NK cells control tumor-promoting function of neutrophils in mice

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Abstract

Although the importance of NK cells as a direct anti-tumor effector is well appreciated, the immuno-regulatory function of NK cells to control cancer-associated inflammation, which facilitate tumor progression, remains unknown. In this study, we demonstrate that the novel function of NK cells to control tumor-promoting inflammation through the functional modification of neutrophils. NK cells control the tumor-promoting function of neutrophils via an IFN-γ-dependent mechanism and the tumor progression in an NK cell-depleted host is totally diminished when the IL-17A-neutrophils axis is absent. In NK cell-depleted mice, neutrophils acquire the tumor-promoting phenotype as seen in the up-regulation of VEGF-A expression to promote tumor growth and angiogenesis. Importantly, a VEGFR inhibitor preferentially suppressed tumor growth in NK cell-depleted mice and such a selective anti-tumor effect in NK cell-depleted mice was a neutrophil-dependent. Furthermore, the systemic neutropenia by an antimetabolite treatment shows a significant anti-cancer effect only in mice with no NK cells. Thus, NK cells likely play an important role in controlling the tumor-promoting and angiogenic function of neutrophils.

Introduction

The tumor microenvironment consists of not only transformed cancer cells, but also various stromal cells that control tumor progression (1,2). Of such tumor stromal cells, innate and adaptive immune cells and their effector molecules are known as important components that regulate tumor development as known as the immune-editing process (3-5). Natural killer (NK) cells are known to play an important role in the immunological surveillance of development and subsequent growth of cancer cells through a variety of effector mechanisms, including perforin/granzymes or death receptor dependent cytotoxicity and anti-tumor cytokine production (6-8). Besides their direct anti-tumor effector function, NK cells are also involved in controlling the subsequent adaptive immune responses (9-11).

In contrast to anti-tumor immune responses, tumor-promoting immune responses, often regarded as an inflammation, are known to be a critical component of cancer malignant progression (2,12,13). The inflammatory immune cells involved in such tumor-promoting immunity includes innate cells as well as adaptive lymphocytes, and they are capable of producing a variety of inflammatory cytokines, chemokines and/or growth factors (2,13). Neutrophils are the most abundant circulating innate inflammatory cells that respond to infection or tissue damage (14-16). While neutrophils mediate the host defense through their multiple functions including phagocytosis, ROS production or release of granules containing antimicrobial peptides and proteases (14-17), it is also appreciated that neutrophils are a key component of tumor-promoting immune responses (18-20). Indeed, many clinical studies support the circulating neutrophil counts or the neutrophil-to-lymphocyte ratios (NLRs) in cancer patients as a predictive biomarker for their poor

outcome and/or distant metastasis (21-25). Furthermore, it has been suggested that the function of neutrophils is converted from tumor-suppressing to tumor-promoting in the tumor-bearing state, and tumor-associated neutrophils (TANs) were reported to be classified into anti-tumor (N1) or tumor-promoting (N2) subpopulations (26). While N2 TANs were driven by the presence of TGF-β (26), IFN-β polarized TANs towards N1 phenotype (27). These studies clearly suggest that an understanding of functional switching of TANs into tumor-promotion is critical for controlling cancer progression.

Although both NK cells and neutrophils are critical components of tumor stroma, there is no clear indication of whether the interaction between NK cells and neutrophils is involved in tumor control. In this study, we demonstrate the novel function of NK cells to control tumor-promoting inflammation through the functional modification of neutrophils. NK cells control the tumor-promoting function of neutrophils via an IFN-γ-mediated mechanism, and therefore the tumor progression in an NK cell-depleted host is totally diminished when the IL-17A-neutrophils axis is absent. In NK cell-depleted mice, neutrophils acquired a tumor-promoting phenotype as seen in their VEGF-A up-regulation to promote tumor growth and angiogenesis. Importantly, the VEGFR inhibitor preferentially suppressed tumor growth in NK cell-depleted mice and such a selective anti-tumor effect in NK cell-depleted mice was the neutrophil-dependent. Furthermore, the systemic neutropenia induced by an antimetabolite treatment showed a significant anti-cancer effect only in mice with no NK cells. Thus, NK cells likely play an important role in controlling the tumor-promoting and angiogenic function of neutrophils.

Materials and Methods

Mice

Wild-type C57BL/6 (WT) mice were purchased from CLEA Japan, Inc. (Tokyo, Japan). IFN-$\gamma^{-/-}$ (IFN-γ KO) and IL-17$^{-/-}$(IL-17 KO) mice on B6 background were kindly provided by Dr. Y. Iwakura (Tokyo University of Science, Chiba, Japan) and maintained at Laboratory Animal Research Center, Institute of Medical Science, The University of Tokyo. In some experiments, groups of mice were treated with either anti-CD4 monoclonal antibody (mAb, clone GK 1.5), anti-CD8 mAb (clone 53.6.2), anti-NK1.1 mAb (clone PK136), anti-asialo-GM1 (asGM1) antibody (Wako Chemicals, Osaka, Japan) on Day -3 and -1, anti-CXCR3 mAb (clone CXCR3-173, Bio X Cell, NH, USA) on Day -1, 0, 2, 4 and 6 or anti-Ly6G antibody (clone 1A8, Bio X Cell) on Day -1 and 3 (where day 0 is the day of primary tumor inoculation). Antibodies against CD4, CD8 and NK1.1 were purified from hybridoma cells. In some experiment, mice were treated with SU5416 (AdooQ BioScience, Irvine, CA, USA, 30 mg/kg i.p.) for 7 days from the day of tumor inoculation. To induce systemic neutropenia, mice were injected intravenously (i.v.) with 150 mg/kg 5-FU (Kyowa Kirin, Tokyo) at Day 0 relative to the tumor implantation. All experiments were approved and performed according to the guidelines of the Animal Care and Use Committee of the Graduate School of Pharmaceutical Sciences of The University of Tokyo, the Care and Use of Laboratory Animals of University of Toyama and the Animal Care and Use Committee of Institute of Medical Science of The University of Tokyo.

Cells and reagents

MCA205, a methylcholanthrene-induced murine fibrosarcoma cell line was kindly provided by Dr. S. A. Rosenberg (National Cancer Institute, Bethesda, MD) and maintained in complete RPMI 1640 medium. MCA205-Luc2 cells stably expressing luciferase with a CMV-promoter were established as previously described (28). Briefly, MCA205 cells (5 x10^5/well) were seeded in a 6 well plate and transfected with pGL4.50 vector using Lipofectamine 2000. The cells were selected with Hygromycin B (200 μg/ml) and cloned by limiting dilution. *In vitro* and in vivo characterization of MCA205-Luc2 cells is shown in Supplementary Figure 1.

The mouse Lewis Lung carcinoma cell line (3LL) was kindly provided by Dr. Kazuyoshi Takeda (Juntendo University), and the luciferase-expressing 3LL cell line (3LL-Luc2) was prepared as previously described (29). pGL4.50 [luc2/CMV/Hygro] vector, and D-luciferin were obtained from Promega (WI, USA). Lipofectamine 2000 was purchased from Invitrogen (Carlsbad, CA). Hygromycin B was obtained from Nacalai Tesque (Kyoto, Japan).

Bioluminescent imaging of in vivo cancer cell growth

MCA205-Luc2 cells or 3LL-Luc2 cells were inoculated subcutaneously (s.c.). To obtain bioluminescent images, mice were injected with D-luciferin (Promega, 150 mg/kg i.p.) and luminescence was measured with an *in vivo* imaging system (IVIS Lumina II, Perkin Elmer, MA, USA) 10 minutes after the D-luciferin injection. Regions of interest analyses was performed using Living Image 4.2 Software (Caliper Life Science, Hopkinton, MA, USA) to determine the light emitted from the tumor. For each mouse, all values were determined as photons per sec (photon/sec).

Tumor-infiltrating lymphocyte (TIL) isolation and flow cytometry

MCA205-Luc2 cells (10^5) were inoculated subcutaneously. Seven days after inoculation, tumor tissues were dissected, minced and digested with 2 mg/mL collagenase (Roche Diagnostics GmbH, Mannheim, Germany) and 0.1 mg/mL DNase I (Roche Diagnostics GmbH) in serum-free RPMI 1640 for 1 hr at 37 ºC. Samples were further homogenized through wire mesh. For collecting tumor culture supernatant, the tumor homogenates were cultured in complete RPMI 1640 media for 24 hrs and the cell-free supernatant were harvested. For flow cytometry analysis, cells were first pre-incubated with anti-CD16/32 (2.4G2) mAb to avoid non-specific binding of antibodies to FcγR. The cells were then incubated with a saturating amount of fluorophore-conjugated mAb. Antibodies against CD3ε (2C11), NK1.1 (PK136), CD11b (M1/70), Gr-1 (RB6-8C5) and Ly6G (1A8) were purchased from Biolegend (San Diego, CA, USA), eBioscience (San Diego, CA, USA) or Tombo Bioscience (San Diego, CA, USA). Flow cytometry analysis was performed with a FACS Canto (BD Bioscience, San Jose, CA, USA) and the data were analyzed with FlowJo software (Tree Star, Ashland, OR, USA).

Matrigel plug angiogenesis assay

Growth factor-reduced Matrigel (BD Biosciences) containing MCA205-Luc2 cells (10^5) was s.c. inoculated and 1% Evans blue was injected intravenously into mice at 7 days after the inoculation. Ten minutes later, Matrigel plugs were removed after perfusion by saline and incubated with formamide for 48 hrs. The amount of Evans blue dye was quantified by the absorbance at 620nm and normalized to the weight of Matrigel.

