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Interleukin-33/ST2 axis promotes breast cancer growth and metastases by facilitating intratumoral accumulation of immunosuppressive and innate lymphoid cells

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The role of IL-33/ST2 pathway in antitumor immunity is unclear. Using 4T1 breast cancer model we demonstrate time-dependent increase of endogenous IL-33 at both the mRNA and protein levels in primary tumors and metastatic lungs during cancer progression. Administration of IL-33 accelerated tumor growth and development of lung and liver metastases, which was associated with increased intratumoral accumulation of $CD11b^+Gr\text{-}1^+$ TGF-$\beta1^+$ myeloid-derived suppressor cells (MDSCs) that expressed IL-13α1R, IL-13-producing $Lin^-Sca\text{-}1^+ST2^+$ innate lymphoid cells (ILCs) and $CD4^+Foxp3^+ST2^+IL\text{-}10^+$ Tregs compared to untreated mice. Higher incidence of monocytic *vs*. granulocytic MDSCs and plasmocytoid *vs*. conventional dendritic cells (DCs) was present in mammary tumors of IL-33-treated mice. Intratumoral $NKp46^+NKG2D^+$ and $NKp46^+FasL^+$ cells were markedly reduced after IL-33 treatment, while phosphate-buffered saline-treated ST2-deficient mice had increased frequencies of these tumoricidal natural killer (NK) cells compared to untreated wild-type mice. IL-33 promoted intratumoral cell proliferation and neovascularization, which was attenuated in the absence of ST2. Tumor-bearing mice given IL-33 had increased percentages of splenic MDSCs, $Lin^-Sca\text{-}1^+$ ILCs, IL-10-expressing $CD11c^+$ DCs and alternatively activated M2 macrophages and higher circulating levels of IL-10 and IL-13. A significantly reduced NK cell, but not $CD8^+$ T-cell cytotoxicity in IL-33-treated mice was observed and the mammary tumor progression was not affected when $CD8^+$ T cells were *in vivo* depleted. We show a previously unrecognized role for IL-33 in promoting breast cancer progression through increased intratumoral accumulation of immunosuppressive cells and by diminishing innate antitumor immunity. Therefore, IL-33 may be considered as an important mediator in the regulation of breast cancer progression.

Interleukin-33 (IL-33), a member of the IL-1 family, is a multifunctional cytokine released upon necrotic cell death, which secretion can also be induced in live cells under biomechanical stress conditions.[1] IL-33 is primarily expressed in nonhematopoietic cells including fibroblasts, epithelial cells and endothelial cells, but is also present in cells of hematopoietic origin, particularly in macrophages and dendritic cells (DCs).[2,3] IL-33 regulates innate and acquired immunity[3–6] through binding to membrane-bound ST2 molecule (ST2L) of the IL-33R complex expressed on murine and human Th2 cells, mast cells, natural killer (NK) cells, myeloid cells and DCs.[3,7] IL-33 promotes Th2 immune response[2,3] and polarization of alternatively activated M2 macrophages.[8] Newly identified Type 2 innate lymphoid cells (ILC2) produce large amounts of IL-5 and IL-13 in response to IL-33 in the intestine,[9] adipose tissue[10] and lungs.[11] However, IL-33 could activate Th1, NK, NKT and $CD8^+$ T cells under certain pathophysiological conditions. IL-33 has a dual role in inflammatory disorders; it has protective effects in obesity, atherosclerosis and experimental fulminant hepatitis[12–14] and proinflammatory role in asthma and antigen-induced arthritis.[15,16] In addition, IL-33 promoted expansion of suppressive myeloid cells and $CD4^+Foxp3^+$ $ST2L^+$ regulatory T cells (Tregs) in cardiac allograft model.[17]

The role of IL-33 in cancer is unclear. We have reported that deletion of the ST2 gene favors innate and acquired antitumor immunity in 4T1 mammary carcinoma model.[18] However, the most recent study demonstrates antitumor effects of IL-33 showing attenuated tumor metastasis in the B16 melanoma and Lewis lung carcinoma metastatic models in mice with transgenic expression of IL-33.[19] Increased circulating IL-33 levels observed in gastric cancer patients may

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What's new?

This study employed a breast cancer cell line to investigate the role of interleukin-33 in cancer progression. They found more IL-33 in the cells as the disease advanced, and they showed that administring IL-33 to mice accelerated growth and metastasis. The increased levels of IL-33 sped cancer progression both by hindering antitumor immunity and by encouraging angiogenesis. Understanding the effects of IL-33 on cancer suggests possible avenues for tumor immunotherapy.

be related to cancer progression by the ability of IL-33 to promote Th2 immune responses, thus creating the microenvironment for tumor growth and progression.[20] The possible dichotomous functions of IL-33 in antitumor immunity in various cancers have yet to be determined.

The role of IL-33/ST2 axis in the regulation of tumor-infiltrating immune cell subsets in breast cancer is not defined. Although Th1 cells and cytotoxic $CD8^+$ T lymphocytes (CTLs) induce effective and durable anti-immune response,[21] the cross talk between myeloid cells and NK cells is crucial in the initiation of innate antitumor immunity.[22] Tumor cells *via* secretion of cytokines and chemokines may facilitate recruitment of multiple cell types that secrete inhibitory and immunosuppressive IL-10 and TGF-β. Myeloid-derived suppressor cells (MDSCs) and $CD4^+Foxp3^+$ Tregs are enriched within tumors and at peripheral sites such as spleen and peripheral blood where they potently suppress antitumor response. Tumor-infiltrating suppressor cells alter the activation and maturation of DCs, promote polarization of M2 macrophages, suppress T-cell-mediated tumor-specific immune responses and have proangiogenic role.[23] Functionally altered intratumoral DCs, MDSCs and Tregs compromise antitumor immunity by reducing NK cell and $CD8^+$ T cells cytotoxicity and IFNγ production.[24–26]

We investigated the effects of exogenously administered IL-33 on murine 4T1 breast cancer progression. We report that IL-33 enhanced mammary carcinoma growth and development of lung and liver metastases by facilitating expansion of immune suppressor cells within tumors and by diminishing innate antitumor immunity. We demonstrate IL-33-mediated increase of $CD11b^+Gr\text{-}1^+TGF\beta1^+$ MDSCs, $CD4^+Foxp3^+ST2^+$ IL-10^+ Tregs and IL-13-producing ILCs within mammary tumors. Endogenous IL-33 at both the mRNA and protein levels increased during 4T1 breast cancer progression in untreated mice. Our findings reveal a novel role for IL-33 in the mechanisms of breast cancer immune escape.

Material and methods

Mice

Female (10–11 weeks old) BALB/c (WT) and ST2 knockout ($ST2^{-/-}$) mice on BALB/c background (generated as described previously by Townsend *et al.*[27] were used in experiments. $ST2^{-/-}$ and WT mice were housed under standard laboratory conditions. The experiments were approved by the Animal Ethics Board of the Faculty of Medical Sciences, University of Kragujevac, Serbia.

4T1 tumor cell line

The weakly immunogenic mouse breast tumor cell line 4T1 that is syngeneic to the BALB/c background was purchased from the American Type Culture Collection (ATCC, USA). 4T1 cells were maintained in Dulbecco's-modified Eagle's medium supplemented with 10% fetal bovine serum (FBS), 2 mmol/L L-glutamine, 1 mmol/L penicillin–streptomycin and 1 mmol/L mixed nonessential amino acids (Sigma-Aldrich, St. Louis, MO) (complete growth medium). Subconfluent monolayers, in log growth phase, were harvested by brief treatment with 0.25% trypsin and 0.02% EDTA in phosphate-buffered saline (PBS, PAA Laboratories GmbH, Etobicoke, Canada) and washed thrice in serum-free PBS before use in all *in vitro* and *in vivo* experiments. The number of viable tumor cells was determined by trypan blue exclusion and only cell suspensions with >95% viable cells were used.

Immunohistochemical staining of IL-33 in mouse primary breast cancer tissues

Breast cancer paraffin tissue sections were incubated with a biotinylated goat anti-mouse IL-33 antibody (Abcam, Cambridge, UK) followed by visualization using Mouse Specific HRP/DAB Detection IHC Kit (Abcam) and photomicrographed at 400× magnification using Nikon eclipse Ti-E inverted research microscope equipped with NIS-Elements Imaging software (Nikon Instruments, Melville, NY).

