Fluorochrome-conjugated antibodies used for flow cytometry

For cell-surface markers, the following monoclonal antibodies from eBioscience (San Diego, CA) were used: CD11b fluorescein isothiocyanate (FITC) (clone M1/70), CD3 FITC (clone 17A2), CD3 phycoerythrin cyanin 5 (PECy5) (clone 17A2), CD103 Brilliant violet 421 (clone 2E7), CD4 Pacific blue (clone RM4-4), CD4 allophycocyanin (APC)/Cy7 (clone RM4-4), CD8 PECy7 (clone 53-5.8), CD11c APC (clone N418), CD95 APC (clone SA367H8), CD19 PECy7 (clone N418), B220 APC (clone RA3-6B2), CD45 PerCp/Cy5.5 (clone 30-F11), CD45 APC/Cy7 (clone 30-F11), CD44 APC/Cy7 (clone IM7), CD279 APC/Cy7 (clone 29F.1A12), NK1.1 APC/Cy7 (clone PK136), F4/80 Pacific blue (clone BM8), CD69 APC (clone H1.2F3), CD107b Alexa Fluor 647 (clone M3/84), CD62L Alexa Fluor 647 (clone MEL-14), Ly6G APC (clone 1A8), Ly6C Alexa Fluor 647 (clone HK1.4), CD25 PerCp/Cy5.5 (clone 3C7), and CD178 APC (clone MFL3).

For intracellular markers, monoclonal antibodies from Biolegend, San Diego, CA, USA) were used as follows: Foxp Alexa Fluor 647 (clone 150D), IL-4 APC (clone 11B11), Ki67 Pacific blue (clone 16A8). Additionally we used the following antibodies: IFNγ Alexa Fluor 700 (clone XMG1.2, from BD Bioscience, San Diego, CA, USA); IL-18 (D048-3, R&D System); Gost Dye violet 450, Gost Dye violet 510 Gost Dye violet 780 (from Tonbo Biosciences, San Diego, CA, USA).

Figure legends

Figure S1. CXCR4 knockout in myeloid cells of C57Bl6 mouse model.

A, to examine the CXCR4 expression in myeloid cells, 1 ml of 4% thioglycolate was peritoneally injected into $CXCR4^{\Delta/\Delta}$ mice and $CXCR4^{WT}$ control mice.. The next day (12 hours later), peritoneal cells were collected and subsequent GFP-labeled myeloid cells were sorted by flow cytometry. RNA was extracted from the GFP-sorted cells and the CXCR4 mRNA level was determined by RT-PCR. CXCR4 expression was greatly reduced in the myeloid cells from $CXCR4^{\Delta/\Delta}$ mice compared to that of the $CXCR4^{WT}$ mice. Actin mRNA was used as a loading control. B, quantitation of the CXCR4 mRNA. C, to examine the protein expression of CXCR4 on the myeloid

