Extracellular stimuli from chemoattractants promote conformational changes in integrin extracellular domains that increase their capacity to bind ligand and therefore attach to endothelium and extracellular matrices (21–22). Although all the steps in integrin activation have yet to be deciphered, integrin activation can depend on Rap1 (Ras-proximate-1), a Ras-like small GTP-binding protein (23–25). Rap1 activates the effector protein, RIAM, which interact closely with the cytoskeletal protein talin, thereby localizing it near the membrane (26–27). Talin binds

to sites in integrin β chain cytoplasmic tails, thereby disrupting electrostatic interactions between amino acids in the integrin α and β chain cytoplasmic tails and altering the packing of integrin transmembrane domains (28–30). These events alter the conformation of the extracellular domains of the integrin heterodimer and increase ligand binding. In addition, paxillin-binding to the integrin $\alpha 4$ cytoplasmic tail also promotes integrin activation, as disruption of the paxillin-binding site in the integrin $\alpha 4$ cytoplasmic tail partially prevents talin binding and inhibits adhesion and trafficking of lymphocytes and myeloid cells (20, 31). Our recent studies showed that PI3K γ activation by a variety of inflammatory stimuli is essential to promote integrin $\alpha 4$ conformational changes, cell adhesion and myeloid cell trafficking in vivo (9–10). However, the exact molecular mechanisms by which myeloid cell PI3K γ regulates integrin $\alpha 4\beta 1$ activation

in vitro and in vivo remained to be elucidated. Here we show that PI3K γ -mediated integrin activation and subsequent myeloid cell recruitment and tumor inflammation depends on PLC γ , CalDAG-GEFI and II, Rap1a and RIAM.

Results

PI3K γ Activates Integrin α 4 β 1 in a Rap1a-dependent Manner

As we recently showed that integrin α 4 β 1 activation in CD11b+ granulocytes and monocytes depends on PI3K γ (p110 γ) (9–10), we sought to determine how PI3K γ activates integrin α 4 β 1. Integrin activation has been shown to depend on members of the Rap1 family of small GTPases (23–25). Therefore, we asked whether myeloid cell PI3K γ could promote integrin α 4 β 1 activation in a Rap1a-dependent manner. Like other small GTPases, Rap1 cycles between GDP-bound inactive and GTP-bound active forms. Thus, we measured Rap1 activity in CD11b+ bone marrow derived myeloid cells using a Ral-RBD (Ras Binding Domain) pull-down assay, which measures GTP-bound Rap in cell lysates. We found that the chemoattractants IL-1 β , IL-6, and SDF-1 α stimulated Rap-GTP loading in CD11b+ myeloid cells, but Rap-GTP loading was inhibited in the presence of the p110 γ inhibitor TG100-115 (Figure 1C). To determine whether integrin α 4 β 1 activation in myeloid cells depends on Rap1a, we evaluated the capacity of Rap1a deficient (Rap1a $^{-/-}$) and Rap1a siRNA transfected WT myeloid cells to adhere to VCAM-1. While Rap1a-deficient mice develop with minimal defects, leukocytes from these mice have exhibited reduced responsiveness to chemoattractants (33–36). Indeed, Rap1a $^{-/-}$ and Rap1a siRNA transfected CD11b+ bone marrow derived myeloid cells exhibited significantly reduced adhesion to VCAM-1 in response to a variety of chemoattractants (Figure 1D), even though Rap1a deficient myeloid cells expressed normal levels of integrin α 4 β 1 (Figure S1A–B). The geranylgeranyltransferase inhibitor GGTI-2147, which prevents the post-translational modification required to target small GTPases such as Rap1 to membranes, also effectively blocked chemoattractant-induced myeloid cell adhesion (Figure S1C). Importantly, p110 γ $^{-/-}$ and Rap1a $^{-/-}$ bone marrow derived CD11bGr1lo monocytes (Figure 1E) and CD11bGr1hi granulocytes (Figure 1F) failed to adhere significantly to VCAM-1. Taken together, these data suggest that p110 γ -mediated activation of integrin α 4 β 1 in myeloid cells depends on Rap1a.

To determine whether Rap1a is sufficient for p110 γ -mediated integrin α 4 β 1 activation in myeloid cells, we expressed constitutively activated PI3K γ (p110 γ CAAX), constitutively activated Rap1 (RapV12) or control plasmid (pcDNA) in CD11b+ myeloid cells. Constitutively activated p110 γ and Rap1 both stimulated adhesion to VCAM-1 in myeloid cells even in the absence of added chemoattractants. However, integrin α 4-targeted siRNA blocked p110 γ CAAX and RapV12 stimulated myeloid cell adhesion to VCAM-1, indicating that p110 γ and Rap1 are both upstream of integrin α 4 β 1 (Figure 2A, Figure S1D–E). Importantly, expression of constitutively activated Rap1, but not activated p110 γ , promoted adhesion in Rap1a $^{-/-}$ myeloid cells (Figure 2B). Furthermore, RapV12 induced constitutive myeloid cell adhesion in both WT and p110 γ $^{-/-}$ myeloid cells and in TG100-115 and p110 γ siRNA treated cells (Figure 2C), indicating that p110 γ is upstream of Rap1a in the integrin activation pathway. Taken together, our results indicate that Rap1a is activated by PI3K γ and is both necessary and sufficient to promote PI3K γ -mediated integrin α 4-dependent myeloid cell adhesion.

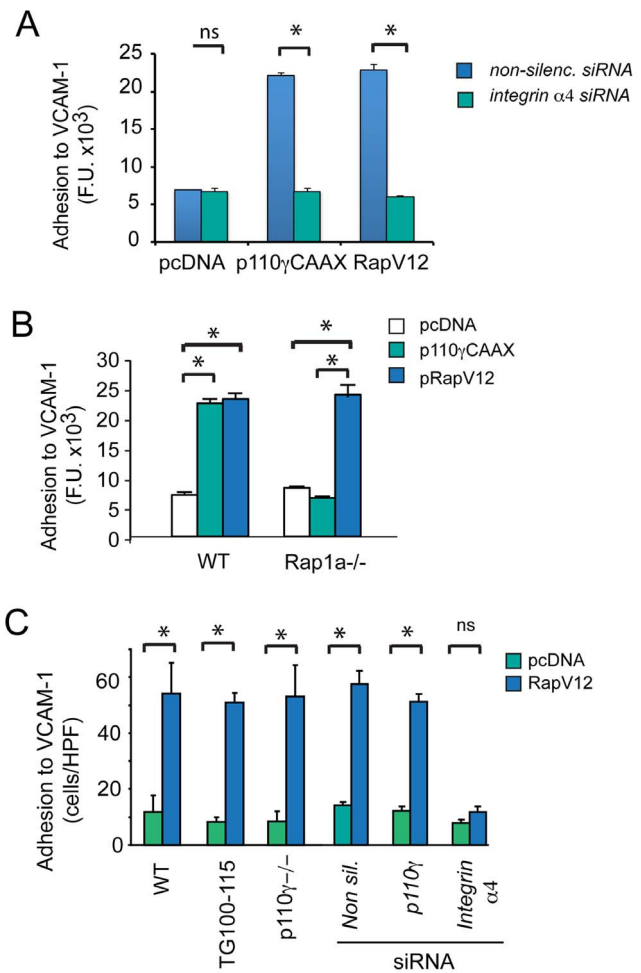


Figure 2. PI3K γ -mediated integrin α 4 β 1 adhesion requires Rap1a. (A) Adhesion to VCAM-1 of p110 γ CAAX, pRapV12 or pcDNA expressing CD11b+ myeloid cells that were also transfected with either integrin α 4 or control siRNA, *P<0.01, integrin α 4 vs non-silencing siRNA (n=3). (B) Adhesion to VCAM-1 of p110 γ CAAX, pRapV12 and pcDNA (control transfected) WT or Rap1a $^{-/-}$ CD11b+ myeloid cells (n=3), *P<0.01, p110 γ CAAX or pRapV12 vs pcDNA. (C) Adhesion to VCAM-1 of pRapV12 and pcDNA chemoattractant-stimulated WT, TG100-115 (PI3K γ inhibitor)-treated, p110 γ $^{-/-}$, non-silencing, p110 γ and integrin α 4 siRNA-transfected CD11b+ myeloid cells (n=3), *P<0.01 Rap1a vs pcDNA. doi:10.1371/journal.pone.0060226.g002

