Supporting Information

Giurisato et al. 10.1073/pnas.1707929115

SI Materials and Methods

Genotype Determination of Mouse Strains. Mice carrying the *erk5*^{F} allele, the *caMEK5*^{F}, the *CMV-Cre*^{ER} or *LysM-Cre* transgenes were identified by PCR on genomic DNA, as described (1–3). The colonies were maintained in a pathogen-free facility at the University of Manchester. All animal procedures were performed under license in accordance with the UK Home Office Animals (Scientific Procedures) Act (1986) and institutional guidelines. In particular, mice with tumors were closely monitored by careful clinical examination to allow detection of deterioration of their physical condition. Animals showing signs of distress were killed before any further deterioration in condition occurred.

Statistical Analysis. We used unpaired *t* tests to calculate two-tailed *P* value to estimate statistical significance of differences between two distinct genotypes or two treatment groups. To compare values between multiple test groups in luciferase reporter experiments, we performed a one-way ANOVA followed by Tukey's test. Data were analyzed by using Prism software (GraphPad). $P < 0.05$ was taken to be statistically significant. For all tissue experiments, images are representative of cohorts of at least three mice.

1. Wang X, et al. (2006) Activation of extracellular signal-regulated protein kinase 5 downregulates FasL upon osmotic stress. *Cell Death Differ* 13:2099–2108.
2. Wang W, et al. (2014) Genetic activation of ERK5 MAP kinase enhances adult neurogenesis and extends hippocampus-dependent long-term memory. *J Neurosci* 34: 2130–2147.
3. Clausen BE, Burkhardt C, Reith W, Renkawitz R, Förster I (1999) Conditional gene targeting in macrophages and granulocytes using LysMcre mice. *Transgenic Res* 8: 265–277.

