The Journal *of* Immunology

Tumoral Expression of IL-33 Inhibits Tumor Growth and Modifies the Tumor Microenvironment through CD8+ T and NK Cells FREE

Xin Gao; ... et. al

Related Content

Cutting Edge: Intravenous Ig Inhibits Invariant NKT Cell-Mediated Allergic Airway Inflammation through FcγRIIIA-Dependent Mechanisms

J Immunol (March,2011)

Cutting Edge: Flt3 Ligand Mediates STAT3-Independent Expansion but STAT3-Dependent Activation of Myeloid-Derived Suppressor Cells

J Immunol (April,2014)

IL-33 Released in the Liver Inhibits Tumor Growth via Promotion of CD4+ and CD8+ T Cell Responses in Hepatocellular Carcinoma

J Immunol (December,2018)

Tumoral Expression of IL-33 Inhibits Tumor Growth and Modifies the Tumor Microenvironment through CD8^{+} T and NK Cells

Xin Gao,*[,†,1] Xuefeng Wang,*[,†,‡,1] Qianting Yang,*[,§,1] Xin Zhao,*[,¶] Wen Wen,[†] Gang Li,*[,†] Junfeng Lu,[‖] Wenxin Qin,[#] Yuan Qi,*[,†] Fang Xie,[†] Jingting Jiang,** Changping Wu,** Xueguang Zhang,[†] Xinchun Chen,[§] Heth Turnquist,[††,‡‡] Yibei Zhu,[†] and Binfeng Lu*[,§§]

Cancer immunotherapy has shown great promise as a new standard cancer therapeutic modality. However, the response rates are limited for current approach that depends on enhancing spontaneous antitumor immune responses. Therefore, increasing tumor immunogenicity by expressing appropriate cytokines should further improve the current immunotherapy. IL-33 is a member of the IL-1 family of cytokines and is released by necrotic epithelial cells or activated innate immune cells and is thus considered a "danger" signal. The role of IL-33 in promoting type 2 immune responses and tissue inflammation has been well established. However, whether IL-33 drives antitumor immune responses is controversial. Our previous work established that IL-33 promoted the function of CD8^{+} T cells. In this study, we showed that the expression of IL-33 in two types of cancer cells potently inhibited tumor growth and metastasis. Mechanistically, IL-33 increased numbers and IFN-γ production by CD8^{+} T and NK cells in tumor tissues, thereby inducing a tumor microenvironment favoring tumor eradication. Importantly, IL-33 greatly increased tumor Ag-specific CD8^{+} T cells. Furthermore, both NK and CD8^{+} T cells were required for the antitumor effect of IL-33. Moreover, depletion of regulatory T cells worked synergistically with IL-33 expression for tumor elimination. Our studies established "alarmin" IL-33 as a promising new cytokine for tumor immunotherapy through promoting cancer-eradicating type 1 immune responses. *The Journal of Immunology*, 2015, 194: 438–445.

Tumor Ag–specific immune responses are either present spontaneously in human cancer patients as a critical component of tumor immune surveillance or can be elicited by cancer vaccination or adoptive T cell transfer (1–3). Type 1 immune responses, mediated by Th1, CD8^{+} T, NK, NKT, and γδ T cells, are thought to be a critical component of cell-mediated immunity against cancer (4). In humans, the presence of Th1 and CD8^{+} T within the tumor can be a favorable prognostic indicator (4). Blockade of immune checkpoint molecules and TIL-based immunotherapy have achieved great success with melanoma (5–7). It is well-known, however, that many tumor-infiltrating Th1 and CD8^{+} T cells are in a state of nonresponsiveness due to local mechanisms of immune suppression in the tumor microenvironment (8, 9). Many mechanisms are responsible for the apparent

failure of antitumor immunity including the active immunosuppression by the tumor microenvironment and the lack of sufficient immune-stimulatory signals. Therefore, reversing immune suppression in the tumor microenvironment is a key step for successful immunotherapy of cancer.

IL-33 is a member of the IL-1 family of cytokines (10). Its receptor complex consists of ST2 (also known as IL1RL1) and IL-1RAcP (11, 12). IL-33 is constitutively produced by structural and lining cells, such as epithelial cells and endothelial cells, where the first line of host defense against pathogens normally arises (13). Besides in epithelial cells, IL-33 can also be induced in myeloid cells and tissue stromal cells during infection. These properties of IL-33 make it a likely candidate "alarmin" for tissue damage and infection (14). IL-33 has been well established as a potent cytokine that promotes

*Department of Immunology, University of Pittsburgh School of Medicine, Pittsburgh, PA 15261; [†]Department of Immunology, Institute of Medical Biotechnology, Soochow University, Suzhou 215007, People's Republic of China; [‡]Department of Biochemistry and Molecular Biology, Soochow University, Suzhou 215007, People's Republic of China; [§]Guangdong Key Laboratory for Emerging Infectious Disease, Shenzhen Key Laboratory of Infection and Immunity, Third People's Hospital, Guangdong Medical College, Shenzhen, Guangdong 518112, China; [¶]The First Affiliated Hospital, Soochow University, Suzhou 215006, People's Republic of China; [‖]Key Laboratory of Cryogenics, Technical Institute of Physics and Chemistry, Chinese Academy of Sciences, Haidian District, Beijing 100190, People's Republic of China; [#]State Key Laboratory of Oncogenes and Related Genes, Shanghai Cancer Institute, Renji Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai 200032, China; **The Third Affiliated Hospital, Soochow University, Changzhou 213003, China; [††]Starzl Transplantation Institute, University of Pittsburgh School of Medicine, Pittsburgh, PA 15261; [‡‡]Department of Surgery, University of Pittsburgh School of Medicine, Pittsburgh, PA 15261; and [§§]University of Pittsburgh Cancer Institute, Pittsburgh, PA 15232

[1]X.G., X.W., and Q.Y. contributed equally to this article.

Received for publication May 27, 2014. Accepted for publication October 31, 2014.

Address correspondence and reprint requests to Dr. Binfeng Lu or Dr. Yibei Zhu, Department of Immunology, University of Pittsburgh School of Medicine, 200 Lothrop Street, Pittsburgh, PA 15261 (B.L.) or Department of Immunology, Soochow University, 708 Renmin Road, Suzhou 215007, People's Republic of China (Y.Z.). E-mail addresses: binfeng@pitt.edu (B.L.) or xlyb99@163.com (Y.Z.)

The online version of this article contains supplemental material.

Abbreviations used in this article: i.d., intradermally; MDSC, myeloid-derived suppressor cell; TIL, tumor-infiltrating lymphocyte; Treg, regulatory T cell.

Th2-mediated immune responses (10). Recent evidence also supports its role in type 1 immune responses defined by the predominant production of IFN-γ. We have shown that IL-33 synergized with both TCR and IL-12 to enhance IFN-γ production by CD8+ T and Th1 cells (15). In addition, IL-33 promotes IFN-γ production by NK cells and NKT cells (16–18). IL-33 signaling has also been shown to be required for eradication of viral infection through CD8+ T cells (19). Therefore, IL-33 is a candidate cytokine for reversing the immunosuppressive tumor microenvironment.

Because IL-33 is a danger signal released at the damaged tissue, we set out to determine whether tumoral expression of active IL-33 can render effective antitumor immune responses. In this study, we expressed IL-33 in two types of tumor cell lines and compared the growth upon transplantation to syngeneic mice. We found that overexpression of IL-33 in these tumor cells strongly inhibited tumor growth. IL-33 greatly increased numbers of tumor-infiltrating NK cells and CD8+ T cells, as well as their IFN-γ production. In addition, we showed that the inhibition of tumor growth by IL-33 was dependent on CD8+ T cells and NK cells, as well as IFN-γ and perforin. Moreover, depletion of regulatory T cells (Tregs) further improved the antitumor effect of IL-33. Taken together, our study establishes IL-33 as a promising cytokine for improving tumor immunotherapy.

