Supporting Information

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SI Materials and Methods

Cell Preparation. Mice were anesthetized with isoflurane, and peripheral blood was collected via terminal cardiac puncture with heparin as an anticoagulant. In adoptive transfer experiments, mice were perfused with 30 mL PBS following blood collection. The spleen and tumor were weighed before being cut into small fragments and digested for 1 h at 37 °C with 0.2% collagenase D (Roche) for the spleen or with a mix of 300 U/mL crude collagenase (Sigma-Aldrich), 1 mg/mL Dispase II (Roche), and 2,000 U/mL DNase I (Calbiochem) for the tumor tissue. In some experiments, spleen cells were prepared by mechanical dissociation only (gentle forcing through a nylon mesh using the rubber tip of a syringe plunger). When present, necrotic tumor tissue (identified as darker tissue with a loose gel-like consistency located at the core of the tumor) was separated from solid tumor tissue by aspiration and gentle scraping. After digestion, tumor tissue was underlaid with 40% (vol/vol) Percoll and subjected to further separation by density centrifugation. Bone marrow (BM) cells were extracted from both femurs (separately for femur photoconversion mice) by flash centrifugation or flushing with a needle and syringe. The resulting blood, spleen, tumor, and BM preparations were triturated by pipette, red blood cells were removed using ammonium-chloride-potassium lysing buffer [150 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM $Na_2(EDTA)$], and single-cell suspensions were obtained by passing each sample through a 70-μm cell strainer. Cells then were stored on ice before same-day analysis by flow cytometry.

Flow Cytometry. Flow-Count fluorospheres (Beckman Coulter) were used to determine cell numbers and normalize cell concentrations before antibody staining. Cells were resuspended in FACS buffer [PBS with 2% (vol/vol) FBS and 0.05% NaN_3], incubated with anti-mouse CD16/CD32 monoclonal antibody (clone 93; BioLegend) for 5 min at room temperature to block nonspecific staining of Fc-receptors, and then stained with combinations of fluorophore- or biotin-conjugated anti-mouse monoclonal antibodies for 30 min at 4 °C, as indicated in Table S2. Biotin-conjugated antibody staining and streptavidin staining were performed sequentially. Stained cells were washed twice and passed through a 70-μm cell strainer before analysis.

Data were acquired on a Gallios flow cytometer (Beckman Coulter) and analyzed using FlowJo software (version 9.6.4; Tree-Star). Depending on the experiment, 100,000–2 million events were acquired. For Kikume Green-Red (KikGR) experiments, the standard Gallios filter configuration was adjusted to 620 dichroic short pass (DCSP)/590 bandpass (BP) for FL2 (KikGR-Red, KikRed), 730 DCSP/675 BP for FL3 (7-aminoactinomycin, 7-AAD), and no DCSP/755 BP for FL4 (phycoerythrin-cyanine 7, PE-Cy7). Monocytes, hematopoietic stem cells (HSCs), and granulocyte-macrophage progenitor (GMP) cells were identified as depicted in Fig. S2. Doublets were excluded using a forward-scatter height (FSC-H) vs. forward-scatter width (FSC-W) gate. Nonviable cells were excluded based on FSC and side-scatter (SSC) profiles and 7-AAD staining (KikGR experiments) or propidium iodide staining [except in experiments using KikGR and fluorescent ubiquitination-based cell cycle indicator (Fucci) mice]. Flow cytometry data were visualized as 5% probability contour plots on linear scales (FSC and SSC channels) or log scales (fluorescence channels), with appropriate compensation and biexponential settings applied. Percentages shown within gates indicate the proportion of the parent population that is contained by the gate. Cell numbers were calculated as the total cell number for a tissue (as determined by Flow-Count fluorospheres) multiplied by the proportion of total cells occupied by the population of interest. KikRed gate positions were determined by calibration against unconverted control samples for each tissue, with a false-positive tolerance of 0.02–0.1%.