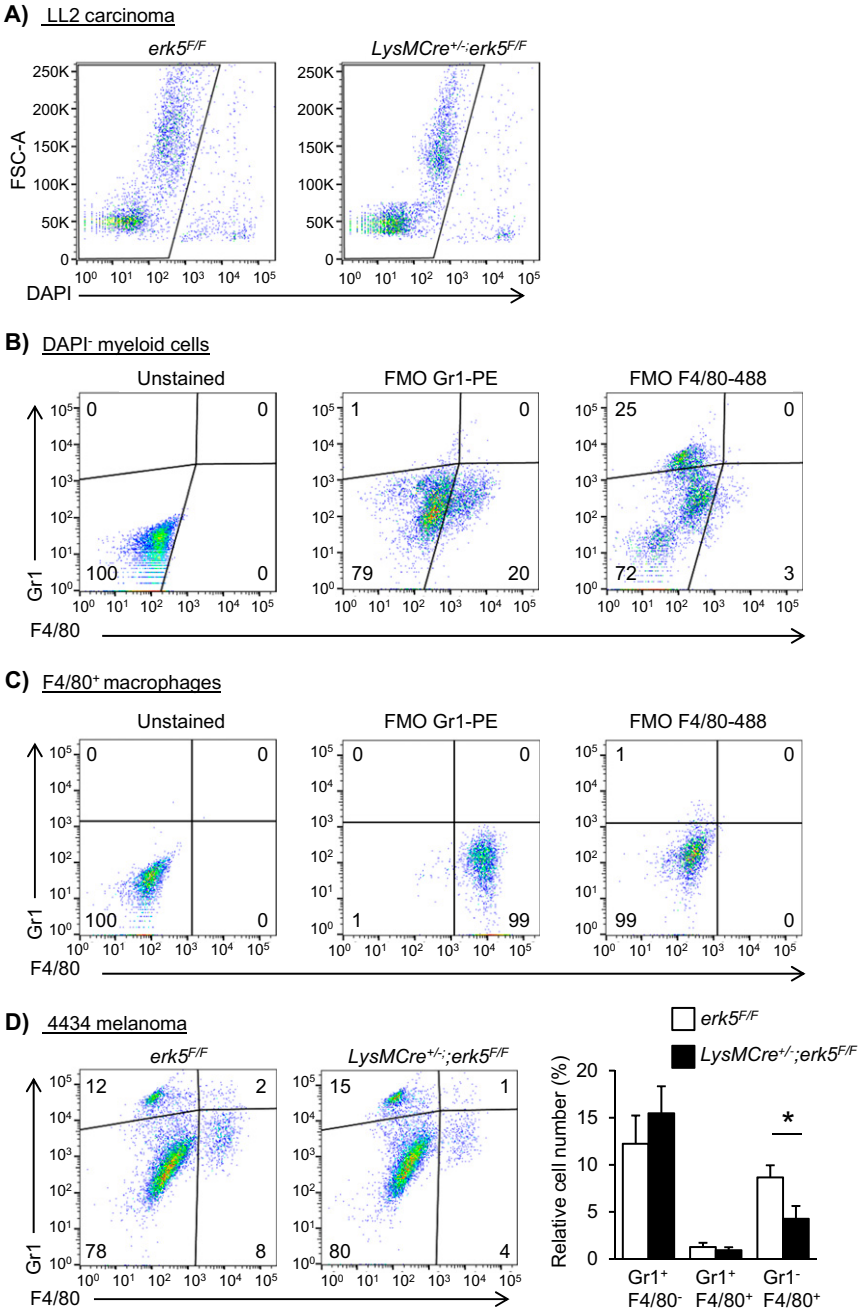

Fig. S1. Flow-cytometry analysis of myeloid cell populations in tumor grafts. (*A*) Representative gating strategy for flow-cytometry analysis of live (DAPI$^-$) cell populations from LL/2 tumor grafts excised from *erk5*$^{F/F}$ and *LysMCre*$^{+/-}$;*erk5*$^{F/F}$ mice. The same strategy was used to gate live (DAPI$^-$) cells from 4434 melanoma grafts. (*B*) Representative gating of unstained live (DAPI$^-$) myeloid cell populations in tumors excised from *erk5*$^{F/F}$ mice and fluorescence minus one (FMO) controls used for Gr1 and F4/80 staining. (*C*) Unstained and FMO controls used to interpret flow-cytometry analyses of tumor-derived macrophages after sorting with magnetic microbeads ultrapure conjugated to antibodies against F4/80. (*D*) Representative flow-cytometry analysis and quantification of live (DAPI$^-$) myeloid cell populations in melanoma 24 d after s.c. inoculation of 4434 cells in *erk5*$^{F/F}$ and *LysMCre*$^{+/-}$;*erk5*$^{F/F}$ mice. Graphical analysis shows a significant reduction of TAM fraction in tumor grafts excised from *LysMCre*$^{+/-}$;*erk5*$^{F/F}$ mice. The data correspond to the mean ± SD (n = 3 tumors). *$P < 0.05$.