Neutrophil isolation and in vitro culture

To isolate tumor-infiltrating neutrophils, tumor samples were harvested 7 days after the inoculation and $CD11b^+$ $Ly\text{-}6G^+$ neutrophils were isolated by cell sorting (>90 % purity, FACS Aria Special Order, BD Bioscience, San Jose, CA, USA). To isolate peritoneal neutrophils, mice were injected interperitoneally (i.p.) with 7.5% casein solution (1mL) and cells were collected from the peritoneum 24 hrs after casein injection. $Ly6G^+$ neutrophils were purified using a Mojosort cell separation kit (Biolegend). Briefly, cells were first pre-incubated with CD16/32 (2.4G2) mAb to avoid non-specific binding of antibodies to FcγR. The cells were then incubated with a saturating amount of biotin-conjugated Ly6G mAb and streptavidin-conjugated magnet beads. $Ly6G^+$ neutrophils were isolated using a Mojosort magnet. Peritoneal neutrophils (10^6 cells) were cultured with tumor supernatant (1:1 dilution with fresh media) for 6 hrs. Tumor supernatant was prepared as described above mentioned and the blocking anti-IFN-γ mAb (clone XMG1.2, 10 μg/ml) was added in some experiments. Gene expression of neutrophils was measured using real-time RT-PCR. RT-PCRs were performed with the following primers: ICAM-1 (f) 5′′-CAATTCACACTGAATGCCAGCTC-3′′ (r) 5′′-CAAGCAGTCCGTCTCGTCCA-3′′; TNF-α (f) 5′′-TTGTCTACTCCCAGGTTCTCT-3′′ (r) 5′′-GAGGTTGACTTTCTCCTGGTATG-3′′; CCL-2 (f) 5′′-CTCACCTGCTGCTACTCATTC-3′′ (r) 5′′-ACTACAGCTTCTTTGGGACAC-3′′; VEGF-A (f) 5′′-GTGCACTGGACCCTGGCTTTA-3′′ (r) 5′′-GGTCTCAATCGGACGGCAGTA-3′′. To standardize the cDNA, the housekeeping gene GAPDH was tested with primer pairs: (f) 5′′-AAATGGTGAAGGTCGGTGTG-3′′; (r) 5′′-TGAAGGGGTCGTTGATGG-3′′. The protein expression of VEGF-A was determined by the specific ELISA kit (RayBiotech, Norcross, GA, USA) according to the manufacturer’s instruction.

Immunohistochemistry and immunofluorescence staining

For frozen section preparation, tumor tissues were immediately fixed with 1% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) at 4 °C, and then transferred to PBS. Tissues were soaked in 30% PBS-buffered sucrose, and then the entire tissue was embedded in optimal cutting temperature (OCT) compound (Sakura Finetek Japan) and frozen at −20 °C. The frozen sections were cut on a cryostat (CM 3050S-IV; Leica Microsystems). The sections were incubated at 4 °C overnight with monoclonal anti-CD31antibody (1:100 MAB1398Z Millipore). For immunofluorescence, the Alexa-Fluor488-conjugated secondary antibodies (Jackson) were used at 1:500 dilutions. Nuclei were stained with Hoechst 33258 (Nacalai Tesque). The immunofluorescent images were randomly obtained by a confocal microscope (TCS-SP5; Leica Microsystems).

Statistical analysis

All data were obtained from a group of 6 to 9 mice and are representative of at least two independent experiments. Data were analyzed for statistical significance using the Student's *t*-test. *P* values less than 0.05 were considered significant.

Results

NK cell absence promotes in vivo cancer cell growth through an IL-17A-dependent mechanism

In order to determine the role of NK cells in controlling cancer cell growth, we monitored the luminescence of MCA205-Luc2 cells implanted in control B6 mice (control) or B6 mice treated with antibodies that deplete immune effector cells. In the absence of NK cells, but not $CD4^+$ or $CD8^+$ T cells, the in vivo growth of MCA205-Luc2 cells was significantly promoted compared with control B6 mice (Fig 1A). Such NK cell-dominant tumor control was preferentially seen for up to 10 days after the tumor inoculation, whereas T cell control, particularly $CD8^+$ cells, became apparent at later time points (data not shown). To further understand the effector pathway by which NK cells control cancer cell growth, we tested IFN-γ-deficient (IFN-γ KO) mice. As shown in Figure 1B, the in vivo growth of MCA205-Luc2 cells was significantly promoted in IFN-γ KO mice, similar to that of NK cell-depleted mice, and NK cell depletion did not further enhance MCA205-Luc2 cell growth in IFN-γ KO mice. To examine whether NK cells systemically or locally control in vivo growth of MCA205-Luc2 cells, we blocked CXCR3 chemokine receptor which is known to regulate tumor infiltration of migratory $CD27^{hi}$ NK cells (30,31). As shown in Figure 1C, the in vivo blocking of CXCR3 showed a mostly similar result in the growth of MCA205-Luc2 cells to NK cell depleted mice, and the CXCR3 blocking did not further enhance MCA205-Luc2 cell proliferation in the NK cell-depleted mice. These results clearly suggest that NK cells control cancer cell growth mainly through IFN-γ- and CXCR3-dependent mechanism.

Critical contribution of IL-17A-neutrophil axis for promoting in vivo cancer cell growth in the absence of NK cells

IL-17A is known as a major cytokine for tumor-promoting immune response and counteracts the anti-tumor function of IFN-γ (32-34). Surprisingly, we found that NK cell-depletion did not affect the in vivo growth of MCA205-Luc2 cells in IL-17A-deficient (IL-17 KO) mice (Fig 2A). This result indicates that the promotion of in vivo cancer cell growth in the absence of NK cells clearly required IL-17A. Considering the IL-17A is a cue for inflammatory response by attracting neutrophils to inflammatory sites (35) and neutrophils are known as a critical component of tumor-promoting inflammation (18-20), we next determined whether neutrophils are involved in the promotion of cancer cell growth in NK cell-depleted mice. We employed anti-Ly6G antibody (clone 1A8) to specifically deplete neutrophils as previously described (36). As shown in Figure 2B, the promotion of MCA205-Luc2 cell growth was diminished by neutrophil depletion in the absence of NK cells. This contribution of neutrophils for promoting cancer cell growth in NK cell-depleted mice was also confirmed in a 3LL-Luc2 model (Fig. 2C). Neither MCA205 cells nor 3LL cells in mice with intact NK cells showed differences in cancer cell growth upon neutrophil-depletion (Fig. 2B and C), suggesting that neutrophils are involved in controlling cancer cell growth only in the context of NK cell absence. In order to understand whether NK cells directly control tumor neutrophil accumulation, we examined the infiltration of neutrophils into MCA205-Luc2 tumors by flow cytometry. Although we found that neutrophil infiltration into the tumor sites was largely impaired in IL-17 KO mice, NK cell-depletion did not affect the proportion of tumor infiltrating neutrophils in both WT and IL-17 KO mice (Fig. 3A and B). Collectively, these results clearly suggest the critical contribution of neutrophils for promoting in vivo cancer cell

growth in the absence of NK cells and the quality, but not quantity, of neutrophils at the tumor site can be controlled by NK cells.

NK cells control neutrophil angiogenic switch by regulating VEGF-A expression

Tumor-associated neutrophils (TANs) are a critical component of tumors, and either anti-tumor (N1) or tumor-promoting (N2) phenotypes of TANs are reported (26). In order to examine the phenotypic difference of TANs with or without NK cells, we isolated TANs and subjected them to morphological or molecular characterization. While we did not see any difference in the maturation status of TANs as determined by their nuclear segments (Fig 4A), TANs in the absence of NK cells significantly up-regulated their mRNA expression of VEGF-A amongst other anti-tumor (ICAM-1, TNF-α) or pro-tumor (CCL2) neutrophil markers (Fig 4B). Importantly, naïve peritoneal neutrophils up-regulated their mRNA expression of VEGF-A upon culture with the supernatant of an NK cell-depleted MCA205 tumor tissue culture (Fig 5A), suggesting that NK cells control VEGF-A expression of TANs by modulating soluble factor(s) within the tumor microenvironment. Considering a direct inhibitory effect of IFN-γ in VEGF production (37,38), we tested the role of NK cells and/or IFN-γ in neutrophil VEGF-A expression. While the blocking of IFN-γ did not affect neutrophil VEGF-A expression upon co-cultured with tumor tissue culture supernatants (Fig. 5B), the supernatant of IFN-γ KO tumor up-regulated neutrophil VEGF-A expression regardless of NK cell status (Fig. 5C). Importantly, the protein expression of VEGF-A within tumor was elevated in the absence of NK cells, and a such elevated VEGF-A production was totally cancelled upon anti-Ly6G treatment (Fig. 5D). These results imply that NK cells control neutrophil VEGF-A expression and the endogenous IFN-γ

controls such neutrophil VEGF-A switching likely in an indirect mechanism. We then assessed whether the absence of NK cells actually affects in vivo tumor angiogenesis by using a Matrigel plug assay. As shown in Figure 6A, vascular formation towards the MCA205/Matrigel plug was enhanced in the absence of NK cells. Importantly, the depletion of neutrophils totally canceled such enhanced angiogenesis in NK cell-depleted mice. In align with those data, we also observed the increased vessel area and diameter as determined by the immunohistochemistry staining of anti-CD31 within MCA205/Matrigel plug in the absence of NK cells and a such blood vessel formation was totally dependent on neutrophils (Fig. 6B-D). Those data clearly and strongly support that NK cells control tumor angiogenesis through neutrophils.

To directly demonstrate an involvement of VEGF-dependent angiogenesis in tumor growth of NK cell-depleted mice, we tested the specific tyrosine kinase inhibitor for VEGFR, SU5416, to block VEGF pathway in the presence or absence of NK cells and neutrophils. As shown in Figure 7, we clearly demonstrated the treatment of SU5416 inhibited the in vivo growth of MCA205-Luc2 cells only in NK cell-depleted mice. Furthermore, such a selective anti-tumor effect of SU5416 in NK cell-depleted mice was neutrophil-dependent because such anti-tumor effect completely diminished upon anti-Ly6G treatment. Collectively, these results clearly indicate the novel function of NK cells to control the neutrophil angiogenic switch by regulating VEGF-A expression.

5-FU-induced systemic neutropenia controls in vivo cancer cell growth in the absence of NK cells

In order to test a clinical application of our findings, we examined whether systemic

neutropenia could control the growth of tumors in mice with impaired or absent NK cells. The antimetabolite 5-fluorouracil (5-FU) is well-known to act as an anti-cancer drug by targeting DNA replication (39) and is also very well-known to induce systemic neutropenia (40). A single treatment of 5-FU significantly reduced the population of neutrophils in the peripheral blood, bone marrow, spleen and tumor (Fig 8A and data not shown). At this dose of 5-FU treatment, we did not see any direct anti-tumor effect as shown in Figure 8B. In contrast, the same dose of 5-FU treatment showed a significant anti-cancer effect in mice with depleted NK cells. These data suggest that chemotherapy-induced systemic neutropenia may be effective for controlling tumor malignancy in addition to its direct anti-tumor effect when NK cells are impaired.