Adoptive Transfer. A pictorial representation of the adoptive transfer procedure is shown in Fig. 2*D*. Cells isolated from the BM or spleens of separate day-14 tumor-bearing $CD45.1^+$ $CD45.2^+$ or $CD45.2^+$ donors, respectively, were stained with biotin-conjugated monoclonal antibodies against CD3 (clone 145–2C11; BD Biosciences), CD11c (clone HL3; BD Biosciences), B220 (clone RA3-6B2; BD Biosciences), CD71 (clone RI7217; BioLegend), c-kit (clone 2B8; eBioscience), NK1.1 (clone PK136; BD Biosciences), Ly6G (clone 1A8; BioLegend), and Ter-119 (clone TER-119; BD Biosciences) and then were enriched by lineage depletion using an AutoMACS machine and Streptavidin MicroBeads (Miltenyi Biotec), according to the manufacturer's instructions. Enriched BM-derived and spleen-derived monocytes were mixed 1:1, labeled with carboxyfluorescein succinimidyl ester (5 μM, 37 °C, 5 min), then transferred i.v. into day-14 tumor-bearing $CD45.1^+$ recipients (5×10^6 cells per mouse). Recipient mouse blood and tumors were collected 4 h after transfer for analysis by flow cytometry.

In Vitro Migration Assay. Transwell migration assays were performed using the ChemoTx system (Neuro Probe) according to the manufacturer's instructions. Briefly, suspensions of 1.25×10^6 spleen cells in 25 μL RPMI medium 1640 with 0.5% BSA and 20 mM Hepes were applied to each well of a polycarbonate membrane plate (5-μm pore size). The membrane plate then was placed on a 96-well microplate containing 29 μL of culture medium per well with or without the chemokine CCL2 (Peprotech). After 2-h incubation at 37 °C, the number of cells in the lower chambers was measured by flow cytometer using Flow-Count fluorospheres and expressed as a proportion of the initially applied cell number.

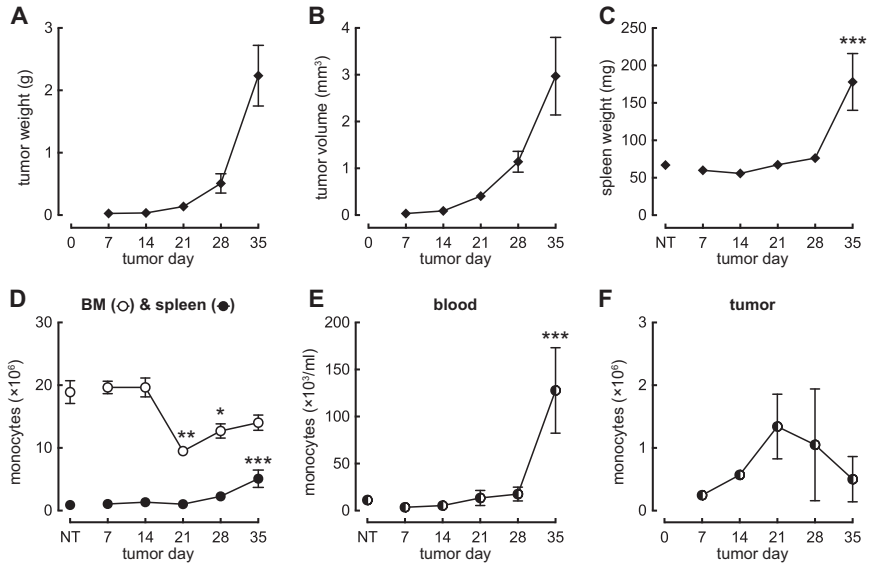

Fig. S1. Spleen monocyte cellularity increases only in late-stage tumor development. Wild-type mice were inoculated s.c. in the right flank with 5×10^5 Lewis lung carcinoma (3LL) tumor cells. Tissues were collected every 7 d for weighing and analysis by flow cytometry. (*A*) Tumor weight. (*B*) Tumor volume. (*C*) Spleen weight. (*D–F*) Number of Ly6C^{hi} monocytes in the BM (*D*), spleen (*D*), blood (*E*), and tumor (*F*) throughout tumor development (see Fig. S2*A* for flow cytometry gating). BM cell numbers represent whole-body totals. NT, non–tumor-bearing (healthy) mice. *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$ (compared with NT control). Data represent means ± SEM ($n = 4$ in *A* and *C–F*; $n = 6$–20 in *B*) and are representative of two independent experiments.