Materials and Methods

Animals and tumor model

C57BL/6 (B6; H2K^b), BALB/c (H2K^d), and Rag2$^{-/-}$ IL2rg$^{-/-}$ mice were purchased from The Jackson Laboratory (Bar Harbor, ME). BALB/c St2$^{-/-}$ mice (20) and B6.CD4-cre T-bet$^{-/-}$ Eomes$^{-/-}$ (double knockout) mice were bred for experimental use at the University of Pittsburgh. All mice were housed in the specific pathogen-free facility of the University of Pittsburgh School of Medicine. Experiments were conducted under an Institutional Animal Care and Use Committee–approved protocol and in accordance with National Institutes of Health guidelines.

B16 cells were injected intradermally (i.d.) to B6 mice, and size of the tumor was monitored every 2 d. 4T1 cells were injected into the mammary fat pad of BALB/c mice, and size of the tumor was monitored every 2 d. Five mice in each group were injected with 200 μg anti-CD8 (Clone 53-6.72; Bioxcell, Lebanon, NH), or 15 μl anti-asialo GM1 (Wako Chemicals) Abs or control normal rat IgG (Bioxcell) four times before and after tumor inoculation (days −2, 1, 7, 14). Metastatic 4T1 tumor nodules were enumerated after the India ink staining procedure, as previously reported (21). India ink solution was injected through the trachea to inflate the lung, and the lung was stained for 5 min. The lungs were then removed and placed in Fekete's solution (70% alcohol, 10% formalin, and 5% acetic acid) for destaining. Tumor nodules did not absorb India ink, which resulted in the normal lung tissue staining black and the tumor nodules remaining white. Tumor nodules were counted blindly by two independent investigators.

Plasmid construction

pcDEF3-Dap10 vector was obtained from Dr. Lawrence Kane (University of Pittsburgh). The full-length IL-33 protein is a nuclear protein and only released by stressed cells. The mature peptide (S109 to I266) sequence of murine IL-33 was then amplified from cDNA generated from mouse lung RNA. The IL-33 expression construct was generated by fusing the nucleotide sequence encoding human CD8α signal sequence to the 5′ end of IL-33 (S109 to I266) sequence, then ligated into pcDEF3-Dap10 vector via BamH1 and EcoR1. IL-12 was generated by fusing the (G4S)3 linker sequence to the 3′ end of murine IL-12p40 sequence and 5′ end of murine IL-12p35 sequence. Then this was ligated into pcDNA3.1$^+$ vector or pcDEF3 via Kpn1 and Xba1 or BamH1 and Not I, respectively. All of the constructs were confirmed to be correct by DNA sequencing analysis (available upon request).

Cell culture

B16 and 4T1 cells were cultured in DMEM plus 10% FCS. IL-33 and IL-12 expression vectors were transfected into B16 and 4T1 cells, respectively, using Lipofectamine 2000 (Invitrogen Life Technologies) per the manufacturer's instructions. Empty vector (pcDNA3.1$^+$ or pcDEF3) was transfected into B16 and 4T1 cells as control. Twenty-four hours posttransfection, cells were diluted into culture plates and selected with G418 (Sigma-Aldrich) at a concentration of 600 mg/l. The stable cell lines were selected by further subcloning. Expression levels of IL-33 and IL-12 in the subclones were

determined by ELISA. Three independently generated B16–IL-33 and three independently generated B16–IL-12 clones, which produced around 5–50 ng/ml/24 h cytokines, were used in our experiments.

ELISA measurement of mouse IL-33

Purified anti-mouse IL-33 Ab (goat polyclonal IgG, Poly5165; BioLegend) was used as the capturing Ab. Biotin anti-mouse IL-33 Ab (Poly5165; BioLegend) was used as the detection Ab. Recombinant mouse IL-33 (ELISA Std.; BioLegend) was used as standard.

Analysis of tumor-infiltrating lymphocytes

Tumor masses were removed, minced, and digested with collagenase and hyaluronidase digestion solution (2.5 mg/ml collagenase I, 1 mg/ml collagenase IV, 0.25 mg/ml hyaluronidase IV-S, 300 μg/ml DNase I, and 0.01% HEPES in RPMI 1640 medium) at 37°C for 2 h. The pieces were then gently pressed between the frosted edges of two sterile glass slides, and the cell suspension was filtered through a 40-μm cell strainer (BD Biosciences). Tumor-infiltrating lymphocytes (TILs) were further purified by using a gradient as per manufacturer's protocol, washed, and resuspended in HBSS for analysis. The various cell populations were analyzed by flow cytometry. Flow cytometric analysis was performed using a FACS flow cytometer (BD Biosciences, San Jose, CA). For intracellular cytokine staining, harvested cells were stimulated with PMA (10 ng/ml) and ionomycin (1 μg/ml) for 4 h and incubated for the last 1 h with brefeldin A (10 μg/ml). Cells were subjected to intracellular cytokine analysis with anti–IFN-γ Ab (clone XMG1.2; eBioscience).

Evaluation of specific CD8+ T cell responses ex vivo

CD8+ T cells were isolated from spleen of tumor-bearing BALB/c mice by positive selection using immunomagnetic beads according to the manufacturer's protocol (CD8α [Ly-2] MicroBeads; Miltenyi Biotec, Auburn, CA) and passed through a magnetic cell sorting column (Miltenyi Biotec, Auburn, CA). APCs were prepared from normal splenocytes by depleting CD4+ and CD8+ T cells and then irradiated at 3000 Gy. Purified CD8+ T cells (2 × 10^6/well) were cocultured with 6000 Gy irradiated 4T1 tumor cells (5 × 10^5/well) and irradiated APCs (5 × 10^4/well) in presence of 20 IU/ml recombinant human IL-2 (obtained from the BRB Preclinical Repository) in 96-well round-bottom plates in a humidified incubator at 37°C and 5% CO$_2$. After 96 h, cell-free supernatants were harvested and assayed for IFN-γ by a murine IFN-γ ELISA kit (BD Pharmingen, San Diego, CA).