surface, peripheral monocytes were prepared from CXCR4$^{\Delta/\Delta}$ and CXCR4^{WT} mice. Cells were stained with anti-CXCR4 antibody (MAB21651-sp, R&D System) at 1:1000 dilution for 2h and subsequently incubated with 2nd AlexaFluor 647-conjugated antibody (312-605-003, Jackson ImmunoResearch Lab, Inc) at a 1:10,000 dilution for 30 min. The CXCR4 expression on GFP- myeloid cells was analyzed by flow cytometry. The number above the outlined area indicates percent myeloid CXCR4^{+} cells. D, PyMT breast cancer cells (1×10^{6}) were intravenously injected into CXCR4$^{\Delta/\Delta}$ (n=6) or CXCR4^{WT} littermate control mice (n=6). Two weeks after injection, the lung weight was determined and normalized to tumor-free lung. Wilcoxon rank-sum test. E, representative photo of lung PyMT tumor from CXCR4$^{\Delta/\Delta}$ or CXCR4^{WT} or tumor-free mouse. F, Cell cycle DNA analysis. neutrophils from bone marrow (a) and peripheral blood (b) of CXCR4$^{\Delta/\Delta}$ and CXCR4^{WT} littermate control mice (n=6) were stained with CD11b-FITC and Ly6G-Apc/cy7, fixed with 70% ethanol overnight, and stained with propodium iodide (PI) solution for 30 min. Cells were subjected to cell cycle analysis by FACS. G, CXCL12 induction of neutrophil proliferation and apoptosis. Bone marrow cells were isolated from C57BL6 mice and cultured in the medium containing increasing concentrations of CXCL12 (0, 6.2, 12.5, 25, 50 and 100 ng/ml). The nuclear protein Ki67 for proliferation and cell surface Annexin V for early apoptosis were analyzed on CD11b+Ly6G+ neutrophils on day 1, day 2 (H). I, CD3 distribution in tumor microenvironment. Immunohistochemistry staining for CD3 cells in inducible melanomas of recipient mice received bone marrow transplantation from CXCR4$^{\Delta/\Delta}$ and CXCR4^{WT} littermate mice. The density of CD3 cells in the tissues was quantified using Image J software. J, representative photos of CD3 staining tumor tissues.

Figure S2. Cell cycle analysis and Fas expression

A, for NK cell depletion**,** peripheral NK cells were examined two days after CXCR4$^{\Delta/\Delta}$ mice were intravenously injected with 300 μg/mouse polyclonal Asialo-GM1 Ab or normal rabbit serum with an equivalent amount of IgG as sham control. Over 93% of NK cells were depleted in the mice

treated with Asialo-GM1 Ab compared with that of mice treated with the same amount of IgG from normal rabbit serum control (10 mice/group). B, CD8 depletion**,** peripheral CD8+ cells were examined two days after $CXCR4^{\Delta/\Delta}$ mice were intraperitoneally injected with 200 µg/mouse anti-CD8a mAb (Cat# BE0117, BioXcell) or isotype IgG2α(Cat#BE0089, BioXcell). Over 98% $CD8^+$ T cells were depleted in the mice treated with CD8 mAb compared with that of mice treated with the same amount of IgG2α mAb as control (6 mice/group). C, antitumor immunity in $CXCR4^{Mye\Delta/\Delta}$ mice was independent $CD8^+$ T cells. After $CXCR4^{Mye\Delta/\Delta}$ mice were treated with CD8 mAb, there was an over 98% depletion of $CD8^+$ T cells as compared to those treated with IgG control antibodies. These $CD8^+$T cell depleted or IgG control treated mice were intravenously implanted with 1×10^5 B16F0 melanoma cells. Two weeks after tumor cell implantation, the tumor weight in the lung was evaluated by subtracting the weight of the lung from a non-tumor bearing mouse from that of lungs from tumor-bearing mice. Data were statistically analyzed by the Wilcoxon rank-sum test. D, response of NK cells to CD8 T cell depletion. Leukocytes were isolated from tumor-bearing lungs treated with either CD8 mAb or IgG2a isotype mAb, stained with fluorescence-conjugated antibodies against CD45, NK1.1, CD69, CD107a, CD178, NKG2A, NKG2D, and subjected to FACS analysis. Two- way ANOVA with model based mean comparisons and BH p value correction.

Figure S3. Kinetic study of functional biomarkers of NK cells

A, cytokine profile. Serum were collected from $CXCR4^{Mye\Delta/\Delta}$ or $CXCR4^{WT}$ mice and a cytokine array analysis (AAM-CYT-G3, Raybiotech) for 62 cytokines was performed on a GenoPix 4000B Scanner (Molecular Devices, Sunnyvale, CA). Cy3-biotin-conjugated anti-cytokines in the serum of $CXCR4^{Mye\Delta/\Delta}$ mice were quantified and normalized to that of $CXCR4^{WT}$ mice, then graphed as fold-decrease compared to $CXCR4^{WT}$ .mice. B, Fas (CD95) expression of immune cells. Peripheral leukocytes from $CXCR4^{\Delta/\Delta}$ and $CXCR4^{WT}$ mice were stained with CD95-Alex Flour647, F4/80-BV421, Ly6G-APC/Cy7, CD8-Alex Flour700,CD4-pacific blue, B220-Alex Four700, Nk1.1-APC/Cy7 and CD11c-AF700. The cells were sorted based upon GFP and Tomato expression

