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An inducible model for specific neutrophil depletion by diphtheria toxin in mice

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Neutrophils are crucial for immunity and play important roles in inflammatory diseases; however, mouse models selectively deficient in neutrophils are limited, and neutrophil-specific diphtheria toxin (DT)-based depletion system has not yet been established. In this study, we generated a novel knock-in mouse model expressing diphtheria toxin receptor (DTR) under control of the endogenous Ly6G promoter. We showed that DTR expression was restricted to $Ly6G^+$ neutrophils and complete depletion of neutrophils could be achieved by DT treatment at 24–48 h intervals. We characterized the effects of specific neutrophil depletion in mice at steady-state, with acute inflammation and during tumor growth. Our study presents a valuable new tool to study the roles of neutrophils in the immune system and during tumor progression.

Ly6G, DTR, neutrophil depletion, tumor

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INTRODUCTION

Neutrophils are the most abundant circulating immune cells in humans; however, there are few genetic models to study the specific roles of neutrophils. Mouse models expressing DTR in regulatory T cells and dendritic cells have been used extensively to dissect the functions of these cells in disease models, but such models targeting neutrophils are currently lacking (Kim et al., 2007; Kissenpfennig et al., 2005; Probst et al., 2005). To date, most studies using neutrophil depletion have been performed using anti-Gr1 or anti-Ly6G antibody treatment (Daley et al., 2008; Hickey, 2012; Moses et al., 2016). However, use of depleting antibodies has several limitations, including high cost and adverse effects associated with antibody-dependent cellular cytotoxicity (ADCC). In addition, FACS-based analysis of neutrophils in antibody-treated mice is not feasible due to the lack of independent antibody clones to label these cells (Yipp and Kubes, 2013). In this study, we generated a knock-in mouse model by expressing human HBEGF, a high affinity receptor for diphtheria toxin receptor (DTR) under control of the endogenous promoter for Ly6G. DTR was expressed via T2A link to the endogenous Ly6G. As observed by FACS, DTR expression was stringently restricted to Ly6G-positive cells and was absent in the other cell types. In our experi-

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ments, Ly6G-DTR heterozygous mice had efficient and thorough depletion of neutrophils following DT treatment. Our data showed that neutrophils were completely absent 24 h after DT treatment, and partially recovered after 48 h. However, the neutrophil numbers did not fully recover to normal levels until 3 days after DT treatment. Using this model for inducible neutrophil depletion, we observed that the absence of neutrophils was not associated with a significant declination in circulating and splenic NK cells. We next grafted neutrophil-depleted mice with Yumm3.3 melanoma cells, to assess the impact on tumor growth. Tumor mass was significantly increased in DT-treated Ly6G-DTR mice, compared with DT-treated WT controls; however, NK cell numbers remained unchanged in tumors. Interestingly, using this model we observed tumor infiltrating $CD25^+$ T cell differentiation was facilitated by neutrophils, as depletion of neutrophils resulted in a significant drop in $CD25^+$ T cells in tumors. In addition to test application of this Ly6G-DTR model, we treated mice first with DT and induced acute lung inflammation in both Ly6G-DTR and wildtype controls. The lung inflammation experiments showed that neutrophil accumulation occurred a few hours following LPS challenge, and such neutrophils were completely absent in the Ly6G-DTR model. Our study presents a novel neutrophil depletion model using the highly sensitive DTR system and reveals an important role for neutrophils in tumor growth, and such a model could also be used to study inflammation.

RESULTS

Ly6G driven expression of human HBEGF by CRISPR/Cas9-mediated knock-in

Diphtheria toxin has high-affinity binding to heparin-binding EGF-like growth factor, which results in potent apoptosis. Human HBEGF is expressed in mouse models as a DT receptor (DTR) for inducible depletion of cells *in vivo* (Kissenpfennig et al., 2005). In a previous model, DTR has been expressed under control of the endogenous promoter for Lat expressed by T cells (Mingueneau et al., 2009), administration of DT at 20 ng per gram body weight resulted in a dramatic depletion of T cells in blood 12 h after DT treatment, and a complete absence of T cells by 24 h (Figure S1A in Supporting Information). In this study, we first validated that the DT treatment did not significantly impact immune cells in peripheral blood of wildtype mice. FACS analyses performed 24 h after DT treatment showed that neutrophils as well as T and B lymphocytes were not affected in wildtype mice after DT treatment, when compared to PBS-treated controls (Figure S1B in Supporting Information). Ly6G is a highly specific marker for mature neutrophils. To obtain DTR knock-in at the Ly6G locus, we induced double-strand break of DNA by CRISPR/Cas9 at the Ly6G locus upstream of the stop codon and inserted the T2A-DTR coding sequence. The founder mice with the knock-in (KI) allele were screened by PCR and validated by sequencing (Figure S2 in Supporting Information, data not shown). Since DTR expressing cells are very sensitive to DT treatment, we aimed to obtain heterozygous mice with one allele of Ly6G-DTR KI. In such heterozygous Ly6G-DTR KI mice, we found that DTR expression was detectable by FACS when cells from peripheral blood were stained with anti-DTR antibody. Notably, DTR expression was only found in $Ly6G^+$ neutrophils (Figure 1A and B). Therefore, we obtained a Ly6G-DTR knock-in line specifically expressing DTR in neutrophils.