Real-time PCR

Total RNA from mouse breast cancer was extracted using Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Two micrograms of total RNA was reverse-transcribed using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). Real-time qRT-PCR was performed in a Mastercycler® ep realplex (Eppendorf, Hamburg, Germany) using Power SYBR MasterMix (Applied Biosystems) and miRNA-specific primers for IL-33 (5′ TCC TTG CTT GGC AGT ATC CA 3′ sense, 5′ TGC TCA ATG TGT CAA CAG ACG 3′ antisense), ST2 (5′ GCA ATT CTG ACA CTT CCC ATG 3′ sense; 5′ ACG ATT TAC TGC CCT CCG TA 3′ antisense) and β-actin (5′ AGC TGC GTT TTA CAC CCT TT 3′ sense; 5′ AAG CCA TGC CAA TGT TGT CT 3′ antisense) as a housekeeping gene, and TaqMan® Universal PCR Master Mix (Roche Applied Science, IN) and miRNA-specific primers for TGFβ1 and β-actin as a housekeeping gene. Cycle threshold (C_T) values for IL-33, T1/ST2 and TGFβ1 were all

normalized to β-actin and analyzed, as previously described.[28]

IL-33 administration

Mice were inoculated with 5×10^4 4T1 tumor cells orthotopically into the fourth mammary fat pad. The naive and 4T1 tumor-bearing WT female BALB/c mice (10–11 weeks old) were injected intraperitoneally twice per week for 2 weeks at 1 μg per injection or five times every other day at 0.4 μg per injection with murine recombinant IL-33 (R&D Systems, Minneapolis, MN) or PBS.[13]

Estimation of *in vivo* 4T1 tumor growth and metastases

The size of primary 4T1 mammary tumors was assessed morphometrically using electronic calipers in two dimensions and tumor volumes (mm^3) were calculated as $L \times W^2/2$, where L represents the major axis of the tumor, while W represents minor axis and pulmonary metastases were enumerated by light microscope.[18]

MTT uptake assay

4T1 tumor cells were cultured in 96-well plate (5×10^4 cells per well) in complete DMEM (Invitrogen) growth medium without and with murine r-IL-33 (50 and 500 ng/mL) for 24 hr and 4-hr MTT (Sigma-Aldrich, St. Louis, MO) assay was performed.[29]

Preparation of tumor-infiltrating leukocytes

Primary 4T1 tumors and metastatic lungs were minced and placed in 5 mL of DMEM containing 1 mg/mL collagenase I, 1 mM EDTA and 2% FBS (all from Sigma-Aldrich) for enzymatic digestion. After incubation for 2 hr at 37°C, 10 mL of 0.25% trypsin was added and incubated for 3 min followed by DNase I (Sigma-Aldrich) solution for 1 min and digests filtered through 40-μm nylon cell strainer (BD Biosciences). Single-cell suspensions of spleens were obtained by mechanical dispersion.

Flow cytometric analyses

Fluorochrome-labeled anti-mouse mAbs specific for CD3 (145-2C11), CD4 (H129.19), CD8 (53-6.7), CD19 (1D3), CD45 (30-F11), NKp46 (29A1.4), CD11b (M1/70), CD11c (N418), MHC class II (14-4-4S), CD44 (IM7), CD80 (16-10A1), CD86 (GL1), CD205 (561118), CD206 (MR5D3), F4/80 (CI:A3-1), TLR2 (T2.5), CD273 (TY25), CD107a (1D4B), CD25 (PC61), CD279 (J43), Ly6G (1A8), Ly6C (HK1.4), Ly-6C/G (RB6/8C5), CD95L (MFL3), NKG2D (CX5), FcεRIa (MAR-1), Siglec-F (E50-2440), ST2 (245707), c-Kit (2B8), Sca-1 (D7), mouse lineage antibody cocktail or isotype-matched controls (BD Pharmingen, NJ/Invitrogen, Carlsbad, CA) were used. Rabbit anti-mouse IL13 receptor alpha 1 antibody (Abcam) followed by goat anti-rabbit IgG PE-Cy5.5 (Abcam) were also used for flow cytometric analyses. For intracellular staining, cells were stimulated with PMA (50 ng/mL)/ionomycin (500 ng/mL) (Sigma-Aldrich) with GolgyStop (BD Pharmingen, NJ) for 4 hr and stained with fluorochrome-labeled anti-mouse mAbs specific for Foxp3 (MF23), IFN-γ (XMG1.2), IL-10 (JES5-16E3), IL-4 (BVD4-1D11), IL-5 (TRFK5), IL-12 (C15.6), IL-13 (eBio13A) and TGF-β (141231) (BD Pharmingen/BioLegend, San Diego, CA/eBiosciences, San Diego, CA). Cells were analyzed with FACSCalibur Flow Cytometer (BD Biosciences, San Jose, CA) and analysis was conducted with FlowJo (Tree Star).

Purification of NK cells

$NK49b^+$ cells were purified from spleens using magnetic cell separation kit (Invitrogen) as previously described.[18]

Purification of $CD8^+$ T cells

$CD8^+$ T cells were purified from spleens by adding the mixture of monoclonal antibodies against non-$CD8^+$ T cells (Invitrogen), followed by Mouse Depletion Dynabeads (Invitrogen). Remaining uncoupled cells were highly enriched mouse $CD8^+$ T cells (>90%).

Cytotoxicity assay

Cytotoxic activity of splenocytes, enriched NK cells and enriched $CD8^+$ T cells against 4T1 cells as targets was measured using the 4-hr MTT (Sigma-Aldrich) assay at various target–effector (T:E) ratios, as previously described.[18] The percentage of cytotoxity was calculated as: cytotoxity (%) = [1 − experimental group (OD)/control group (OD)] × 100. Lytic units, $LU_{20}/10^7$ cells, were calculated from means of triplicate percentages of killing obtained in four different T:E ratios.

In vivo depletion of $CD8^+$ cells

For *in vivo* depletion of $CD8^+$ cells, mice were treated i.p. with 100 μg of anti-CD8 mAb (YTS 169.4) 1 day before and 5 days after 4T1 tumor cell inoculation and the efficacy of depletion of $CD8^+$ was >95%.

Intratumoral cell proliferation and microvessel density

Paraffin-embedded primary 4T1 tumor tissue sections were stained with Rb anti-mouse Ki-67 antibody (Abcam) and goat anti-rabbit IgG PE-Cy5.5 (Abcam) for detection of proliferative marker Ki-67.[30] For the detection of microvessels Rb anti-mouse CD31 (Abcam) and Ms anti-mouse alpha-smooth muscle actin (αSMA) (Abcam) were applied followed by secondary mAbs: FITC-conjugated goat anti-rabbit IgG and PE-conjugated goat anti-mouse IgG (Abcam) added.[31] The sections were mounted with ProLong Gold antifade reagent with DAPI (Invitrogen) and analyzed at 100× magnification, using Nikon eclipse Ti-E inverted research microscope equipped with NIS-Elements Imaging software (Nikon Instruments). Only brightness and contrast were adjusted.

Measurement of cytokines

Serum levels of IL-10 and IL-13 were measured using highly sensitive enzyme-linked immunosorbent assay (hs-ELISA) kits (R&D Systems).

Statistical analysis

The data were analyzed using statistical package SPSS, version 13. The normality of distribution was tested by Kolmogorov–Smirnov test. The two-tailed Student's *t*-test, Fisher's exact test or nonparametric Mann–Whitney rank-sum test were used. The results were considered significantly different when $p < 0.05$ and highly significantly different when $p < 0.01$.

Results

IL-33 accelerates 4T1 breast cancer progression

Mice were challenged with 4T1 mammary carcinoma cells orthotopically into the fourth mammary fat pad and recombinant murine IL-33 was intraperitoneally administered either twice per week for 2 weeks at 1 μg per injection or five times every other day at 0.4 μg per injection. Tumor appearance, tumor volume and tumor diameter were monitored and the development of lung and liver metastases was assessed. The appearance of palpable tumors was significantly accelerated ($p < 0.05$) and primary tumor diameters and tumor volumes were significantly increased in mice treated with IL-33 compared to PBS-treated mice at Day 16 after tumor challenge (both $p < 0.05$; Fig. 1*a*). Moreover, five repeated injections of IL-33 at 0.4 μg per injection resulted in more pronounced differences in tumor appearance and tumor diameters with significantly increased tumor volumes compared to other schedule of IL-33 administration ($p < 0.05$; Fig. 1*a*).

Importantly, exogenous IL-33 (4 × 1 μg per injection) facilitated metastatic dissemination of 4T1 cells; 4 weeks after 4T1 tumor challenge 75 and 25% of mice given IL-33 developed lung and liver metastases, respectively, in contrast to none among PBS-treated mice (both $p < 0.05$; Fig. 1*b*).

Endogenous IL-33 mRNA and protein levels increase during 4T1 breast cancer progression

We next sought to assess endogenous levels of IL-33 in mammary carcinoma and metastatic lungs. Immunohistochemical data demonstrate expression of IL-33 in mammary carcinoma tissue sections in untreated wild-type BALB/c mice at Day 16 after 4T1 tumor challenge (Fig. 1*c*). Endogenous IL-33 and ST2 mRNA levels in mammary tumors showed significant time-dependent increase with the highest levels detected at Day 28 after tumor challenge (Fig. 1*d*). Similarly, flow cytometric analyses revealed time-dependent increase of IL-33 expression in mammary tumors during cancer progression (Fig. 1*e*). IL-33 was expressed by $CD45^+$ leucocytes and tumor cells in the mammary carcinoma (Fig. 1*f*, upper panel) and in the metastatic lungs (Fig. 1*f*, lower panel). Inoculated mouse breast tumor cell line 4T1 did not express IL-33 as evaluated by flow cytometry (data not shown).