Results

IL-33 exerts a strong antitumor effect when expressed in the tumor cells

Our results, as well as others', have shown that IL-33 enhanced the function of effector CD8+ T and NK cells (15). We then postulated that IL-33 might have potent antitumor effects in vivo through CD8+ T and NK cells. To test this hypothesis, we sought to determine whether expression of IL-33 in tumor cells could inhibit tumor growth in vivo. We used the B16 melanoma cells because they represent an aggressive murine tumor model and are highly resistant to various immunotherapies. Thus, cytokines that have a significant effect on B16 tumor growth should have greater efficacy on other more immunogenic tumor cell lines. We generated B16 cell clones that express mouse IL-33. As control for the therapeutic efficacy, we also generated B16 clones that express mouse IL-12. Because full-length IL-33 is normally expressed in the nucleus, with the exception of neutrophils and macrophage that secret IL-33 by unknown mechanisms (22), we designed a secretable IL-33 by adding a leader sequence to IL-33. This allowed for IL-33 secretion from tumor cells (data not shown). The expression plasmid was transfected into B16F0 cells to generate stable cell lines, which were subjected to the limiting dilution culture to obtain B16–IL-33 clones. IL-33 was not detected in B16 cells, but 2–50 ng/ml IL-33 was made by B16–IL-33 clones (Supplemental Fig. 1). IL-33 did not inhibit B16 proliferation and survival in vitro during culture (Supplemental Fig. 2). Expression of IL-33 did not increase levels of MHC class I on B16 cells either. We injected control B16, B16–IL12, or B16–IL-33 into C57/
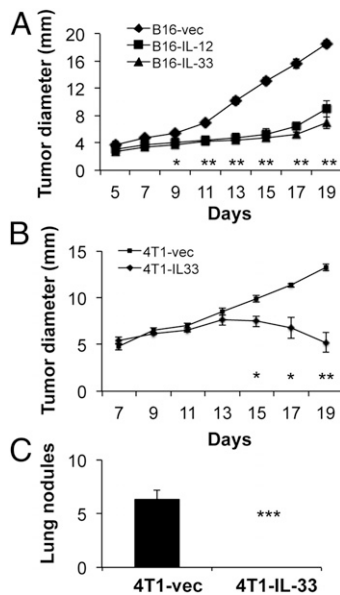
FIGURE 1. Expression of IL-33 in tumor cell lines inhibited tumor growth and metastasis in vivo. (**A**) A total of 2×10^5 B16-vector, B16–IL-12, or B16–IL-33 cells was injected i.d. into B6 mice, and the size of the tumor was monitored every 2 d. Data (mean ± SEM) are representative of three independent experiments. Five mice were in each group. Comparison was performed between the B16-vec and B16–IL-33 groups. (**B**) A total of 1×10^5 4T1-vector or 4T1–IL-33 cells was injected into the mammary fat pad of BALB/c mice, and the size of the tumor was monitored every 2 d. Data (mean ± SEM) are representative of three independent experiments. Five mice were in each group. (**C**) Metastatic tumor nodules in the lung were quantified 30 d after 4T1-vector and 4T1–IL-33 tumor inoculation. Data (mean ± SEM) are representative of three independent experiments. Five mice were in each group. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ determined by Mann–Whitney U test.

BL6 mice i.d. and monitored tumor growth every 2 d. Consistent with the established Th1-promoting function of IL-12, B16–IL-12 tumor grew much slower than the control B16 tumor (Fig. 1A). Importantly, tumor growth was greatly inhibited upon IL-33 expression (Fig. 1A). Thus, tumoral expression of IL-33 showed a potent antitumor effect similar to that of IL-12.

In addition to B16, we also tested IL-33 on the growth of transplanted 4T1 breast cancer cell line in BALB/c mice. IL-33 did not inhibit 4T1 proliferation and survival in vitro during culture (Supplemental Fig. 2). Nevertheless, similar to what we observed in the B16 model, 4T1 cells overexpressing IL-33 grew at a much slower rate than 4T1-vector controls (Fig. 1B). Thus, our data indicated that IL-33 exerted potent antitumor effects when expressed in tumor cells in BALB/c mice. 4T1 breast cancer cells metastasize mainly to the lung. We then examined lung metastasis after we sacrificed the mice around 31 d after tumor cell inoculation. Many tumor nodules were observed in lungs from 4T1-bearing mice. In sharp contrast, no tumor nodule was found in the lungs of 4T1–IL-33–bearing mice (Fig. 1C). These data support the idea that IL-33 has pronounced antitumor function when expressed and secreted from tumor cells.

Antitumor effect of IL-33 is dependent on ST2 expression on host cells and the presence of lymphocytes

Our RT-PCR analysis showed no ST2 expression on B16 or 4T1 cells (data not shown), thus supporting the hypothesis that IL-33 exerts its antitumor effect through direct action on host cells. To further confirm this, we inoculated WT and ST2$^{-/-}$ mice with 4T1 and 4T1–IL-33 cells and examined tumor growth. In wild type mice, the growth of 4T1–IL-33 tumors was inhibited compared

with 4T1 tumors. In contrast, 4T1 and 4T1–IL-33 grew at similar rates in ST2$^{-/-}$ mice. In addition, there was no difference in the growth rate between 4T1 cells inoculated in WT and ST2$^{-/-}$ mice (Fig. 2A). Thus, host cell ST2 signaling was crucial for the antitumor effect of IL-33. To further determine the cell types that are important for the tumor-inhibitory function of IL-33, we inoculated WT and Rag2$^{-/-}$IL2rg$^{-/-}$ mice, which lack T, B, and NK cells, with 4T1 and 4T1–IL-33 cells. 4T1–IL-33 tumors grew at a faster rate than that of 4T1 tumors in Rag2$^{-/-}$IL2rg$^{-/-}$ mice (Fig. 2B). These data suggest that lymphoid cells were elicited by IL-33 to inhibit tumor growth. IL-33 accelerated tumor growth in the absence of lymphocytes.

IL-33 promotes type 1 immune responses in the tumor site

Type 1 lymphocytes, particularly CD8$^+$ T and NK cells, are involved in tumor immunosurveillance. To further understand the underlying mechanisms of the tumor-inhibitory effect of IL-33, we characterized tumor-infiltrating CD8$^+$ and NK cells in B16-vec and B16–IL-33 tumors by flow cytometry. We first examined the CD8$^+$ and NK TILs from tumors around 10 d after inoculation. At this time point, the sizes of B16–IL-33 tumors and control B16 tumors were similar. Strikingly, we found that CD45$^+$ TILs were present at much greater number in B16–IL-33 tumors when compared with B16 tumors (Fig. 3A). This is consistent with a proinflammatory role of IL-33. In addition, within the CD45$^+$ TILs, the percentage of NK cells was much greater in B16–IL-33
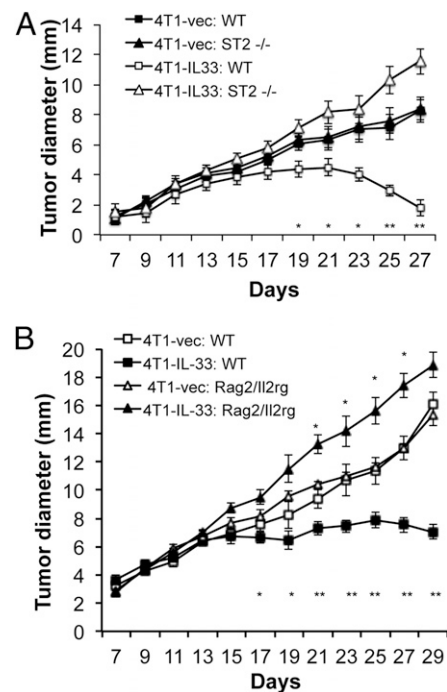

FIGURE 2. The antitumor effect of IL-33 is dependent on ST2 signaling and lymphocytes. (**A**) A total of 1×10^5 4T1-vector or 4T1-IL-33 cells was injected into the mammary fat pad of BALB/c and ST2$^{-/-}$ mice, and the size of the tumor was monitored every 2 d. Data (mean ± SEM) are representative of three independent experiments. Five mice were in each group. Significance difference was found between 4T1-IL-33: WT and 4T1-vector: WT groups. (**B**) A total of 1×10^5 4T1-vector or 4T1-IL-33 cells was injected into the mammary fat pad of BALB/c and Rag2$^{-/-}$IL2rg$^{-/-}$ mice, and the size of the tumor was monitored every 2 d. Data (mean ± SEM) are representative of three independent experiments. Five mice were in each group. Significant difference was found between 4T1-IL-33: WT and 4T1-vector: WT groups, as well as between 4T1-IL-33: Rag2$^{-/-}$ IL2rg$^{-/-}$ and 4T1-vector: Rag2$^{-/-}$ IL2rg$^{-/-}$ groups. *$p < 0.05$, **$p < 0.01$ determined by Mann–Whitney U test.