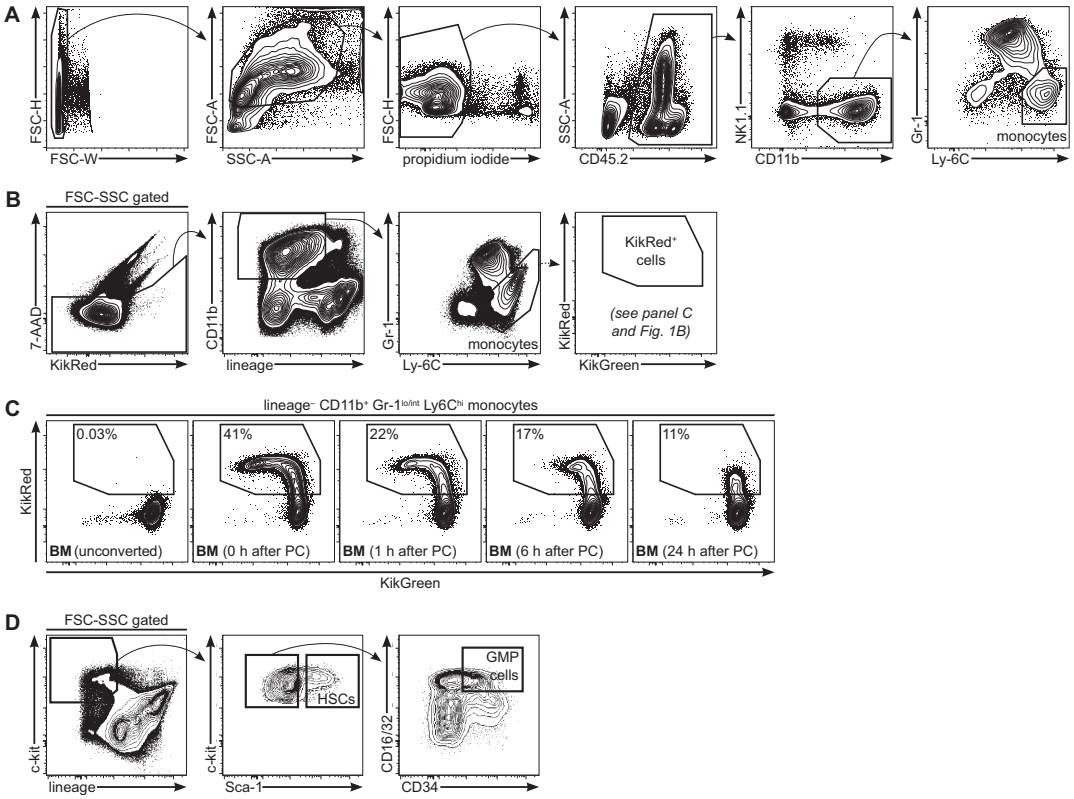

Fig. S2. Identification of myeloid cells by flow cytometry. (*A*) Gating scheme used to identify CD45$^+$ NK1.1$^-$ CD11b$^+$ Gr-1$^{lo/int}$ Ly6C^{hi} monocytes in tissue preparations from wild-type mice. Although the Gr-1 antibody reacts with both Ly6G and Ly6C molecules, it binds with higher affinity to Ly6G and thus effectively differentiates Gr-1$^{lo/int}$ (Ly6G^{lo} Ly6C^{hi}) monocytes from Gr-1^{hi} (Ly6G^{hi} Ly6C^{int}) neutrophils. Doublets were excluded using a FSC-H vs. FSC-W gate; nonviable cells were excluded by propidium iodide staining. (*B*) Gating scheme used to identify lineage-negative CD11b$^+$ Gr-1$^{lo/int}$ Ly6C^{hi} monocytes in cell preparations from KikGR mice. Nonviable cells were excluded by 7-AAD staining to avoid interference with the detection of the KikRed and KikGreen proteins. Lineage markers were CD11c, B220, NK1.1, and TCRβ. (*C*) Representative flow cytometry plots showing the egress of photoconverted (KikRed$^+$) Ly6C^{hi} monocytes from the femurs of day-35 3LL tumor-bearing mice. PC, photoconversion. (*D*) Gating scheme used to identify lineage-negative c-kit$^+$ Sca-1$^+$ HSCs and lineage-negative c-kit$^+$ Sca-1$^-$ CD16/32$^+$ CD34$^+$ GMP cells in cell preparations from Fucci mice. Lineage markers were CD3, CD11b, B220, NK1.1, Gr-1, and Ter-119. Data are representative of at least two independent experiments.