Rap1a Promotes Integrin α 4 β 1 Conformational Changes and Ligand Binding

Extracellular stimuli induce integrin conformational changes that result in increased ligand-binding and cell adhesion, a process that is called “integrin activation”. Chemoattractants rapidly (within seconds) induce PI3K γ -dependent myeloid cell integrin β 1 conformational changes, as measured by cell surface binding of HUTS21, an antibody that recognizes an epitope expressed only on activated human β 1 integrin (10, 22). These chemoattractants also rapidly stimulate the binding of recombinant VCAM-1 to myeloid cell integrin α 4 β 1 in WT but not PI3K γ or integrin α 4 deficient myeloid cells (10). To determine whether Rap1a is required for PI3K γ -mediated integrin α 4 β 1 conformational changes, chemoattractant-stimulated myeloid cells from WT, p110 γ $^{-/-}$ and Rap1a $^{-/-}$ mice were incubated with soluble VCAM-1/Fc. Bound VCAM-1/Fc was then detected with

fluorescently-labeled antibodies (Figure 3A). Chemoattractants markedly stimulated the binding of soluble VCAM-1/Fc to WT but not PI3K γ ^{-/-} or Rap1a^{-/-} myeloid cells (Figure 3B). Mn2⁺, which binds to the integrin-ligand binding pocket and directly promotes ligand binding and thus integrin activation, stimulated VCAM-1/Fc binding to WT, PI3K γ ^{-/-} and Rap1a^{-/-} cells, demonstrating that integrin α 4 β 1 is capable of being activated in all three genotypes. In support of these findings, we found that pharmacological blockade of Rap1 by GGTI-2147 suppressed chemoattractant-induced VCAM-1-binding to myeloid cells (Figure S2). Importantly, expression of activated RapV12 restored ligand binding to p110 γ ^{-/-} (Figure 3C) and Rap1a^{-/-} myeloid cells (Figure 3D). As expression of activated PI3K γ (p110 γ CAAX) failed to stimulate ligand binding in Rap1a^{-/-} myeloid cells (Figure 3D), these results further support the concept that Rap1a is downstream of PI3K γ and is essential for PI3K γ -stimulated ligand binding to integrin α 4 β 1.

We also found that while chemoattractants stimulated binding of HUTS21 on human myeloid cells, the Rap1 selective inhibitor GGTI-2147 inhibited HUTS21 binding, confirming that integrin activation and conformational changes depend on Rap1 (Figure 3E-F). Taken together, our results show that Rap1a is necessary and sufficient to activate integrin α 4 β 1 in both mouse and human myeloid cells.

CalDAG-GEFs and PLC γ are Required for PI3K γ Mediated Integrin Activation

As it is not clear how PI3K γ activates Rap1a, we postulated that PI3K γ might activate Rap1a exchange factors. Guanine nucleotide exchange factors (GEFs) exchange GDP for GTP to generate the active form of monomeric GTPase (37). As some RapGEFs are ubiquitously expressed while others are expressed only in select tissues (25), we examined the expression of RapGEFs in primary murine myeloid cells and found that four RapGEFs, CalDAG-GEFI/RasGRP2, CalDAG-GEFII/RasGRP, Epac1 and Epac2 (37–40) are expressed in primary bone marrow derived myeloid cells. While all four GEFs were expressed, mRNA levels of Epac1 were most abundant (Supplementary Figure 3A). To determine if any of these RapGEFs play a role in integrin activation, we suppressed their gene expression in myeloid cells by siRNA mediated gene knockdown. siRNA knockdown of CalDAG-GEFI or II but not Epac1 or 2 blocked chemoattractant-induced integrin α 4 β 1-mediated cell adhesion (Figure 4A; Figure S3B). Combined inhibition of both CalDAG-GEFI and II blocked myeloid cell adhesion to the same extent as blockade of CalDAG-GEFI alone, suggesting that CalDAG-GEFI may play a primary role in PI3K γ -mediated Rap1a activation (Figure 4A). Inhibition of CalDAG-GEFI and II together blocked myeloid cell adhesion induced by p110 γ CAAX but did not inhibit adhesion induced by pRapV12, indicating that RapGEFs act upstream of Rap1 in integrin

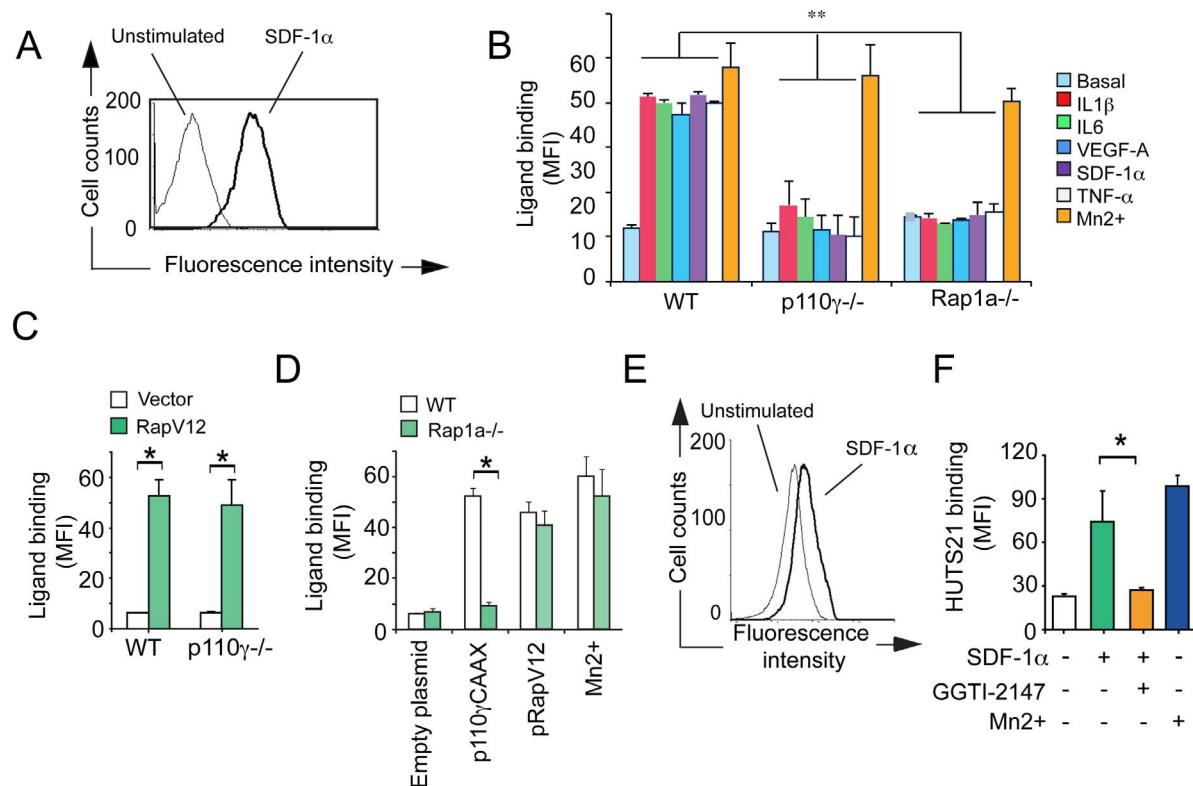


Figure 3. Rap1 promotes myeloid cell integrin α 4 β 1-ligand binding and conformational changes. (A) Representative histogram of soluble, fluorescently labeled VCAM-1/Fc bound to CD11b⁺ myeloid cells 3 min after treatment with (black line) or without (grey line) IL-1 β . (B) Mean fluorescence intensity (MFI) of VCAM-1/Fc bound to WT, p110 γ ^{-/-} and Rap1a^{-/-} myeloid cells in the absence (basal) or presence of chemoattractants or the positive control stimulus Mn2⁺ (n=3), *P<0.01, p110 γ ^{-/-} or Rap1a^{-/-} vs WT. (C) MFI of VCAM-1/Fc bound to pRapV12- and control-transfected WT and p110 γ ^{-/-} myeloid cells (n=3), *P<0.01, pRapV12 vs control. (D) MFI of VCAM-1/Fc bound to WT and Rap1a^{-/-} myeloid cells transfected with p110 γ CAAX, pRapV12 or control plasmid or treated with Mn2⁺ (n=3), *P<0.01, Rap1a^{-/-} vs WT. (E) Representative histogram of HUTS21 antibody binding to unstimulated (grey line) or SDF-1 α stimulated (black line) human CD11b⁺ myeloid cells. (F) MFI of HUTS21 binding to unstimulated or SDF-1 α stimulated human myeloid cells in the presence of absence of 10 μ M geranylgeranyltransferase inhibitor (GGTI-2147) or Mn2⁺ (n=3), *P<0.01, +GGTI vs -GGTI. doi:10.1371/journal.pone.0060226.g003