IL-33 treatment increases immunosuppressive cells in spleens of tumor-bearing mice

IL-33 administration markedly increased spleen cellularity at Day 13 after 4T1 tumor challenge compared to PBS-treated mice ($136.7 \pm 8.2 \times 10^6$ *vs.* $119.6 \pm 5.5 \times 10^6$; $p < 0.05$). Both naïve and tumor-bearing mice given IL-33 exhibited pronounced increase in $CD11b^+CD11c^-Gr\text{-}1^+$ MDSCs in spleens compared with PBS-treated mice (both $p < 0.05$; Fig. 2*a*). In contrast, both naïve and tumor-bearing ST2-deficient mice had significantly lower percentages of MDSCs compared to wild-type mice (both $p < 0.05$; Fig. 2*a*). Tumor-bearing mice treated with IL-33 had significantly increased monocytic MDSCs while significantly decreased granulocytic MDSCs compared to PBS-treated mice. Moreover, IL-33-treated mice exhibited increased percentages of $CD11b^+Gr\text{-}1^+$MDSCs that expressed IL-13Rα1 and TGF-β1 (Fig. 2*a*).

Exogenous IL-33 increased the percentage of IL-10-producing $CD4^+Foxp3^+ST2^+$ cells in spleens of tumor-bearing mice ($p < 0.01$; Fig. 3*c*) in contrast to tumor-naive mice and PBS-treated tumor-bearing mice (Fig. 2*b*).

The number of $CD11c^+CD11b^-$ DCs and the percentage of DCs expressing $MHCII^+$ were significantly increased in the spleens of IL-33-treated tumor-bearing mice ($p < 0.01$; $p < 0.05$; Fig. 2*c*). IL-33 treatment reduced splenic conventional DCs (cDCs), subpopulation of $CD8^+CD205^+$ cDCs (not shown) and plasmocytoid DCs (pDCs) in tumor-bearing mice (Fig. 2*c*). DCs were of immature phenotype as assessed by decreased expression of costimulatory molecules CD80 and CD86 (both $p < 0.05$; Fig. 2*c*). An increase of suppressive $CD11c^+IL\text{-}10^+$ DCs was observed only in tumor-bearing mice given IL-33 (Fig. 2*c*), whereas no difference in the frequencies of IL-12 p40/p70-expressing $CD11c^+$ cells was observed (data not shown).

IL-33 treatment increased the frequency of $F4/80^+$ macrophages in spleens of tumor-bearing mice, in comparison to PBS-treated animals ($11.69\% \pm 0.56\%$ *vs.* $7.31\% \pm 1.01\%$; $p < 0.05$). Further phenotypic analyses revealed that IL-33-treated mice had increased percentage of $CD206^+$ while decreased percentage of $TLR2^+$ M1 macrophages (both, $p < 0.05$; data not shown), indicative of their polarization toward alternatively activated M2 phenotype.

Administration of IL-33 increased the incidence of Lin^- $Sca\text{-}1^+$ ILCs in the spleens of tumor-bearing mice (Fig. 2*d*). Splenic $CD45^+Lin^-$ cells (negative for lineage markers CD3ϵ, CD11b, CD45R/B220, Ly-76, Ly-6G and Ly-6C) expressed a progenitor cell-associated marker Sca-1, IL-2Rα-chain CD25, adhesion molecule CD44, ST2, IL-5 and IL-13 and the cell phenotype characteristic of Type 2 ILCs (Supporting Information Fig. 1). $Lin^-Sca\text{-}1^+$ cells expressed IL-13 in response to IL-33 only in tumor-bearing mice and were IL-4, IL-10 and IFN-γ negative (data not shown). $IL\text{-}5^+$ cells were negative for mast cell marker FcϵRIa or eosinophil marker Siglec-F (data not shown). Systemic effect of IL-33 administration in tumor-bearing mice was reflected by significantly increased serum levels of IL-10 and IL-13 compared to tumor-bearing mice that received PBS (Fig. 2*e*).

IL-33-mediated progression of mammary tumors is associated with diminished NK cell cytotoxicity

In vitro cytotoxicity of splenic NK cells isolated from IL-33-treated tumor-bearing mice was significantly decreased ($p <$

0.05; Fig. 3*a*). The lesser presence of CD107a$^+$NK cells in both naive and tumor-bearing mice given IL-33 in comparison with PBS-treated mice was observed (both $p < 0.01$; Fig. 3*a*). IL-33 increased PD-1-expressing NKp46$^+$ cells ($p < 0.01$; Fig. 3*b*) and markedly reduced the number of IFN-γ-expressing NKp46$^+$ cells in tumor-bearing mice ($p < 0.05$;

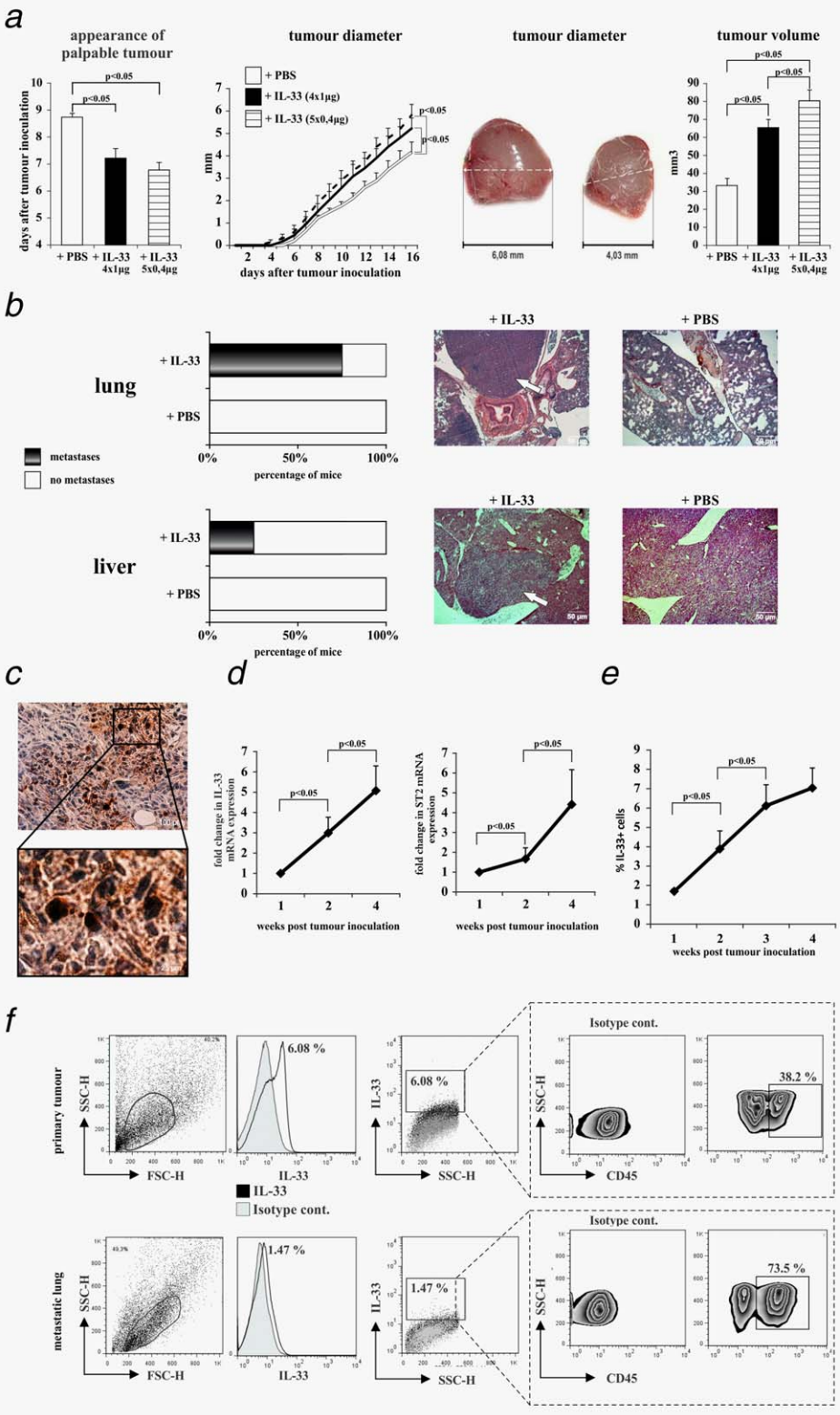

Figure 1.

Fig. 3*b*). In addition, IL-33 increased the number of $NKp46^+IL\text{-}10^+$ cells in both naive and tumor-bearing mice (both, $p < 0.01$; Fig. 3*b*).