Ly6G-DTR mice have normal development of neutrophils

Even though Ly6G is a highly specific marker for neutrophils, previous studies found that Ly6G itself did not impact neutrophil development (Hasenberg et al., 2015). In further experiments we validated by FACS analyses the numbers of neutrophils in the peripheral blood and spleen of the Ly6G-DTR mice. In peripheral blood, we found that neutrophil development was normal in Ly6G-DTR mice when compared to wildtype B6 mice as were T and B lymphocytes, as expected (Figure 2A and B). In the spleen, Ly6G-DTR KI mice also had normal counts of total white blood cells, neutrophils and lymphocytes (Figure 2C and D). In the bone marrow, the total white blood cells, neutrophils, lymphocytes as well as SSC^{low} $CD11b^+$ myeloid cells mainly composing macrophages and monocytes were equivalent in numbers between Ly6G-DTR KI mice and the wildtype B6 mice. In lymph nodes, we did not observe significant differences between Ly6G-DTR KI mice and their controls (Figure S3 in Supporting Information). It is also important to note that Ly6G-DTR KI mice still allowed for labeling of Ly6G to track neutrophils, as shown in our experiments that Ly6G could be readily detected by FACS.

Efficient neutrophil depletion upon DT treatment in Ly6G-DTR mice

To deplete neutrophils, we injected a single dose of DT (20 ng g^{-1} body weight) in Ly6G-DTR mice, and blood analyses were performed at 24 h intervals after DT treatment. As expected, we saw complete absence of neutrophils 24 h after DT injection (Figure 3A and B). Interestingly, the kinetics of neutrophil recovery after a single dose of DT showed that circulating neutrophils were partially restored 48 h after DT treatment and reached normal levels by day 5 (Figure 3B). In absolute count, the neutrophils in circulation reached normal levels by day 4 following DT treatment (Figure 3C). Interestingly, after DT treatment we found that during recovery of neutrophils in Ly6G-DTR mice, Ly6G

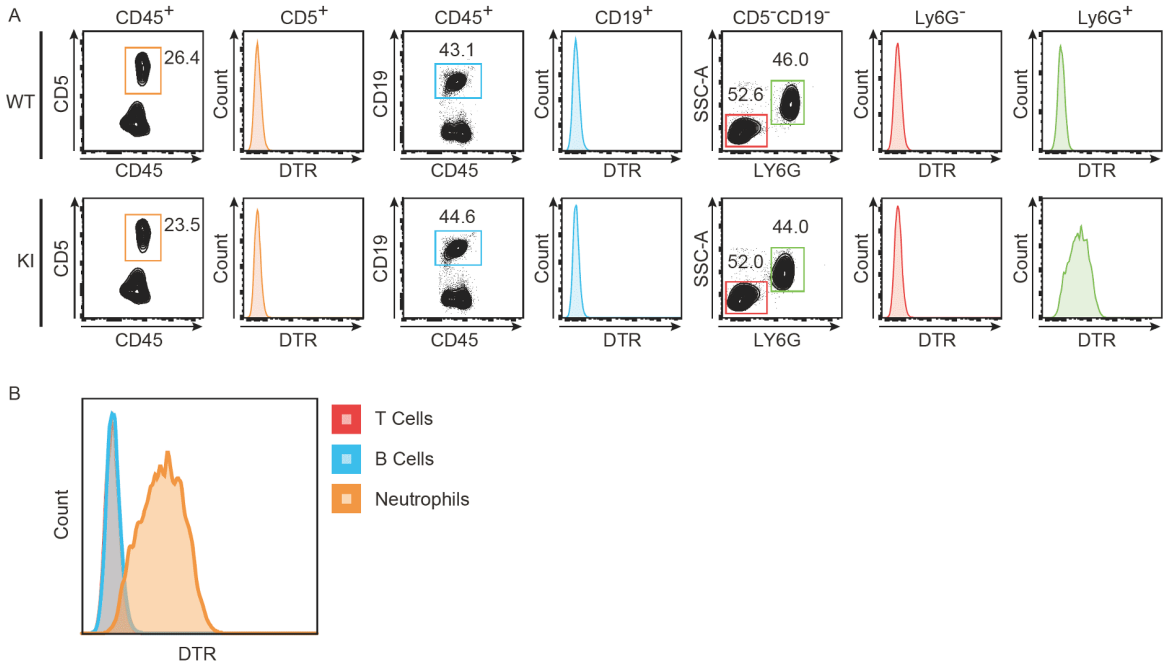

Figure 1 Expression of DTR in Ly6G-DTR knock-in mice. A, $CD45^+$ cells were white blood cells; T/B lymphocytes were gated by CD5 and CD19 expression; neutrophils were gated on $Ly6G^+$ and SSC^{high} cells out of $CD5^-CD19^-$ population. Histograms of T cells, B cells and neutrophils for DTR expression in wildtype mice (WT) and Ly6G-DTR knock-in (KI) mice were representative ones from three independent experiments involving six mice aged 8–10 weeks for each genotype. B, Histogram overlay of T cells, B cells and $Ly6G^+$ neutrophils for comparison of DTR expression using blood cells.

surface expression as measured by mean fluorescence intensity was significantly lower during the recovery phase, which suggests that Ly6G could be a maturation marker for neutrophils (Figure 3D). In parallel, we also treated B6 wildtype mice with the same dosage of DT, and observed no significant fluctuation in neutrophil numbers. Even though neutrophils in heterozygous Ly6G-DTR mice could be readily labeled by anti-Ly6G antibody, and such cells are extremely sensitive to DT treatment, we observed that Ly6G surface expression in wildtype B6 was higher than that in heterozygous Ly6G-DTR mice (Figure 3D). These data showed that DT treatment in Ly6G-DTR heterozygous mice led to very efficient neutrophil depletion for up to 48 h. Therefore, DT treatment at 24–48 h intervals in Ly6G-DTR mice maintains efficient and specific depletion of neutrophils.