activation (Figure 4B). As CalDAG-GEFI knockout mice also exhibit defects in leukocyte integrin activation (40–41), these results indicate that CalDAG-GEFI and II promote PI3K γ -mediated adhesion but are not required once Rap has been activated. Importantly, knockdown of CalDAG-GEFI and CalDAG-GEFII, but not Epac1 and 2, prevented Rap1 GTP-loading in response to SDF1 α (Figure 4C). These findings show that the myeloid cell GEFs CalDAG-GEFI and CalDAG-GEFII activate Rap1 and thereby promote integrin $\alpha 4\beta 1$ -mediated adhesion in response to PI3K γ activation.

CalDAG-GEFs can be activated by calcium or diacylglycerol (DAG), two second messengers produced by the actions of phospholipase C γ (PLC γ) (42). Each PLC isoform contains a plextrin-homology domain, which allows it to bind to phosphatidylinositol in the plasma membrane (43). Importantly, as PI(3,4,5)P $_3$ can activate PLC γ (44), it is possible that PI3K γ activates Rap1 by stimulating PLC γ in myeloid cells. Therefore, we asked whether PLC γ is necessary for Rap1 activation in myeloid cells. Two isoforms of PLC γ are expressed in primary myeloid cells, PLC $\gamma 1$ and PLC $\gamma 2$. We found that siRNA-mediated knockdown of PLC $\gamma 1$ blocked Rap1 GTP-loading induced by SDF-1 α stimulation (Figure 4D). siRNA-mediated knockdown of PLC $\gamma 1$ (Figure 4E–F, Figure S3C) or pharmacological inhibition of PLC γ (Figure S3D) completely inhibited myeloid cell adhesion to VCAM-1 in response to stimulation by chemoattractant factors or expression of activated PI3K γ (p110 γ CAAX) (Figure 4E–F). As knockdown of PLC $\gamma 1$ expression does not block adhesion in pRapV12 expressing cells (Figure 4F), our results indicate that PLC $\gamma 1$ functions downstream of PI3K γ and upstream of Rap1a. PLC $\gamma 1$ thus is required for PI3K γ activation of Rap1a and $\alpha 4\beta 1$ dependent myeloid cell adhesion. Although calcium and diacylglycerol can activate some isoforms of protein kinase C (45), we observed no role for PKC in integrin activation in myeloid cells, as neither broad spectrum nor selective PKC inhibitors affected myeloid cell adhesion to VCAM-1 (Figure S4A–B).

Integrin $\alpha 4$ -mediated myeloid cell adhesion depends on the interaction of the adapter proteins talin and paxillin with the cytoplasmic tails of integrin $\alpha 4\beta 1$ (9,20,31). Previous studies implicated the Rap1 effector RIAM in facilitating talin binding to the integrin β cytoplasmic tail and immune cell adhesion events (26–28). As the Rap1 effector RIAM is strongly expressed in myeloid cells, to determine whether RIAM promotes integrin $\alpha 4\beta 1$ activation, we evaluated the effect of siRNA-mediated knockdown of RIAM on myeloid cell adhesion. RIAM siRNA blocked cell adhesion in response to chemoattractants (Figure 5A, Figure S4C) and expression of activated Rap1 or PI3K γ (Figure 5B). Moreover, inhibition of RIAM expression prevented integrin $\alpha 4\beta 1$ ligand-binding in response to p110 γ CAAX or RapV12 expression (Figure 5C), suggesting that RIAM directly regulates integrin $\alpha 4$ activation and myeloid cell adhesion downstream of both PI3K γ and Rap1. Our studies thus indicate that RIAM is required for integrin $\alpha 4$ activation in myeloid cells.

As RIAM promotes integrin activation by facilitating interaction of talin with the integrin $\beta 1$ cytoplasmic tail (26), we next asked whether PI3K γ activation promotes talin and paxillin binding to integrin $\alpha 4\beta 1$. To accomplish this goal, we immunoprecipitated integrin $\alpha 4\beta 1$ from unstimulated and chemoattractant stimulated WT, $\alpha 4Y991A$, and PI3K γ -/- cells and determined whether talin and paxillin co-immunoprecipitated with the integrin. We found that talin and paxillin co-immunoprecipitated with integrin $\alpha 4\beta 1$ in stimulated WT but not in stimulated $\alpha 4Y991A$ or p110 γ -/- myeloid cells (Figure 5D). Taken together, these findings indicate that Rap1 and its effector RIAM facilitate integrin $\alpha 4\beta 1$ activation in response to PI3K γ activation

by stimulating association of the adapter proteins talin and paxillin with integrin $\alpha 4\beta 1$.

In previous studies, we found that PI3K γ -mediated integrin $\alpha 4\beta 1$ activation is required for trafficking of myeloid cells to the tumor microenvironment (10). To investigate whether Rap1a is required for this recruitment of myeloid cells to the tumor microenvironment, we adoptively transferred fluorescently labeled myeloid cells into mice bearing established subcutaneous Lewis lung carcinoma (LLC) tumors and within 2 h, we could quantitatively identify these cells in tumors using flow cytometry. We found that Rap1a-/-, Rap1a siRNA transfected and GGTI-2147 treated myeloid cells failed to accumulate in the tumor microenvironment (Figure 5E), indicating that Rap1a, like p110 γ and integrin $\alpha 4\beta 1$ (9–10), is required for myeloid cell trafficking to tumors.

To determine the impact of myeloid cell Rap1a on tumor inflammation and progression, we evaluated the growth of LLC tumors in WT mice that were lethally irradiated and transplanted with bone marrow from WT or Rap1a-/- mice. Importantly, mice transplanted with Rap1a-/- BM exhibited significantly reduced tumor growth (Figure 6A–B). Tumors from mice with Rap1a-/- bone marrow showed significant reductions in the recruitment of total CD11b+Gr1+ myeloid cells (Figure 6C), in the recruitment of monocytic and granulocytic subpopulations (Figure 6D–E) and CD11b+F4/80+ macrophages (Figure 6F) to the tumor microenvironment, as determined by protease-mediated dissolution of tumors and flow cytometric quantification of cell subpopulations. Similar reductions in tumor growth and inflammation were also observed in Rap1a-/- animals (Figure S5A–D). We also observed reduced recruitment of macrophages and blood vessels in mice with Rap1a-/- bone marrow cells (Figure 6G–I). Together, these results indicate that Rap1a is an important mediator of tumor inflammation and progression through its actions on integrin activation during the recruitment of myeloid cells to the tumor microenvironment, with subsequent effects on tumor angiogenesis.