As shown in Figure 3*c*, 4T1 tumor inoculation led to a significant increase in $CD8^+$ T-cell cytotoxicity *in vitro*, but no significant difference was found between IL-33- *vs*. PBS-treated mice including the incidence of $CD8^+CD107a^+$ T cells (Fig. 3*c*). After *in vivo* depletion of $CD8^+$ cells (Fig. 3*d*) mammary tumor growth remained accelerated in mice treated with IL-33 to a similar extent as observed in PBS-treated mice (Fig. 3*d*).

IL-33 administration facilitates accumulation of immunosuppressive cells within mammary tumors

In mammary tumors, IL-33 treatment increased the incidence of $CD11b^+CD11c^-Gr\text{-}1^+$ MDSCs, albeit not significantly. Of note, MDSCs were significantly decreased in tumors in mice lacking ST2 ($p < 0.05$; Fig. 4*a*). The frequencies of monocytic MDSCs were significantly increased while the frequencies of granulocytic MDSCs were significantly decreased in mammary tumors in mice given IL-33 (Fig. 4*a*, Supporting Information Fig. 2). IL-33 treatment led to a significant increase of $CD11b^+CD11c^-Gr\text{-}1^+$ myeloid suppressor cells expressing IL-13α1 and TGF-β1 ($p < 0.05$; Fig. 4*a*). TGF-β1 mRNA levels in mammary tumors showed significant time-dependent increase reaching the highest level at day 28 after tumor challenge (Fig. 4*b*).

IL-33 administration influenced the incidence of intratumoral DCs subpopulations; the decrease of the percentage of $CD11c^+CD11b^+B220^-MHCII^+$ cDCs while the increase of the percentage of $CD11c^+CD11b^-B220^+MHCII^-$ pDCs (both, $p < 0.05$; Fig. 4*c*) and subpopulation of $CD8^+CD205^+$ cDCs were observed (data not shown).

IL-33 treatment did not influence accumulation of $F4/80^+$ macrophages in mammary tumors (data not shown), nor the percentage of $CD206^+$, $TLR2^+$ and $IL\text{-}10^+$ $F4/80^+$ macrophages compared to PBS-treated mice (data not shown).

IL-33 significantly increased the percentages of intratumoral $CD4^+Foxp3^+$ Tregs (Fig. 4*d*). The most pronounced increase was observed in the percentage of $ST2^+IL\text{-}10^+$ subset of $CD4^+Foxp3^+$ Tregs in mice given IL-33 ($p < 0.01$; Fig. 4*d*, Supporting Information Fig. 2).

Mice given IL-33 had significantly increased $CD45^+Lin^-Sca\text{-}1^+$ ILCs at tumor sites ($p < 0.05$; Fig. 4*e*), which exhibited activated phenotype as assessed by upregulated expression of CD44, CD25 and ST2 (Fig. 4*e*). $Lin^-Sca\text{-}1^+$ ILCs expressed IL-13 in response to IL-33 (Fig. 4*e*) and were IL-4, IL-10 and IFN-γ negative (data not shown).

IL-33 profoundly affects the incidence and functional phenotype of tumor-infiltrating NK cells

Administration of IL-33 significantly reduced the percentage of intratumoral $NKp46^+$ NK cells, while the incidence of these cells was significantly increased in mice lacking ST2 compared to PBS-treated tumor-bearing mice (Fig. 5*a*). Mice given IL-33 had significantly decreased percentages of NK cells that expressed activation marker NKG2D compared to PBS-treated controls, while non-significant trend toward increased incidence of $NKp46^+NKG2D^+$ cells was observed in ST2-deficient mice (Fig. 5*b*). Similarly, markedly reduced $NKp46^+FasL^+$ tumoricidal cells were observed in mammary tumors of IL-33-treated mice ($p < 0.05$, Fig. 5*c*) in contrast to significantly increased presence of $NKp46^+FasL^+$ cells within mammary tumors in ST2-deficient mice. The increased presence of PD-1-expressing $NKp46^+$ cells was found in mammary tumors after IL-33 administration, while $NKp46^+PD\text{-}1^+$ cells were significantly decreased in tumors in the absence of ST2 (both $p < 0.05$, Fig. 5*d*).

IL-33 increases mammary tumor cell proliferation and blood vessel density

We next assessed intratumoral cell proliferation and angiogenesis in mice treated with IL-33. Higher numbers of Ki-67-positive cells in mammary tumors were observed in mice given IL-33 compared to PBS-treated controls ($p < 0.05$; Fig. 6*a*). ST2-deficient mice had significantly lower numbers of Ki-67-positive cells within the primary tumor tissues ($p < 0.05$; Fig. 6*a*). Whether IL-33 affects proliferation of 4T1 cells

Figure 1. Exogenously administered IL-33 accelerates mammary tumor appearance, growth and metastases in murine 4T1 breast cancer. (*a*) Accelerated tumor growth and metastases after IL-33 treatment. On Day 16 primary tumor volumes were calculated after exogenously administered PBS (black bars) or IL-33 injected intraperitoneally twice per week for 2 weeks with 1 μg of murine recombinant IL-33 (white bars) or 0.4 μg of mr-IL-33 five times every other day (patterned bars). The difference in tumor diameters is shown on the representative photograph of the excised mammary tumors. Data are presented as mean ± SEM from three independent experiments, each carried out with six mice per group. Statistical significance was tested by Mann–Whitney rank-sum test or Student's unpaired *t*-test, where appropriate. (*b*) IL-33 facilitates metastases to lungs and liver. Lung and liver metastases were assessed at Day 28 after 4T1 tumor challenge (arrows). Data are presented as percentages from eight mice per group. Statistical significance was tested by Fisher's exact test. H&E staining of lung and liver tissues showing representative metastatic colony (magnification at ×100). (*c*) Immunohistochemical detection of IL-33 in murine breast cancer. Murine breast cancer tissue sections were stained with anti-IL-33 antibodies and IL-33-positive cells (brown) are shown. Original magnification, ×400. (*d*) PCR detection of IL-33 and ST2 in primary tumor. Endogenous IL-33 and ST2 mRNA expression was determined by RT-PCR. β-Actin mRNA expression was used as internal control. IL-33 and ST2 mRNA levels were evaluated against β-actin mRNA expression (IL-33/β-actin mRNA expression value ×100). (*e*) Flow cytometric detection of IL-33 in primary tumor. Flow cytometric analysis of IL-33-expressing cells in cell suspensions of digested mammary tumors during breast cancer progression. (*f*) Representative FACS plots of IL-33-expressing cells within primary tumor (upper panel) and metastatic lung (lower panel) 28 days after 4T1 tumor challenge. Data present results from two independent experiments, each carried out with four mice per group. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