Validation of neutrophil depletion in acute inflammation and cellular crosstalk using Ly6G-DTR mice

Neutrophils are among the most immediate responders to inflammatory stimuli. To model neutrophil absence in acute inflammation, we treated B6 wildtype mice and Ly6G-DTR mice with DT for 24 h (Figure 4A). After DT treatment, the mice were subjected to i.p. injection of LPS for 6 h to induce acute lung inflammation with PBS-treated controls (Wang et al., 2019). By making single cell suspension from lungs, we analyzed alveolar macrophages, eosinophils and neutrophils in lungs, and found that neutrophil depletion was complete in lungs of Ly6G-DTR mice (Figure 4B). After DT treatment, the alveolar macrophages and eosinophils remained comparable between B6 wildtype and Ly6G-DTR mice in both LPS group and PBS group (Figure 4C). However, LPS induced surge of neutrophil accumulation in lungs of DT-treated B6 wildtype mice, but not Ly6G-DTR mice (Figure 4C and D). Such results indicated that Ly6G-DTR mice extremely sensitive to DT treatment could be used in study of acute inflammation in lungs.

Neutrophils are themselves essential for immunity against infection, but they may also interact with the other types of immune cells. As shown in previous study using both a genetic model deficient in transcription factor Gfi1 and antibody-mediated depletion of neutrophils *in vivo*, which described decreased NK cell number and function was associated with neutropenia (Jaeger et al., 2012; Ordoñez-Rueda et al., 2012). Therefore, we assessed the impact of neutrophil depletion on NK cells in our independent Ly6G-DTR mouse model. As we saw a minute amount of Ly6G low neutrophils in the blood 48 h after DT, to deplete the mice completely with neutrophils, we injected DT every 24 h which rendered absence of neutrophils for 7 days, and FACS analyses of blood were performed at different time points (Figure 4E). By comparing DT-treated B6 wildtype mice and Ly6G-DTR mice, as shown in Figure 4F, even though neutrophils were completely depleted for 7 days, we did not find a significant declination of NK cell numbers in the blood.

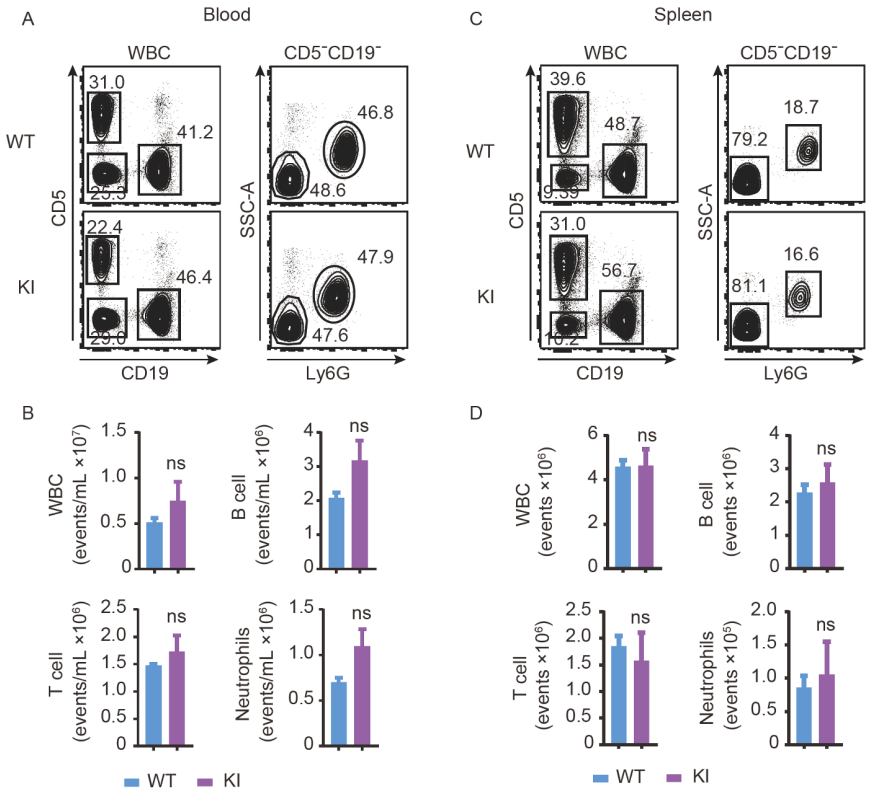

Figure 2 Characterization of Ly6G-DTR mice without DT treatment by FACS. A and B, Blood cell analyses by FACS using CD19 as B cell marker, and $CD5^+CD19^-$ cells were T cells. Neutrophils were a $Ly6G^+SSC^{high}$ population gated on CD5 and CD19 negative cells. C and D, Analyses of splenocytes of Ly6G-DTR mice by FACS using CD19 as B cell marker, and $CD5^+CD19^-$ cells were T cells. Neutrophils were a $Ly6G^+SSC^{high}$ population gated on CD5 and CD19 negative cells. Each group C57BL/6 control (WT) or Ly6G-DTR (KI) involved six mice. Data were analyzed by two-tailed Student's *t*-test and presented as mean±SEM, and ns stands for no statistical significance.