Discussion

Tumor inflammation promotes angiogenesis, immunosuppression and metastasis. Targeting the causes and consequences of chronic inflammation is likely to provide significant benefit in the treatment and prevention of a wide variety of cancers. Our previous studies have investigated the mechanisms demonstrated that myeloid derived suppressor cells are recruited from the circulation by tumor-derived chemoattractants, which stimulate PI3-kinase γ (PI3K γ)-mediated integrin $\alpha 4$ activation and myeloid cell extravasation into tumors (9). We have show here that PI3K γ activates PLC γ , leading to RasGrp/CalDAG-GEF-I&II mediated, Rap1a-dependent activation of integrin $\alpha 4\beta 1$, extravasation of myeloid cell, and inflammation-associated tumor progression. Our studies demonstrate that chemoattractants stimulate myeloid cell RTK, GPCR and TIR-receptor-mediated integrin $\alpha 4\beta 1$ activation, myeloid cell tumor trafficking and tumor inflammation and growth by promoting PI3K γ -dependent Rap1a activation. PI3K γ mediated Rap1a and integrin $\alpha 4\beta 1$ activation depend on PLC γ , Rap-GEFs CalDAG-GEFI and II and RIAM. RIAM promotes integrin $\alpha 4\beta 1$ conformational changes through its interactions with talin. Talin then disrupts the packing of the integrin cytoplasmic tails, leading to integrin unfolding and activation (Figure 7). In vivo, tumor-derived chemoattractants promote the recruitment and infiltration of myeloid cells to the tumor microenvironment by increasing PI3K γ -Rap1a-integrin $\alpha 4\beta 1$ -dependent adhesion to the tumor endothelium. As myeloid cells in

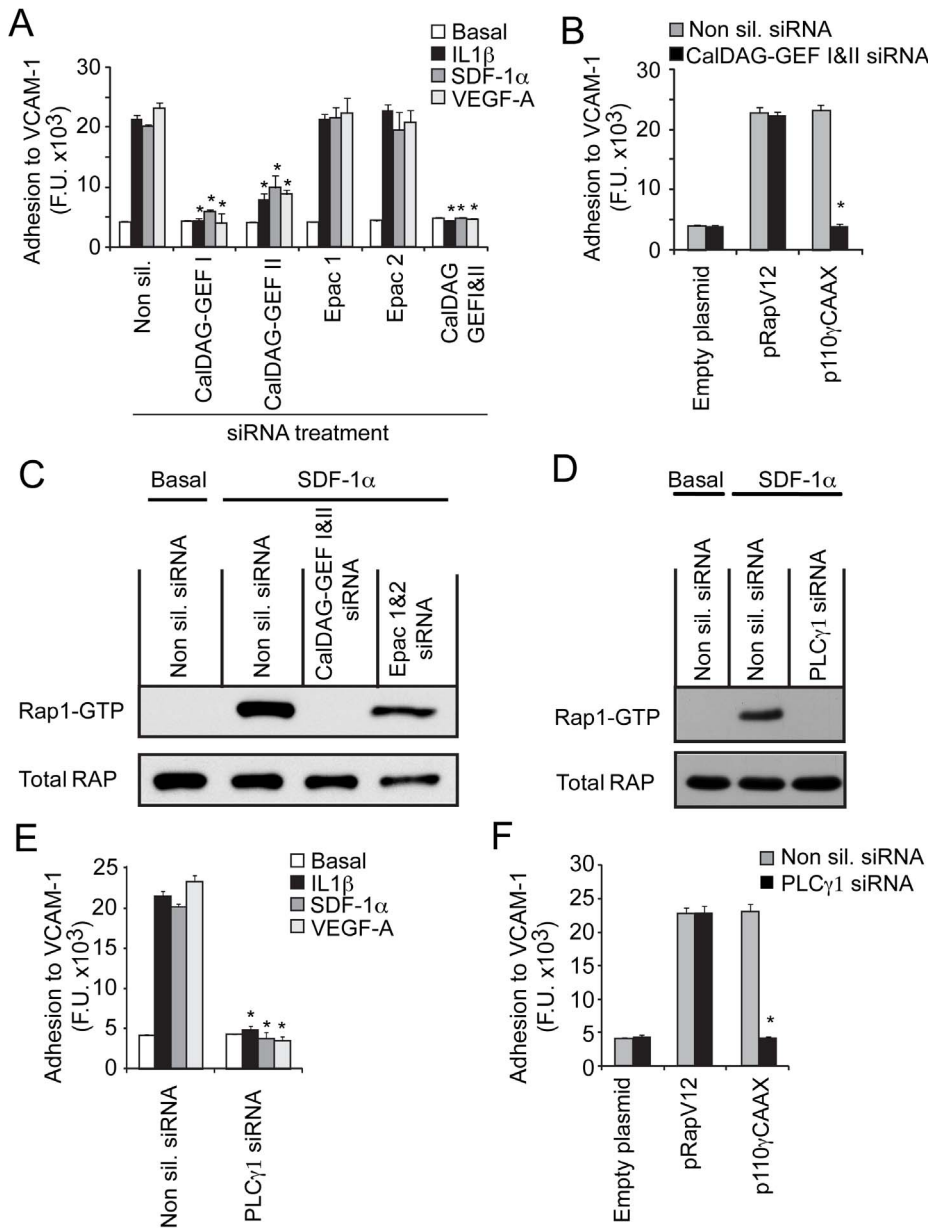


Figure 4. CaIDAG-GEFs are required for PI3K γ -mediated Rap1 and integrin α 4 β 1 activation in myeloid cells. (A) Adhesion of chemoattractant-treated CD11b⁺ myeloid cells to VCAM-1 after transfection with non-silencing, CalIDAG-GEFI, CalIDAG-GEFII, Epac1, Epac2 and CalIDAG-GEFI+CalIDAG-GEFII siRNAs (n = 3), *P<0.01, CalIDAG-GEFI, CalIDAG-GEFII vs non silencing siRNA. (B) Adhesion to VCAM-1 of myeloid cells after transfection with CalIDAG-GEFI and II or control siRNAs in combination with pRapV12, p110 γ CAAX, or empty plasmid (n = 3), *P<0.01 CalIDAG-GEFI and II vs control siRNAs. (C) Rap1 GTP and total Rap immunoblot of unstimulated, non-silencing siRNA-transfected (Basal) myeloid cells and SDF-1 α stimulated CalIDAG-GEFI and II, Epac 1 and 2, and non-silencing siRNA-transfected myeloid cells. (D) Rap1 GTP and total Rap immunoblot of unstimulated non-silencing siRNA-transfected (basal) and SDF-1 α -stimulated myeloid cells after transfection with PLC γ 1 or control siRNA. (E) Adhesion of PLC γ 1 or control siRNA-transfected, chemoattractant-stimulated myeloid cells (n = 3), *P<0.01 PLC γ 1 vs non-silencing siRNA. (F) Adhesion to VCAM-1 of myeloid cells after transfection with PLC γ 1 or control siRNA in combination with pRapV12, p110 γ CAAX, or vector control (empty plasmid) (n = 3), *P<0.01 vs PLC γ 1 non-silencing siRNA. doi:10.1371/journal.pone.0060226.g004

the tumor microenvironment suppress T-cell mediated anti-tumor immunity (3–5), promote tumor angiogenesis (7–13), and contribute to resistance to chemotherapy (9), our studies thus suggest novel targets for the development of therapeutic approaches for cancer and inflammatory diseases.

Tumors release a diverse array of chemoattractants that promote myeloid cell infiltration of tumors; these chemoattractants activate G protein coupled receptors, receptor tyrosine kinases or

Toll-like/interleukin-1 receptors to initiate myeloid cell recruitment (9–10,12–15). Using competitive inhibitors of chemoattractants and PI3K genetic mutations, siRNA-mediated knockdown and chemical inhibitors of PI3K isoforms, we recently showed that a variety of tumor-derived chemoattractants stimulate myeloid cell recruitment by activating PI3K γ , but not other PI3K isoforms, in circulating myeloid cells (9–10). We showed here that PI3K γ activity alone is sufficient to promote integrin α 4 β 1 activation, as