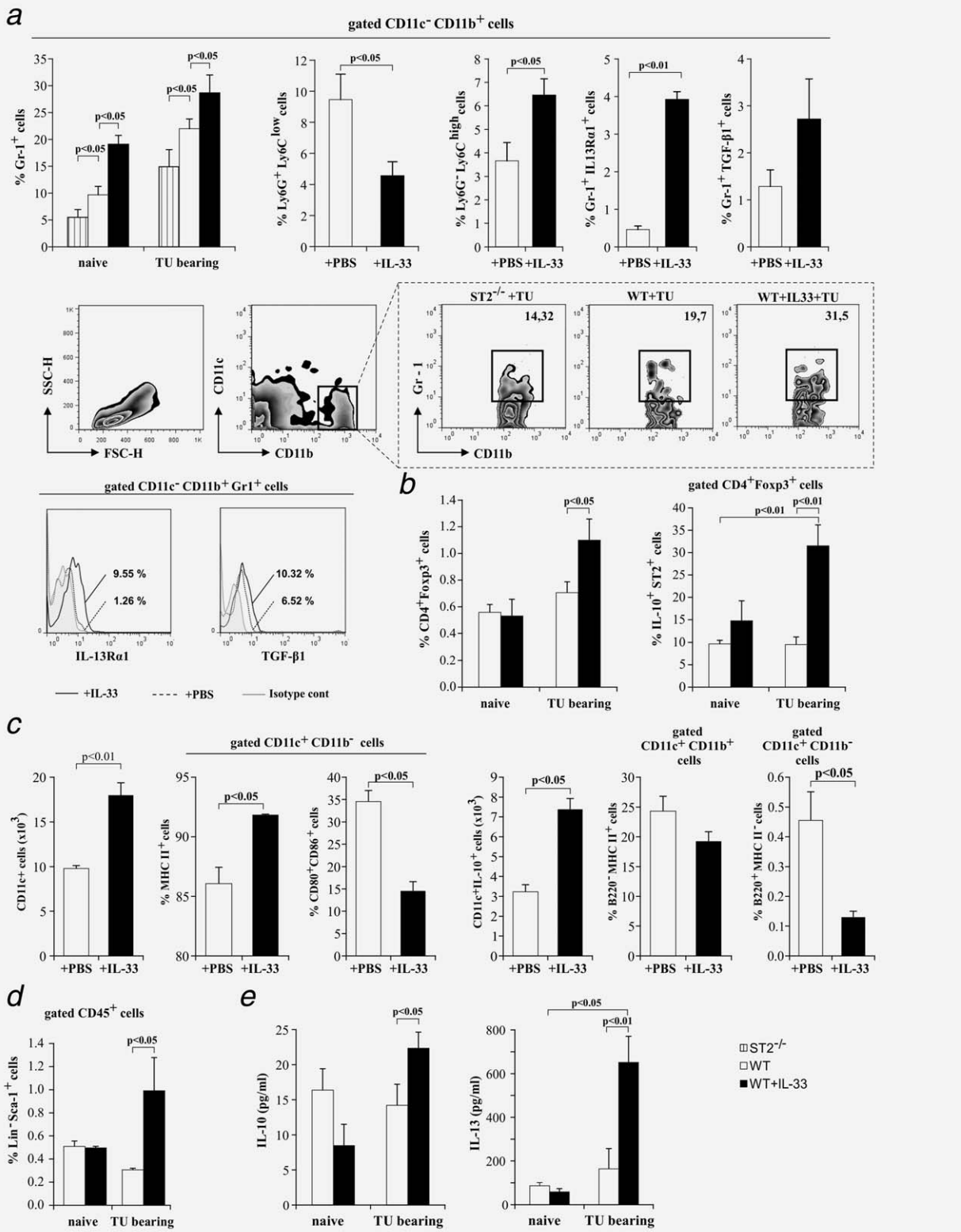

Figure 2. IL-33 increases immunosuppressive cells in spleens of tumor-bearing mice. (*a*) IL-33 treatment increases the accumulation and changes the functional phenotype of MDSCs in spleens of tumor-bearing mice. Flow cytometric analyses of Gr-1, Ly6G, Ly6C, IL-13Rα1 and TGF-β1 expression in gated $CD11c^{-}CD11b^{+}$ cells. Representative FACS dotplots of $CD11c^{-}CD11b^{+}Ly6G^{+}Ly6C^{low}$ granulocytic and $CD11c^{-}CD11b^{+}Ly6G^{-}Ly6C^{high}$ monocytic MDSCs are shown. (*b*) IL-33 induces accumulation of $CD4^{+}Foxp3^{+}IL\text{-}10^{+}ST2^{+}$ cells in spleens of tumor-bearing mice. Flow cytometric analysis of surface ST2 and intracellular IL-10 in gated $CD4^{+}Foxp3^{+}$ T-regulatory cells. (*c*) IL-33 increases numbers of $CD11c^{+}$ and $CD11c^{+}IL\text{-}10^{+}$ cells, while decreases the percentage of $CD11c^{+}CD11b^{+}B220^{-}MHCII^{+}$ conventional, $CD11c^{+}CD11b^{-}B220^{+}MHCII^{-}$ plasmocytoid and $CD11c^{+}CD11b^{-}CD80^{+}CD86^{+}$ dendritic cells. Flow cytometric analyses of $CD11c^{+}$, $CD11c^{+}IL\text{-}10^{+}$ cells and $B220^{-}MHCII^{+}$ cells in gated $CD11c^{+}CD11b^{+}$ cell population, $MHCII^{+}$, $CD80^{+}CD86^{+}$ and $B220^{+}MHCII^{-}$ cells in gated $CD11c^{+}CD11b^{-}$ cells in spleens of IL-33- or PBS-treated mice, at Day 13 after 4T1 tumor challenge. (*d*) IL-33 treatment induces accumulation of $Lin^{-}Sca\text{-}1^{+}$ cells in spleens of tumor-bearing mice. Flow cytometric analysis of $Lin^{-}Sca\text{-}1^{+}$ cells in gated $CD45^{+}$ cells in spleen. (*e*) IL-33 increases systemic concentrations of IL-10 and IL-13, 13 days after 4T1 tumor inoculation. Serum levels of IL-10 and IL-13 were determined by ELISA. Data are presented as mean ± SEM of two independent experiments, each carried out with four mice per group. Statistical significance was determined by Student's *t*-test.

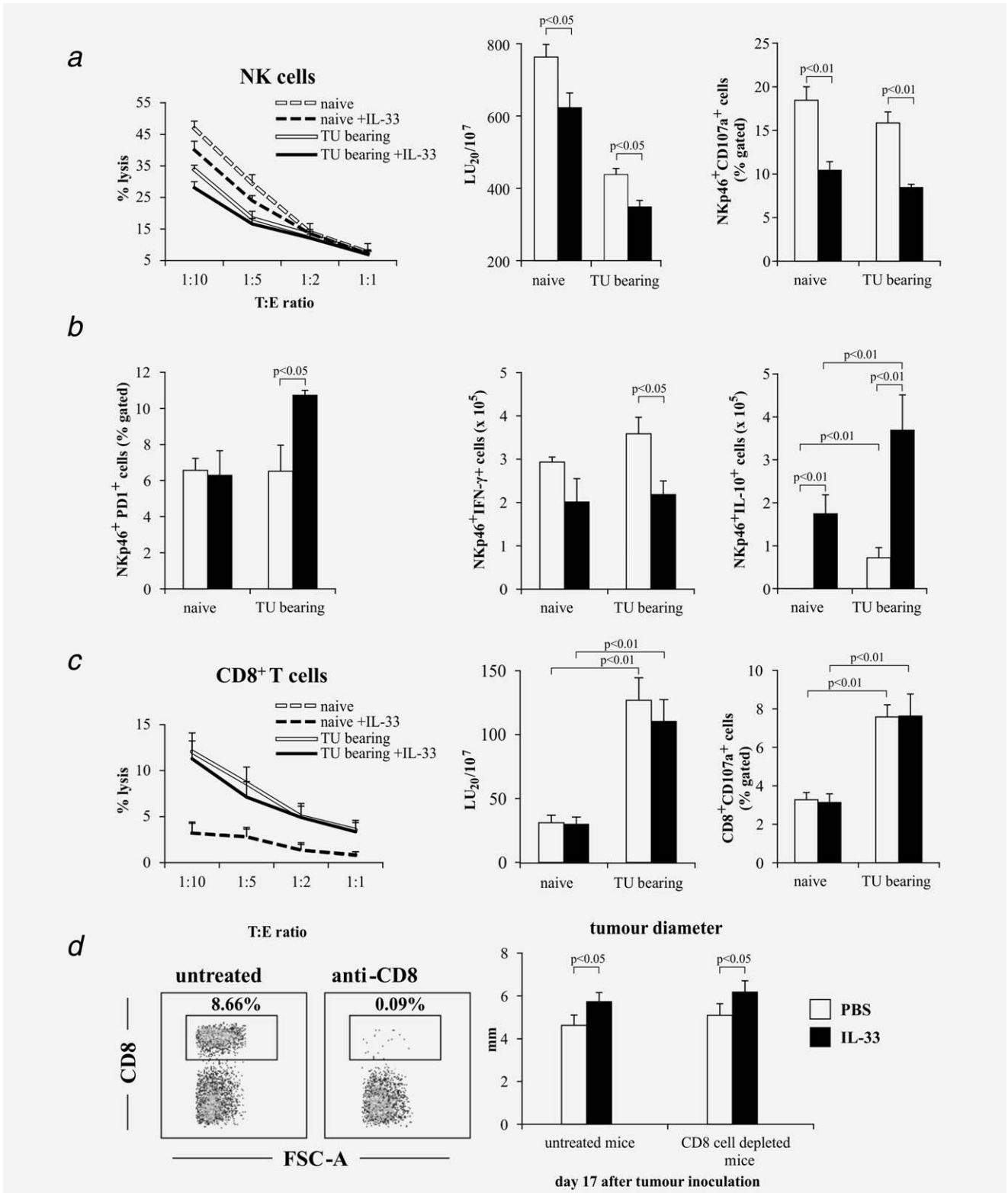

Figure 3. IL-33 affects cytotoxic activity of NK cells, but not $CD8^+$ T cells. (*a*) IL-33 markedly decreased cytotoxicity of NK cells isolated from spleen of naive and tumor-bearing mice. The cytotoxic activity of NK cells was tested in 4-hr MTT assay against 4T1 cell targets. Cytotoxic capacity was also evaluated by flow cytometric analysis of CD107a expression on $NKp46^+$ cells. Data are means ± SEM of two independent experiments, each carried out with three mice per group. Statistical significance was tested by Student's *t*-test. (*b*) IL-33 markedly reduced the number of IFN-γ-producing $NKp46^+$ cells, whereas increased the number of IL-10-producing $NKp46^+$ cells as well as percentage of PD-1-expressing $NKp46^+$ cells in spleens of tumor-bearing mice at Day 13 after 4T1 tumor challenge. Flow cytometric analyses of PD-1-, IFN-γ- and IL-10-expressing splenic $NKp46^+$ cells. Data are means ± SEM of two independent experiments, each carried out with three mice per group. Statistical significance was tested by Student's *t*-test. (*c*) Tumor inoculation led to a significant increase in $CD8^+$ T-cell-mediated cytotoxicity with no difference between IL-33- and PBS-treated mice. The cytotoxicity of $CD8^+$ T cells was evaluated by MTT assay and flow cytometric analysis of CD107a expression. Data are means ± SEM of two independent experiments, each carried out with three mice per group. Statistical significance was tested by Student's *t*-test. (*d*) Depletion of $CD8^+$ cells had no influence on accelerated tumor growth in IL-33-treated mice. Mice were depleted of $CD8^+$ T cells and representative FACS plot shows the percentages of $CD8^+$ cells after *in vivo* administration with anti-CD8 mAb (YTS 169.4) (left panel). On Day 17 after 4T1 tumor challenge the tumor diameters were evaluated in IL-33- and PBS-treated CD8-depleted mice. Data are means ± SD from four mice per group. Statistical significance was tested by Student's *t*-test.