FIGURE 3. Expression of IL-33 in B16 cells enhanced type 1 immune responses in the tumor microenvironment. A total of 2×10^5 B16-vector or B16–IL-33 cells was injected i.d. into B6 mice. On day 10, tumors were resected and processed to generate single-cell suspension. (**A**) Percentages of CD45$^+$ cells in tumor cell suspension. (**B**) Flow cytometric plots showing NK and CD8$^+$ T cells in tumor and percentage of CD8$^+$ or NK1.1$^+$ cells within the CD45$^+$ population. (**C**) Representative flow cytometric plots showing IFN-γ$^+$ CD8$^+$ T cells in tumor and percentage of IFN-γ$^+$ cells in CD8$^+$ TILs. (**D**) Representative flow cytometric plots showing granzyme B$^+$CD8$^+$ T cells in tumor and percentage of granzyme B$^+$ cells in CD8$^+$ TILs. (**E**) Quantitative RNA analysis of IFN-γ, IL-12, perforin, and granzyme B in B16–IL-33 tumors compared with B16 control tumors. Results are mean ± SEM of three independent experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ two-tailed unpaired Student t test.
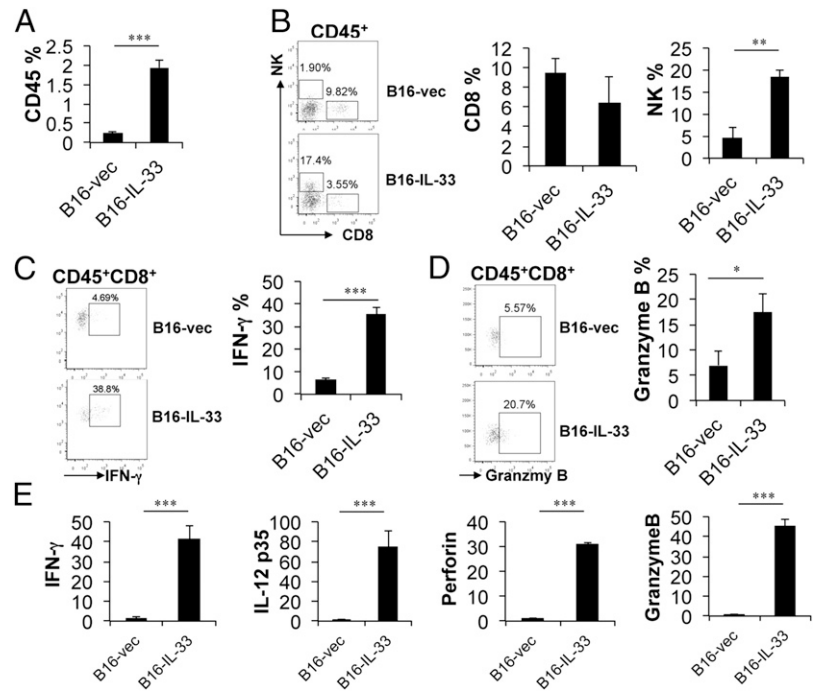
tumors when compared with control B16 tumors (Fig. 3B). Although the percentage of CD8$^+$ T cells was not significantly different between these tumors (Fig. 3B), much higher percentages of CD8$^+$ TILs in B16–IL-33 tumors produced IFN-γ and granzyme B when compared with those in B16 tumors (Fig. 3C, 3D). This effect was local, as no systematic changes of CD8$^+$ T or NK cells were found in the spleen, lymph nodes, or tumor draining lymph nodes. Quantitative RNA analysis further revealed that the mRNA levels of type 1 effector genes such as IFN-γ, IL-12, perforin, and granzyme B were highly upregulated in B16–IL-33 tumors when compared with B16 tumors (Fig. 3E). In addition, we found similar increases in CD8 and NK cells, as well as IFN-γ, in B16–IL-33 tumors around 20 d after tumor cell inoculation (Supplemental Fig. 3). These data suggest that IL-33, when overexpressed in tumor cells, promotes type 1 immune responses in tumor tissues.

T-bet and Eomes are master transcriptional factors essential for the antitumor activities of CD8$^+$ T cells (23–25). We have shown that T-bet and Eomes were critical for ST2 expression in activated CD8$^+$ T cells in vitro, suggesting ST2 expression is tightly linked to effector CD8$^+$ T cell differentiation. To determine whether T-bet and Eomes regulate ST2 expression in vivo, particularly during antitumor immune responses, we inoculated WT and T-bet$^{-/-}$ Eomes$^{-/-}$ mice with 4T1–IL-33 cells and analyzed the expression of ST2 on tumor-infiltrating CD8$^+$ T cells. ST2 was highly upregulated in CD8$^+$ TILs isolated from WT mice. In contrast, ST2 levels were greatly reduced in TIL from T-bet$^{-/-}$ Eomes$^{-/-}$ (DKO) mice (Fig. 4A). Thus, ST2 expression on CD8$^+$ TIL required T-bet and Eomes.

We then further investigated whether ST2 expression was required for tumor infiltration by CD8$^+$ T cells. We inoculated WT and ST2$^{-/-}$ mice with 4T1–IL-33 cells and analyzed the tumor-infiltrating CD8$^+$ T cells by flow cytometry. We found both the percentage of lymphocytes and percentage of CD8$^+$ T cells was greater in WT mice than that of ST2$^{-/-}$ mice (Fig. 4B). In addition, the percentage of NK cells was also increased in tumors from WT mice when compared with those from ST2$^{-/-}$ mice (Fig. 4B). These data are consistent with the idea that IL-33 signaling is required for the accumulation of CD8$^+$ T and NK cells in tumor.

IL-33 elicits tumor Ag-specific adaptive immune responses

We showed that tumor cell expression of IL-33 elicited greatly increased tumor-infiltrating CD8$^+$ T and NK cells. These findings prompted us to explore whether systemic tumor Ag-specific T cells can be induced to a higher level upon tumoral expression of IL-33. To address this question, we isolated CD8$^+$ T cells from the spleen of 4T1 or 4T1–IL-33 tumor-bearing mice. We then cocultured these CD8$^+$ T cells with irradiated tumor cells in the presence of APCs isolated from non–tumor-bearing mice. We then measured the levels of IFN-γ in these cultures by ELISA. We
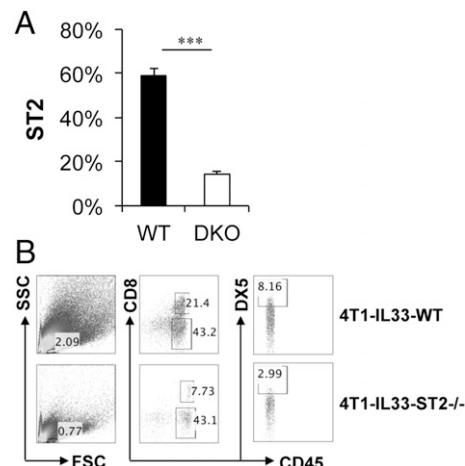

FIGURE 4. ST2 expression was regulated by T-bet and Eomes in vivo and required for CD8$^+$ T cells and NK cell infiltration of IL-33–expressing tumors. (**A**) A total of 2×10^5 B16–IL-33 cells was injected i.d. into B6 and T-bet$^{-/-}$ Eomes$^{-/-}$ (DKO) mice. Twenty days after inoculation, tumors were harvested and processed to generate a single-cell suspension. The surface expression of ST2 was analyzed by flow cytometry. Results are mean ± SEM of three independent experiments. ***$p < 0.001$ two-tailed unpaired Student t test. (**B**) A total of 1×10^5 4T1-vector or 4T1–IL-33 cells was injected into the mammary fat pad of BALB/c and ST2$^{-/-}$ mice. Twenty days after inoculation, tumors were harvested and processed to generate a single-cell suspension. Representative flow cytometric plots showing CD8$^+$ T and NK cells in tumor (TIL).

found <10 ng/ml IFN-γ was produced by CD8$^+$ T cells from 4T1-bearing mice. In contrast, ~60 ng/ml IFN-γ was produced by CD8$^+$ T cells isolated from 4T1-IL-33–bearing mice (Fig. 5A). These data demonstrated that tumoral expression of IL-33 led to a significant increase of tumor-specific CD8$^+$ T cells, supporting the role of IL-33 in eliciting a tumor-specific adaptive immune response.