Discussion

Although the importance of NK cells in anti-tumor immune responses has been widely appreciated, their role in tumor-promoting immune responses remains unknown. In this study, we demonstrated that the tumor-infiltrating NK cells control in vivo cancer cell growth through IFN-γ- and CXCR3-dependent mechanism in concert with our previous finding (31), and further that the IL-17A-neutrophil axis is critically involved in such promotion of in vivo cancer cell growth when NK cells are absent. NK cells suppressed the expression of VEGF-A in the tumor-infiltrating neutrophils, and therefore neutrophil-dependent tumor-induced angiogenesis was promoted in the absence of NK cells. The VEGFR inhibitor preferentially suppressed tumor growth in NK cell-depleted mice and such a selective anti-tumor effect in NK cell-depleted mice was the neutrophil-dependent. Importantly, systemic neutropenia by anti-metabolite 5-FU treatment was effective to control cancer cell growth separately from its direct anti-tumor effect when NK cells are absent. These results indicate the novel function of NK cell in angiogenic switching of neutrophils to control tumor-promoting immune responses within the tumor microenvironment.

In addition to the suppressive arm of immune responses known as cancer immune-surveillance or the immune-editing process, mounting evidences indicate the importance of the inflammatory tumor microenvironment in cancer promotion or malignant progression (2,12,13). Amongst the various immune cells involved in tumor-promoting immune responses, neutrophils are regarded as important innate immune cells for tumor-associated inflammation (13,41). A number of studies suggest that the infiltration of TANs correlates with poor prognosis in both pre-clinical and clinical tumor settings (19,20,42,43). In regards to the functional or phenotypic

character of TANs, it is reported that TANs display plasticity in their function either as an anti-tumor (N1) or as a tumor-promoting (N2) phenotype and TGF-β has been known to induce TANs into the tumor-promoting N2 phenotype (26,27). Although we observed higher production of TGF-β in NK cell-depleted tumors (data not shown), we did not see any bias in N2 population in NK cell-depleted tumors as determined by their nuclear segmentation (Fig. 4A). Instead, TANs in the absence of NK cells significantly up-regulated their expression of VEGF-A amongst the N2-associated molecule (Fig. 4B). Considering that the culture supernatant of NK cell-depleted tumors also up-regulated VEGF-A expression in naïve peritoneal neutrophils (Fig. 5A), we assumed that a soluble factor produced within the tumor microenvironment must be responsible for such polarization of TANs in the absence of NK cells. Corresponding with the suppressive role of IFN-γ in VEGF expression (37,38), we found that the NK cell IFN-γ regulate the VEGF expression on neutrophils by an unknown indirect mechanism. While the role of NK cell IFN-γ in the suppression of neutrophil migration to inflammation sites has been reported (44,45), NK cell depletion did not affect the tumor infiltration of neutrophils (Fig. 3) suggesting that NK cells control the tumor-promoting function of neutrophils, but not the tumor-infiltration, in an IFN-γ-dependent mechanism. Considering that the endogenous type I IFN (IFN-β) also regulates tumor angiogenesis and growth in a mouse model through neutrophils (27), endogenous IFNs are generally important for controlling tumor angiogenesis through the functional modulation of neutrophils.

Two components of the innate immune system, neutrophils and NK cells, were found in this study to counter-regulate tumor progression. However, the precise mechanism by which NK cells control the neutrophil tumor-promoting function has yet

to be proven. In the context of the anti-inflammatory role of NK cells, it is reported that NK cells regulate neutrophil function through the NKG2A receptor pathway in a colitis model (46). Furthermore, human NK cells have been shown to trigger neutrophil apoptosis through activating NK cell receptor NKp46- and Fas-dependent pathways (47,48). The NK cell-producing cytokines, IFN-γ and TNF-α, are known to convert neutrophil function from tumor-promoting to tumor-suppressing (49). TANs are also known to influence tumor control by coordinating with other components of immune cells mainly through their suppressive function of anti-tumor immunity (43,50,51). Although a suppressive function of TANs in the activity of anti-tumor $CD8^+$ T cells was reported (43), we did not see any difference in the status of tumor-infiltrating $CD8^+$ T cells as determined by their effector/memory marker CD62L/CD44 expression or activation marker PD-1 expression (Supplementary Fig. 2). Moreover, NK cells, but not $CD8^+$ T cells, temporary and dominantly control in vivo cancer cell proliferation by modifying neutrophil function in an IFN-γ-dependent mechanism. Clearly, further study is required to ascertain the relevance of our current findings in later stage tumors. Nevertheless, we conclude that TANs in the absence of NK cells are actively involved in tumor promotion rather than suppressing anti-tumor $CD8^+$ T cell responses.

Regarding the clinical implication of our findings, there are correlations between neutrophil-to-lymphocyte ratios (NRLs) and the prognosis of cancer patients (21-25). We demonstrated that systemic neutropenia induced by 5-FU treatment effectively controlled cancer cell proliferation separately from its direct anti-tumor effect in NK cell-depleted mice. In alignment with our pre-clinical findings, a recent clinical study indicate that peripheral NK-mediated cytotoxicity was negatively associated with the survival of patients treated with 5-FU (52). Most importantly, we

propose a novel approach that the functional impairment of neutrophils in cancer patients may improve their prognosis particularly when lack intact NK cell activity or accumulation into the tumor. We believe that our present findings offer a new therapeutic opportunity for cancer patients and for predicting the therapeutic outcome in the context of NK cell-neutrophil counter-regulation.

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References

1. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell **2011**;144:646-74

2. Grivennikov SI, Greten FR, Karin M. Immunity, inflammation, and cancer. Cell **2010**;140:883-99

3. Dunn GP, Bruce AT, Ikeda H, Old LJ, Schreiber RD. Cancer immunoediting: from immunosurveillance to tumor escape. Nature immunology **2002**;3:991-8

4. Schreiber RD, Old LJ, Smyth MJ. Cancer immunoediting: integrating immunity's roles in cancer suppression and promotion. Science **2011**;331:1565-70

5. O'Sullivan T, Saddawi-Konefka R, Vermi W, Koebel CM, Arthur C, White JM*, et al.* Cancer immunoediting by the innate immune system in the absence of adaptive immunity. The Journal of experimental medicine **2012**;209:1869-82

6. Smyth MJ, Hayakawa Y, Takeda K, Yagita H. New aspects of natural-killer-cell surveillance and therapy of cancer. Nature reviews Cancer **2002**;2:850-61

7. Vivier E, Ugolini S, Blaise D, Chabannon C, Brossay L. Targeting natural killer cells and natural killer T cells in cancer. Nature reviews Immunology **2012**;12:239-52

8. Hayakawa Y, Smyth MJ. NKG2D and cytotoxic effector function in tumor immune surveillance. Seminars in immunology **2006**;18:176-85

9. Scharton TM, Scott P. Natural killer cells are a source of interferon gamma that drives differentiation of CD4+ T cell subsets and induces early resistance to Leishmania major in mice. The Journal of experimental medicine **1993**;178:567-77

10. Martín-Fontecha A, Thomsen LL, Brett S, Gerard C, Lipp M, Lanzavecchia A*, et al.* Induced recruitment of NK cells to lymph nodes provides IFN-γ for TH1 priming. Nature immunology **2004**;5:1260-5

11. Watt SV, Andrews DM, Takeda K, Smyth MJ, Hayakawa Y. IFN-gamma-dependent recruitment of mature CD27(high) NK cells to lymph nodes primed by dendritic cells. Journal of immunology **2008**;181:5323-30

12. Coussens LM, Werb Z. Inflammation and cancer. Nature **2002**;420:860-7

13. Mantovani A, Allavena P, Sica A, Balkwill F. Cancer-related inflammation. Nature **2008**;454:436-44

14. Kruger P, Saffarzadeh M, Weber AN, Rieber N, Radsak M, von Bernuth H*, et al.* Neutrophils: Between host defence, immune modulation, and tissue injury. PLoS pathogens **2015**;11:e1004651

15. Nauseef WM, Borregaard N. Neutrophils at work. Nature immunology **2014**;15:602-11

16. Kolaczkowska E, Kubes P. Neutrophil recruitment and function in health and inflammation. Nature reviews Immunology **2013**;13:159-75

17. Borregaard N. Neutrophils, from marrow to microbes. Immunity **2010**;33:657-70

18. Pekarek LA, Starr BA, Toledano AY, Schreiber H. Inhibition of tumor growth by elimination of granulocytes. The Journal of experimental medicine **1995**;181:435-40

19. Scapini P, Morini M, Tecchio C, Minghelli S, Di Carlo E, Tanghetti E*, et al.* CXCL1/macrophage inflammatory protein-2-induced angiogenesis in vivo is mediated by neutrophil-derived vascular endothelial growth factor-A. Journal of immunology **2004**;172:5034-40

20. Nozawa H, Chiu C, Hanahan D. Infiltrating neutrophils mediate the initial angiogenic switch in a mouse model of multistage carcinogenesis. Proceedings of the National Academy of Sciences of the United States of America **2006**;103:12493-8

21. Walsh S, Cook E, Goulder F, Justin T, Keeling N. Neutrophil‐lymphocyte ratio as a prognostic factor in colorectal cancer. Journal of surgical oncology **2005**;91:181-4

22. Sarraf KM, Belcher E, Raevsky E, Nicholson AG, Goldstraw P, Lim E. Neutrophil/lymphocyte ratio and its association with survival after complete resection in non–small cell lung cancer. The Journal of thoracic and cardiovascular surgery **2009**;137:425-8

23. Shimada H, Takiguchi N, Kainuma O, Soda H, Ikeda A, Cho A*, et al.* High preoperative neutrophil-lymphocyte ratio predicts poor survival in patients with gastric cancer. Gastric Cancer **2010**;13:170-6

24. Bhatti I, Peacock O, Lloyd G, Larvin M, Hall RI. Preoperative hematologic markers as independent predictors of prognosis in resected pancreatic ductal adenocarcinoma: neutrophil-lymphocyte versus platelet-lymphocyte ratio. The

American Journal of Surgery **2010**;200:197-203

25. Halazun K, Aldoori A, Malik H, Al-Mukhtar A, Prasad K, Toogood G*, et al.* Elevated preoperative neutrophil to lymphocyte ratio predicts survival following hepatic resection for colorectal liver metastases. European Journal of Surgical Oncology (EJSO) **2008**;34:55-60

26. Fridlender ZG, Sun J, Kim S, Kapoor V, Cheng G, Ling L*, et al.* Polarization of tumor-associated neutrophil phenotype by TGF-beta: "N1" versus "N2" TAN. Cancer cell **2009**;16:183-94

27. Jablonska J, Leschner S, Westphal K, Lienenklaus S, Weiss S. Neutrophils responsive to endogenous IFN-beta regulate tumor angiogenesis and growth in a mouse tumor model. The Journal of clinical investigation **2010**;120:1151-64