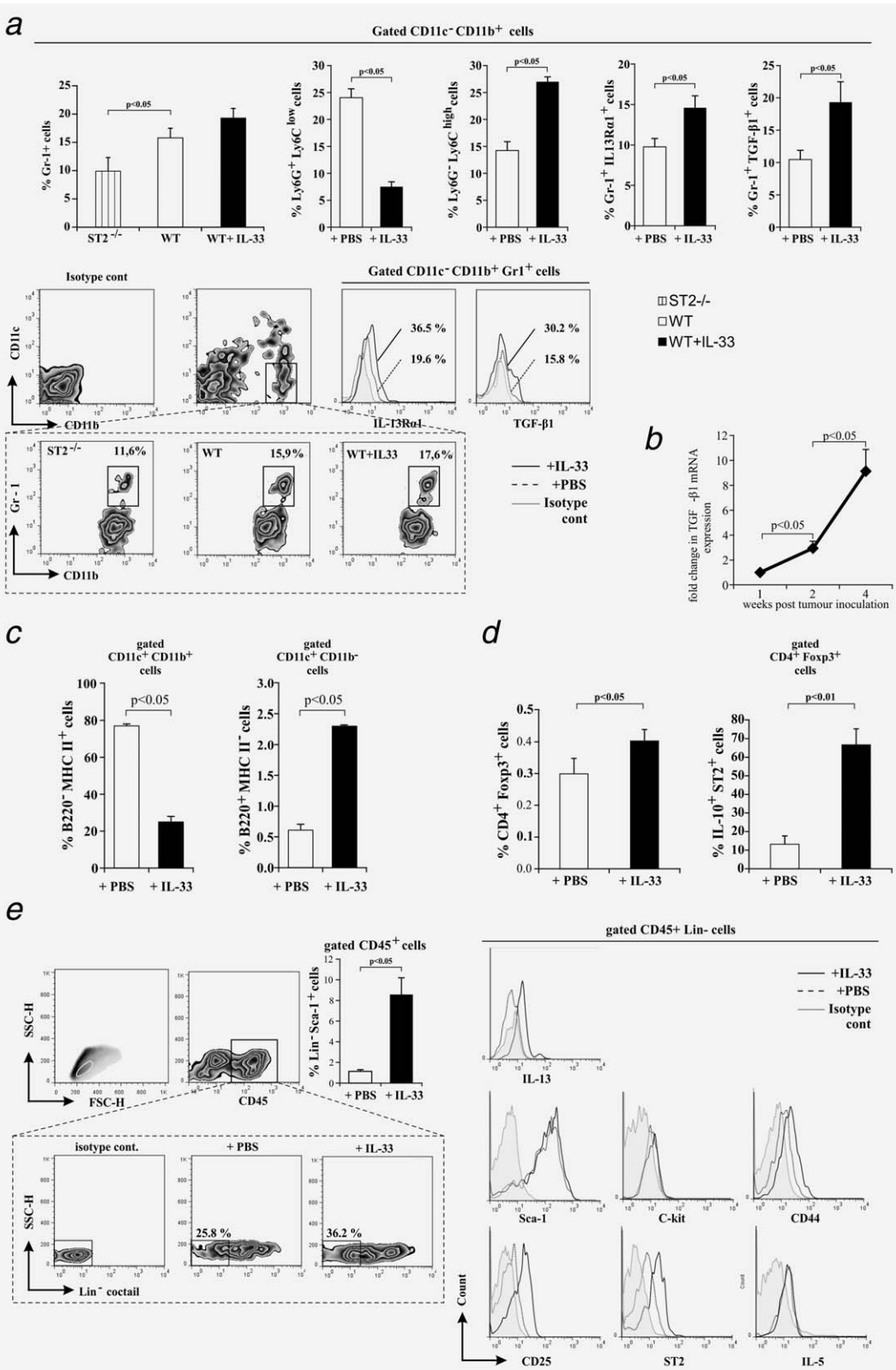

Figure 4. IL-33 administration affects cellular makeup in mammary tumor microenvironment. (*a*) IL-33 increases intratumoral accumulation of immunosuppressive $CD11c^{-}CD11b^{+}Gr\text{-}1^{+}$ cells that express IL-13Rα1 and TGF-β1; while in the absence of ST2 the percentage of MDSCs is decreased. Primary mammary tumors were isolated at Day 13, digested and single-cell suspensions stained and gated for $CD11c^{-}CD11b^{+}$ cells. Typical example of flow cytometry analysis of Gr-1, Ly6G, Ly6C, IL-13Rα1 and TGF-β1 expression in $CD11c^{-}CD11b^{+}$ cells is shown. (*b*) RT-PCR detection of TGF-β1 mRNA levels in primary tumors. TGF-β1 mRNA levels were evaluated against β-actin mRNA expression. (*c*) IL-33 decreases the percentage of conventional $CD11c^{+}CD11b^{+}B220^{-}MHCII^{+}$ while increases the percentage of plasmocytoid $CD11c^{+}CD11b^{-}B220^{+}MHCII^{-}$ dendritic cells. Flow cytometric analysis of $B220^{-}MHCII^{+}$ cells in gated $CD11c^{+}CD11b^{+}$ cells and $B220^{+}MHCII^{-}$ cells in gated $CD11c^{+}CD11b^{-}$ cells. (*d*) IL-33 facilitates infiltration of immunosuppressive $CD4^{+}Foxp3^{+}IL\text{-}10^{+}ST2^{+}$ Tregs. Flow cytometric analysis of $CD4^{+}Foxp3^{+}$ Tregs. Analysis of surface ST2 and intracellular IL-10 in the gated $CD4^{+}Foxp3^{+}$ Tregs. (*e*) Increased accumulation of $CD45^{+}Lin^{-}Sca\text{-}1^{+}$ innate lymphoid cells with activated phenotype in tumor microenvironment. Flow cytometric analysis of $CD45^{+}Lin^{-}Sca\text{-}1^{+}$ ILCs and expression of Sca-1, c-kit, CD44, CD25, ST2, IL-5 and IL-13. Representative FACS plots and histograms are shown. Results are presented as mean ± SEM from four mice per group, in two separate experiments. Statistical significance was determined by Student's *t*-test.