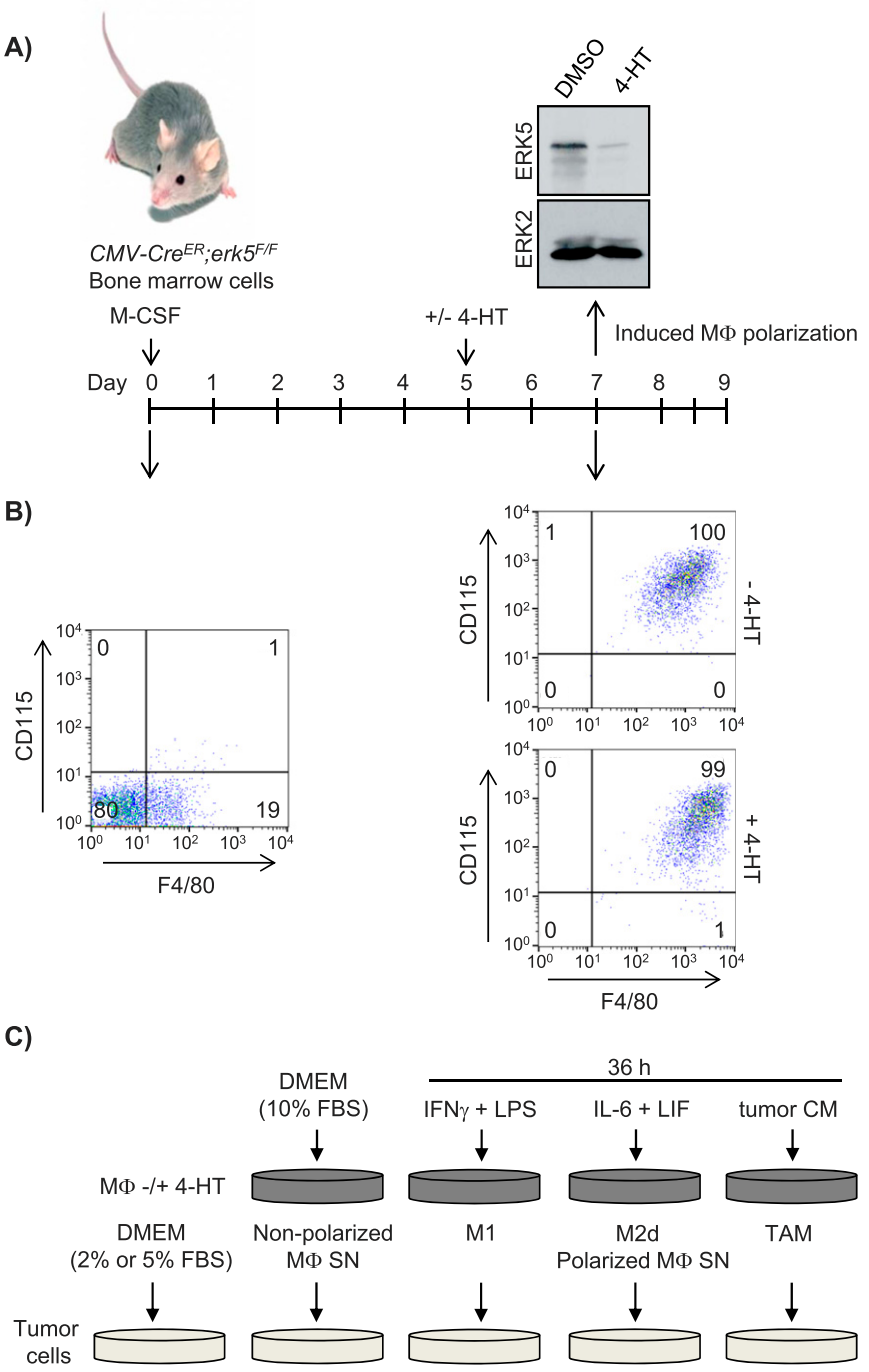

Fig. S2. In vitro model to study the role of ERK5 in protumor macrophage polarization. (*A*) Time schedule of cell treatment. Bone marrow cells were isolated from femurs of *erk5*$^{F/F}$ mice carrying an inducible form of the Cre recombinase, namely, Cre^{ER}, under an ubiquitous CMV promoter. The cells were cultured in DMEM containing FBS and CSF-1 to obtain differentiated macrophages, as described (1). In vitro Cre-mediated recombination of the *erk5*^{F} allele was induced by incubation with 4-HT (0.1 μM). Macrophages treated with DMSO were used as controls. Immunoblot analysis with an antibody to ERK5 demonstrates decreased ERK5 expression in *CMV-Cre*^{ER}*;erk5*$^{F/F}$ macrophages incubated with 4-HT for 48 h before being harvested at day 7. Tubulin expression was used as loading control. In subsequent experiments, *CMV-Cre*^{ER}*;erk5*$^{F/F}$ BMDMs mock-treated with DMSO or treated with 4-HT were referred to as *erk5*$^{F/F}$ and *erk5*$^{Δ/Δ}$ macrophages, respectively. (*B*) Flow-cytometry analyses at days 0 and 7 demonstrated similar level of F4/80 and CD115 staining, between mock and 4-HT–treated cells. Notably, *erk5*-deleted macrophages exhibited a slightly higher level of F4/80 and CD115 compared with WT cells, indicative of advanced differentiation. (*C*) Schematic representation of experimental design. Macrophage (MΦ) polarization was induced by incubation for 36 h in DMEM supplemented with 10% FBS together without or with IL-4 (25 ng/mL), IL-6 (50 ng/mL), and LIF (25 ng/mL), or IFNγ (50 ng/mL) and LPS (100 ng/mL), or by exposure to tumor-cell-conditioned medium (CM; 1/2 dilution). The conditioned medium was collected from subconfluent (80%) flasks of carcinoma (LL/2) or melanoma (4434) murine cell lines incubated for 24 h in DMEM supplemented with 10% FBS, centrifuged for 5 min at 200 × *g*, and filtered through a 0.45-μm filter, before being used fresh. After 36 h, polarized macrophages were either harvested or rinsed twice with PBS and cultured in DMEM supplemented with 5% FBS for a further 24 h to obtain nonpolarized and polarized macrophage supernatants (SN).

1. Young HL, et al. (2017) An adaptive signaling network in melanoma inflammatory niches confers tolerance to MAPK signaling inhibition. *J Exp Med* 214:1691–1710.

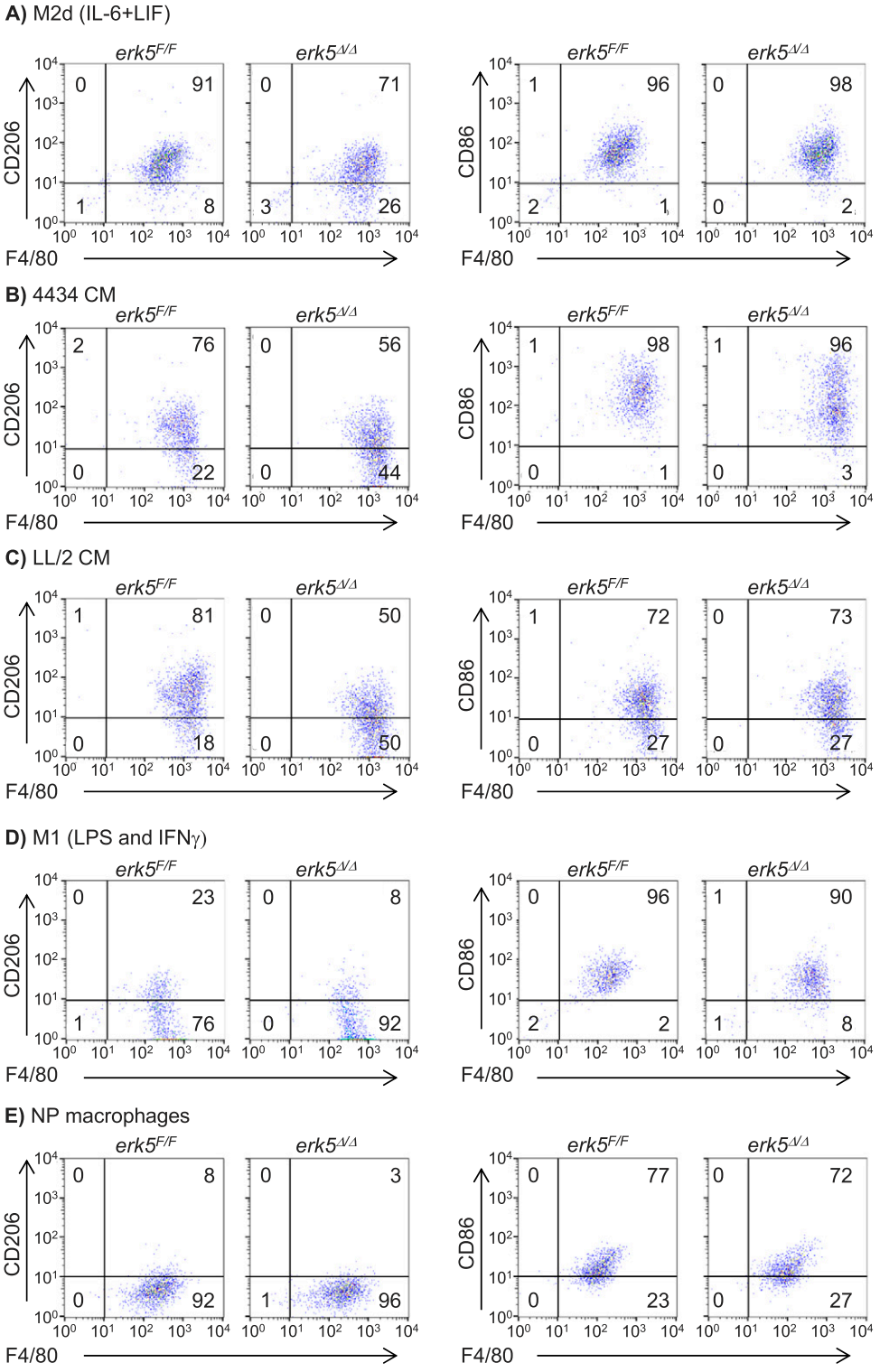

Fig. S3. Flow-cytometry analysis of polarized macrophages. (*A–D*) *erk5*$^{F/F}$ and *erk5*$^{\Delta/\Delta}$ macrophages were stimulated for 36 h with IL-6 and LIF (*A*), tumor-conditioned medium from 4434 cells (*B*) or LL/2 cells (*C*), or LPS and IFNγ (*D*) to generate M2d, TAM, and M1 phenotypes, respectively. (*E*) Nonpolarized (NP) cells were cultured in 10% FBS-containing medium. Flow-cytometry analyses confirmed that M2d macrophages and TAMs were positive for CD206 and CD86, whereas M1 and nonpolarized macrophages were positive for CD86, but exhibited a very low CD206 level.