Such comparison was also made for splenocytes, and we did not find significant declination of NK cells in number as a result of neutrophil depletion in the spleen (Figure 4G). These results indicated that Ly6G-DTR mice are a valuable model to study neutrophil function and interactions between neutrophils and other immune cells.

Increased tumor growth in neutrophil-depleted Ly6G-DTR mice

Neutrophils have been shown to infiltrate tumors in experimental models; however, their specific roles in shaping the tumor microenvironment are poorly studied. We applied the Ly6G-DTR model to analyze how neutrophil deficiency could affect tumor growth *in vivo*. Yumm3.3 melanoma cells were transplanted subcutaneously into isogenic C57BL6 and Ly6G-DTR mice. Tumor growth was monitored, and immunophenotyping of tumor-infiltrating immune cells was performed by FACS. Following tumor engraftment, DT was injected every other day from day 9 to day 13, and tumors and the spleen were harvested and analyzed at day 14 (Figure 5A). Firstly, we observed that the tumor weights were significantly higher in neutrophil-depleted mice (Figure 5B and C). As expected, DT treatment depleted neutrophils in tumors from Ly6G-DTR mice that were grafted with Yumm3.3 cells (Figure 5D). We compared tumor-infiltrating T cells (TIL) in DT-treated Ly6G-DTR mice to control mice and found that the total numbers of both CD4 and CD8 T cells in tumors were not significantly changed (Figure 5E). However, there was a striking decrease in the percentage of CD25-expressing CD4 T cells tumors from DT-treated Ly6G-DTR mice in comparison to DT-treated WT controls (Figure 5F). In addition, we analyzed NK cells in tumors, but did not find any significant change in their numbers when neutrophils were depleted by DT treatment (Figure 5G). Furthermore, in the spleen from DT-treated Ly6G-DTR mice, we did not observe the same drop in CD25-expressing T cells (data not shown). Our results from short-term neutrophil depletion in Ly6G-DTR mice suggest that neutrophils could exert an anti-tumor function, and their presence was correlated with $CD25^+$ CD4 T cell accumulation in tumors.

DISCUSSION

Neutrophils are the foremost defense against microbial infection, but animal models allowing for highly specific depletion of these cells are still limited (Deniset and Kubes,