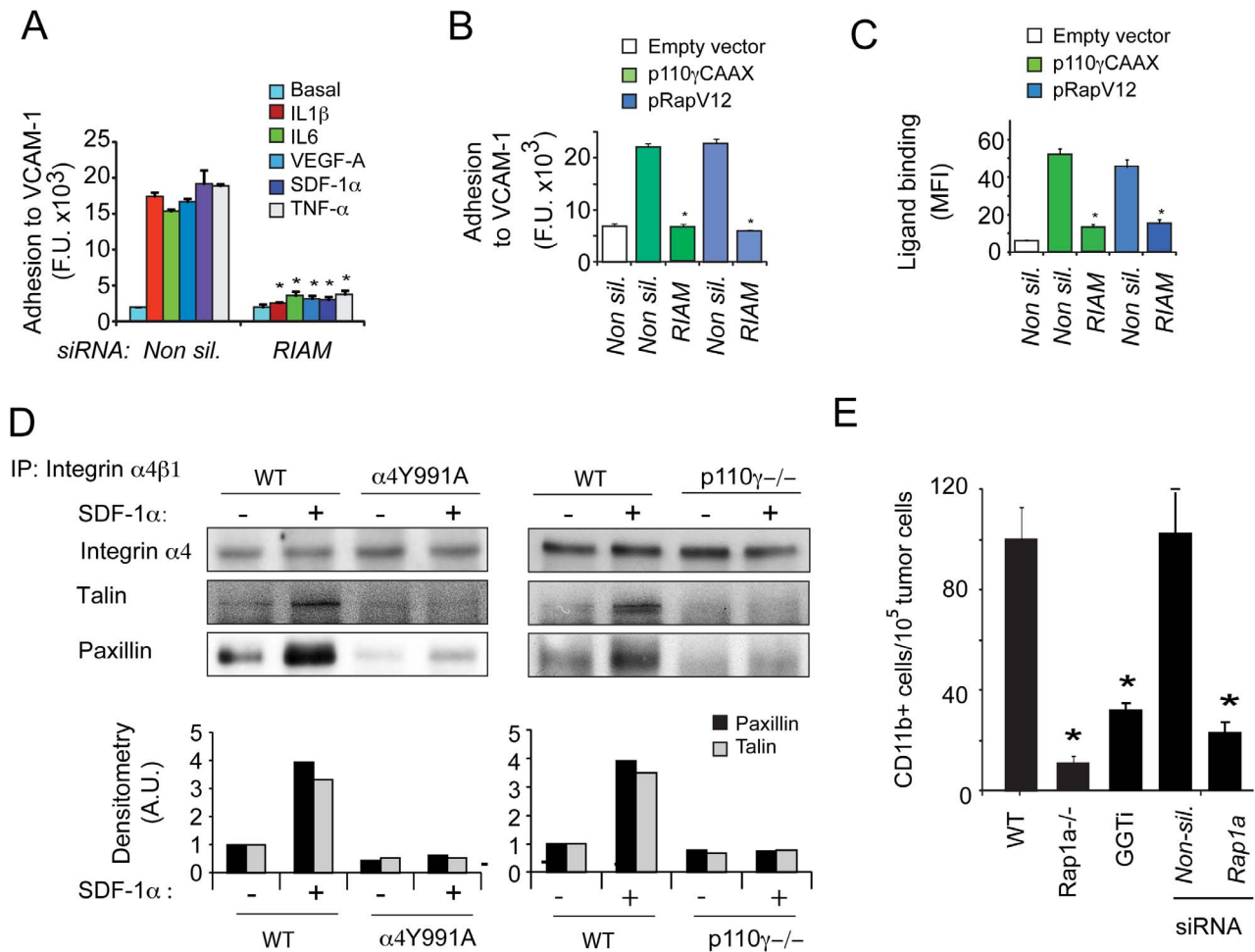


Figure 5. RIAM is necessary for PI3K γ -mediated integrin α 4 β 1 activation. (A) Adhesion to VCAM-1 of chemoattractant-treated myeloid cells after transfection with RIAM or control siRNA (n = 3), *P < 0.01 vs RIAM vs. non-silencing siRNA. (B) Adhesion to VCAM-1 of RIAM or non-silencing siRNA-transfected myeloid cells that were also transfected with p110 γ CAAX, pRapV12 or control plasmid (n = 3), *P < 0.01 RIAM vs non-silencing siRNA. (C) VCAM-1/Fc binding to RIAM or non-silencing siRNA-transfected myeloid cells that were also transfected with p110 γ CAAX, pRapV12 or control plasmid (n = 3), *P < 0.01 RIAM vs non-silencing siRNA. (D) Immunoprecipitates of integrin α 4 β 1 from WT, α 4Y991A, and p110 γ ^{-/-} bone marrow derived cells with (+) or without (-) SDF-1 α stimulation and immunoblotted to detect the integrin α 4 subunit, talin, and paxillin. Histograms: densitometry of talin and paxillin levels normalized to integrin α 4 levels. (E) Trafficking to LLC tumors of WT or Rap1a^{-/-} myeloid cells, myeloid cells treated with 10 μ M GGTI-2147, and myeloid cells transfected with Rap1a or non-silencing siRNAs, (n = 3), *P < 0.01 Rap1a^{-/-} or 10 μ M GGTI-2147 vs WT and Rap1a vs non-silencing siRNAs. doi:10.1371/journal.pone.0060226.g005

expression of membrane targeted PI3K γ (p110 γ CAAX) alone increases myeloid cell adhesion and α 4 ligand binding. Our studies have shown that in primary myeloid cells and macrophages, PI3K γ is the only PI3K isoform that is expressed at significant levels. However, preliminary studies indicate that myeloid leukemia cells exhibit higher levels of other PI3K isoforms, such as PI3K δ , than PI3K γ (not shown). These cells exhibit constitutive adhesion to VCAM-1, suggesting that once activated, other PI3K isoforms can promote integrin α 4 β 1 activation. Using p110 γ CAAX expression, we were able to show that once activated, PI3K γ is sufficient to activate integrin α 4 β 1 and that this event depends on Rap1 activity.

Two Rap isoforms, Rap1a and Rap1b, have been implicated in the regulation of integrin activation (33–36, 47). Rap1b has a clear role in regulating platelet-mediated integrin α IIB β 3 activation (36), while Rap1a has been implicated in regulating leukocyte and endothelial cell migration (33–35). In the studies described here, we found that Rap1a is required to promote integrin α 4 β 1

activation and adhesion. Our prior studies showed that co-localization of integrin α 4 β 1, N/K Ras, p110 γ and specific PI3K regulatory subunits in plasma membranes is required for integrin α 4 β 1 activation in response to inflammatory signals. Our current results suggest that the Rap1a isoform may also co-localize in these membrane microdomains to promote integrin α 4 β 1 activation in myeloid cells.

In the studies described here, we found that Rap1a is required to promote integrin α 4 β 1-dependent myeloid cell trafficking during inflammation and tumor growth in vivo, as we observed substantial suppression of tumor growth and inflammation in mice transplanted with Rap1a^{-/-} bone marrow. Although several reports have shown distinct functional roles within cells for Rap1a and Rap1b (34, 36, 48), knockdown of Rap1a in primary myeloid cells resulted in almost complete loss of PI3K γ -mediated integrin α 4 β 1 activity in vitro, suggesting that Rap1a is the predominant form of Rap1 that is activated by PI3K γ in these cells.