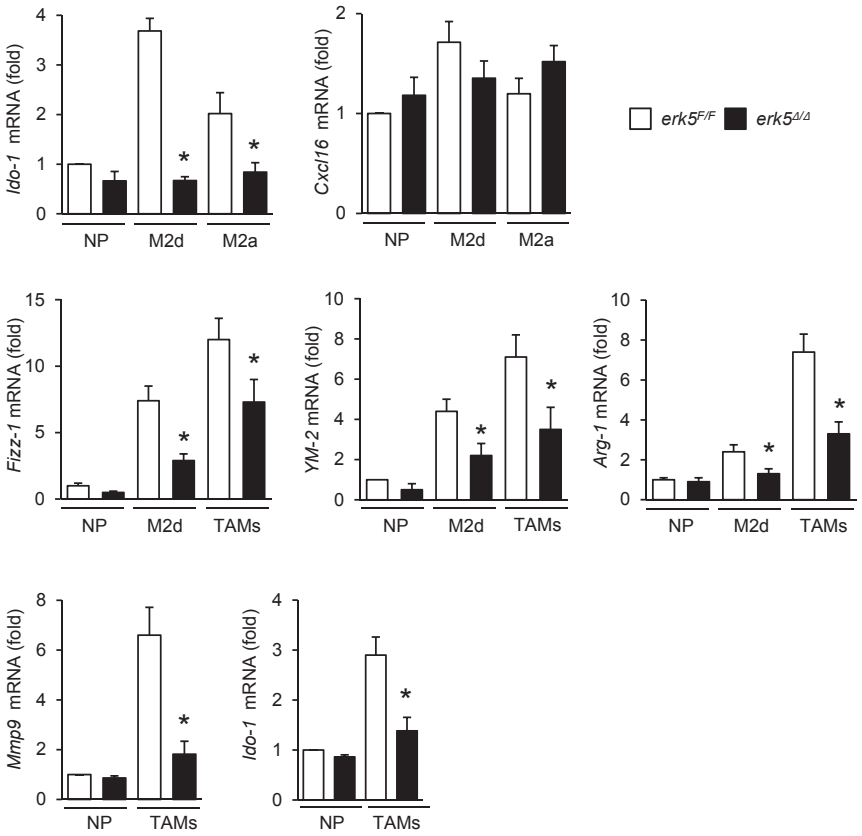

Fig. S4. ERK5 deficiency in macrophages impairs the production of protumoral factors. *erk5*$^{F/F}$ and *erk5*$^{\Delta/\Delta}$ macrophages were stimulated with IL-6 + LIF (M2d), IL-4 (M2a), or 4434 cell-conditioned medium (TAMs). Nonpolarized (NP) macrophages were used as controls. The expression of M2 markers was analyzed by qPCR. The data correspond to the mean ± SD of three independent experiments performed in triplicate. *$P < 0.05$ (compares polarized *erk5*$^{F/F}$ vs. *erk5*$^{\Delta/\Delta}$ macrophages).