28. Takahashi K, Nagai N, Ogura K, Tsuneyama K, Saiki I, Irimura T*, et al.* Mammary tissue microenvironment determines T cell-dependent breast cancer-associated inflammation. Cancer science **2015**;106:867-74

29. Fushiki H, Kanoh-Azuma T, Katoh M, Kawabata K, Jiang J, Tsuchiya N*, et al.* Quantification of mouse pulmonary cancer models by microcomputed tomography imaging. Cancer science **2009**;100:1544-9

30. Wendel M, Galani IE, Suri-Payer E, Cerwenka A. Natural killer cell accumulation in tumors is dependent on IFN-gamma and CXCR3 ligands. Cancer research **2008**;68:8437-45

31. Hayakawa Y, Sato-Matsushita M, Takeda K, Iwakura Y, Tahara H, Irimura T. Early activation and interferon-gamma production of tumor-infiltrating mature CD27 high natural killer cells. Cancer science **2011**;102:1967-71

32. Wang L, Yi T, Kortylewski M, Pardoll DM, Zeng D, Yu H. IL-17 can promote tumor growth through an IL-6-Stat3 signaling pathway. The Journal of experimental medicine **2009**;206:1457-64

33. He D, Li H, Yusuf N, Elmets CA, Li J, Mountz JD*, et al.* IL-17 promotes tumor development through the induction of tumor promoting microenvironments at tumor sites and myeloid-derived suppressor cells. Journal of immunology **2010**;184:2281-8

34. Kimura Y, Nagai N, Tsunekawa N, Sato-Matsushita M, Yoshimoto T, Cua DJ*, et al.* IL-17A-producing CD30(+) Vdelta1 T cells drive inflammation-induced cancer progression. Cancer science **2016**;107:1206-14

35. Iwakura Y, Ishigame H, Saijo S, Nakae S. Functional specialization of

interleukin-17 family members. Immunity **2011**;34:149-62

36. Carr KD, Sieve AN, Indramohan M, Break TJ, Lee S, Berg RE. Specific depletion reveals a novel role for neutrophil-mediated protection in the liver during Listeria monocytogenes infection. European journal of immunology **2011**;41:2666-76

37. Nosaka M, Ishida Y, Kimura A, Kuninaka Y, Inui M, Mukaida N*, et al.* Absence of IFN-gamma accelerates thrombus resolution through enhanced MMP-9 and VEGF expression in mice. The Journal of clinical investigation **2011**;121:2911-20

38. Kommineni VK, Nagineni CN, William A, Detrick B, Hooks JJ. IFN-gamma acts as anti-angiogenic cytokine in the human cornea by regulating the expression of VEGF-A and sVEGF-R1. Biochemical and biophysical research communications **2008**;374:479-84

39. Longley DB, Harkin DP, Johnston PG. 5-fluorouracil: mechanisms of action and clinical strategies. Nature reviews Cancer **2003**;3:330-8

40. Moore MA, Warren DJ. Synergy of interleukin 1 and granulocyte colony-stimulating factor: in vivo stimulation of stem-cell recovery and hematopoietic regeneration following 5-fluorouracil treatment of mice. Proceedings of the National Academy of Sciences of the United States of America **1987**;84:7134-8

41. Powell DR, Huttenlocher A. Neutrophils in the Tumor Microenvironment. Trends in immunology **2016**;37:41-52

42. Shen M, Hu P, Donskov F, Wang G, Liu Q, Du J. Tumor-associated neutrophils as a new prognostic factor in cancer: a systematic review and meta-analysis. PloS one **2014**;9:e98259

43. Coffelt SB, Kersten K, Doornebal CW, Weiden J, Vrijland K, Hau CS*, et al.* IL-17-producing gammadelta T cells and neutrophils conspire to promote breast cancer metastasis. Nature **2015**;522:345-8

44. Wu HJ, Sawaya H, Binstadt B, Brickelmaier M, Blasius A, Gorelik L*, et al.* Inflammatory arthritis can be reined in by CpG-induced DC-NK cell cross talk. The Journal of experimental medicine **2007**;204:1911-22

45. Figueiredo F, Commodaro AG, de Camargo MM, Rizzo LV, Belfort R, Jr. NK1.1 cells downregulate murine endotoxin-induced uveitis following intraocular administration of interleukin-12. Scandinavian journal of immunology **2007**;66:329-34

46. Hall LJ, Murphy CT, Quinlan A, Hurley G, Shanahan F, Nally K*, et al.* Natural killer cells protect mice from DSS-induced colitis by regulating neutrophil function via the NKG2A receptor. Mucosal immunology **2013**;6:1016-26

47. Thoren FB, Riise RE, Ousback J, Della Chiesa M, Alsterholm M, Marcenaro E*, et al.* Human NK Cells induce neutrophil apoptosis via an NKp46- and Fas-dependent mechanism. Journal of immunology **2012**;188:1668-74

48. Robinet P, Baychelier F, Fontaine T, Picard C, Debre P, Vieillard V*, et al.* A polysaccharide virulence factor of a human fungal pathogen induces neutrophil apoptosis via NK cells. Journal of immunology **2014**;192:5332-42

49. Sun R, Luo J, Li D, Shu Y, Luo C, Wang SS*, et al.* Neutrophils with protumor potential could efficiently suppress tumor growth after cytokine priming and in presence of normal NK cells. Oncotarget **2014**;5:12621-34

50. Rotondo R, Barisione G, Mastracci L, Grossi F, Orengo AM, Costa R*, et al.* IL-8 induces exocytosis of arginase 1 by neutrophil polymorphonuclears in nonsmall cell lung cancer. International journal of cancer Journal international du cancer **2009**;125:887-93

51. Youn JI, Collazo M, Shalova IN, Biswas SK, Gabrilovich DI. Characterization of the nature of granulocytic myeloid-derived suppressor cells in tumor-bearing mice. Journal of leukocyte biology **2012**;91:167-81

52. Karakhanova S, Ryschich E, Mosl B, Harig S, Jager D, Schmidt J*, et al.* Prognostic and predictive value of immunological parameters for chemoradioimmunotherapy in patients with pancreatic adenocarcinoma. British journal of cancer **2015**;112:1027-36

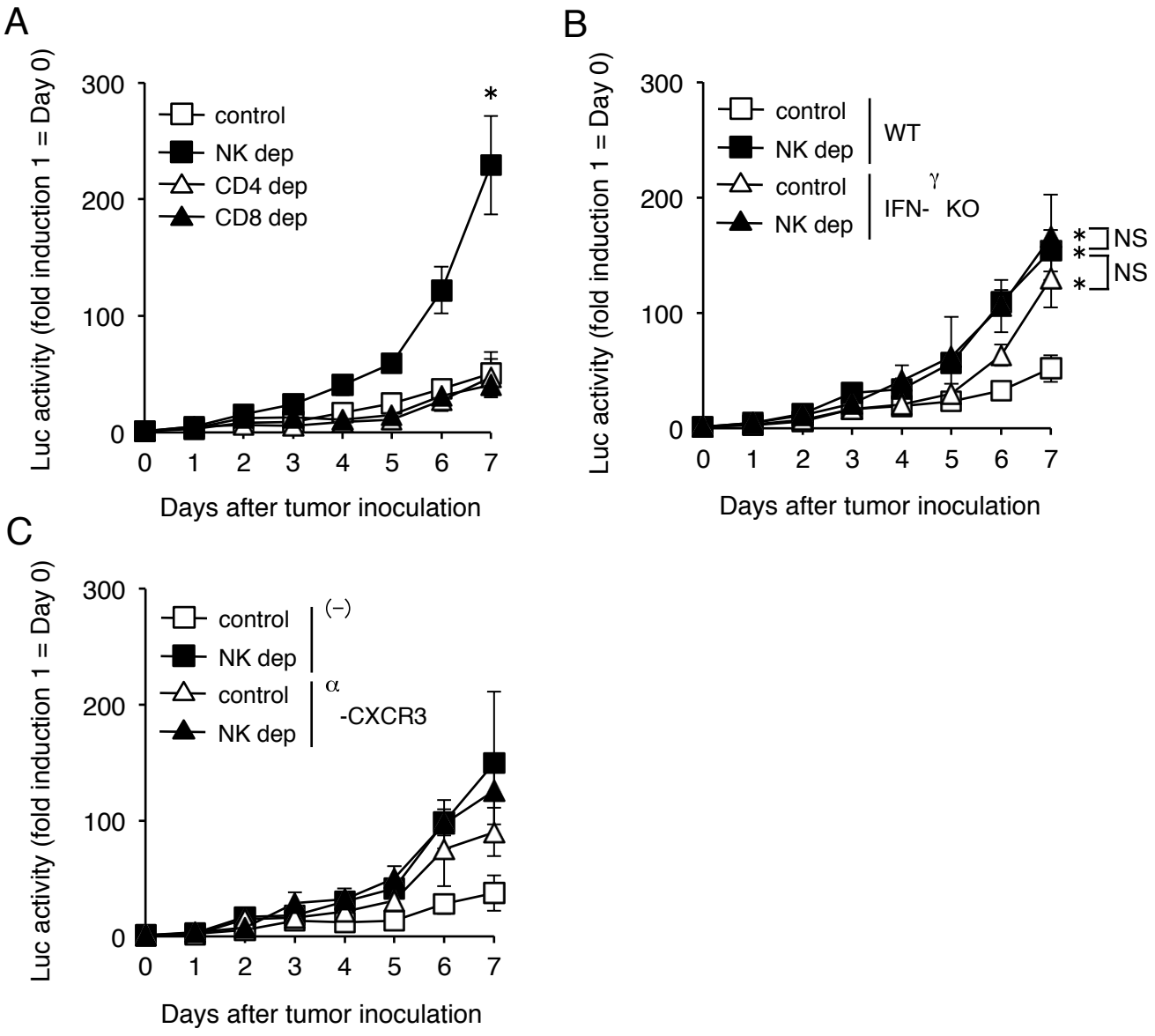


Figure 1. Tumor-infiltrating NK cells controls tumor growth in IFN-γ-dependent manner.