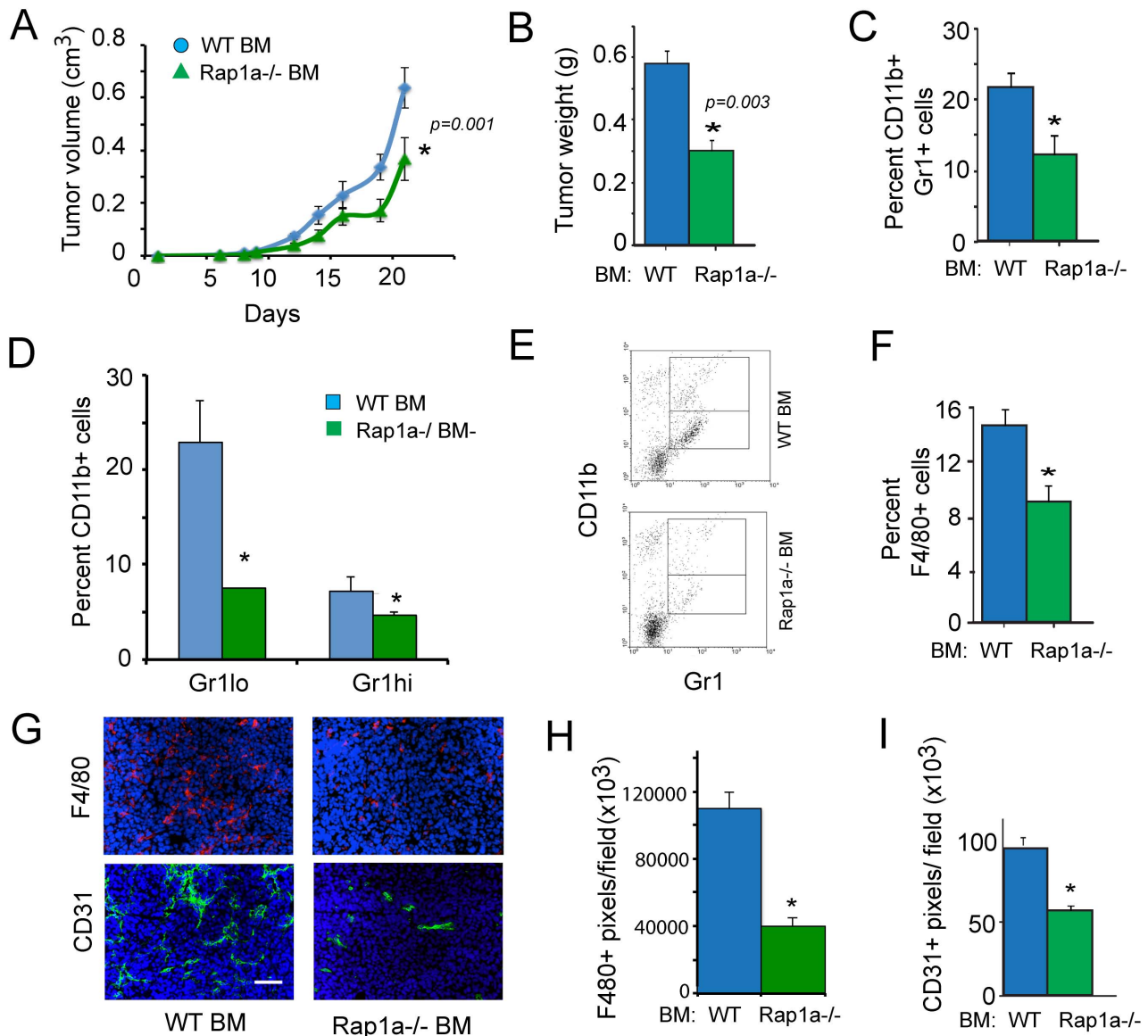


Figure 6. Myeloid cell Rap1a promotes tumor inflammation and growth. (A) Tumor volume and (B) weight of LLC tumors grown in WT mice transplanted with WT or Rap1a^{-/-} bone marrow (n = 10); *P = 0.001 to 0.003, WT vs Rap1a^{-/-} BM, as determined by ANOVA. (C) Percentage of CD11b+Gr1⁺ tumor-infiltrating myeloid cells in WT and Rap1a^{-/-} bone marrow transplanted (BMT) tumors, *P < 0.01, Rap1a^{-/-} vs WT (ANOVA). (D) Percentage of CD11b+Gr1^{lo} monocytic and CD11b+Gr1^{hi} granulocytic lineage cells in WT and Rap1a^{-/-} BMT tumors, *P < 0.01 Rap1a^{-/-} vs WT (ANOVA). (E) Representative FACS profiles from D, with upper quadrant corresponding to CD11b+Gr1^{hi} granulocytic and lower quadrant corresponding to CD11b+Gr1^{lo} monocytic lineage cells. (F) Percentage of F4/80⁺ tumor-infiltrating myeloid cells in WT and Rap1a^{-/-} BMT tumors, *P < 0.01 Rap1a^{-/-} vs WT (ANOVA). (G) Representative images of tumor cryosections that were immunostained to detect F4/80⁺ macrophages (red) and CD31⁺ blood vessels (green) and counterstained with Dapi. Scale bar = 40 μ m. (H) Quantification of F4/80⁺ pixels/field in cryosections from G, *P < 0.01 Rap1a^{-/-} vs WT. (I) CD31⁺ pixels/field in cryosections from G, *P < 0.01 Rap1a^{-/-} vs WT. doi:10.1371/journal.pone.0060226.g006

Our studies demonstrate that two RapGEFs, CalDAG-GEFI and CalDAG-GEFII, but not Epac 1 or 2, promote integrin $\alpha 4\beta 1$ activation in myeloid cells. Importantly, mice and patients lacking CalDAG-GEFI exhibit leukocyte adhesion deficiencies (40–41) as do mice lacking Rap1a (33). Although Epac1, a cAMP-activated GTP exchange factor (38–39), is the most highly expressed RapGEF in myeloid cells, we found that it plays no role in PI3K γ -mediated integrin activation, as siRNA-mediated knockdown of Epac1 did not affect integrin activation or myeloid cell adhesion. As Epac1 has been shown to play roles in cAMP-dependent Rap1 activation and cell adhesion in various cell types (38–39), our

results suggest that PI3K γ -mediated Rap1 activation is regulated separately from cAMP-mediated Rap1 activation by the use of different Rap-GEFs.

Our studies implicating two calcium and diacylglycerol activated Rap-GEFs in Rap-dependent integrin activation suggested a role for PLC γ in integrin $\alpha 4\beta 1$ activation. PLC γ hydrolyzes 4,5 biphosphate (PI(4,5)P₂) into two second messengers, inositol 1,4,5 triphosphate (IP₃) and diacylglycerol (DAG)(42–43). While IP₃ releases calcium from intracellular stores, PLC generated DAG activates PKC and guanine exchange factors (GEFs) such as CalDAG-GEFI and CalDAG-GEFII (40–41). As PLC γ can be

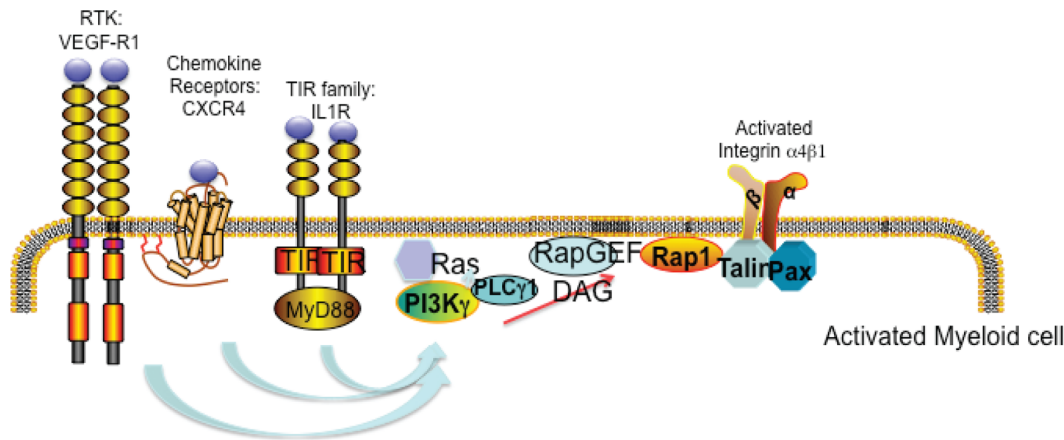


Figure 7. PI3K γ mediated activation of integrin $\alpha 4\beta 1$ requires Rap1a and other co-factors. Schematic of stimulated myeloid cells demonstrating that diverse receptor types activate PI3K γ , thereby promoting integrin $\alpha 4\beta 1$ conformational changes, leading to integrin activation in a PLC $\gamma 1$, RAP-GEF, Rap1, RIAM, talin and paxillin-dependent manner. doi:10.1371/journal.pone.0060226.g007

activated by PI3-Kinase (44), we tested PLC $\gamma 1$ siRNA and pharmacological inhibitors and found that these reagents completely blocked Rap activation, integrin activation and myeloid cell adhesion.

Our studies clearly identified a role for the Rap effector RIAM in myeloid cell integrin activation, as RIAM knockdown suppressed integrin $\alpha 4\beta 1$ -mediated adhesion and ligand binding. Rap can also activate RAPL, another Rap effector protein found in lymphocytes. Rap1-GTP can bind to RIAM and promote talin localization to the leading edge of cells, since Rap1 is prenylated and thereby localized to the membrane.