IL-33 exerts its antitumor effects through CD8$^+$ T and NK cells, as well as IFN-γ and perforin effector molecules

Because CD8$^+$ T cells and NK cells were increased in 4T1–IL-33 tumors and tumor-specific CD8$^+$ T cells were increased in 4T1–IL-33–bearing mice, we then determined whether CD8$^+$ T cells and/or NK cells are required for the antitumor effect of IL-33. We used anti-CD8 and NK Abs to deplete CD8$^+$ T cells and NK cells, respectively, and then examined tumor progression in these mice compared with mice injected with control Abs. 4T1-IL-33–inoculated mice survived much longer compared with 4T1-inoculated mice (Fig. 5B). Injection of either anti-NK or anti-CD8 mAbs shortened the survival of 4T1-bearing mice compared with mice injected with control mAbs. Importantly, injection of either anti-NK or anti-CD8 mAbs into 4T1-IL-33–bearing mice greatly reduced the survival of these mice (Fig. 5B). Thus, both NK and CD8$^+$ T cells are required for the antitumor function of IL-33.

Because IFN-γ, perforin, and granzyme B were all increased in B16–IL-33 tumors compared with B16 tumors during both acute and chronic phases, we sought to determine whether these type 1 effector molecules were required for the antitumor effect of IL-33. We injected control B16 or B16-IL-33 into WT, IFN-$\gamma^{-/-}$, or perforin$^{-/-}$ mice i.d. and monitored tumor growth every 2 d. The growth inhibition by IL-33 was partially reversed in IFN-$\gamma^{-/-}$ mice. Perforin$^{-/-}$ mice also showed a more modest reversal of suppression (Fig. 5C). Therefore, IFN-γ played an important role in mediating antitumor effect of IL-33.

IL-33 regulates tumor-associated myeloid cells and promotes MHC II expression

To gain further insight into how IL-33 impacts the cellular network within tumor, we examined the myeloid compartment with particular focus on monocytes, neutrophils, and macrophages using CD11b and Gr1 markers. We first examined cells from tumors that

formed about 10 d after inoculation, and sizes were comparable between B16 and B16–IL-33 tumors. We found that percentage of Gr1^{high} CD11b$^+$ cells, largely neutrophils, was much higher in B16–IL-33 tumors than B16 tumors (Fig. 6A). The percentage of other CD11b$^+$ myeloid subsets (Gr1^{int}CD11b$^+$ monocytic cells and Gr1$^-$CD11b$^+$ macrophages) was not different between these tumors. Taking into account an increase in total CD45$^+$ immune cells, all CD11b$^+$ myeloid cells were increased in B16–IL-33 tumors compared with B16 control tumors. Our data support previous reports showing that tumor-associated neutrophils in the type 1 environment inhibit tumor growth (26, 27). We also characterized the CD11b$^+$ myeloid cells at a later time point (20 d after inoculation). Although we observed lower percentages of Gr1^{high} CD11b$^+$ myeloid cells within CD45$^+$ immune cells in the B16–IL-33 tumor, the number of these cells was actually higher in B16–IL-33 tumors due to greater numbers of CD45$^+$ TILs in B16–IL-33 tumors compared with B16 tumors. The percentage of other subsets of CD11b$^+$ cells was also higher in B16–IL-33 tumors if taking into account increased levels of CD45$^+$ immune cells in B16–IL-33 tumors compared with B16 tumors. In the mouse tumor microenvironment, many Gr1$^+$CD11b$^+$ (including both Gr1^{high} and Gr1^{int}) cells are considered myeloid-derived suppressor cells (MDSCs), and these cells do not express MHC class II (28). Our analysis revealed that the level of MHC II was much higher in Gr1^{high} and Gr1^{int} cells in the B16–IL-33 tumor when compared with the B16 tumor, consistent with an increase of immunogenicity of the B16–IL-33 tumor microenvironment (Fig. 6B, 6C). This is likely due to IFN-γ produced by CD8$^+$ T cells and NK cells. ST2 was highly expressed in all subpopulations of CD11b$^+$ cells isolated from B16–IL-33, suggesting that IL-33 might directly modulate the function of these cells (Fig. 6D). Thus, we showed in this study that IL-33 promoted type 1 immune responses in the tumor microenvironment, and increased the flux of myeloid cells and their MHC class II expression.

Elimination of Tregs further improves antitumor function of IL-33

Despite the apparent presence of strong type 1 immune responses and inhibition of tumor growth, the B16–IL-33 tumor continues to increase in size. This prompted us to examine whether self-limiting