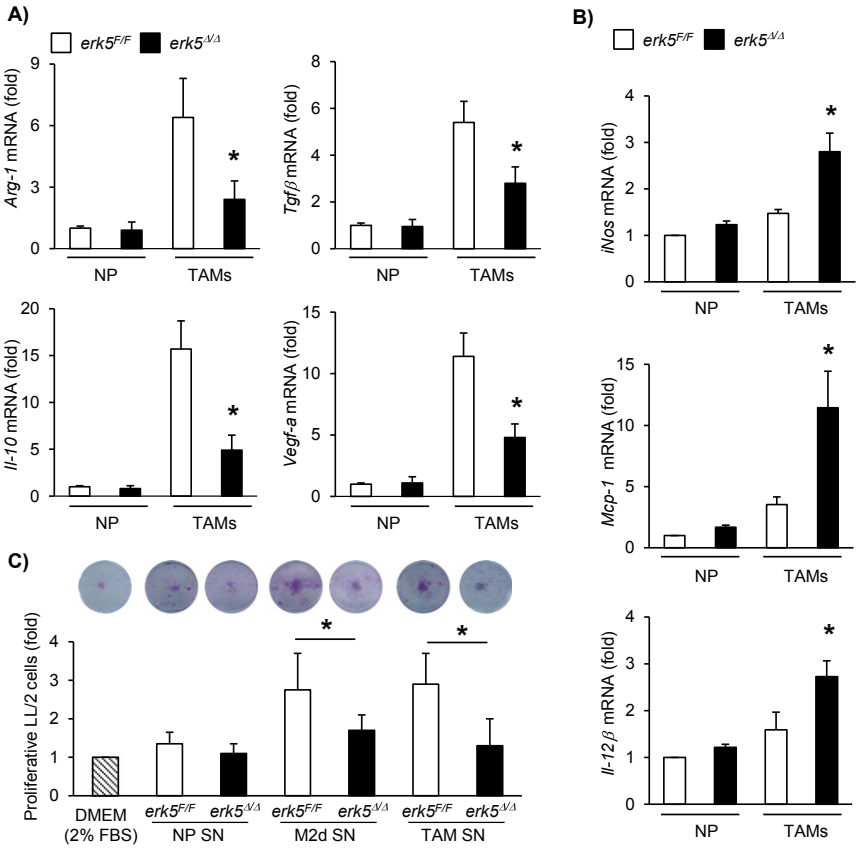

Fig. S5. ERK5 is required for macrophage-induced carcinoma cell proliferation. (*A* and *B*) Total RNA was extracted from *erk5*$^{F/F}$ and *erk5*$^{\Delta/\Delta}$ macrophages polarized by exposure to LL/2-conditioned medium (TAMs). Nonpolarized (NP) macrophages were used as controls. The amount of transcripts was measured by qPCR. The data correspond to the mean ± SD of three (*A*) or two (*B*) independent experiments performed in triplicate. *$P < 0.05$ (compares *erk5*$^{F/F}$ vs. *erk5*$^{\Delta/\Delta}$ TAMs). (*C*) Colony formation assay of carcinoma LL/2 cells incubated for 7 d with supernatants (SN) from *erk5*$^{F/F}$ and *erk5*$^{\Delta/\Delta}$ nonpolarized macrophages or macrophages activated with IL-6 + LIF (M2d) or LL/2-conditioned medium (TAMs). LL/2 cells growing in low-serum medium (2% FBS) were used as controls. The data correspond to the mean ± SD of three independent experiments performed in duplicate.

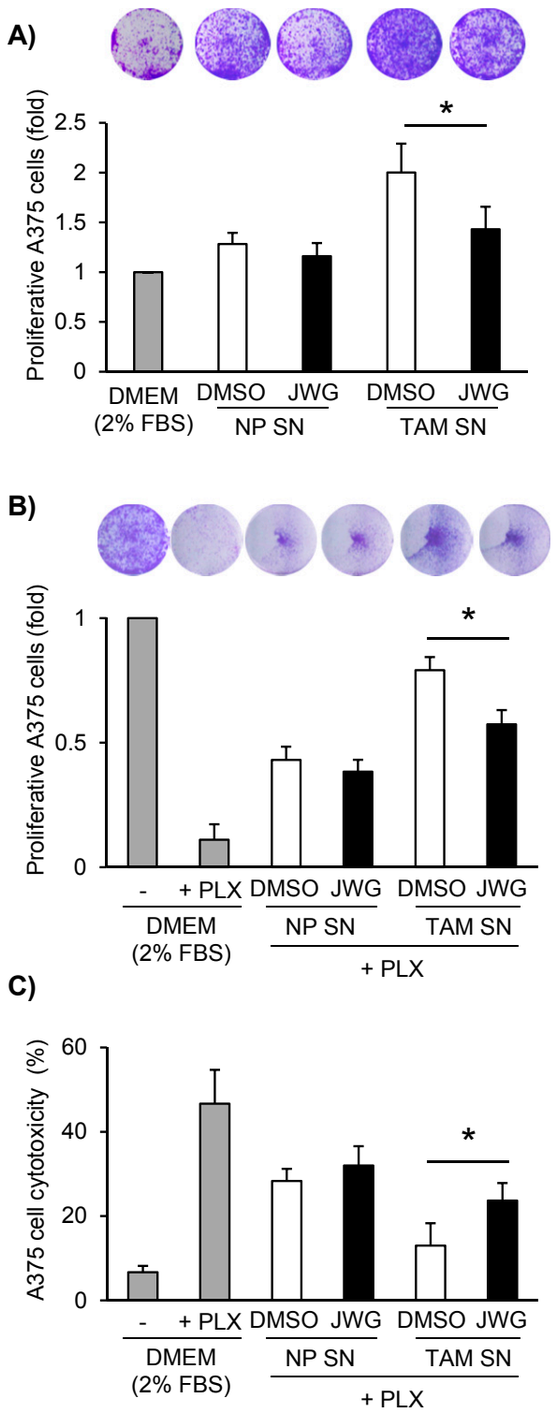

Fig. S6. ERK5 activity is required for human TAMs to support melanoma cell proliferation and to protect melanoma cells against PLX4032 toxicity. Human macrophages were obtained from in vitro differentiation of monocytes isolated from leukocyte cones provided from healthy blood donors, as described (1). Human blood was purchased through the National Health Service Blood and Transplant. According to the University of Manchester Research Ethics Committee, this approach did not raise any ethical issues, considering that the donated material is anonymized and is surplus to clinical requirement or unsuitable for therapeutic use. Where indicated, macrophages were mock-treated with DMSO or pretreated with 2.5 μM JWG-045 for 2 h, together with or without PLX (1 μM), before being incubated with RPMI supplemented with A375 melanoma-conditioned medium (1/4 dilution) for 24 h to induce TAM polarization. To produce human melanoma-conditioned medium, A375 cells were grown to 80% confluency in RPMI 1640 Glutamax medium supplemented with 10% FBS, after which the medium was exchanged. Two days later, the conditioned medium was collected, centrifuged, and filtered, before being used fresh. Polarized human macrophages were stimulated for a further 24 h with LPS (20 ng/mL) directly added to the RPMI-supplemented medium. Nonpolarized macrophages were incubated with nonsupplemented RPMI medium. Cells were thoroughly washed in PBS before incubating for 24 h in nonsupplemented RPMI medium to produce conditioned medium to be used with A375 cells in vitro. (*A* and *B*) Colony formation assay of human A375 melanoma cells incubated for 7 d with SN from NP human macrophages or from human TAM. A375 cells growing in low-serum medium (2% FBS) were used as controls. (*C*) A375 cell cytotoxicity was determined by using CellTox green and analyzed with Gen5. The data correspond to the mean ± SD of two independent experiments performed in triplicate. *$P < 0.05$ (compares tumor cell proliferation induced by SN from human TAMs mock-treated with DMSO or treated with JWG-045).

1. Young HL, et al. (2017) An adaptive signaling network in melanoma inflammatory niches confers tolerance to MAPK signaling inhibition. *J Exp Med* 214:1691–1710.