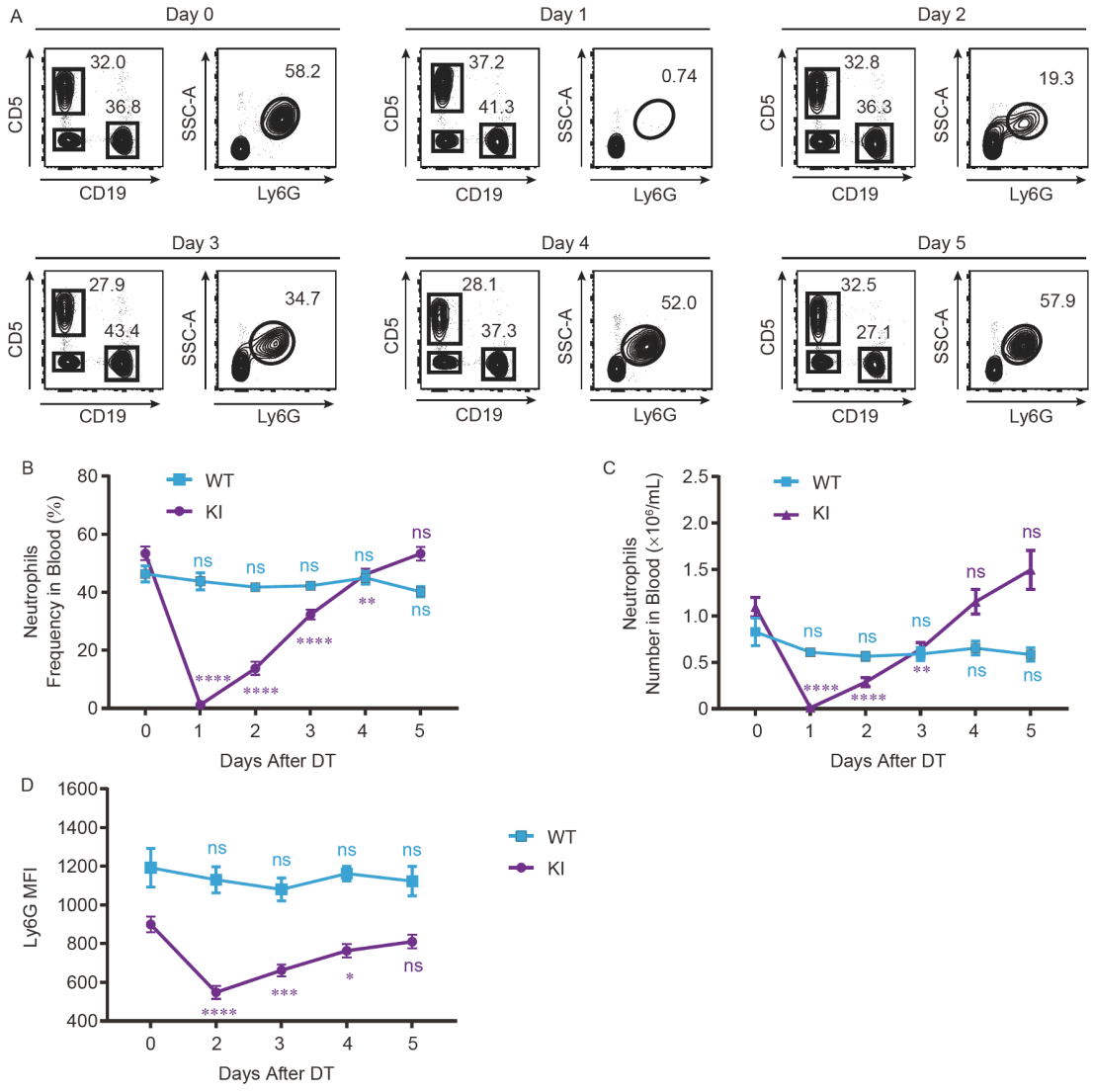

Figure 3 Depletion of neutrophils in Ly6G-DTR mice. A, Gating of neutrophils in the peripheral blood of Ly6G-DTR mice after a single dose of DT treatment. A single dose of DT (20 ng g^{-1} body weight) was injected intra-peritoneally at day 0 before blood test, and blood tests by FACS were performed consecutively for 5 days. Representative contour plots were used to assess neutrophils as a $Ly6G^{+}SSC^{high}$ population which was gated from CD5 and CD19 negative cells. B, Frequencies of neutrophils in the blood after DT treatment in Ly6G-DTR mice with C57BL/6 mice (WT) controls. C, Absolute count of neutrophils in the blood after DT treatment in Ly6G-DTR mice with C57BL/6 mice (WT) controls. D, Expression level of Ly6G measured by mean fluorescence intensity in blood neutrophils in Ly6G-DTR mice with C57BL/6 mice (WT) controls. Each time point involved six samples for each genotype and the data of B, C and D were from the same batch of animals, and DT-treated C57BL/6 mice (WT) were used as controls for statistic comparisons. Data were analyzed by two-tailed Student's *t*-test and presented as mean±SEM, ns for no statistical significance, $*P<0.05$, $**P<0.01$, $***P<0.001$, $****P<0.0001$.

2016). Diphtheria toxin-mediated cellular depletion is highly sensitive, and careful selection of specific promoters driving expression of the DT receptor is essential to achieve cell type-specific depletion (Buch et al., 2005). Ly6G is a commonly used marker for mouse neutrophils and the monoclonal antibody 1A8 against this surface molecule has higher specificity in labeling neutrophils than the previously used anti-Gr1 antibody, which recognizes both Ly6G and Ly6C (Bruhn et al., 2016; Wojtasiak et al., 2010). DT treatment does not noticeably deplete any specific immune cells in wildtype mice; however, doses of DT as low as a few ng per gram of body weight can completely and specifically remove DTR-expressing cells, as shown in previous studies (Bar-On and Jung, 2010). Therefore, cost effective and thorough depletion of neutrophils could be achieved if DTR was expressed under a lineage-specific gene such as Ly6G in mice. Therefore, using fertilized eggs of B6 mice we performed CRISPR/Cas9-mediated DNA double-strand break at the Ly6G locus, followed by homologous recombination of the template DNA which included T2A splice linker and DTR coding sequences. Such a construct resulted in highly specific expression of DTR in neutrophils, and only the Ly6G-positive cells were found expressing DTR in the heterozygous knock-in mice. Even though the homozygous knock-

