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Blocking senescence and tolerogenic function of dendritic cells induced by γδ Treg cells enhances tumor-specific immunity for cancer immunotherapy

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ABSTRACT

Background Regulatory T (Treg) cells are a key component in maintaining the suppressive tumor microenvironment and immune suppression in different types of cancers. A precise understanding of the molecular mechanisms used by Treg cells for immune suppression is critical for the development of effective strategies for cancer immunotherapy.

Methods Senescence development and tolerogenic functions of dendritic cells (DCs) induced by breast cancer tumor-derived γδ Treg cells were fully characterized using real-time PCR, flow cytometry, western blot, and functional assays. Loss-of-function strategies with pharmacological inhibitor and/or neutralizing antibody were used to identify the potential molecule(s) and pathway(s) involved in DC senescence and dysfunction induced by Treg cells. Impaired tumor antigen HER2-specific recognition and immune response of senescent DCs induced by γδ Treg cells were explored in vitro and in vivo in humanized mouse models. In addition, the DC-based HER2 tumor vaccine immunotherapy in breast cancer models was performed to explore the enhanced antitumor immunity via prevention of DC senescence through blockages of STAT3 and programmed death-ligand 1 (PD-L1) signaling.

Results We showed that tumor-derived γδ Treg cells promote the development of senescence in DCs with tolerogenic functions in breast cancer. Senescent DCs induced by γδ Treg cells suppress Th1 and Th17 cell differentiation but promote the development of Treg cells. In addition, we demonstrated that PD-L1 and STAT3 signaling pathways are critical and involved in senescence induction in DCs mediated by tumor-derived γδ Treg cells. Importantly, our complementary in vivo studies further demonstrated that blockages of PD-L1 and/or STAT3 signaling can prevent γδ Treg-induced senescence and reverse tolerogenic functions in DCs, resulting in enhanced HER2 tumor-specific immune responses and immunotherapy efficacy in human breast cancer models.

Conclusions These studies not only dissect the suppressive mechanism mediated by tumor-derived γδ Treg cells on DCs in the tumor microenvironment but also provide novel strategies to prevent senescence and dysfunction in DCs and enhance antitumor efficacy mediated by tumor-specific T cells for cancer immunotherapy.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Different types of regulatory T (Treg) cells create immunosuppressive microenvironments in patients with cancer that are a major obstacle to effective antitumor immunity and successful tumor immunotherapy. Accumulated myeloid dendritic cells (DCs) with tolerogenic functions are also critical components within the suppressive tumor microenvironment in different types of cancers.

WHAT THIS STUDY ADDS

⇒ We used both in vitro studies and in vivo in breast cancer humanized mouse tumor models to demonstrate that tumor-derived γδ Treg cells can promote senescence in DCs, leading to tolerogenic functions and impaired antitumor immunity of DCs. We also identified that programmed death-ligand 1 (PD-L1) and STAT3 signaling are important for controlling DC senescence mediated by Treg cells, and blockages of these signaling in DCs can prevent DC senescence and enhance antitumor immunity and immunotherapy.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ These studies indicate that prevention of the development of DC senescence and tolerogenic functions induced by Treg cells within the tumor microenvironment is a novel and promising strategy for cancer immunotherapy.

INTRODUCTION

Regulatory T (Treg) cells induce immune tolerance by suppressing host immune responses against self-antigens or non-self-antigens, thus playing critical roles in preventing autoimmune diseases. However, different types of Treg cells also create immunosuppressive microenvironments in patients with cancer, which are a major obstacle to effective antitumor immunity and successful tumor immunotherapy.[1 2] We have demonstrated that accumulated γδ Treg cells exist in the human breast

tumor microenvironment[3 4] and that patients with a high proportion of γδ Treg cells have advanced cancer stage, high lymph node metastasis, poor survival, and high risk of relapse.[4 5] We further discovered that breast cancer-derived γδ Treg cells use a novel suppressive mechanism of senescence induction on T cells and DCs in the tumor suppressive microenvironment.[6 7] However, how those effector T cells and dendritic cells (DCs) become senescent immune cells with dysfunctional antitumor immunity in the suppressive tumor microenvironment is still under investigation. Therefore, it is critical to dissect the molecular changes responsible for dysfunctional antitumor immunity mediated by Treg cells in order to develop effective approaches for clinical immunotherapy.