FIGURE 5. NK and CD8$^+$ T cells, as well as IFN-γ and perforin, were required for the antitumor effect of IL-33. (**A**) A total of 1×10^5 4T1-vector or 4T1–IL-33 cells was injected into the mammary fat pad of BALB/c mice. Thirty days after inoculation, CD8$^+$ T cells were purified from the spleens of these mice and restimulated with irradiated 4T1 cells for 72 h. The level of IFN-γ was then measured by ELISA. Results are mean ± SEM of three independent experiments. *$p < 0.05$ two-tailed unpaired Student t test. (**B**) Mice were inoculated i.d. with 1×10^5 4T1 tumor cells ($n = 5$). These mice were injected i.p. with anti-CD8, or anti-asialo GM1 Abs, or control IgG four times before and after tumor inoculation (days −2, 1, 7, 14). Kaplan–Meier survival curves are shown and the log-rank test was performed. $p = 0.0018$. (**C**) A total of 2×10^5 B16-vector or B16–IL-33 cells was injected i.d. into WT B6, IFN-$\gamma^{-/-}$ B6, or perforin$^{-/-}$ B6 mice, and the size of the tumor was monitored every 2 d. Tumor diameters (mean ± SEM) are presented. Five mice were in each group. Comparison was performed between the B16–IL-33: WT and B16–IL-33: IFN-$\gamma^{-/-}$ groups. The difference between the B16–IL-33: WT and B16–IL-33: perforin$^{-/-}$ groups was significance from days 13–19. *$p < 0.05$ determined by Mann–Whitney U test.
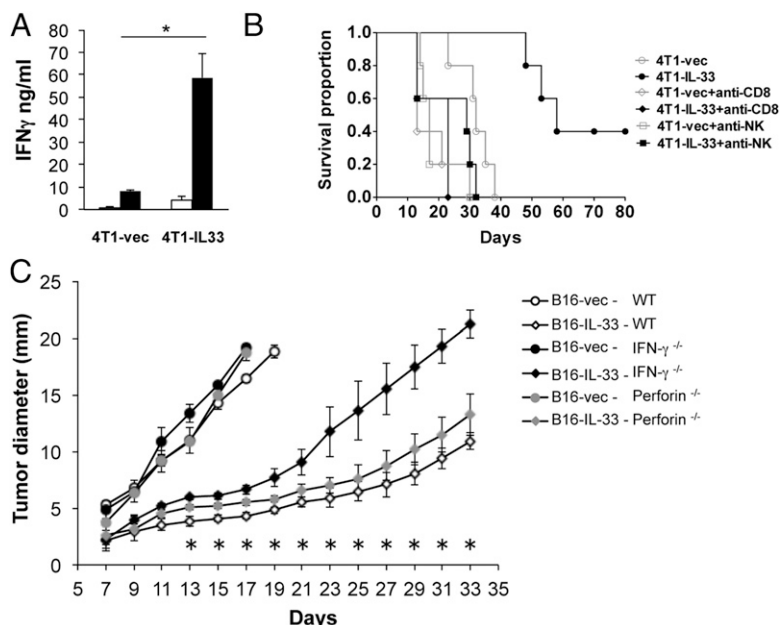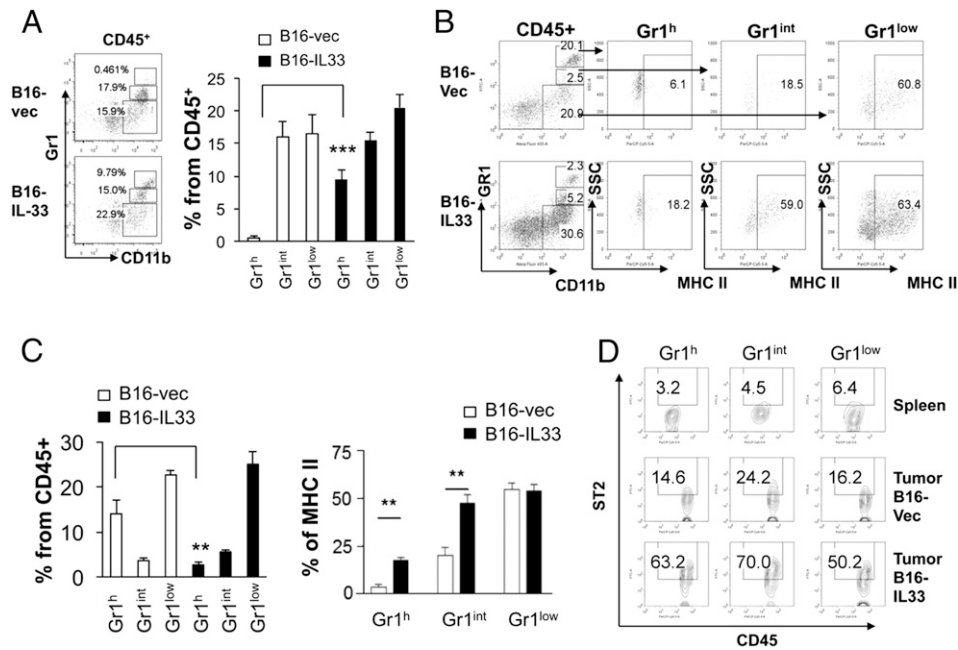
FIGURE 6. IL-33 regulated tumor-associated myeloid cells and upregulated MHC II on tumor-associated myeloid cells. A total of 2×10^5 B16-vector or B16–IL-33 cells was injected i.d. into B6 mice. On day 10 or 20, tumors were resected and processed to generate a single-cell suspension. (**A**) Representative flow cytometric analysis of CD11b$^+$ subpopulations and percentages of CD11b$^+$ subpopulations within CD45$^+$ cells on day 10. (**B**) Representative flow cytometric analysis of CD11b$^+$ subpopulations on day 20. (**C**) Percentages of CD11b$^+$ subpopulations in CD45$^+$ cells and percentages of MHC class II expression on three CD11b$^+$ subpopulations on day 20. Results are mean $\pm$ SEM of three independent experiments. (**D**) Surface ST2 expression on Gr1^{high} CD11b$^+$, Gr1^{int} CD11b$^+$, and Gr1^{low} CD11b$^+$ cells from spleen and tumor. Results are mean $\pm$ SEM of three independent experiments. $**p < 0.01$, $***p < 0.001$ two-tailed unpaired Student t test.

immune regulatory mechanisms were also induced in the B16–IL-33 tumor. We focused on CD4 T cells for two reasons. First, Th2 cells have been shown to be upregulated by IL-33 (29). Second, Tregs are a dominant group of CD4$^+$ T cells within the tumor, and we showed that IL-33 promoted Treg proliferation in the spleen (30–32). Despite reports that IL-33 induces Th2 responses (11), we did not find significant numbers of IL-4$^+$, IL-5$^+$, or IL-13$^+$ CD4$^+$ TILs (data not shown). In contrast, we found >60% of CD4$^+$ TILs were Foxp3$^+$, presumably Tregs (Fig. 7A, Supplemental Fig. 4). Interestingly, ~30% of these Tregs from B16–IL-33 tumors were ST2$^+$. Similar percentages of Tregs from B16 tumor also expressed ST2. However, levels seemed lower. The fact that many Tregs express ST2 suggested that IL-33 might have a direct effect on these cells (Fig. 7A); thus, the antitumor effect of IL-33 could be inhibited by IL-33–influenced Tregs. To test this hypothesis, we depleted Tregs in mice using anti-CD25 (PC61) Ab. We found B16 grew at a similar rate in B6 mice regardless of anti-CD25 application, consistent with previous findings (33). In contrast, Treg depletion resulted in a much greater shrinkage of B16–IL-33 tumors (Fig. 7B). These data indicated that IL-33 induced type 1 antitumor immune responses, but such responses were kept in check by Tregs.

Discussion

Tumor growth can trigger inflammatory responses by releasing danger signals and expression of tumor Ags. Tumor progression is inhibited by type 1 lymphocytes such as Th1, CD8$^+$ T, NK, and $\gamma\delta$ T cells, as well as collaborating innate cells such as dendritic cells and type 1 macrophages and neutrophils (M1 and N1) (4, 23, 26, 27). Nevertheless, tumors progress by enlisting and driving the dominance of immune-suppressive cell types such as Tregs and MDSCs, as well as myeloid cells that produce cancer-promoting factors. Our study showed that high-level expression of IL-33 in tumors led to increases in antitumor immune responses and promoted the type 1 lymphocyte-dominated tumor immunogenic

microenvironment. This was due to IL-33–induced increases in the production of type 1 effector molecules, such as IFN-γ and granzymes, by CD8$^+$ and NK cells in the tumor microenvironment.

Our study establishes an antitumor function of IL-33. This finding is in keeping with recent studies that also show a potent function of IL-33 in driving type 1 immune responses in tumor,
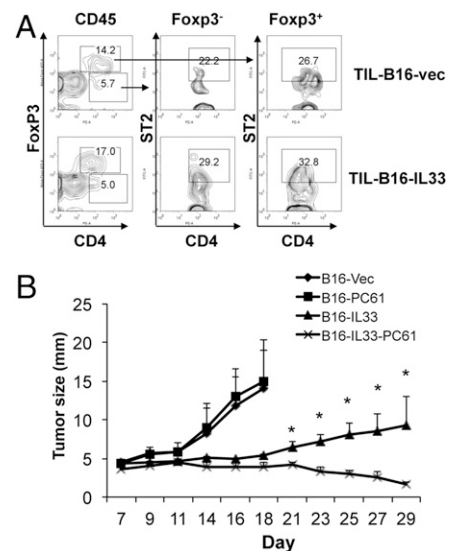

FIGURE 7. Synergistic effect of IL-33 expression and depletion of Tregs for eliminating tumors. (**A**) Representative flow cytometric analysis of ST2 on Foxp3$^+$CD4$^+$ and Foxp3$^-$CD4$^+$ in B16 tumor and B16–IL-33 tumor. (**B**) B6 mice were injected with control Rat IgG or PC61, 200 μg per mouse twice a week. One week after Ab injection, 2×10^5 B16-vector (B16) or B16–IL-33 cells were injected i.d. into these mice. Tumor sizes were measured every 2 d. Tumor diameters (mean $\pm$ SEM) are presented. Five mice were in each group. Comparison was performed between B16–IL-33 and PC61 B16–IL-33 groups. $*p < 0.05$ determined by Mann–Whitney U test.