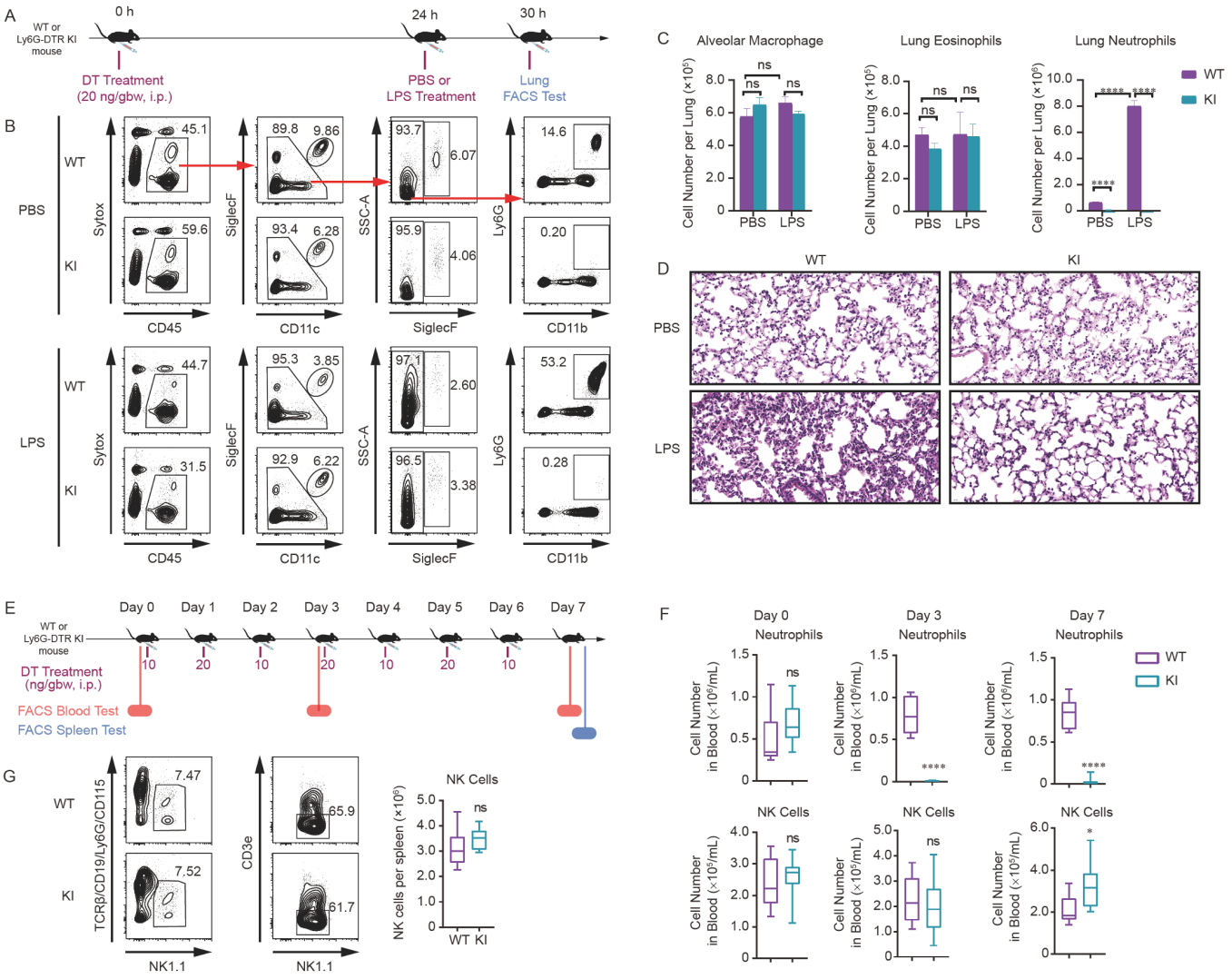

Figure 4 Neutrophil depletion in inflamed lungs and NK cell phenotyping in neutrophil-depleted Ly6G-DTR mice. A, Overview of the experiment to analyze LPS challenged lungs in DT-treated C57BL/6 (WT) and Ly6G-DTR mice. Both C57BL/6 (WT) mice and Ly6G-DTR mice were treated with DT at 20 ng g^{-1} body weight. 24 h after DT treatment, PBS or LPS (12.5 mg kg^{-1} body weight) was i.p. injected, and lungs were analyzed 6 h after LPS challenge. B, Gating of alveolar macrophages, eosinophils, and neutrophils in lungs of C57BL/6 (WT) mice and Ly6G-DTR mice after DT treatment, followed by PBS or LPS injection. C, Statistical comparisons of alveolar macrophages, eosinophils, and neutrophils in lungs of C57BL/6 (WT) mice and Ly6G-DTR mice. Six mice were used in each group. D, Staining of lung sections from C57BL/6 (WT) mice and Ly6G-DTR mice that were treated with DT, followed by i.p. injection of PBS or LPS (H&E staining, 400× total magnification). E, Overview of the experiment to analyze neutrophil deficiency and NK cell phenotype in C57BL/6 (WT) and Ly6G-DTR mice receiving 7 days of DT treatment consecutively. Mice were treated with alternative dosages of 10 ng and 20 ng g^{-1} body weight in each group of mice. FACS analyses of the blood and splenocytes were indicated. F, Neutrophil and NK cell counts at day 0, 3 and 7 in the blood of C57BL/6 (WT) and Ly6G-DTR mice. G, NK cell gating in splenocytes and absolute number of NK cells in the spleen of DT-treated C57BL/6 (WT) mice and Ly6G-DTR mice. Each group of mice involved six animals. Data were analyzed by two-tailed Student's *t*-test and presented as mean±SEM, ns for no statistical significance, $*P<0.05$, $****P<0.0001$.

in mice had undermined expression of surface Ly6G, the heterozygotes were sufficient for detection of Ly6G and DTR expression. More importantly, the heterozygous knock-in mice allowed for complete depletion of neutrophils following DT treatment. Therefore, we succeeded in obtaining an independent model for neutropenia studies. Using the Ly6G-DTR model, we found that neutrophil accumulation during LPS-induced acute inflammation was completely absent, while the other myeloid lineages such as alveolar macrophages and eosinophils in the lungs remained unchanged. Therefore, this new model provided a valuable tool for efficient and specific depletion of neutrophils for inflammation studies. Intriguingly, our results from DT-treated Ly6G-DTR mice showed that both in the spleen and tumor tissue, neutrophil depletion was not associated with a drastic phenotypic change in NK cells, suggesting it may be necessary to study the impact of neutrophils on NK cells during a longer term of neutropenia, and to assess the possibility of under-investigated mechanisms involved in the interactions between neutrophils and NK cells. It is also important to note