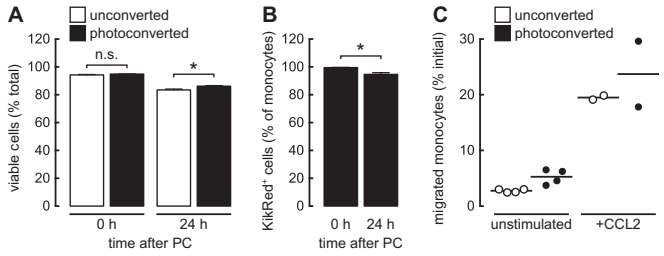

Fig. S3. KikGR photoconversion does not affect cell viability or migration potential, and KikRed protein levels remain stable over 24 h in monocytes. (*A* and *B*) BM cells from unconverted KikGR mice were photoconverted in vitro and analyzed by flow cytometry immediately (0 h) or after culture for 24 h at 37 °C. (*A*) Cell viability was assessed by measuring the proportion of cells staining negative for 7-AAD. (*B*) The stability of KikRed protein levels in Ly6C^{hi} monocytes was assessed by measuring the proportion of KikRed^{+} cells over time. (*C*) Spleen cells from unconverted wild-type mice or photoconverted KikGR mice were applied to the upper chamber of a Transwell migration assay in the presence or absence of CCL2 stimulation. Data represent the proportion of initially applied monocytes detected in the lower chamber after 2 h incubation at 37 °C. PC, photoconversion; n.s., not significant. *$P \leq 0.05$. Data represent means ± SEM ($n = 3$ in *A* and *B* or technical replicates in *C*) and are representative of two independent experiments.