MCA205-Luc2 cells (10^5 cells/mouse) were s.c. inoculated into WT mice or IFN-γ KO mice. To deplete NK cells (NK dep), mice were treated with anti-asGM1 antibody (200 μg/mouse, i.p.) on Day -3 and -1 (Day 0=tumor inoculation). To deplete CD4 T cells (CD4 dep) and CD8 T cells (CD8 dep), mice were treated with anti-CD4 mAb (clone GK1.5, 250 μg/mouse, i.p.) or anti-CD8 mAb (clone 53-6.7, 250 μg/mouse, i.p.) on Day -3 and -1, respectively. To block CXCR3, mice were treated with anti-CXCR3 mAb (clone CXCR3-173, 500 *μ*g/mouse, i.p.) on Day -1, 0, 2, 4 and 6. The bioluminescence of MCA205-Luc2 tumor was monitored in WT mice (A, B, C) or IFN-γ KO mice (B). Luminescence was normalized by that of the individual mouse on Day 0. Data are presented as the mean ± SEM. *P < 0.05 compared with control mice. NS, not significant.

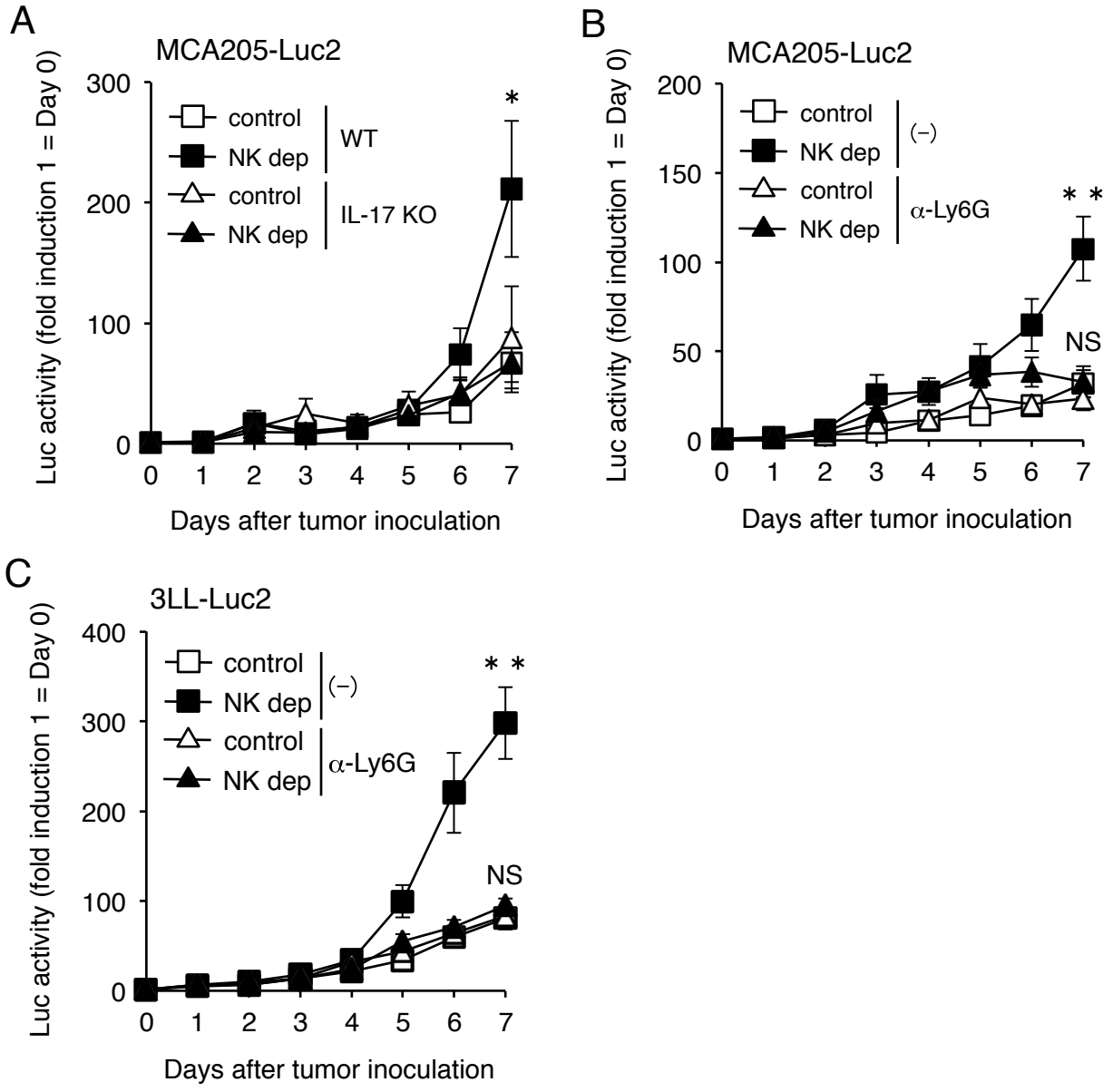


Figure 2. Requirement of IL-17A-Ly-6G$^+$ neutrophil axis for enhanced tumor growth in NK-cell depleted mice.

MCA205-Luc2 (A and B) or 3LL-Luc2 (C) cells (10^5 cells/mouse) were s.c. inoculated into WT mice or IL-17 KO mice. To deplete NK cells and/or neutrophils, mice were treated with anti-asGM1 antibody (200 μg/mouse, i.p.) on Day -3 and -1 (Day 0=tumor inoculation), and/or anti-Ly6G mAb (500 μg/mouse, i.p.) on Day -1 and 3, respectively. The bioluminescence of MCA205-Luc2 tumors was monitored. Luminescence was normalized by that of the individual mouse on Day 0. Data are presented as the mean ± SEM. *$P < 0.05$, **$P < 0.01$ compared with untreated control mice. NS, not significant.

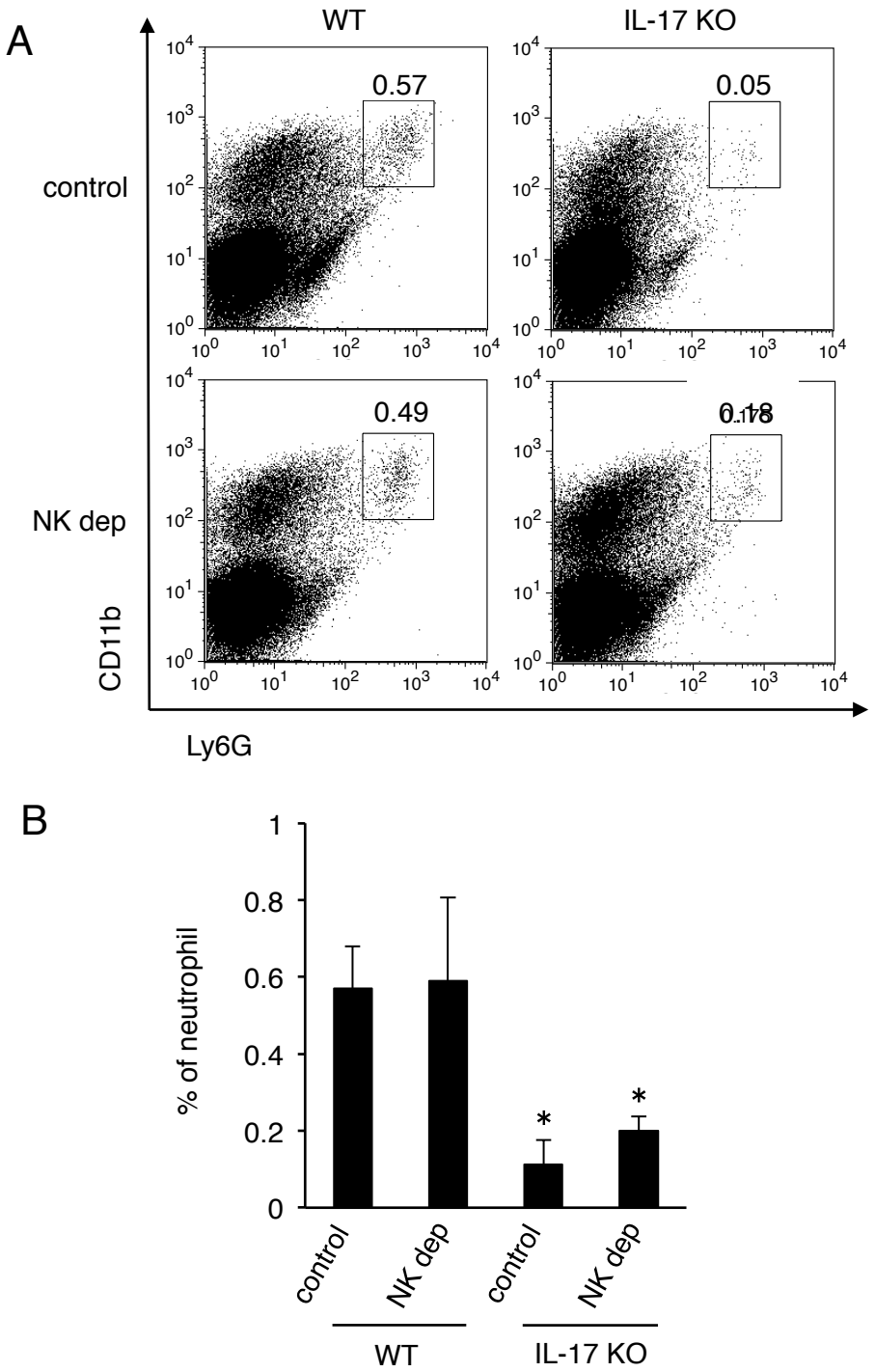


Figure 3. Impaired tumor neutrophil infiltration in IL-17-deficient, but not in NK cell-depleted mice.

MCA205-Luc2 cells (10^5 cells/mouse) were s.c. inoculated subcutaneously into WT or IL-17 KO mice. To deplete NK cells, mice were treated with anti-asGM1 antibody (200 μg/mouse, i.p.) on Day -3 and -1 (Day 0=tumor inoculation). (A) Dot plots of flow cytometry analysis are shown. The numbers are the percentage of cells electronically gated on neutrophils (CD11b+ Ly-6G+) in tumors. (B) Summary of percentage of neutrophils in tumors. Data are presented as the mean ± SEM. *$P < 0.05$ compared with WT mice.