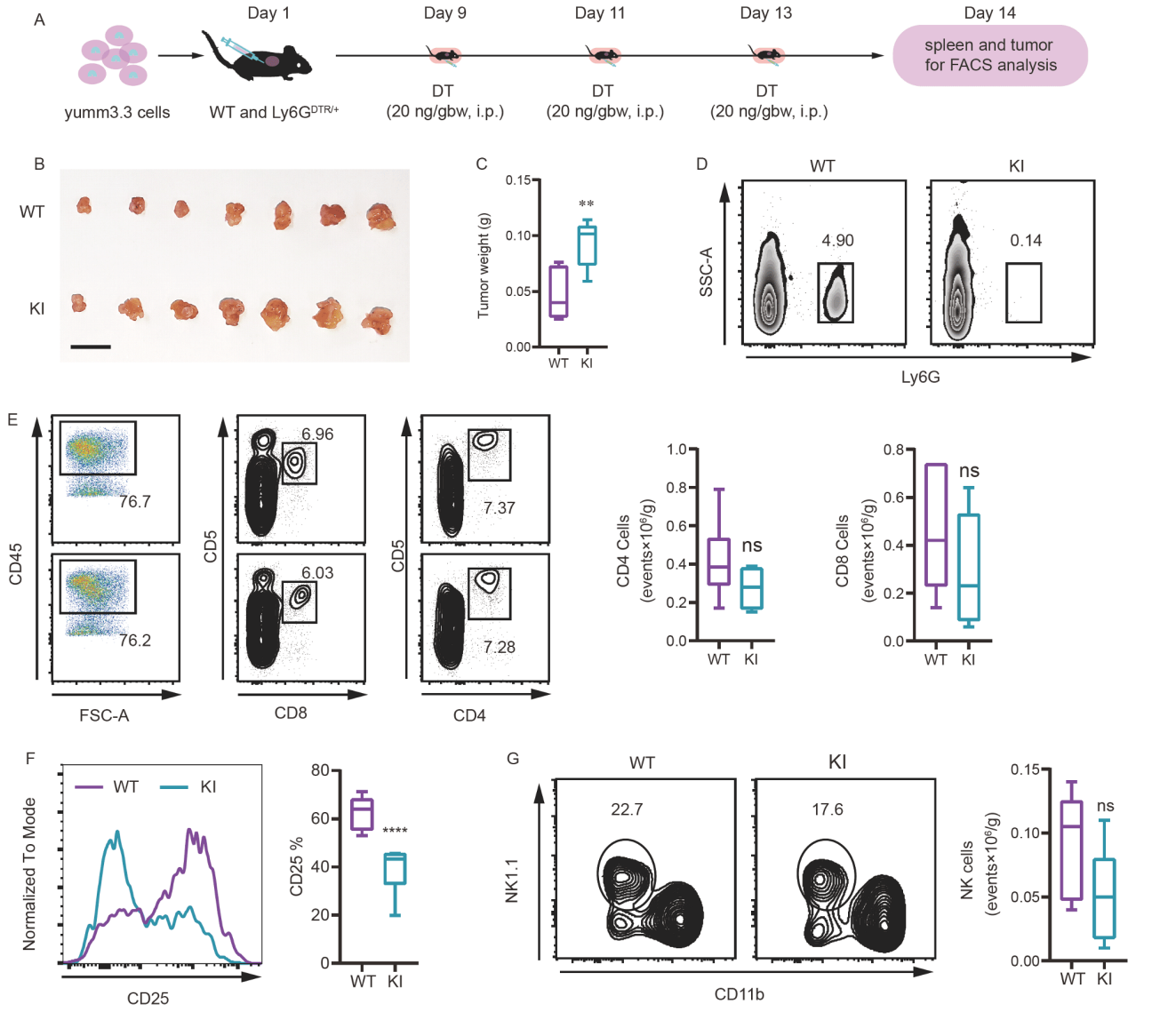

Figure 5 Tumor graft in neutrophil-deficient Ly6G-DTR mice. A, Overview of the experiments. Yumm3.3 melanoma cells were injected at day 1 subcutaneously into both C57BL/6 control (WT) and Ly6G-DTR (KI) mice. DT treatment started at day 9 after tumor grafts were visible and three doses of DT 20 ng g^{-1} body weight of mice were injected intra-peritoneally and mice were analyzed at day 14. B and C, Sizes of tumors from C57BL/6 control (WT) and Ly6G-DTR (KI) mice after DT treatment. D, Neutrophils in the tumor from C57BL/6 control (WT) and Ly6G-DTR (KI) mice after DT treatment. Neutrophils were gated on lineage (CD5, CD19, F4/80, Ly6C) negative cells. E, T cells in the tumor. CD4 and CD8 T cells were gated out of $CD45^+$ cells. F, CD25 expression in CD4 T cells in tumors from C57BL/6 control (WT) and Ly6G-DTR (KI) mice after DT treatment. G, NK cell count in tumors from C57BL/6 control (WT) and Ly6G-DTR (KI) mice after DT treatment. NK cells were gated on lineage (CD5, CD19, F4/80) negative CD11b intermediate to high cells. Each group of mice with DT involved seven animals. Data were analyzed by two-tailed Student's *t*-test and presented as mean±SEM, ns for no statistical significance, $**P<0.01$, $****P<0.0001$.

that depletion of neutrophils using our Ly6G-DTR model led to enhanced tumor growth, despite the fact that $CD25^+$ CD 4 T cells, which may represent regulatory T cells, were significantly decreased in tumors from neutrophil-depleted mice. We demonstrate that a single dose of DT in our model results in the complete absence of neutrophils 24 h after treatment and a significantly reduced neutrophil count was maintained 48 h after treatment. In further studies, it could be of interest to analyze the kinetics of neutrophil recruitment and dynamics in tissues after depletion using Ly6G-DTR mice (Kolaczkowska and Kubes, 2013). Therefore, we present a new model that could be very useful for study of neutrophils in various settings including tumor progression and infectious diseases.