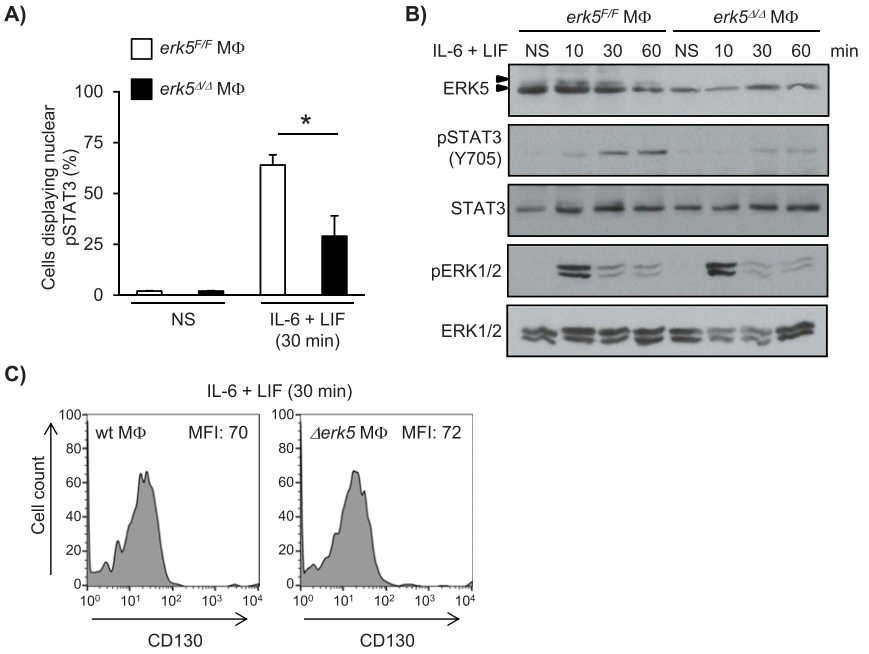

Fig. S7. ERK5 is required for mediating STAT3 phosphorylation at Tyr705 in macrophages stimulated by IL-6 and LIF. $erk5^{F/F}$ and $erk5^{\Delta/\Delta}$ macrophages were treated with IL-6 and LIF for the indicated times. Nonstimulated (NS) macrophages were used as controls. (*A*) The immunofluorescent signal of pSTAT3(Y705) from Fig. 6*B* was quantitated with ImageJ. The data correspond to the mean ± SD of three independent experiments. $*P < 0.01$ (compares $erk5^{F/F}$ vs. $erk5^{\Delta/\Delta}$ macrophages). (*B*) Protein lysates were analyzed by immunoblot with indicated antibodies. Similar results were obtained in two independent experiments. (*C*) Histogram plots comparing CD130 expression by mean fluorescence intensity (MFI) are shown.