viral infection, and autoimmune models. Published work by our laboratory and others showed that activated CD8^{+} T cells and Th1 cells produce larger amounts of IFN-γ in response to IL-33 (15, 34). In addition, antiviral immune responses require IL-33 signaling in CD8^{+} T cells (19). Furthermore, Th1, NK, and NKT cells respond to IL-33 in a similar fashion to increase their IFN-γ production (16, 17, 35). Overexpression of IL-33 systemically in transgenic mice promotes NK and CD8^{+} T cell function globally and inhibits tumor growth (36). IL-33 has also been used to further boost antitumor vaccination (37). In addition, in the Con A–induced hepatitis model, IL-33 has been shown to be pathogenic (38) by inducing type 1 immune responses. These studies clearly established a critical role of IL-33 in promoting type 1 immune responses. Nevertheless, there are studies that show an opposite function of IL-33. One study shows that systemic injection of low levels of IL-33 leads to increases in tumor metastasis (39). In the Con A–induced hepatitis model, IL-33 has also been shown to be protective (40). In the transplantation setting, i.p. injection of IL-33 protects cardiac allografts through expansion regulatory cells, particularly Tregs (32). We believe that differences in the level of IL-33 and the primary target cells of IL-33 in these experiments are accountable for the different in vivo effects. Persistent and higher levels of IL-33 in tumor tissues, as well as during infection, promote type 1 immune responses. In addition, other cytokines, such as IL-2 and IL-12, both of which were shown to synergize with IL-33 to induce IFN-γ, can help shape the nature of IL-33–driven immune responses (15, 34). In contrast, low-level and systemic delivery of IL-33 protein into resting or tumor-bearing mice resulted in immune tolerance (32, 39, 40). In the Con A–induced hepatitis model, IL-33 has been shown to be protective in one and pathogenic in another (38, 40). In the Con A–induced hepatitis model, higher doses and frequencies of IL-33 injection elicited strong type 1 immune responses, whereas lower doses of IL-33 resulted in immune tolerance. Thus, the immune phenotype of IL-33 is likely shaped by dose, the local environment, and the target cells. Regulatory effects may be achieved through the systemic actions of IL-33, which expand and activate MDSCs, tolerogenic dendritic cells, and Tregs (32, 41). However, high-level and tumor-site delivery of IL-33 can mediate potent antitumor responses mediated by CD8^{+} T cells, NK and NKT, and IFN-γ. This knowledge will be key for the clinical application of this cytokine for cancer immunotherapy. This can potentially be achieved by inducible expression of IL-33 in tumor-specific T cells for enhancing adoptive T cell therapy or tumor Ag-specific, Ab-mediated IL-33 delivery (42).

CTLA-4 and PD-1 mAb-based immunotherapies have achieved great clinical success for melanoma (5–7). Nevertheless, the response rate for this type of therapy for other solid cancers will likely be limited because of its dependence on spontaneous T cell responses to tumor. Introducing the right inflammation to the tumor microenvironment should help further increase the response rate of immunotherapy in solid cancer by making the tumor more immunogenic. Cytokine-mediated immune therapy of cancer should provide new opportunities for enhancing the immune "checkpoint"-based approach; thus, IL-33 should be a promising cytokine for enhancing cancer immunotherapy.

Acknowledgments

Disclosures

The authors have no financial conflicts of interest.

References

1. Dunn, G. P., A. T. Bruce, H. Ikeda, L. J. Old, and R. D. Schreiber. 2002. Cancer immunoediting: from immunosurveillance to tumor escape. *Nat. Immunol.* 3: 991–998.
2. Finn, O. J. 2008. Cancer immunology. *N. Engl. J. Med.* 358: 2704–2715.
3. Rosenberg, S. A., J. C. Yang, R. M. Sherry, U. S. Kammula, M. S. Hughes, G. Q. Phan, D. E. Citrin, N. P. Restifo, P. F. Robbins, J. R. Wunderlich, et al. 2011. Durable complete responses in heavily pretreated patients with metastatic melanoma using T cell transfer immunotherapy. *Clin. Cancer Res.* 17: 4550–4557.
4. Galon, J., A. Costes, F. Sanchez-Cabo, A. Kirilovsky, B. Mlecnik, C. Lagorce-Pagès, M. Tosolini, M. Camus, A. Berger, P. Wind, et al. 2006. Type, density, and location of immune cells within human colorectal tumors predict clinical outcome. *Science* 313: 1960–1964.
5. Wolchok, J. D., H. Kluger, M. K. Callahan, M. A. Postow, N. A. Rizvi, A. M. Lesokhin, N. H. Segal, C. E. Ariyan, R. A. Gordon, K. Reed, et al. 2013. Nivolumab plus ipilimumab in advanced melanoma. *N. Engl. J. Med.* 369: 122–133.
6. Brahmer, J. R., S. S. Tykodi, L. Q. Chow, W. J. Hwu, S. L. Topalian, P. Hwu, C. G. Drake, L. H. Camacho, J. Kauh, K. Odunsi, et al. 2012. Safety and activity of anti-PD-L1 antibody in patients with advanced cancer. *N. Engl. J. Med.* 366: 2455–2465.
7. Topalian, S. L., F. S. Hodi, J. R. Brahmer, S. N. Gettinger, D. C. Smith, D. F. McDermott, J. D. Powderly, R. D. Carvajal, J. A. Sosman, M. B. Atkins, et al. 2012. Safety, activity, and immune correlates of anti-PD-1 antibody in cancer. *N. Engl. J. Med.* 366: 2443–2454.
8. Zou, W. 2005. Immunosuppressive networks in the tumour environment and their therapeutic relevance. *Nat. Rev. Cancer* 5: 263–274.
9. Zou, W. 2006. Regulatory T cells, tumour immunity and immunotherapy. *Nat. Rev. Immunol.* 6: 295–307.
10. Liew, F. Y., N. I. Pitman, and I. B. McInnes. 2010. Disease-associated functions of IL-33: the new kid in the IL-1 family. *Nat. Rev. Immunol.* 10: 103–110.
11. Schmitz, J., A. Owyang, E. Oldham, Y. Song, E. Murphy, T. K. McClanahan, G. Zurawski, M. Moshrefi, J. Qin, X. Li, et al. 2005. IL-33, an interleukin-1-like cytokine that signals via the IL-1 receptor-related protein ST2 and induces T helper type 2-associated cytokines. *Immunity* 23: 479–490.
12. Chackerian, A. A., E. R. Oldham, E. E. Murphy, J. Schmitz, S. Pflanz, and R. A. Kastelein. 2007. IL-1 receptor accessory protein and ST2 comprise the IL-33 receptor complex. *J. Immunol.* 179: 2551–2555.
13. Moussion, C., N. Ortega, and J. P. Girard. 2008. The IL-1-like cytokine IL-33 is constitutively expressed in the nucleus of endothelial cells and epithelial cells in vivo: a novel 'alarmin'? *PLoS ONE* 3: e3331.
14. Haraldsen, G., J. Balogh, J. Pollheimer, J. Sponheim, and A. M. Küchler. 2009. Interleukin-33 - cytokine of dual function or novel alarmin? *Trends Immunol.* 30: 227–233.
15. Yang, Q., G. Li, Y. Zhu, L. Liu, E. Chen, H. Turnquist, X. Zhang, O. J. Finn, X. Chen, and B. Lu. 2011. IL-33 synergizes with TCR and IL-12 signaling to promote the effector function of CD8+ T cells. *Eur. J. Immunol.* 41: 3351–3360.
16. Smithgall, M. D., M. R. Comeau, B. R. Yoon, D. Kaufman, R. Armitage, and D. E. Smith. 2008. IL-33 amplifies both Th1- and Th2-type responses through its activity on human basophils, allergen-reactive Th2 cells, iNKT and NK cells. *Int. Immunol.* 20: 1019–1030.
17. Bourgeois, E., L. P. Van, M. Samson, S. Diem, A. Barra, S. Roga, J. M. Gombert, E. Schneider, D. Dy, P. Gourdy, et al. 2009. The pro-Th2 cytokine IL-33 directly interacts with invariant NKT and NK cells to induce IFN-gamma production. *Eur. J. Immunol.* 39: 1046–1055.
18. Bourgeois, E. A., A. Levescot, S. Diem, A. Chauvineau, H. Bergès, P. Milpied, A. Lehuen, D. Damotte, J. M. Gombert, E. Schneider, et al. 2011. A natural protective function of invariant NKT cells in a mouse model of innate-cell-driven lung inflammation. *Eur. J. Immunol.* 41: 299–305.
19. Bonilla, W. V., A. Fröhlich, K. Senn, S. Kallert, M. Fernandez, S. Johnson, M. Kreutzfeldt, A. N. Hegazy, C. Schrick, P. G. Fallon, et al. 2012. The alarmin interleukin-33 drives protective antiviral CD8^{+} T cell responses. *Science* 335: 984–989.
20. Townsend, M. J., P. G. Fallon, D. J. Matthews, H. E. Jolin, and A. N. McKenzie. 2000. T1/ST2-deficient mice demonstrate the importance of T1/ST2 in developing primary T helper cell type 2 responses. *J. Exp. Med.* 191: 1069–1076.
21. Lewis, J. D., M. H. Shearer, R. C. Kennedy, and R. K. Bright. 2005. Surrogate tumor antigen vaccination induces tumor-specific immunity and the rejection of spontaneous metastases. *Cancer Res.* 65: 2938–2946.
22. Lefrançais, E., and C. Cayrol. 2012. Mechanisms of IL-33 processing and secretion: differences and similarities between IL-1 family members. *Eur. Cytokine Netw.* 23: 120–127.
23. Chen, L. J., X. Zheng, Y. P. Shen, Y. B. Zhu, Q. Li, J. Chen, R. Xia, S. M. Zhou, C. P. Wu, X. G. Zhang, et al. 2013. Higher numbers of T-bet(+) intratumoral lymphoid cells correlate with better survival in gastric cancer. *Cancer Immunol. Immunother.* 62: 553–561.
24. Lu, B., L. Chen, L. Liu, Y. Zhu, C. Wu, J. Jiang, and X. Zhang. 2011. T-cell-mediated tumor immune surveillance and expression of B7 co-inhibitory molecules in cancers of the upper gastrointestinal tract. *Immunol. Res.* 50: 269–275.
25. Zhu, Y., S. Ju, E. Chen, S. Dai, C. Li, P. Morel, L. Liu, X. Zhang, and B. Lu. 2010. T-bet and eomesodermin are required for T cell-mediated antitumor immune responses. *J. Immunol.* 185: 3174–3183.
26. Fridlender, Z. G., J. Sun, S. Kim, V. Kapoor, G. Cheng, L. Ling, G. S. Worthen, and S. M. Albelda. 2009. Polarization of tumor-associated neutrophil phenotype by TGF-beta: "N1" versus "N2" TAN. *Cancer Cell* 16: 183–194.
27. Granot, Z., E. Henke, E. A. Comen, T. A. King, L. Norton, and R. Benezra. 2011. Tumor entrained neutrophils inhibit seeding in the premetastatic lung. *Cancer Cell* 20: 300–314.