In the studies presented here, we found that PI3K γ -mediated Rap1a and integrin $\alpha 4\beta 1$ activation depend on PLC γ , Rap-GEFs CalDAG-GEFI and II and RIAM. In vivo, tumor-derived chemoattractants promote the recruitment and infiltration of myeloid cells to the tumor microenvironment in PI3K γ -Rap1a-integrin $\alpha 4\beta 1$ -dependent adhesion to the tumor endothelium. Blockade of either integrin $\alpha 4\beta 1$ or Rap1a suppresses tumor inflammation, angiogenesis and tumor progression in murine models of cancer (9–10). These results suggest that antagonists of integrin $\alpha 4\beta 1$ or Rap1 could offer clinical benefit for cancer patients. An antagonist of integrin $\alpha 4\beta 1$, the humanized antibody natalizumab (Tysabri), has been developed for clinical applications and is currently in use for the treatment of multiple sclerosis and inflammatory bowel disorders (48). While this antagonist offers significant clinical benefit in these autoimmune diseases, it also elevates the risk to 0.2% for complications associated with immunosuppression, including progressive multifocal leukoencephalopathy (PML), a neurotoxic JC virus infection of the brain (49–50). Natalizumab use in cancer therapy has been considered based on observations that $\alpha 4\beta 1$ promotes angiogenesis and inflammation in pre-clinical models of solid tumors (9–10) and that it promotes the survival of B cell leukemias and other hematological cancer cells (51), but it is currently unclear if the potential benefits will outweigh the potential of long-term health risks. Rap1a might serve as an alternative target for cancer therapy. The small GTPases Ras and Rap are post-translationally modified by isoprenylation, the addition of farnesyl or geranylgeranyl lipids to C-terminal CAAX moieties on Ras and Rap, respectively (52). Farnesyltransferase and geranylgeranyltransferase inhibitors have been investigated for cancer and immune disease therapy, as small GTPases play key roles in cell proliferation and invasion pathways

(52). Interestingly, bisphosphonates, which are in clinical practice as inhibitors of bone resorption due to osteoporosis, are potent inhibitors of geranylgeranyl biosynthesis, and thus Rap1, and have proven effective in the therapy of multiple myeloma and breast carcinoma (52–54). As further details of the Rap1a-integrin $\alpha 4\beta 1$ pathway are revealed, it is likely that new targets and therapies for the suppression of tumor inflammation will be developed.

Methods

Ethics

All animal studies were carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All protocols were approved by the Institutional Committee on the Use and Care of Animals of the University of California, San Diego. Discomfort and injury to animals was limited to that which was unavoidable in the conduct of scientifically valuable research. Analgesics, anesthetics, and tranquilizing drugs were as determined by our veterinarians and according to the guidelines set down in the NIH Guide for the Care and Use of Laboratory Animals. All experiments utilized overdose of the inhalant isoflurane (followed by cervical dislocation) for euthanasia, as consistent with the recommendations of the Panel on Euthanasia of the American Veterinary Medical Association. Use of donated human peripheral blood cells from the San Diego Blood Bank was approved by the Institutional Review Board for human subjects research of the University of California, San Diego.

Statistical Analysis

All error bars indicate standard error of the mean (SEM) of 3 replicates (in vitro studies) or 10 replicates (animal studies). All studies were performed a minimum of three times. Statistical significance was assessed with the Student *t* test for in vitro studies and ANOVA for in vivo studies using SigmaXL 6.02. A value of $P < 0.05$ was considered significant and signified by an asterisk.

Purification of Myeloid Cells

Myeloid cells were purified from murine bone marrow or human buffy coats obtained from the San Diego Blood Bank using anti-CD11b magnetic bead affinity chromatography according to manufacturer's directions (Miltenyi Biotec). To assess the purity of

the CD11b⁺ cell population, allophycocyanin-labelled anti-CD11b antibodies was added to cells, and flow cytometry was performed. Cell were >95% CD11b⁺.

Adhesion Assays

1 \times 10⁵ calcein-AM labelled murine CD11b⁺ cells were incubated on plastic plates coated with 5 μ g/ml recombinant soluble VCAM-1 (R&D Systems) for 30 min at 37°C in basal media, culture media from Lewis lung carcinoma cells (TCM) or DMEM containing 200 ng/ml SDF1 α , IL-1 β , IL-6, TNF α or VEGF-A (R&D Systems) and 1 μ M inhibitors directed against PI3K γ (TG100-115), PLC γ (U73122), Rap1-selective geranylgeranyl-transferase inhibitor (GGTI-2147, GGTI-298), PKC (Ro-32-0432, G6976). U73122, GGTI-2147, R0-32-0432 and G6976 were from Calbiochem. TG100-115 was from Sanofi-Aventis. After washing three times with warmed medium, adherent cells were quantified using a plate fluorimeter (GeniosPro, TECAN). CD11b⁺ myeloid cells from WT, Rap1^{-/-}, p110 γ ^{-/-}, α 4Y991A mice as well as siRNA-transfected and RapV12 and p110 γ CAAX-transfected cells were serum-starved for 4 h, and then incubated for 20 min on 5 μ g/ml rsVCAM-1. Adherent cells were automatically quantified using a plate fluorimeter (GeniosPro, TECAN).

Ligand Binding Assay

1 \times 10⁶ CD11b⁺ cells isolated from WT, p110 γ ^{-/-}, or Rap1a^{-/-} mice were incubated with 200 ng/ml IL-1 β , SDF-1 α , IL-6, TNF α , VEGF-A or medium together with 1 mg/ml mouse VCAM-1/humanFc fusion protein (R&D Systems) for 3 min. Cells were washed twice and incubated with donkey anti-human-FC-PE antibody (Jackson ImmunoResearch) then analyzed by FACs Calibur. Mean fluorescence intensity of treated cells was compared to that of unstimulated cells (basal). These ligand-binding assays were performed on WT CD11b⁺ cells and cells transfected with GFP/RapV12, GFP/p110 γ CAAX or siRNAs and on CD11b⁺ cells that were treated with 1 μ M GGTI-2147 for 30 min at 37°C.

Human Integrin Activation Assay

Expression of integrin α 4 on CD11b⁺ cells was determined by flow cytometry to detect PE-conjugated R1/2 (rat anti-murine α 4, eBioscience) or HP2/1 (mouse anti-human α 4, Biogen Idec). To quantify integrin activation, 2.5 \times 10⁶ freshly isolated human myeloid cells/ml were incubated in culture medium containing 10 μ g/ml normal human immunoglobulin (12000C, Caltag) for 45 min on ice. These cells were then incubated in 200 ng/ml SDF-1 α or 1 mM Mn2⁺ in the presence or absence of 1 μ M GGTI-2147 plus 2.5 μ g HUTS21 (β 1 activation epitope, BD-Bioscience), P4C10 (total β 1, Chemicon), or IgG2 control for 10 min at 37°C, followed by Alexa 488 goat-anti-mouse antibodies for 20 min on ice. Bound antibody was quantified by flow cytometry.

siRNA and Plasmid Transfections

Purified CD11b⁺ cells were transfected with 2 μ g/1 \times 10⁶ cells pcDNA 3.1p110 γ CAAX or pcDNA3.1 RapV12 or pGFPMax (Lonza) using AMAXA Mouse Macrophage Nucleofection Kits or with 100 nM of siRNA targeting integrin α 4 (Mm_Itga4_1; Mm_Itga4_2), Rap1a (Mm_Rap1a_1; Mm_Rap1a_7), RIAM (Mm_Apbb1ip_1; Mm_Apbb1ip_3), CalDAG-GEFI (Mm_Rasgrp2_1; Mm_Rasgrp2_2), CalDAG-GEFII (Mm_Rasgrp1_1; Mm_Rasgrp1_2), Epac1 (Mm_Rapgef3_4; Mm_Rapgef3_5), Epac2 (Mm_Rasgrp4_3; Mm_Rasgrp4_5),

PLC γ (Mm_Plcg1_1; Mm_Plcg1_2) or Non-silencing siRNA (Ctrl_AllStars_1), which were purchased from Qiagen. After transfection, cells were cultured for 24 h in culture media containing 20% serum. Each siRNA was tested individually for efficient knockdown of protein expression and for inhibition of adhesion or ligand binding. To control for off-target effects of siRNAs, we used siRNAs that had been previously validated to have no major off-target effect, and we used two or more distinct siRNAs per target.