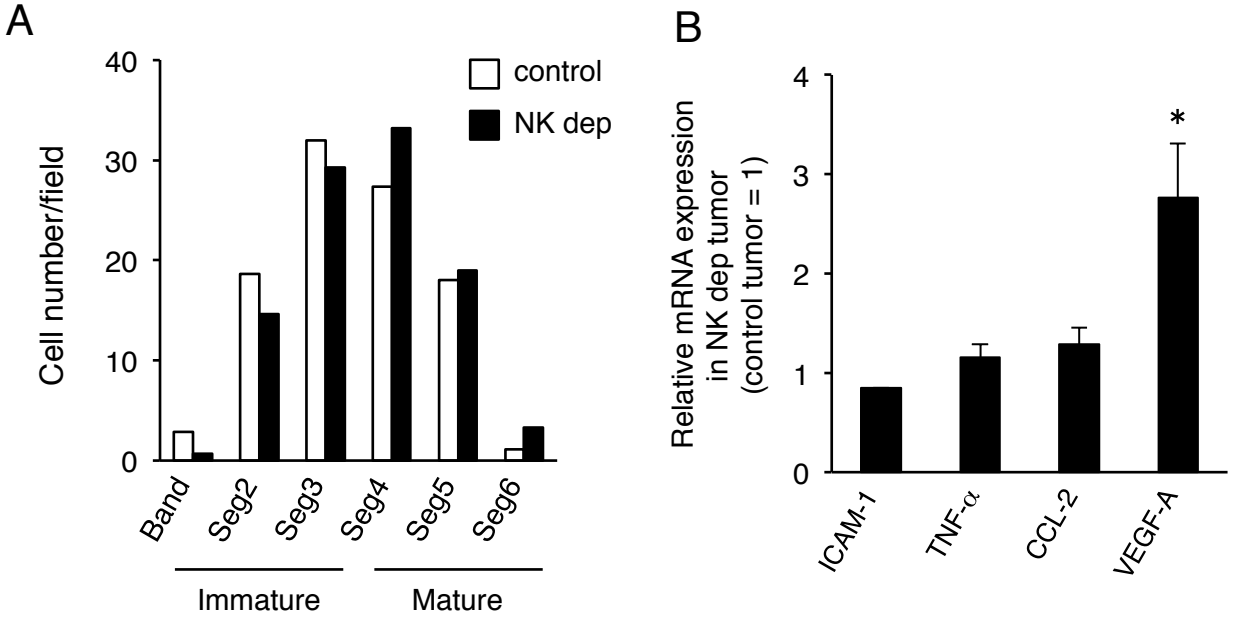


Figure 4. Characterization of tumor-infiltrating neutrophils in NK-depleted mice.

MCA205-Luc2 cells (10^5 cells/mouse) were inoculated subcutaneously into WT mice. To deplete NK cells, mice were treated with anti-asGM1 antibody (200 μg/mouse, i.p.) on Day -3 and -1 (Day 0=tumor inoculation). Tumor samples were harvested on day 7 and CD11b+ Ly-6G+ neutrophils were isolated by cell sorting. (A) Nucleus segmentation of neutrophils from control or NK cell-depleted (NK dep) mice is shown. (B) Relative mRNA expressions of neutrophils are shown. The data indicate normalized mRNA expression of neutrophils in NK cell-depleted mice to control mice. Data are presented as the mean ± SEM. *$P < 0.05$ compared with control tumor group.

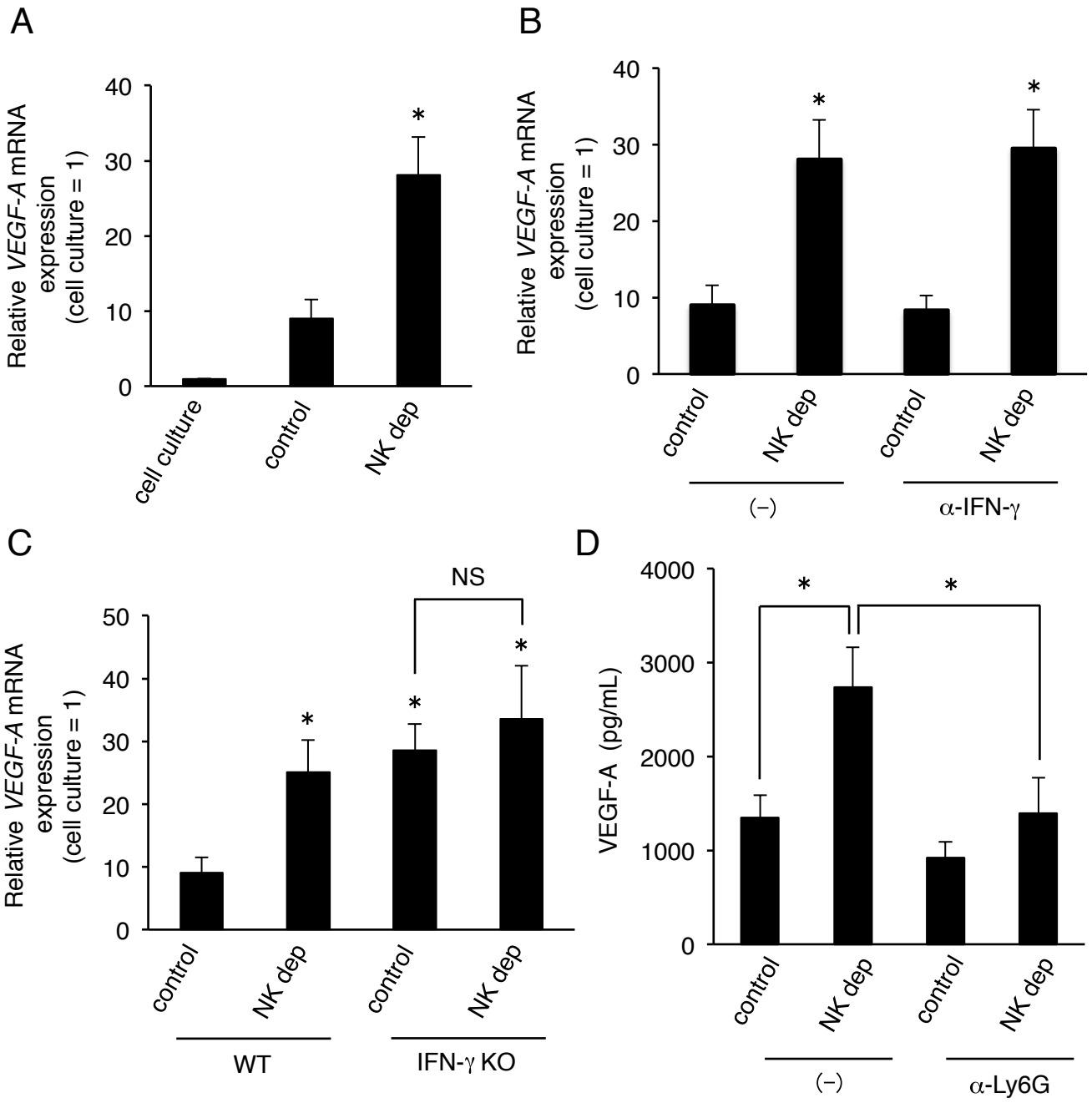


Figure 5. NK cells control angiogenic switching of Ly6G+ tumor-infiltrating neutrophils.

(A) Purified peritoneal Ly6G$^+$ neutrophils were cultured with the supernatant of MCA205-Luc2 cells (cell culture), tumors from WT mice (control), or tumors from NK cell-depleted mice (NK dep) for 6 hrs. (B) To block IFN-γ, anti-IFN-γ mAb (α-IFN-γ, 10 μg/ml) was added into the culture of peritoneal neutrophils with tumor supernatants. (C) Purified peritoneal Ly6G$^+$ neutrophils were cultured with the supernatant of control tumors or NK cell-depleted tumors (NK dep) from WT or IFN-γ KO mice for 6 hrs. Relative mRNA expressions of neutrophils are shown. The data indicate normalized mRNA expression of neutrophils cultured with MCA205-Luc2 cell supernatant. (D) MCA205-Luc2 cells (10^5) were s.c. inoculated into WT mice. Mice were treated with anti-asGM1 antibody (200 μg/mouse, i.p.) and/or treated with anti-Ly6G mAb (α-Ly6G, 500 μg/mouse, i.p.) on Day -1 and 3. Tumor samples were harvested on day 7 and cells were cultured for 24 hrs to collect the supernatant. The VEGF production was measured by ELISA. Data are presented as the mean ± SEM. *$P < 0.05$ compared with control tumor sup group. NS, not significant.

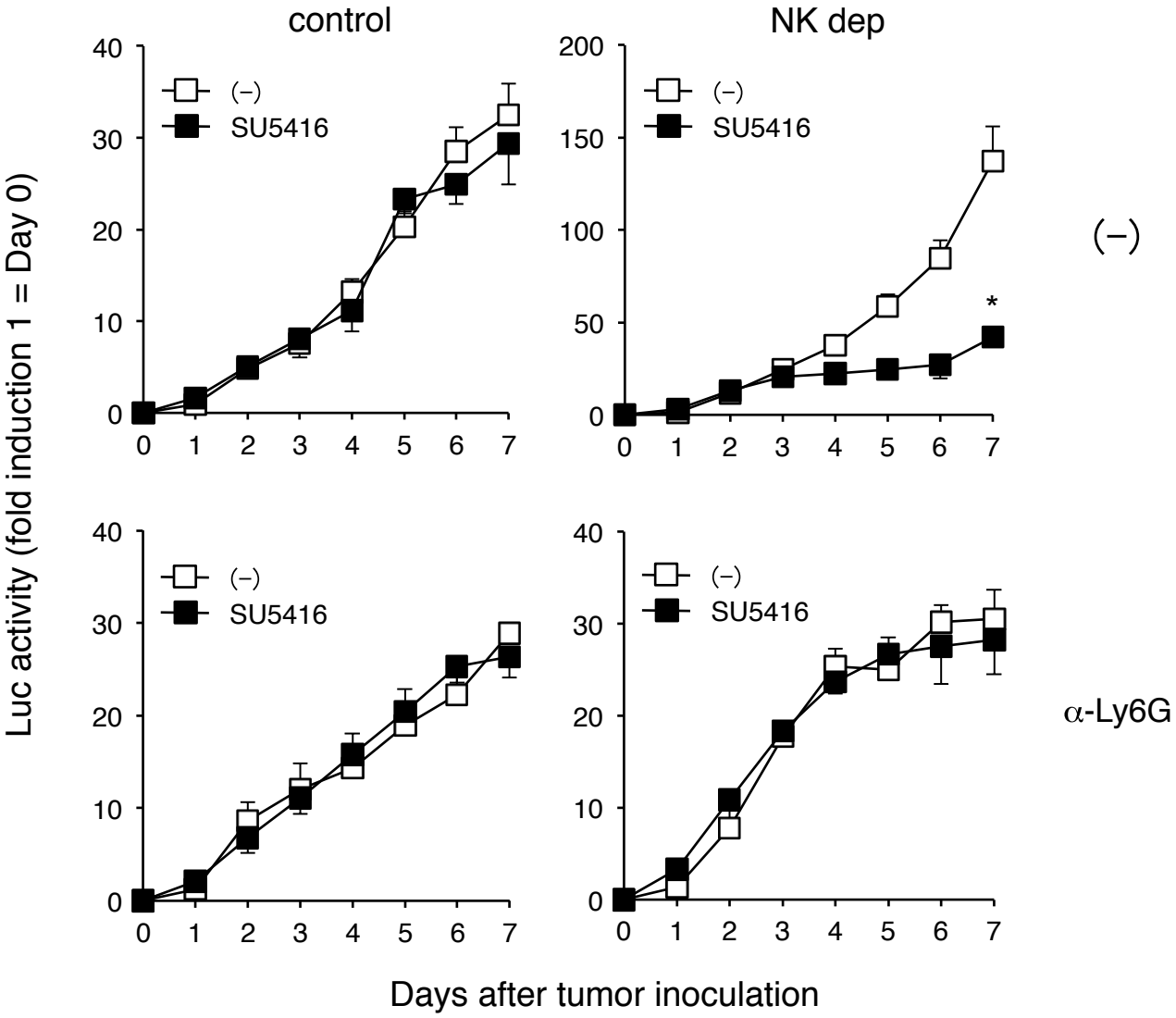


Figure 7. Ly6G$^+$ neutrophil-VEGF axis controls tumor growth in NK-cell depleted mice.