28. Gabrilovich, D. I., and S. Nagaraj. 2009. Myeloid-derived suppressor cells as regulators of the immune system. *Nat. Rev. Immunol.* 9: 162–174.

29. Xu, D., W. L. Chan, B. P. Leung, Fp. Huang, R. Wheeler, D. Piedrafita, J. H. Robinson, and F. Y. Liew. 1998. Selective expression of a stable cell surface molecule on type 2 but not type 1 helper T cells. *J. Exp. Med.* 187: 787–794.

30. Tanchot, C., M. Terme, H. Pere, T. Tran, N. Benhamouda, M. Strioga, C. Banissi, L. Galluzzi, G. Kroemer, and E. Tartour. 2013. Tumor-infiltrating regulatory T cells: phenotype, role, mechanism of expansion in situ and clinical significance. *Cancer Microenviron.* 6: 147–157.

31. Gao, X., Y. Zhu, G. Li, H. Huang, G. Zhang, F. Wang, J. Sun, Q. Yang, X. Zhang, and B. Lu. 2012. TIM-3 expression characterizes regulatory T cells in tumor tissues and is associated with lung cancer progression. *PLoS ONE* 7: e30676.

32. Turnquist, H. R., Z. Zhao, B. R. Rosborough, Q. Liu, A. Castellaneta, K. Isse, Z. Wang, M. Lang, D. B. Stolz, X. X. Zheng, et al. 2011. IL-33 expands suppressive CD11b+ Gr-1(int) and regulatory T cells, including ST2L+ Foxp3+ cells, and mediates regulatory T cell-dependent promotion of cardiac allograft survival. *J. Immunol.* 187: 4598–4610.

33. Matsushita, N., S. A. Pilon-Thomas, L. M. Martin, and A. I. Riker. 2008. Comparative methodologies of regulatory T cell depletion in a murine melanoma model. *J. Immunol. Methods* 333: 167–179.

34. Ngoi, S. M., M. C. St Rose, A. M. Menoret, D. E. Smith, M. G. Tovey, A. J. Adler, and A. T. Vella. 2012. Presensitizing with a Toll-like receptor 3 ligand impairs CD8 T-cell effector differentiation and IL-33 responsiveness. *Proc. Natl. Acad. Sci. USA* 109: 10486–10491.

35. Seltmann, J., T. Werfel, and M. Wittmann. 2013. Evidence for a regulatory loop between IFN-γ and IL-33 in skin inflammation. *Exp. Dermatol.* 22: 102–107.

36. Gao, K., X. Li, L. Zhang, L. Bai, W. Dong, K. Gao, G. Shi, X. Xia, L. Wu, and L. Zhang. 2013. Transgenic expression of IL-33 activates CD8(+) T cells and NK cells and inhibits tumor growth and metastasis in mice. *Cancer Lett.* 335: 463–471.

37. Villarreal, D. O., M. C. Wise, J. N. Walters, E. L. Reuschel, M. J. Choi, N. Obeng-Adjei, J. Yan, M. P. Morrow, and D. B. Weiner. 2014. Alarmin IL-33 acts as an immunoadjuvant to enhance antigen-specific tumor immunity. *Cancer Res.* 74: 1789–1800.

38. Chen, J., L. Duan, A. Xiong, H. Zhang, F. Zheng, Z. Tan, F. Gong, and M. Fang. 2012. Blockade of IL-33 ameliorates Con A-induced hepatic injury by reducing NKT cell activation and IFN-γ production in mice. *J. Mol. Med.* 90: 1505–1515.

39. Jovanovic, I. P., N. N. Pejnovic, G. D. Radosavljevic, J. M. Pantic, M. Z. Milovanovic, N. N. Arsenijevic, and M. L. Lukic. 2014. Interleukin-33/ST2 axis promotes breast cancer growth and metastases by facilitating intratumoral accumulation of immunosuppressive and innate lymphoid cells. *Int. J. Cancer* 134: 1669–1682.

40. Volarevic, V., M. Mitrovic, M. Milovanovic, I. Zelen, I. Nikolic, S. Mitrovic, N. Pejnovic, N. Arsenijevic, and M. L. Lukic. 2012. Protective role of IL-33/ST2 axis in Con A-induced hepatitis. *J. Hepatol.* 56: 26–33.

41. Liu, Q., and H. R. Turnquist. 2013. Implications for Interleukin-33 in solid organ transplantation. *Cytokine* 62: 183–194.

42. Zhang, L., S. P. Kerkar, Z. Yu, Z. Zheng, S. Yang, N. P. Restifo, S. A. Rosenberg, and R. A. Morgan. 2011. Improving adoptive T cell therapy by targeting and controlling IL-12 expression to the tumor environment. *Mol. Ther.* 19: 751–759.