Supporting Information

Meyer et al. 10.1073/pnas.1108121108

SI Materials and Methods

Mice. C57BL/6 mice expressing human ret oncogene in melanocytes (1) were provided by I. Nakashima (Chubu University, Aichi, Japan). OT-1 mice expressing a transgenic TCR specific for OVA-derived peptide SIINFEKL were provided by B. Arnold (German Cancer Research Center, Heidelberg, Germany). Experiments were performed in accordance with government and institute guidelines and regulations.

Reagents and Antibodies. We used RPMI-1640 medium with 2 mM l-glutamine (PAA Laboratories), 10% FCS (PAN Biotech), and 50 μM β-mercaptoethanol (Sigma). Sildenafil was purchased from Pfizer. Rat anti-mouse directly conjugated mAbs (CD4-PE, CD8-FITC, CD8-PE, CD11b-APC, CD11b-FITC, Gr1-APC, Gr1-PE-Cy7, Ly6C-FITC, Ly6G-PE, IL-4Ra-PE, IL-4Ra-biotin, CD45.2-PerCP-Cy5.5, CD45.2-FITC, and isotype-matched control mAbs), streptavidin-PE, Cytofix/Cytoperm kit, purified rat anti-mouse CD16/CD32 (Fc-block), mouse anti-human Ki67, purified mouse anti-human ARG-1 (both cross-reacting with mouse molecules), rat anti-mouse IgG-FITC, and Armenian hamster anti-mouse antibodies (CD3-PerCP, CD3-APC) were from BD Bioscience. FoxP3 fixation/permeabilization kit was from eBioscience. Rat antimouse F4/80-PE (Invitron), purified S100A9 (Abcam), Armenian hamster antimouse TCR ζ-chain mAbs (biotinylated and FITC-conjugated; Santa Cruz), and goat anti-rat IgG-Alexa Fluor 647 were also used. Mouse APC-conjugated tetramers containing K6 and peptide SVYDFFVWL derived TRP-2 were provided by T. Schumacher (The Netherlands Cancer Institute, Amsterdam, The Netherlands). Rabbit antimouse PDE-6α mAbs and Alkaline Phosphatase Rabbit IgG ABC Kit were from GeneTex. Antimouse PDE-5A polyclonal Abs were from Abnova. Rat antimouse CD8 depleting mAbs were from Serotec, and IgG from rat serum was from Sigma.

Preparation of Single Cell Suspensions. Organ samples were cut into small pieces and filtered. Tumor, BM, and spleen samples were depleted of erythrocytes by ammonium chloride lysis.

Flow Cytometry. Single-cell suspensions were treated with Fc-block and mAbs for 20 min at 4 °C. For intracellular staining, samples were preincubated with the Cytofix/Cytoperm or FoxP3 fixation/permeabilization kit. Acquisition was performed by flow cytometry by using FACSCalibur with CELL-Quest software or FACSCantoII with FACSDiva Software (BD Biosciences), with dead cell exclusion based on scatter profile or propidium iodide inclusion. FlowJo software (Tree Star) was used to analyze at least 100,000 events.

Western Blot Analysis. The expression of the PDE-5 protein in MDSCs isolated from tumors and in tumor cells was measured as described previously (3).

Immunohistology. Eyeballs from tumor-bearing mice were fixed in Davidson solution and embedded in paraffin, and 5-μm sections were stained for PDE-6 as previously reported (5).

Bio-Plex Assay. Snap-frozen primary tumor and LN samples were mechanically disrupted and treated by lysis solution (Bio-Rad). Protein concentration in lysates was determined by using Bradford assay (Bio-Rad). Inflammatory factors in tissue lysates were measured by multiplex technology (Bio-Rad).

In Vitro Proliferation Assay. CD11b+ cells were isolated from skin tumors and BM of transgenic and WT mice by using CD11b MicroBeads isolation kit (Miltenyi Biotec). The proportion of Gr1+CD11b+ MDSC in selected population was 80%. In some experiments, BM cells were sorted for Gr1+CD11b+ MDSCs by using FACSARia (BD Bioscience) with the purity of 99%. Splenocytes were isolated from C57BL/6 or OT-1 mice, stimulated, and cocultured with MDSCs in triplicates (at splenocyte: MDSC ratios of 1:0.6, 1:1, or 1:2). C57BL/6 splenocytes were labeled with the CD pEFluor 670 (eBioscience) and stimulated with soluble anti-CD3 and anti-CD28 mAbs (0.5 μg/mL each); splenocytes from OT-1 mice were activated by 250 μg OVA. T-cell proliferation was measured by the reduction of CD expression or by Ki67 level. ζ-Chain expression was detected upon the overnight coculture of nonsimulated C57BL/6 splenocytes with MDSCs.

Sildenafil Treatment. Transgenic tumor-bearing mice were treated orally with sildenafil dissolved in drinking water as published before (2) and monitored daily for tumor progression. Control group of mice with tumors of similar size received usual drinking water. Some sildenafil-treated and untreated mice were injected intraperitoneally with CD8+ T-cell depleting mAbs or IgG from rat serum (both 100 μg per mouse), respectively, at days 0, 2, 14, and 28 after the start of the experiment.

Biochemical Assays. cGMP concentration was measured in tumor lysates with the HitHunter cGMP assay (Amersham Bioscience) (3), and NO was measured by the colorimetric NO assay kit (PromoKine). Tumor samples were prepared as described before (4). For intracellular NO detection, staining with dianiofluorescein-2 diacetate (Cell Technology) was performed.


Meyer et al. www.pnas.org/cgi/content/short/1108121108 1 of 3
**Fig. S1.** Analysis of MDSCs in lymphatic organs of ret transgenic mice. Cells from mice with (ret tu) or without (ret) macroscopic tumors and from non-transgenic littermates (wt) were assessed by flow cytometry. Cumulative data for Gr1^+^CD11b^+^ MDSC in the BM (A) and spleen (B) are expressed as the percentage within leukocytes (mean and SE from 10–31 mice; *P* < 0.05).
**Fig. S4.** PDE-5 expression in MDSCs isolated from skin tumors and in tumor cells established from primary melanoma of transgenic mice (Ret cells). PDE-5 protein was measured in lysates of Ret cells (“2”) and MDSCs isolated from primary tumors of untreated (“3”) or sildenafil-treated (“4”) mice using Western blot analysis with corresponding Abs. Results show PDE-5 and β-actin blots. PDE-5 expression in mouse lungs (“1”) was used as a positive control.

**Fig. S5.** Sildenafil modulates the levels of cGMP and NO in melanoma lesions. (A) cGMP amounts in primary tumors and metastatic LNs were measured with the HitHunter cGMP assay (mean and SE; n = 4–5 samples per group). (B) NO concentrations in skin tumor lysates were detected by colorimetric NO assay kit (mean and SE; n = 4–5 samples per group; *P < 0.05).

**Fig. S6.** Sildenafil reduces the activity of MDSCs from melanoma lesions. (A and B) NO production was detected in MDSCs from metastatic LNs from treated and untreated mice by flow cytometry (mean and SE; n = 5 mice per group). Results are presented as the percentage of NO-producing cells among total MDSCs (A) or as NO MFI characterizing the production rate (B). (C) ARG-1 expression was measured in MDSCs (mean and SE; n = 5 mice per group) and shown as the percentage of ARG-1+ cells within total MDSCs (*P < 0.05).