MATERIALS AND METHODS

Mice

C57BL/6 mice were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. The $Lat^{fl\text{-}dtr}$ knock-in mice or Lat-DTR mice were kindly provided by Drs

Bernard Malissen and Marie Malissen from Centre d′immunophénomique, Inserm in France. For generation of Ly6G-DTR mice, knock-in experiments were performed on the background of C57BL/6. All animal procedures were performed according to guidelines approved by the committee on animal care at Xinxiang Medical University.

Preparation of template DNA for Ly6G-DTR knock-in

Template DNA used for knock-in were prepared from plasmids including 5′ and 3′ homologous recombination arms, sequences encoding T2A linker and DTR protein. DNA synthesis was provided by GenScript in China. In our experiment, the T2A and DTR coding sequences were inserted before the endogenous stop codon of Ly6G, which was flanked by 1 kb 5′ homologous recombination arm, and 1 kb 3′ homologous recombination arm. Double-strand DNA were amplified from plasmids by PCR and purified by QIAquick PCR Purification Kit (Qiagen, Germany), and stocked at –20°C before use.

CRISPR/Cas9-mediated knock-in

To target the 3′ prime UTR of Ly6G, sgRNA (TCTGCAGAAGGACTGAAACCAGG, CAAGGATGAAGGTCATTGAATGG) were prepared according to our previous studies (Chao et al., 2019; Wang et al., 2018). *In vitro* fertilization was performed by superovulation of B6 female mice following the same protocol as described in our previous studies (Huang et al., 2019; Voisinne et al., 2019). Microinjection of sgRNA (50 ng μL^{-1}), Cas9mRNA (50 ng μL^{-1}), and template DNA (10 ng μL^{-1}) were conducted for fertilized eggs at one-cell stage.

Mouse genotyping

In the 44 F0 mice from microinjected eggs, we isolated seven mice with knock-in allele. The genotyping was performed using PCR primers which were flanking beyond the recombination arms or in the insertion fragment. Three primer pairs were used (1-F: AACTGCTGAGCCATGTCTCC, 1-R: TTTTCCCGTGCTCCTCCTTG; 2-F: CATCGTGGGGCTTCTCATGT, 2-R: ACCCAAACTACCAAGGCCAG; h-DTR-F: TTCTGGCTGCAGTTCTCTCG, h-DTR-R: ACATGAGAAGCCCCACGATG) for genotyping the KI allele by amplifying genomic DNA from tail tip biopsies.

Flow cytometry

Cells from the peripheral blood or spleen of the B6 mice or Ly6G-DTR mice were prepared and stained with antibodies as described in our previous studies. All samples concerning cell count were done by the Attune NxT Flow Cytometer (Thermo Fisher Scientific, USA). FACS analyses were performed by software Flowjo 10.0 following sample acquisition with BD FACSCanto™ (BD, USA).

Antibodies

In this study, we used fluorochrome conjugated monoclonal antibodies to label multiple subsets of immune cells in the peripheral blood, splenocytes and single cell suspension prepared from tumors. The antibodies to detect surface markers were listed in Table S1 in Supporting Information.

Lipopolysaccharides preparation and treatment

LPS were purchased from Sigma-Aldrich (Louis, USA). The product was resolved in PBS and stocked in aliquots at –80° C. LPS 12.5 mg kg^{-1} body weight by i.p. injection was used to induce acute inflammation in lungs of mice.

Single cell preparation from lungs

According to the manufacturer's protocol, mouse lungs were perfused with PBS and digested by GentleMACS Dissociator (Miltenyi Biotec, Germany) with 0.5 mg mL^{-1} Collagen type D (Cat. No. 110-8888-2001) and 0.05 mg mL^{-1} DNAse Ⅰ (Cat. No. DN25) in 10% serum-DMEM. Cells were resuspended in FACS medium containing 2 mmol L^{-1} EDTA before antibody labeling.

Tumor inoculation and single cell preparation from tumor mass

2×10^6 Yumm3.3 melanoma cells were injected subcutaneously for each mouse. Tumors were harvested at day 14. Tumor mass was dissociated by GentleMACS Dissociator (Miltenyi Biotec, Germany) with Tumor Dissociation Kit (Order No. 130-096-730, Miltenyi Biotec) according to the manufacturer's protocol. Cells were resuspended in FACS medium containing 2 mmol L^{-1} EDTA before antibody labeling.

Diphtheria toxin preparation and treatment

The diphtheria toxin was purchased from Calbiochem (D00170675, Millipore). The products were resolved in pure water and stocked in 10 μL aliquots at –80°C. Mice were intra-peritoneally injected DT (10 or 20 ng g^{-1} body weight) diluted in PBS.

Statistics

The statistics were performed using GraphPad Prism (version 8.0). Unpaired two-tailed Student's *t*-test was used for

all the grouped comparisons. Data were presented as mean ±SEM. Statistical significance was assessed by *P<0.05, **P<0.01, ***P<0.001, and ****P<0.0001.

Compliance and ethics *The author(s) declare that they have no conflict of interest.*

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