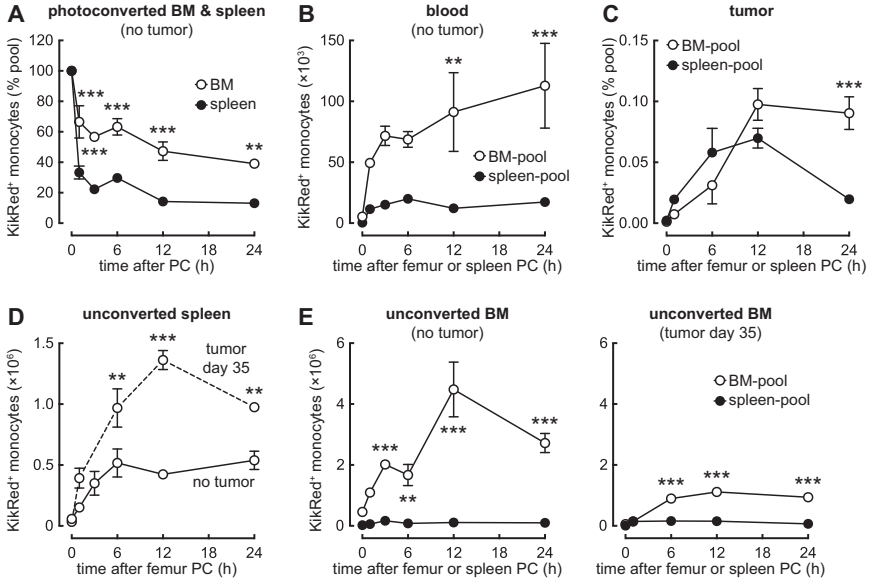

Fig. S4. Time-course graphs showing the redistribution of photoconverted Ly6C^{hi} monocytes in non–tumor-bearing and tumor-bearing mice. (*A*) Egress of KikRed^{+} Ly6C^{hi} monocytes from the BM and spleen following photoconversion in non–tumor-bearing mice. Data are expressed as a proportion of the initial pool of photoconverted cells in each tissue. (*B–E*) Accumulation of KikRed^{+} BM-pool and spleen-pool Ly6C^{hi} monocytes in the blood of non–tumor-bearing mice (*B*), in the tumor (expressed relative to availability in respective source pools) (*C*), in the unconverted spleen in non–tumor-bearing mice and in day-35 tumor-bearing mice (*D*), and in the unconverted BM of non–tumor-bearing and day-35 tumor-bearing mice (*E*). Data from the 24-h time point in *C* are reproduced in Fig. 2*A*; data from the 12-h time points in *D* and *E* are reproduced in Fig. 4. PC, photoconversion. **$P \leq 0.01$; ***$P \leq 0.001$ (BM-pool vs. spleen-pool). Data represent means ± SEM ($n = 3$) and are representative of two independent experiments.

Table S1. Distribution of monocytes in KikGR BM chimeras

	Total monocytes* (×10^3)	
	No tumor	Tumor day 35
Blood†	188 ± 16	183 ± 21
Bone marrow†	27,300 ± 2,700	26,300 ± 2,000
Spleen	1,890 ± 200	4,220 ± 550
Tumor	—	165 ± 57

*Mean cell number data ± SEM ($n = 6$) from nonphotoconverted or 0-h group mice (killed before photoconversion).
†Blood and BM data represent whole-body values.

Table S2. Anti-mouse monoclonal antibodies used for flow cytometry

Marker/ antigen	Clone (manufacturer*)	Fluorophore† or biotin conjugate				
		Wild-type mouse experiments	KikGR experiments	Adoptive transfer experiments	Fucci experiments—monocytes	Fucci experiments—HSCs and GMP cells
B220	RA3-6B2 (BD)		Biotin		PerCP-Cy5.5	PerCP-Cy5.5
CD3	145–2C11 (BD)					PerCP-Cy5.5
CD11b	M1/70 (BD)	PE-Cy7	PacificBlue	PE-Cy7	PE-Cy7	PerCP-Cy5.5
CD11c	HL3 (BD)		Biotin			
CD16/CD32	93 (BD)					PE-Cy7
CD34	RAM34 (BD)					Biotin
CD45.1	A20 (BL)			PacificBlue		
CD45.2	104 (BL/eBio)	PacificBlue		APC-eFluor780		
c-kit	2B8 (eBio)					APC-A750
Ly6A/E (Sca-1)	E13-161.7 (BL)					PacificBlue
Ly6C	HK1.4 (BL)	PerCP-Cy5.5	A647	A647	A647	
Ly6G/C (Gr-1)	RB6-8C5 (BD/BL)	PE	APC-Cy7	PerCP-Cy5.5	APC-Cy7	PerCP-Cy5.5
NK1.1	PK136 (BD)	APC	Biotin		PerCP-Cy5.5	PerCP-Cy5.5
Streptavidin	(BL)		PE-Cy7			APC
TCRβ	H57-597 (eBio)		Biotin			
Ter-119	TER-119 (BD)					PerCP-Cy5.5

*Manufacturers: BD, BD Biosciences; BL, BioLegend; eBio, eBioscience.
†Fluorophores: A647, Alexa Fluor 647; APC, allophycocyanin; Cy, cyanin; FITC, fluorescein isothiocyanate; PerCP, peridinin chlorophyll; PE, phycoerythrin.