and analyzed for Fas (CD95) expression on the different cell populations except for F480, B220 and CD11c. Two-way ANOVA with model based mean comparisons and BH p value correction. C, kinetic study of degranulation during NK cell killing *in vivo*. Yac-1 cells (8×10^6) were intravenously injected into the $CXCR4^{Mye\Delta/\Delta}$ or $CXCR4^{WT}$ littermate control mice. Peripheral blood was collected over a time frame of 0h, 1h, 4h and 24h post-injection and blood cells were isolated. The peripheral NK cells were stained with NK1.1-APC/Cy7 and CD107a-Percep/Cy5.5. The stained cells were subjected to FACS analysis. Wilcoxon rank-sum test with BH p value correction. D, Yac-1 cells (8×10^6) were intravenously injected into the $CXCR4^{mye\Delta/\Delta}$ and $CXCR4^{WT}$ mice. Peripheral leukocytes were collected over a time frame of 0h, 1h, 4h and 24h after injection. The peripheral population of NK cells was determined by FACS analysis. Data were expressed as mean ± SD and statistically analyzed by Student's t-test. ***p<0.01* or **p<0.05* vs. control wild type, n=5. Wilcoxon rank-sum test with BH p value correction. E, NK cells expressing granzyme. 2×10^5 B16F0 melanoma cells were intravenously injected into $CXCR4^{Mye\Delta/\Delta}$ mice and $CXCR4^{WT}$ mice (8/group). Sixteen days after injection of tumor cells, the NK cells infiltrating the lung were analyzed for intracellular granzyme B expression by FACS. Wilcoxon rank-sum test.

Figure S4. Impact of myeloid cells on NK cytotoxicity. A, depletion of neutrophils in mice. Myeloid CXCR4Δ/Δ mice were peritoneally injected with 250 μg of Ly6G mAb (#BE0075, clone 1A8, Bio X Cell) or IgG2a isotype control mAb (#BE0089, clone 2A3, Bio X Cell). Two days after injection, the peripheral leukocytes were stained with Pacific Blue-conjugated Ly6G Ab and subjected to FACS analysis. GFP^+ myeloid cells expressing pacific blue were counted as neutrophils. The depletion rate was over 95%. B, abrogation of macrophages in mice. After 10 days of daily oral gavage of mice with 200 μg of CSF1R inhibitor (Novartis) or 20% captisol vehicle control, 1×10^8 Yac1-Fluc cells were intravenously injected into $CXCR4^{mye\Delta/\Delta}$ or $CXCR4^{WT}$ mice. Images were taken at 1h, 4h or 24h post Yac1 cell injection. Animals without Yac1 cell injection served as negative imaging background control (Ctrl). V, mice treated with vehicle; T, mice treated with CSF1R inhibitor. C, depletion of macrophages in mice. C57Bl6 mice were

intravenously injected with 1 mg of clodronate or vehicle encapsome. Twenty-four hours after clodronate or encapsome treatment, mice were intravenously injected with 8×10^6 Yac1-Luci cells and subsequently subjected to luciferin imaging at 1h, 4h, 24h post-injection of the cells. Ctrl, Control mouse without Yac1-Luci cell injection; E, Encapsome; C Clodronate