Gene Expression

Total RNA was isolated from primary myeloid cells using RNeasy Kit (Qiagen). cDNA was prepared from 500 ng RNA from each sample and qPCR was performed using primer sets for integrin α 4 (*Itga4*), CalDAG-GEFI (*Rasgrp2*), CalDAG-GEFII (*Rasgrp1*), Epac1 (*Rapgef3*), Epac2 (*Rapgef4*), PLC γ (*Plcg1*), RIAM (*Apbb1ip*) from Qiagen (QuantiTect Primer Assay) or GAPDH (*Gapdh*) sense primers 5'CATGTTCCAGTATGACTCCACTC3' and anti-sense primers 5'GGCCTCACCCCATTTGATGT'. Relative expression levels were normalized to *gapdh* expression according to the formula <2⁻(Ct *gene of interest* - Ct *gapdh*)>. Fold inhibition in expression levels were calculated by comparative Ct method <2⁻(ddCt)> (47).

Rap1 Activation Assay

Rap activity was measured in BM cells after incubation for 30 min at 37°C in serum free media in the presence or absence of 1 μ M TG100-115, followed by stimulation with basal medium or medium containing 200 ng/ml IL-1 β , IL-6, SDF-1 α (R&D Systems). GTP-Rap was purified using an RapGTPase pulldown assay kit (Thermo Scientific) from 1 mg cell lysate by addition of RalGDS-GST fusion proteins and glutathione-conjugated sepharose beads. GTP-Rap and total Rap were detected by immunoblotting with anti-Rap antibodies.

Bone Marrow Transplantation

Bone marrow derived cells were aseptically harvested from 6–8 week-old female mice by flushing leg bones of euthanized mice with phosphate buffered saline containing 0.5% BSA and 2 mM EDTA, incubating cells in red cell lysis buffer (155 mM NH₄Cl, 10 mM NaHCO₃ and 0.1 mM EDTA) and centrifuging over Histopaque 1083. Approximately 5 \times 10⁷ BMDC were purified by gradient centrifugation from the femurs and tibias of a single mouse. Two million cells were intravenously injected into tail veins of each lethally irradiated (1000 rad) 6-week old syngeneic recipient mouse. After 4 weeks of recovery, tumor cells were injected in C57BL/6 mice transplanted with BM from Rap1a^{-/-} or WT.

Tumor Studies

C57BL/6 LLC cells were obtained from the American Type Culture Collection (ATCC). Cells were cultured in antibiotic- and fungicide-free DMEM media containing 10% serum; cells tested negative for mycoplasma using the Mycoplasma Plus PCR primer set from Stratagene (La Jolla, CA). 5 \times 10⁵ LLC cells were injected subcutaneously into syngeneic 6- to 8-week old wildtype, Rap1a^{-/-} or BMT mice. Tumors dimensions were recorded at regular intervals. Tumors were excised at 21 days. Tumor weights were obtained, and tumors were cryopreserved in O.C.T., cryosectioned and immunostained for F4/80+ using BM8 (eBioscience) and for CD31 using MEC13.3 (BD Bioscience). Slides were counterstained with DAPI (Invitrogen). Tissues were analyzed using Metamorph (Version 6.3r5, Molecular Devices).

Quantification of Myeloid Cells in Tissues by Flow Cytometry

To quantify myeloid cells in tissues, tumors were excised, minced and digested to single cell suspensions for 1 h at 37°C in 5 ml of Hanks Balanced Salt Solution (HBSS, GIBCO) containing 1 mg/ml Collagenase type IV, 10 μ g/ml Hyaluronidase type V and 20 units/ml DNase type IV (Sigma). Red blood cells were solubilized with RBC Lysis Buffer (eBioscience) and then cells were incubated in FC-blocking reagent (BD Bioscience), followed by anti-CD11b-APC (M1/70), anti-Gr1-FITC (RB6-8C5) and anti F4/80 (BM8) (eBioscience). To exclude dead cells, 0.5 μ g/ml propidium iodide was added.

Supporting Information

Figure S1 Efficiency of integrin and Rap1a gene knock-down and inhibition in primary myeloid cells. (A) Integrin $\alpha 4$ expression (dark line) in myeloid cells from WT and Rap1a $^{-/-}$ bone marrow cells was quantified by flow cytometry. IgG control staining is shown in grey. (B) Validation of siRNA mediated knockdown of Rap1a in myeloid cells by Western blotting. Actin levels were used as loading control. Non-silencing control was set to 1. (C) Percent adhesion of WT chemoattractant-treated myeloid cells to VCAM-1 exposed to increasing concentrations of the geranylgeranyltransferase inhibitor (GGTI-2147). (D) Surface expression of $\alpha 4\beta 1$ in myeloid cells after transfection with integrin $\alpha 4$ (black) or non-silencing (gray) siRNA. Histogram was acquired by flow cytometry. (E) Relative integrin $\alpha 4$ mRNA levels in myeloid cells after siRNA-mediated gene knockdown. Error bars indicate S.E.M. (TIF)

Figure S2 Rap inhibitor blocks $\alpha 4\beta 1$ ligand binding. Mean fluorescence intensity (MFI) of VCAM-1/Fc bound to myeloid cells derived from WT -treated with medium or 10 μ M geranylgeranyltransferase inhibitor (GGTI-2147), in the absence (basal) or presence of IL-1 β and SDF-1 α (n = 3). *P<0.01 vs basal. (TIF)

Figure S3 RapGEFs in myeloid cell adhesion. (A) Relative mRNA expression levels of CalDAG-GEFI, CalDAG-GEFII, Epac1, and Epac2 in myeloid cells. (B) Left: Relative RapGEF

mRNA levels in myeloid cells after siRNA mediated knockdown. Non silencing control was set to 1. (C) Relative mRNA levels of PLC γ in myeloid cells after transfection with PLC γ or control siRNA. Non-silencing control was set to 1 (n = 3). (D) Percent adhesion of chemoattractant-treated WT myeloid cells to VCAM-1 in the presence of increasing concentrations of the PLC γ inhibitor U73122.

(TIF)

Figure S4 Myeloid cell integrin $\alpha 4\beta 1$ activation is PKC independent but RIAM dependent. (A) Adhesion of WT chemoattractant-treated myeloid cells to VCAM-1 in the presence of 1 μ M panPKC inhibitor Ro-32-0432 (n = 3), *P<0.01 vs basal. (B) Adhesion of WT chemoattractant-treated myeloid cells and WT myeloid cells ectopically expressing active p110 γ (p110 γ CAAX), active Rap (RapV12), or empty vector (control) in the absence (empty) or presence (filled) of 1 μ M PKC- α / β inhibitor (Go6976) (n = 3), *P<0.01 vs basal. (C) Relative mRNA levels of RIAM in myeloid cells after transfection with RIAM or control siRNA. Non-silencing control was set to 1 (n = 3).

(TIF)

Figure S5 Rap1a promotes myeloid cell trafficking during tumor inflammation, thereby supporting tumor growth. (A-B) Representative experiment showing (A) tumor volume and (B) weight of LLC tumors grown over 21 days in WT and Rap1a $^{-/-}$ mice (n = 10). (C) Percentage of Gr1+CD11b+ and (D) F4/80+ tumor-infiltrating myeloid cells in WT and Rap1a $^{-/-}$ tumors, *P<0.01 vs WT.

(TIF)

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Author Contributions

Reviewed and revised manuscript: MCS IF SWK EH LAQ JAV. Conceived and designed the experiments: MCS SWK JAV. Performed the experiments: MCS SWK. Analyzed the data: MCS IF SWK EH LAQ JAV. Contributed reagents/materials/analysis tools: MCS IF SWK EH LAQ JAV. Wrote the paper: MCS JAV.

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