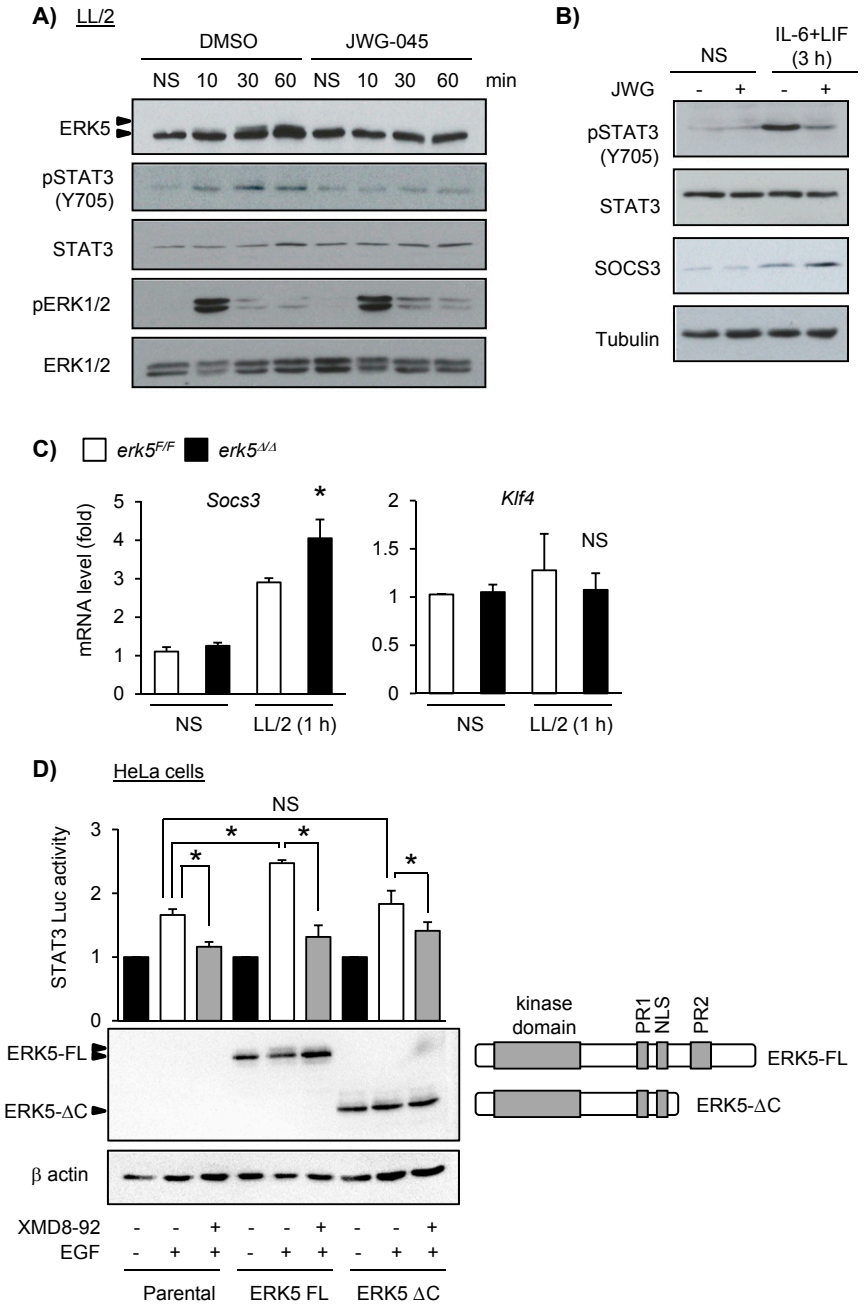

Fig. S8. ERK5 activity is required for STAT3 phosphorylation. Macrophages were starved overnight before being stimulated with LL/2-conditioned medium (LL/2) or with IL-6 and LIF for the indicated times. Where indicated, cells were mock-treated with DMSO or pretreated with JWG-045. Nonstimulated (NS) macrophages were used as controls. (*A* and *B*) Protein lysates were analyzed by immunoblot with indicated antibodies. The efficacy of JWG-045 to inhibit ERK5 is demonstrated by the loss of the migratory shift characteristic of ERK5 phosphorylation in macrophages exposed to LL/2-conditioned medium. Similar results were obtained in two independent experiments. (*C*) Total RNA was extracted, and the amount of transcripts was measured by qPCR. The data correspond to the mean ± SD of two independent experiments performed in triplicate. $*P < 0.05$ (compares stimulated *erk5*$^{F/F}$ vs. *erk5*$^{\Delta/\Delta}$ macrophages). (*D*) Flp-In HeLa cells carrying a tetracycline inducible Flag-tagged FL ERK5 or a Flag-tagged C-terminal truncated mutant of ERK5 (ERK5-ΔC) were transiently cotransfected with 1 μg of a luciferase reporter plasmid containing three STAT3 binding sites (3xGAS-Luc; ref. 1) and 50 ng of Renilla reporter (firefly), in the presence of tetracycline (2 μg/mL; Sigma) for 24 h. Parental cells were used to monitor the effect of endogenous ERK5. After 24 h, the medium was changed, and, where indicated, cells were pretreated with 2.5 μM XMD8-92 in the presence of tetracycline. The day after, cells were stimulated with EGF (20 ng/mL) for 6 h. STAT3-dependent transcriptional activity was measured by the Dual-Luciferase reporter assay system (E1910; Promega) using an Orion microplate luminometer. The data correspond to the mean ± SE of three independent experiments performed in duplicate. Expression level of ERK5-FL and ERK5-ΔC was analyzed by immunoblotting. β-actin was used as loading control.