MCA205-Luc2 cells (10^5 cells/mouse) were s.c. inoculated into WT mice. To deplete NK cells and/or neutrophils, mice were treated with anti-asGM1 antibody (200 μg/mouse, i.p.) on Day -3 and -1 (Day 0=tumor inoculation), and/or anti-Ly6G mAb (α-Ly6G, 500 μg/mouse, i.p.) on Day -1 and 3, respectively. Group of mice were treated with SU5416 (30 mg/kg i.p.) for 7 days from the day of tumor inoculation. The bioluminescence of MCA205-Luc2 tumors was monitored. Luminescence was normalized by that of the individual mouse on Day 0. Data are presented as the mean ± SEM. *P < 0.05 compared with untreated group.

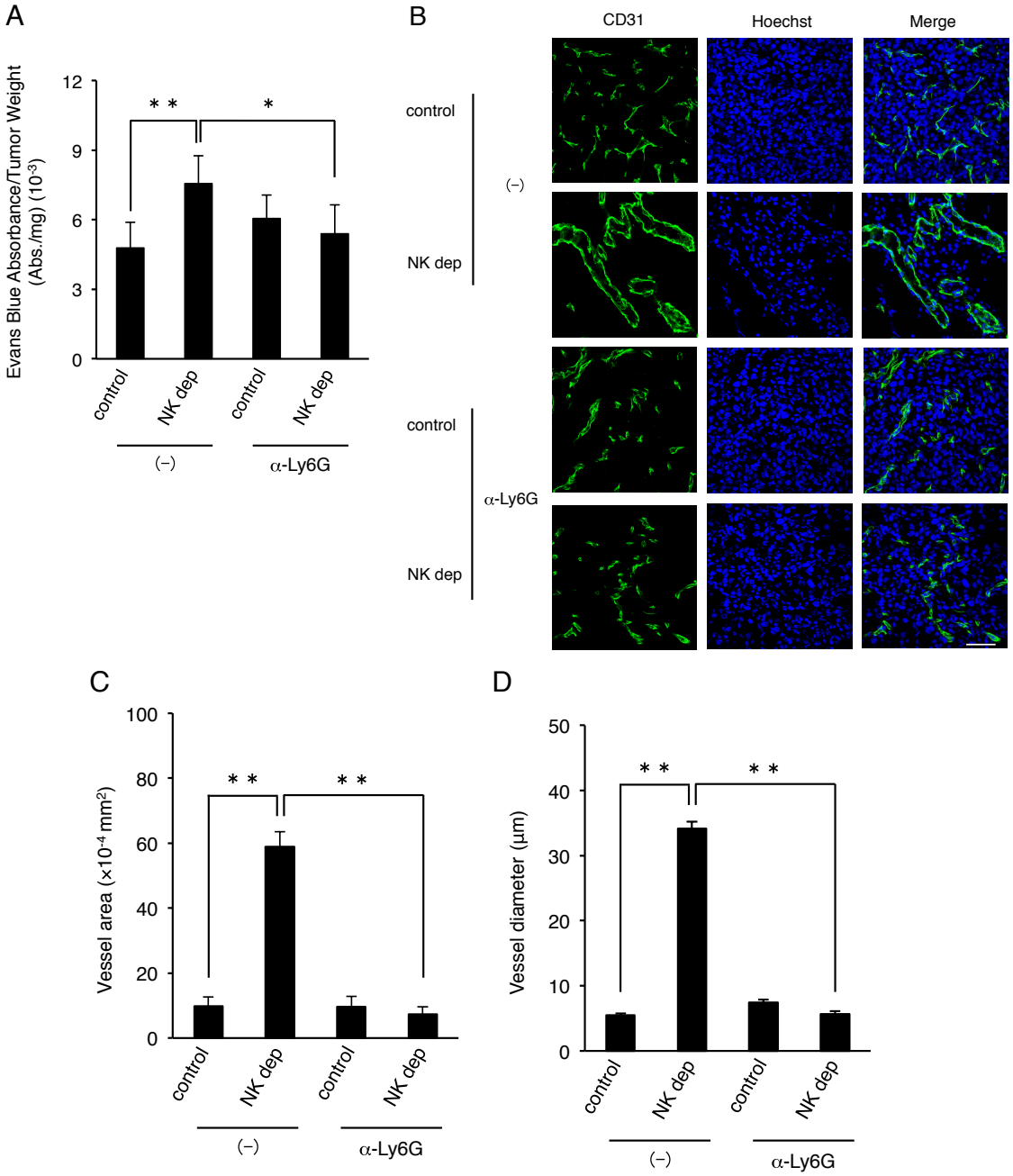


Figure 6. Requirement of Ly6G$^+$ neutrophil-dependent for enhanced tumor angiogenesis in NK-cell depleted mice.

Growth factor-free Matrigel containing MCA205-Luc2 cells (10^5) was injected subcutaneously into WT mice. To deplete NK cells and neutrophils, mice were treated with anti-asGM1 antibody (200 μg/mouse, i.p.) on Day -3 and -1 (Day 0=tumor inoculation), and/or anti-Ly6G mAb (500 μg/mouse, i.p.) on Day -1 and 3, respectively. (A) Mice were i.v. injected with 1% Evans blue 7 days after the tumor inoculation and Matrigel plugs were removed and perfused 10 minutes later, then incubated in formamide for 48 hrs. The amount of Evans blue dye eluted was quantified by measuring the absorbance at 620nm and normalized to the weight of Matrigel. (B) Immunofluorescence of CD31 (green) in tumor with nuclear staining (Hoechst, blue). Scale bar indicates 50 μm. (C) Morphometric analysis of angiogenesis in tumor tissue. (D) Quantitative analysis of blood vessel areas and diameters are shown. Data are presented as the mean ± SEM. *P < 0.05, **P < 0.01.