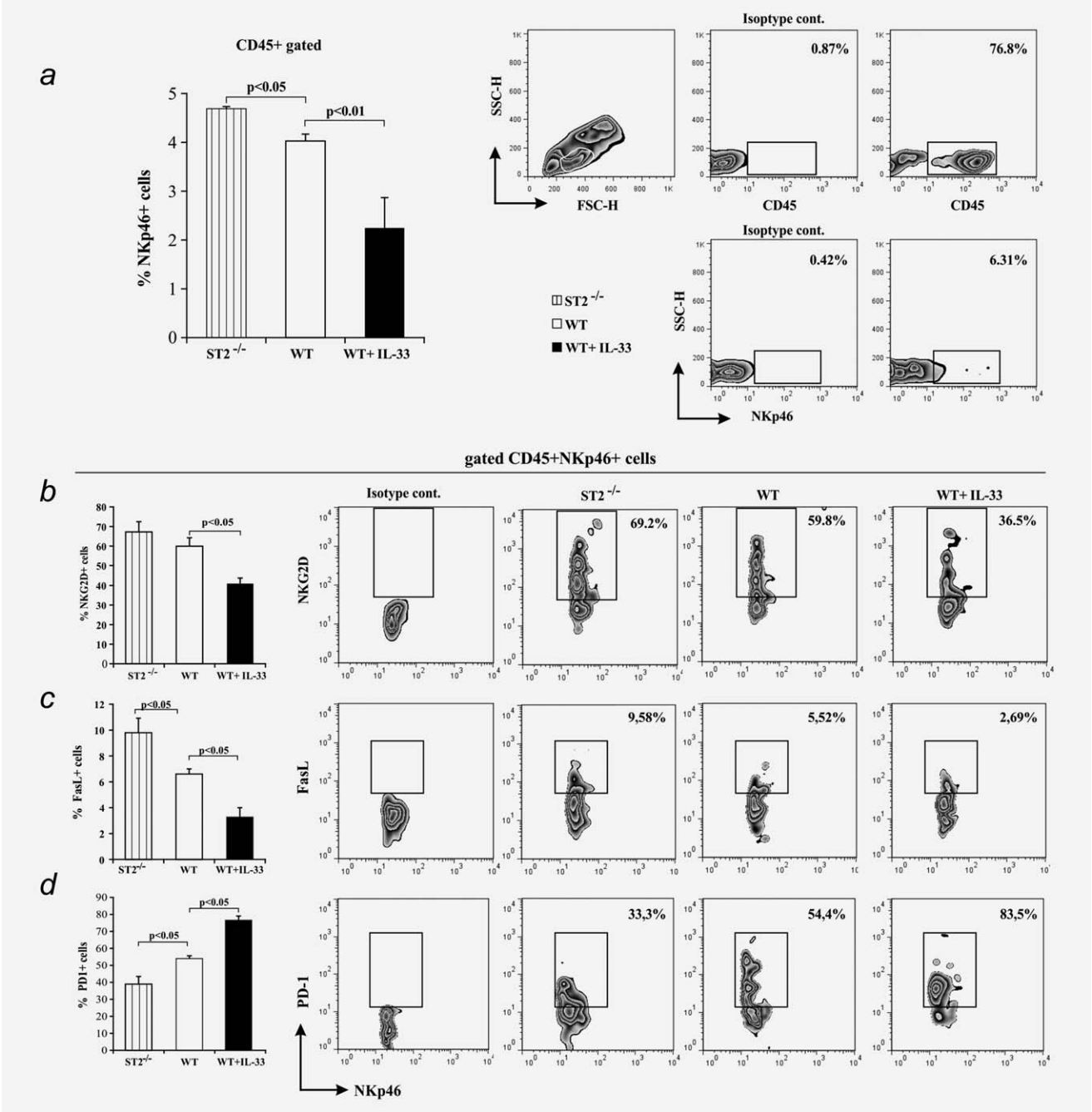

Figure 5. IL-33 changes intratumoral accumulation and functional phenotype of NK cells. (*a*) IL-33 decreases intratumoral accumulation of NK cells; while in the absence of ST2 the percentage of NK cells is significantly higher. Primary tumors were isolated at Day 13, digested and single-cell suspensions stained for $CD45^+$ and $NKp46^+$. (*b*) Administration of IL-33 significantly decreases the percentage of intratumoral $NKp46^+NKG2D^+$ cells. Representative FACS plots of surface NKG2D expression on gated $NKp46^+$ cells are shown. (*c*) IL-33 significantly decreases the percentage of intratumoral $NKp46^+FasL^+$ tumoricidal cells; while their presence in ST2-deficient mice was significantly enriched. Representative FACS plots of surface FasL expression on gated $NKp46^+$ cells are shown. (*d*) Administration of IL-33 increases intratumoral PD-1-expressing $NKp46^+$cells; while in ST2-deficient mice the percentage of $NKp46^+PD\text{-}1^+$ cells was decreased. Representative FACS plots of surface PD-1 expression on gated $NKp46^+$ cells are shown. Data are presented as mean ± SEM of two independent experiments, each carried out with four mice per group. Statistical significance was determined by Student's *t*-test.

was assessed using MTT uptake assay. 4T1 cells incubated *in vitro* with IL-33 did not exhibit increased proliferation (data not shown), thus the direct effect of IL-33 on 4T1 tumor cells was excluded.

The number of CD31-positive endothelial cells in primary tumors was significantly higher in IL-33-treated mice (30.80 ± 4.35 *vs.* 13.29 ± 3.19; $p < 0.05$), similar to the increased number of α-SMA-positive cells (25.67 ± 4.45 *vs.* 10.57 ±

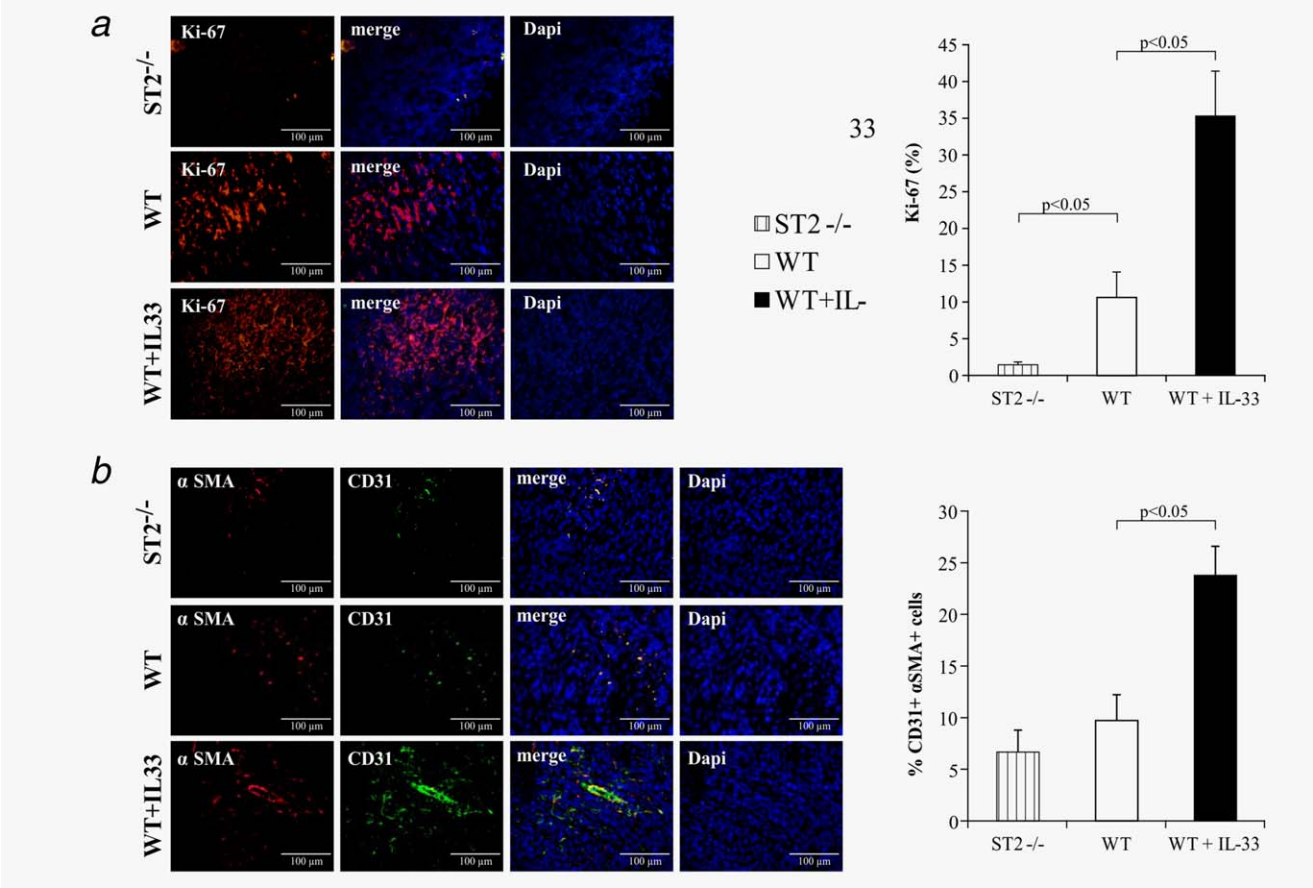

Figure 6. IL-33 increases mammary tumor cell proliferation and microvessel density. (*a*) The proliferation index was significantly higher in IL-33-treated mice and significantly lower in primary tumor tissues of ST2^{-}/$^{-}$ compared to WT mice. Proliferation index was determined by counting Ki-67-positive cells in three microscopic fields per tumor section. Data represents mean ± SEM from six tumors per group. Statistical significance was determined by Student's *t*-test. Original magnification, ×400. (*b*) IL-33 treatment increased microvessel density in primary tumors. Microvessels were stained with CD31 and α-SMA as markers of endothelial and vascular smooth muscle cells, respectively. Data represent mean ± SEM from six tumors per group. Statistical significance was determined by Student's *t*-test. Original magnification, ×400.]Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

3.72; $p < 0.05$) in this group of mice. Compared to PBS-treated mice, the mean counts of CD31^{+}α-SMA^{+} cells were significantly higher in primary tumors of mice given IL-33 (Fig. 6*b*), which also had higher CD31/α-SMA expression ratio (data not shown).

Discussion

To our knowledge, this is the first report to show the time-dependent increase of endogenous IL-33 in primary 4T1 mammary tumors in BALB/c mice during cancer progression as assessed by increased IL-33 mRNA and protein levels accompanied with the time-dependent increase of ST2 mRNA levels (Fig. 1). Endogenous IL-33 was expressed in CD45^{+} leucocytes and tumor cells in primary breast carcinoma and in metastatic lungs. Repeated administration of IL-33 enhanced tumor progression as evaluated by significantly accelerated growth of mammary tumors and metastatic spread to lungs and livers (Fig. 1).