1. Wellbrock C, et al. (2005) STAT5 contributes to interferon resistance of melanoma cells. *Curr Biol* 15:1629–1639.

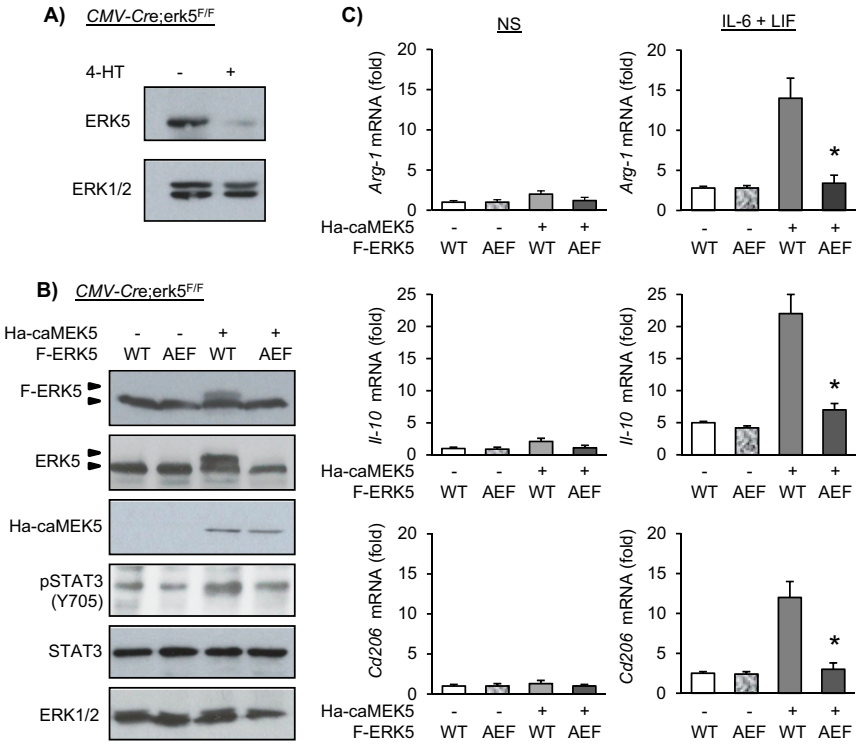

Fig. S9. ERK5 activation by MEK5 enhances expression of M2 markers. BMDMs from femurs of *CMV-Cre:erk5$^{F/F}$* mice were incubated with 4-HT (0.1 μM) for 5 d before being infected with a control adenovirus Ad-Ctrl (–) or with Ad-HA-caMEK5 together with Ad-F-ERK5-WT or Ad-F-ERK5-AEF at MOI 10 for 24 h. Recombinant adenoviruses were generated as described (1). Where indicated, cells were stimulated with IL-6 (50 ng/mL) and LIF (25 ng/mL) or exposed to LL/2-conditioned medium for 24 h. Nonstimulated cells were used as control. (*A*) Immunoblot analysis demonstrates decreased ERK5 expression in *CMV-Cre^{ER};erk5$^{F/F}$* macrophages incubated with 4-HT. (*B*) Immunoblot analysis of protein lysates showed that HA-caMEK5 induced hyperphosphorylation of F-ERK5-WT. This correlated with enhanced phosphorylation of STAT3 at Tyr705. As expected, F-ERK5-AEF did not display the mobility retardation shift by SDS/PAGE, characteristic of ERK5 hyperphosphorylation by caMEK5. An M2 antibody was utilized to confirm similar ectopic expression of F-ERK5-WT and F-ERK5-AEF. Similar results were obtained in two independent experiments. (*C*) Twenty-four hours after infection, cells were stimulated with IL-6 and LIF for 24 h. Nonstimulated (NS) cells were used as controls. Total RNA was purified, and the amount of transcripts was measured by qPCR. The data correspond to the mean ± SD of two independent experiments performed in triplicate. *$P < 0.05$ (compares cells expressing F-ERK5-WT vs. F-ERK5-AEF).

1. Wilkinson EL, Sidaway JE, Cross MJ (2018) Statin regulated ERK5 stimulates tight junction formation and reduces permeability in human cardiac endothelial cells. *J Cell Physiol* 233: 186–200.

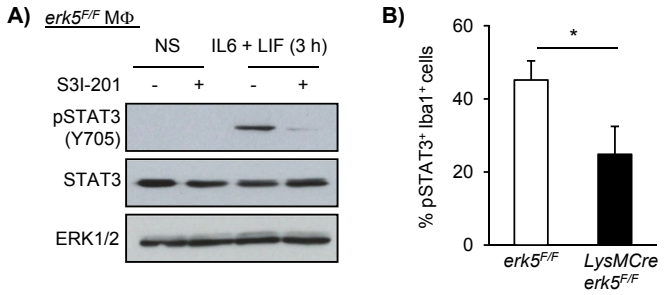

Fig. S10. Analysis of STAT3 in TAMs. (*A*) Macrophages were preincubated with S3I-201 for 2 h before being stimulated with IL-6 and LIF for 3 h. Nonstimulated (NS) macrophages were used as controls. Protein lysates were analyzed by immunoblot with the indicated antibodies. Similar results were obtained in two independent experiments. (*B*) The immunofluorescence signals corresponding to Iba1 or pSTAT3(Y705) from Fig. 7*B* were quantitated with ImageJ. The data expressed as a percentage of Iba1 positive cells expressing pSTAT3(Y705) correspond to the mean ± SD of three independent experiments. *$P < 0.01$ (compares tumors isolated from *erk5$^{F/F}$* and *LysMCre$^{+/-}$;erk5$^{F/F}$* mice).

Table S1. Sequences of primers used for quantitative real-time PCR

Gene	Primer sequences
ARG1	Forward 5′ CAGAAGAATGGAAGAGTCAG
	Reverse 5′ CAGATATGCAGGGAGTCACC
TGFβ	Forward 5′ TGACGTCACTGGAGTTGTACGG
	Reverse 5′ GGTTCATGTCATGGATGGTGC
iNOS	Forward 5′ TGA GGC TCC TCA CGC TTG GGT
	Reverse 5′ ACT TCC AGG GGC AAG CCA TGT
VEGF	Forward 5′ GGAGATCCTTCGAGGAGCACTT
	Reverse 5′ GGCGATTTAGCAGCAGATATAAGAA
IL-12β	Forward 5′ GGAAGCACGGCAGCAGAATA
	Reverse 5′ AACTTGAGGGAGAAGTAGGAATGG
MCP-1	Forward 5′ CAGCTCTCTCTTCCTCCACC
	Reverse 5′ GTGAGTGGGGCGTTAACTG
IL-10	Forward 5′ ATT TGA ATT CCC TGG GTG AGA AG
	Reverse 5′ CAC AGG GGA GAA ATC GAT GAC A
CD206	Forward 5′ TTGGACGGATAGATGGAGGG
	Reverse 5′ CCAGGCAGTTGAGGAGGTTC
SOCS3	Forward 5′ GCTCCAAAAGCGAGTACCAGC
	Reverse 5′ AGTAGAATCCGCTCTCCTGCAG
Fra-1	Forward 5′ CCA GGG CAT GTA CCG AGA CTA
	Reverse 5′ GAT GCT TGG CAC AAG GTG GA
FIZZ1	Forward 5′ CCAGCTAACTATCCCTCCACT
	Reverse 5′ ACACCCAGTAGCAGTCATCC
KLF4	Forward 5′ GTGCCCCGACTAACCGTTG
	Reverse 5′ GTCGTTGAACTCCTCGGTC
YM-2	Forward 5′ CAGAACCGTCAGACATTCATTA
	Reverse 5′ ATGGTCCTTCCAGTAGGTAATA
MMP-9	Forward 5′ CTGGACAGCCAGACACTAAAG
	Reverse 5′ CTCGCGGCAAGTCTTCAGAG
CXCL10	Forward 5′ GACGGTCCGCTGCAACTG
	Reverse 5′ CTTCCCTATGGCCCTCATTCT
CCL5	Forward 5′ AGATCTCTGCAGCTGCCCTCA
	Reverse 5′ GGAGCACTTGCTGCTGGTGTAG
CXCL16	Forward 5′ GCT TTG GAC CCT TGT CTC TTG C
	Reverse 5′ GTG CTG AGT GCT CTG ACT ATG TGC
IDO-1	Forward 5′ CACTGAGCACGGACGGACTGAGA
	Reverse 5′ TCCAATGCTTTCAGGTCTTGACGC
GAPDH	Forward 5′ AACGACCCCTTCATTGAC
	Reverse 5′ TCCACGACATACTCAGCAC
PGK1	Forward 5′ GAAGATTACCTTGCCTGTTGAC
	Reverse 5′ GCTCTCAGTACCACAGTCCA