Figure S5. Gating strategy for identifying NK cells, type I NKT cells and type II NKT cells. A, lymphocytes were first excluded for doublets and single cells were further gated to obtain live cells. Live cells were gated further to obtain $B220^-CD8^-$ lymphocytes. NK cell are characterized as $NK1.1^+CD3\varepsilon^{neg}$ lymphocytes. Type I NKT cells are characterized as CD1d-$tet^+CD3\varepsilon^+$ cells. To determine the frequency of type II NKT cells, $NK1.1^+CD3\varepsilon^+$ cells were gated to exclude type I CD1d-tet^+ NKT cells. All the three populations, viz., NK cells, and type I and type II NKT cells were gated to segregate the interstitial (IST, $CD45^{neg}$) and marginated vascular (MV, $CD45^+$) populations. FACS plots are representative of a single lung sample. B, intravenous anti-Asialo-GM1 mAb treatment efficiently depletes NK cells. Mouse spleen mononuclear cells were stained and analyzed as described in Methods. Interstitial (IST; $CD45^{neg}$) and marginated vascular (MV: $CD45^+$) NK cells and type I and type II NKT cells were gated and characterized as described in above Figure S5A. Absolute numbers of each cell type are indicated. C, CXCL12 inhibited IL-18 secretion. Bone marrow cells from C57BL6 mice were cultured with 10 ng/ml of CXCL12 or PBS vehicle for two days, the supernatant IL18 was determined by ELISA.

Figure S6. The CXCR4 antagonist, LY2510924, reduces CD103+ CD8+ T cells in tumor-bearing mice. A, CD4+ T cell surface protein expression. C57Bl6 mice (9 mice/group) were intravenously injected with B16F0 melanoma cells (1×10^5) and treated subcutaneously with 3 mg/kg of the CXCR4 antagonist, LY2510924, or PBS vehicle twice a day for 2 weeks. After two weeks of treatment, mice were sacrificed and the tumor-infiltrated leukocytes were isolated from lungs. CD4 cell surface markers were stained with specific fluorochrome conjugated antibodies as indicated and subjected to FACS analysis. B, CD4+ T cell intracellular protein expression. The

tumor-infiltrated leukocytes were processed with fix/perm buffer. The expression of CD25/Foxp3 and intracellular cytokines IL-4, IL-12, IFNγ in CD4+T cells were determined by FACS following staining with specific antibodies. C, CD8+ T cell surface markers. The tumor-infiltrated leukocytes above were stained with indicated fluorochrome conjugated antibodies for CD69, CD103, CD107b, PD-1, and analyzed by flow cytometry. Two-way ANOVA with model based mean comparisons and BH p value correction. D, LY2510924 anti-tumor activity. C57Bl6 mice (6 mice/group) were subcutaneously injected with B16F0 melanoma cells (5×10^5) and treated subcutaneously with 3 mg/kg of LY2510924, or PBS vehicle twice a day. Tumor size was measured every five days. Data were plotted and statistically analyzed by the Wilcoxon rank-sum test. E, peripheral immune cell profile. Blood samples were collected 10 days after PBS or LY2510924 treatment. Erythrocytes were excluded with lysis buffer and leukocytes were stained with specific antibodies for FACS analysis using two-way ANOVA with model based mean comparisons and BH p-value correction. F, NK cell distribution and activation. Leukocytes were isolated from the xenograft tumors, stained with CD45-APC/Cy7, NK1.1-PE, and CD69-APC and analyzed by FACS (Wilcoxon rank-sum test). G, Intracellular IL-18 expression in leukocytes that infiltrated into xenograft tumors was analyzed by flow cytometry. Data were statistically analyzed by two-way ANOVA with model based mean comparisons and BH p-value correction.

Figure S7. High CXCR4 expression is associate with poor survival in patients with myeloid cell-enriched tumors. The TCGA dataset of 469 skin cutaneous melanoma samples were analyzed using CBio portal. **A.** Progression-free survival in tumors with high and low CXCR4 expression was performed in a subset of tumors that had high mRNA expression of myeloid cell marker ITGAM. Tumors with high ITGAM expression were selected based on mRNA expression z-score threshold of +1. **B.** Progression-free survival in tumors with high and low CXCR4 expression was performed in all TCGA tumor samples that had RNAseq expression data available.