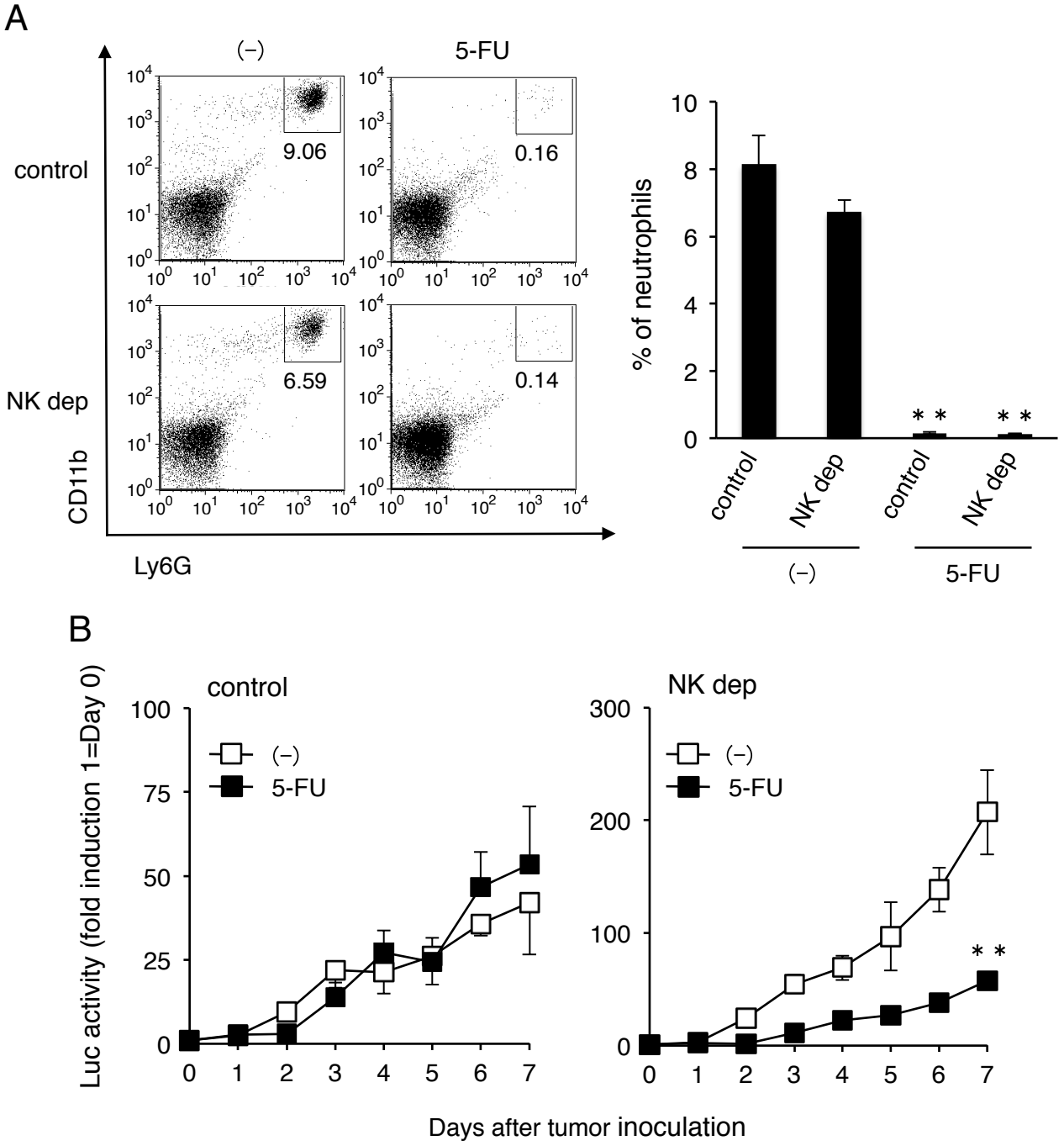
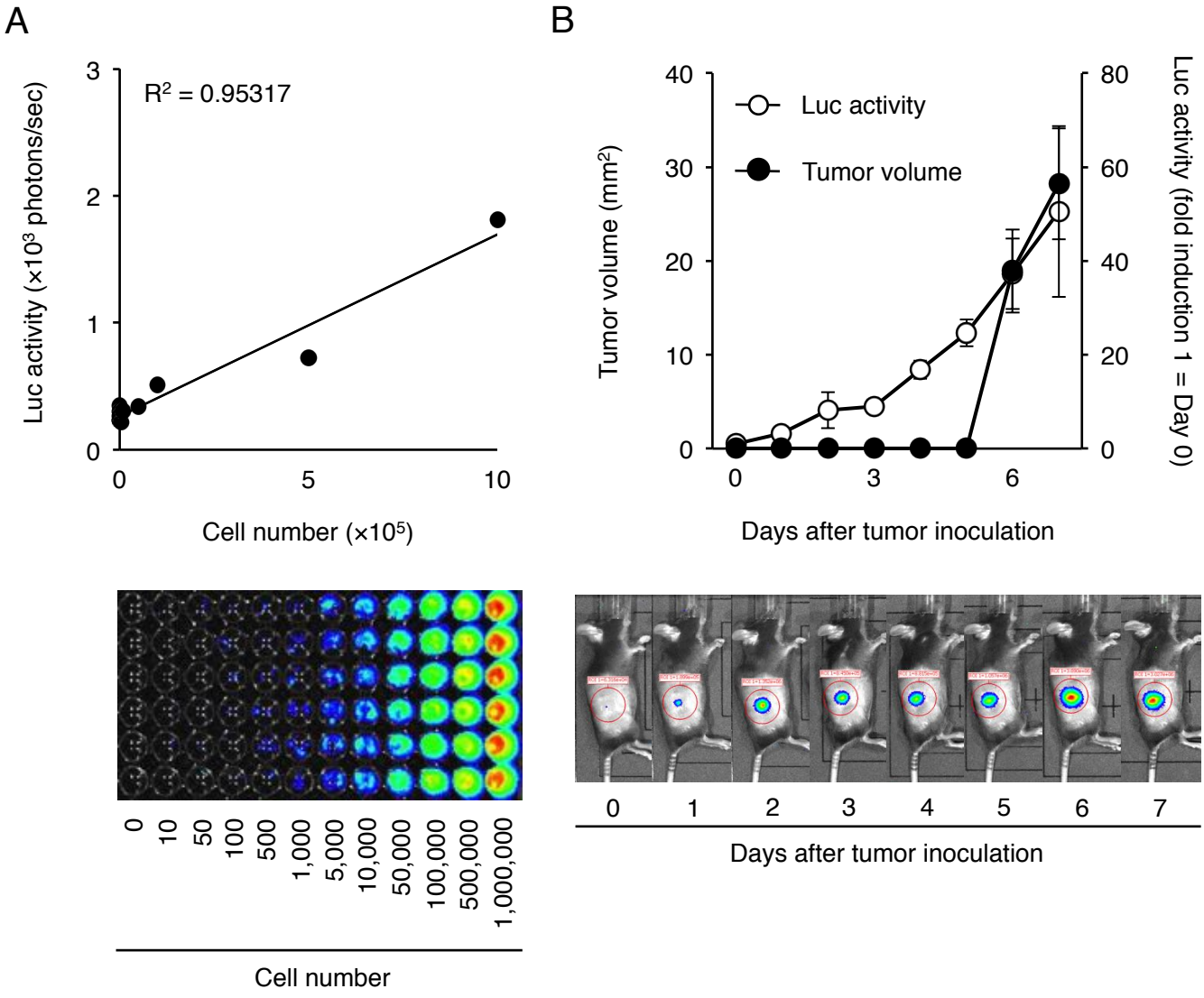


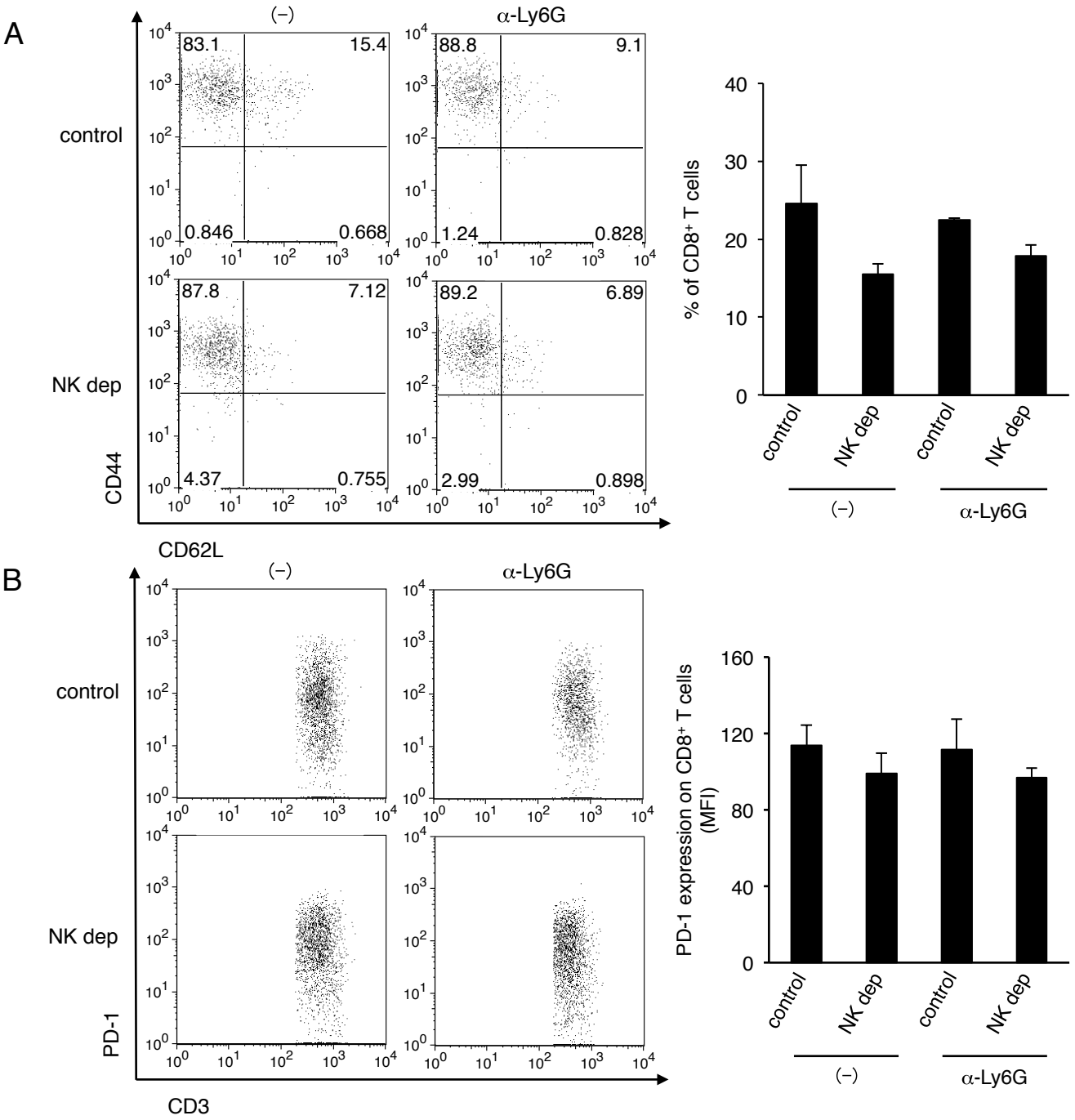
Figure 8. 5-FU-induced systemic neutropenia controls cancer cell proliferation in NK-cell depleted mice.

MCA205-Luc2 cells (10^5 cells) were s.c. inoculated in WT mice (control) or NK cell-depleted mice (NK dep). Mice were treated with anti-asGM1 antibody (200 μg/mouse, i.p.) on Day -3 and -1 (Day 0=tumor inoculation) and/or 5-FU (150 mg/kg, i.v.) on Day 0. (A) Peripheral blood mononuclear cells (PBMCs) were collected on day 7 and the dot plots (left) and summary (right) of flow cytometry analysis are shown. The numbers are the percentage of cells electronically gated on neutrophils (CD11b+ Ly-6G+) in PBMCs. (B) The bioluminescence of MCA205-Luc2 tumors was monitored. Luminescence was normalized by that of the individual mouse on Day 0. Data are presented as the mean ± SEM. ** $P < 0.01$ compared with 5-FU untreated mice.



Supplementary Figure 1. Characterization of MCA205-Luc2 cells.

(A) The graphs are plotted as luminescence (photons/second) against cell number of MCA205-Luc2 cells, and the linear correlation of the plots is shown as R2 (upper panel). MCA205-Luc2 cells were plated at the indicated cell number and luminescent images are shown (lower panel). (B) MCA205-Luc2 cells (10^5 cells) were s.c. inoculated into syngeneic C57BL/6 mice. Tumor growth monitored by bioluminescent imaging (open circle) or conventional caliper measurement (closed circle) is shown (upper panel). Images of bioluminescence on Day 0 to 5 after tumor inoculation are shown (lower panel). Data are represented as the mean ± SEM.



Supplementary Figure 2. No alteration in the phenotype of tumor-infiltrated CTL.

MCA205-Luc2 cells (10^5) were s.c. inoculated into syngeneic C57BL/6 mice. Mice were treated with anti-asGM1 antibody (200 μg/mouse, i.p.) on Day -3 and -1 (Day 0=tumor inoculation) and/or treated with anti-Ly6G mAb (500 μg/mouse, i.p.) on Day -1 and 3. On Day 7, tumor-infiltrating lymphocytes were isolated and subjected to flow cytometry analysis. Representative dot plots of the expressions of CD44 and CD62L (A) or PD-1 (B) on electronically gated $CD3^+$ $CD8^+$ T cells in tumors are shown. The presented graphs show (A) the proportion of CD8 T cells and (B) the PD-1 expression on $CD8^+$ T ($NK1.1^-$ $CD3^+$ $CD8^+$) cells in tumor.