Administration of IL-33 facilitated accumulation of suppressive CD11b^{+}Gr-1^{+} MDSCs both in mammary tumors and spleens (Figs. 2*a* and 4*a*). The importance of endogenous IL-33 in the regulation of these immunosuppressor cells is revealed by their markedly reduced presence in tumors and spleens in the absence of IL-33R, ST2 molecule. IL-33 treatment influenced the frequencies of monocytic *vs.* granulocytic MDSCs, as CD11c^{-}CD11b^{+}Ly6G^{-}Ly6C^{high} monocytic MDSCs were significantly increased while the frequencies of CD11c^{-}CD11b^{+}Ly6G^{+}Ly6C^{low} granulocytic MDSCs were significantly decreased in both mammary tumors and spleens in mice given IL-33 (Figs. 2*a* and 4*a*). There remains no consensus whether any of these MDSCs subpopulations are more immunosuppressive.[32] MDSCs are recruited to tumor sites from peripheral lymphoid organs where they promote the generation of CD4^{+}Foxp3^{+} Tregs[33] and exert immunosuppressive effects *via* production of TGF-β. Indeed, IL-33 treatment led to a significant increase of TGF-β1-producing CD11b^{+}CD11c^{-}Gr-1^{+} MDSCs (Figs. 2*a* and 4*a*). Moreover,

TGF-β1 mRNA levels in primary mammary tumors increased during cancer progression (Fig. 4*b*). It is well known that MDSCs require the presence of IL-13 for their activity, *i.e.*, expression of arginase and nitric oxide synthase II.[34] IL-33 administration expanded IL-13-producing $Lin^-Sca\text{-}1^+ST2^+$ ILCs within mammary tumors (Fig. 4*e*), the findings that suggest possible direct effect of ILCs on MDSCs activity, *via* IL-13. Intratumoral $CD45^+Lin^-$ cells were more numerous and highly expressed in Sca-1, CD25 and ST2, membrane markers indicative of their activated state after IL-33 treatment (Fig. 4*e*). Type 2 ILCs (ILC2) have been shown to facilitate Th2 immune response while preventing Th1-mediated immunity,[9–11,35,36] but their roles in cancer progression are not defined. We believe that tumor infiltrating ILCs were affected in the same manner as it has been described in the gut- and lung-associated mucosal tissues in mice given IL-33.[9–11] Namely, in our study, IL-33 increased the frequencies of IL-5- and IL-13-expressing ILCs and circulating levels of IL-13 in tumor-bearing hosts (Fig. 2*e*). Recent report shows IL-33-mediated increase of IL-5-producing ILCs that regulate antitumor activity of eosinophils in lung tumor metastasis model.[35] However, eosinophils possess both protumor and antitumor activities that are dependent on the tumor microenvironment and tumor type.[37,38] In our study, the increased accumulation of IL-13-producing $Lin^-Sca\text{-}1^+$ cells in spleens and primary tumors mediated by IL-33 might affect immunosuppressive functionality of MDSCs, which expressed IL-13α1 receptor in higher percentages.

We demonstrate increased frequencies of IL-10-producing Tregs that expressed ST2 in spleens and in mammary tumors (Figs. 2*b* and Fig. 4*d*) and poorly stimulatory IL-10-producing $CD11c^+$ DCs (Fig. 2*c*), the findings that are in agreement with the recent study showing amplification of these cell populations by IL-33 in a model of cardiac allograft survival.[17] We show a decrease of conventional $CD11c^+CD11b^+B220^-MHCII^+$ and an increase of plasmocytoid $CD11c^+CD11b^-B220^+MHCII^-$ DCs in tumor microenvironment in response to IL-33 (Fig. 4*c*). DCs phenotype is not necessarily related to their function that depends on experimental conditions, but pDCs might exhibit potent immunosuppressive and tolerogenic properties by affecting the function of $CD4^+$ and $CD8^+$ T cells and supporting polarization and activation of Tregs.[39,40] IL-33 is known to promote the generation of mature DCs that were poorly stimulatory and expressed less MHC or costimulatory molecules, but had increased expression of PD-1 ligands PD-L1 and PD-L2.[41] However, we found increased percentages of $CD11c^+MHCII^+$ cells in IL-33-treated tumor-bearing mice (Fig. 2*c*). Akbari *et al.*[42] showed that semimature IL-10-producing $MHCII^+$ DCs have regulatory properties and could induce $CD4^+$ T-cell unresponsiveness and IL-10-producing Tregs.

MDSCs, Tregs and immature DCs have been associated with suppressed antitumor immunity and enhanced neoangiogenesis in cancer.[24,26,43] Exogenous IL-33 decreased NK cells cytotoxicity against 4T1 tumor cells *in vitro*, while $CD8^+$ T cells cytotoxicity was not affected (Figs. 3*a* and 3*c*). Similarly, IL-33 reduced $NKp46^+CD107a^+$ NK cells in spleens and did not change the percentages of $CD8^+CD107a^+$ T cells (Figs. 3*a* and 3*c*). The effect of IL-33 on NK cells was also demonstrated by reduced presence of IFN-γ-expressing NK cells and increased NK cells that expressed IL-10 in tumor-bearing mice (Fig. 3*b*). The role of IL-33/ST2 axis in NK cells functions is not fully elucidated. IL-33 has been shown to directly stimulate or indirectly amplify IFN-γ production by iNKT and NK cells, but this effect was only seen in the presence of IL-12.[44] Increased $PD\text{-}1^+$ NK cells in spleens and tumors (Fig. 5*d*) and decreased FasL (Fig. 5*c*) and NKG2D-expressing NK cells (Fig. 5*b*) found in mice given IL-33 indicate downregulation of antitumor functions of NK cells.[45] There are evidences that PD-1 expression on NK cells facilitated metastasis in mouse melanoma model.[46] Of note, we demonstrate the opposite phenomenon in mice lacking ST2 gene, the findings that reflect the important pathophysiological role for endogenous IL-33 in NK cell functions. CD8-depleted IL-33-treated mice exhibited accelerated 4T1 tumor growth to a similar extent as observed in control animals (Fig. 3*d*), the findings that suggest a nonessential role of CD8 T-cells-mediated cytotoxic antitumor response in 4T1 mammary carcinoma. These data are in agreement with the study showing the crucial role for IL-33 in innate immunity.[47] Recent study by Bonilla *et al.*[5] revealed that IL-33 released from damaged virus-infected cells facilitates antiviral state, but this model is mainly dependent on cytotoxic $CD8^+$ T cells. In addition, the released IL-33 could be full-length molecule that exerts different effects than cleaved IL-33 molecule that was used in our study.[48] In the most recent study by Gao *et al.*,[19] transgenic expression of IL-33 stimulated NF-κB signaling and promoted the activation and infiltration of $CD8^+$ T cells and NK cells, which inhibited B16 melanoma and Lewis lung carcinoma pulmonary metastases. In both animal models, $CD8^+$ T cells have been shown to play an important antitumor role. In our study, we used 4T1 mammary carcinoma in BALB/c mice with weak immunogenicity and mainly innate antitumor immune response, in contrast to the study by Gao *et al.* in which C56/BL6 mice were used.

Angiogenesis is crucial for tumor growth and progression[31] and increased proliferative activity as well as microvessel density has been associated with accelerated progression of breast cancer.[31,49] IL-33 has the capacity to increase proliferation and migration of endothelial cells *in vitro*, and to induce angiogenesis in both *ex vivo* and *in vivo* models.[50] We found decreased tumor cells proliferation in ST2-deficient mice, while administration of IL-33 increased intratumoral cell proliferation with the significantly increased percentages of Ki-67-positive cells in mammary tumor tissue sections (Fig. 6*a*). IL-33 did not induce proliferation of 4T1 cells *in vitro*, which excludes the direct effect of IL-33 on tumor cells. We show increased blood vessel density in mammary tumors by using endothelial cell marker CD31 and α-SMA, marker

of pericytes and smooth muscle cells.[31] IL-33 enhanced neovascularization in mammary tumors as revealed by the increased number of $CD31^+$ α-SMA^+ cells and increased CD31/α-SMA expression ratio, the finding that suggests the increased density of immature blood vessels (Fig. 6*b*).

Collectively, IL-33 accelerates 4T1 breast cancer progression in at least two manners: through suppression of antitumor immunity and enhanced neoangiogenesis. IL-33 treatment facilitated the expansion of IL-13-producing $Lin^-Sca\text{-}1^+$ ILCs in spleens and mammary tumors, which might amplify immunosuppressive activities of IL-13α1-positive MDSCs. The subsequent induction of $CD4^+Foxp3^+$ $IL\text{-}10^+$ Tregs may promote the generation of tolerogenic or immature DCs resulting in suppression of NK cell cytotoxicity and tumor growth and metastases. The effects of IL-33 in breast cancer progression might thereby be controlled by the development of new biologics for tumor immunotherapy.

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