In addition to the dysfunctional T cells, accumulated myeloid DCs with tolerogenic functions are also critical components within the suppressive tumor microenvironment in different types of cancers.[8–10] However, the mechanisms responsible for the development of tolerogenic DCs mediated by malignant tumor is unclear. We have shown that breast cancer-derived γδ Treg cells can suppress DC maturation and functions, resulting in DC senescence.[3 6 7] We have more recently identified that tumor-derived Treg cells mediate the acceleration of glucose consumption and trigger cell senescence and DNA damage in effector T cells during their crosstalk.[6 11–14] Furthermore, MAPK signaling and STAT3 are key signaling pathways that involve T-cell senescence induction during the Treg-effector T interactions.[6 11–14] However, whether Treg-induced suppression and senescence on DCs in the tumor microenvironment are also through similar molecular actions as on effector T cells is unclear. Improved understating of these molecular mechanisms is urgently needed to develop novel and effective tumor vaccines and immunotherapies.

In our efforts to dissect how tumor-derived γδ Treg cells promote the development of senescent DCs with tolerogenic functions in breast cancer, we found that senescent DCs induced by breast cancer-derived γδ Treg cells have impaired effector functions, leading to suppressing Th1 and Th17 cell differentiation but promoting the development of Treg cells. In addition, we demonstrated that activated programmed death-ligand 1 (PD-L1) and STAT3 signaling pathways are responsible for senescence development in DCs mediated by tumor-derived γδ Treg cells. Importantly, our complementary in vivo studies further demonstrated that PD-L1 and STAT3 signaling blockages could reverse γδ Treg-induced senescence in DCs and enhance HER2 tumor-specific immune responses and antitumor immunotherapy efficacy in breast cancer models. These studies not only dissect the suppressive mechanism mediated by tumor-derived γδ Treg cells on innate immune cells, but also provide novel strategies to prevent senescence in DCs and enhance antitumor efficacy mediated by tumor-specific T cells for breast cancer immunotherapy.

RESULTS

Breast cancer-derived γδ Treg cells induce senescence in DCs with tolerogenic phenotypes

To understand the interaction between γδ Treg cells and DCs, we continued to use our already established human γδ Treg cells from the primary breast cancer tissues of different patients with cancer for the studies.[3 4 7] These γδ Treg cells are $CD8^+$γδ1 T cells, secreting interferon (IFN)-γ and granulocyte macrophage colony-stimulating factor (GM-CSF), but not suppressive cytokines interleukin (IL)-10 or transforming growth factor (TGF)-β. They express little or no CD25 and FoxP3, which are highly expressed in $CD4^+$ Treg cells.[3] Our previous studies have shown that breast tumor-derived γδ Treg cells can block the maturation and function of DCs.[3 7] We further identified that γδ Treg cells can promote the induction of senescence in DCs expressing senescence-associated beta-galactosidase (SA-β-Gal) (figure 1A). Senescent DCs induced by γδ Treg cells had altered phenotypes and functions, including downregulation of maturation molecule CD83, and costimulatory molecules CD80, CD86, and human leukocyte antigen (HLA)-DR isotype expression, but upregulation of PD-L1 expression (online supplemental figure S1A), which were consistent with our previous findings.[3 7] Furthermore, senescent DCs induced by γδ Treg cells suppressed allogeneic naïve $CD4^+$ and $CD8^+$ T-cell proliferation and growth after co-culture for 14 days, but mature DCs pretreated with or without effector CD4-C1 T cells promoted the naïve T-cell growth (figure 1B). In addition, senescent DCs strongly inhibited the proliferation of responding $CD4^+$ T cells stimulated by anti-CD3 antibody, while mature DCs pretreated with or without effector T cells increased the proliferation of anti-CD3-stimulated responding T cells (figure 1C). We also found that senescent DCs induced by γδ Treg cells decreased the release of proinflammatory cytokines IL-1β, IL-6, IL-12, and tumor necrosis factor (TNF)-α after stimulation with lipopolysaccharides (LPS) compared with untreated mature DCs or treated with control effector T cells, which were consistent to our previous findings (figure 1D).[3 7] Toll-like receptors (TLRs) are central receptors on DCs, which can recognize pathogen-derived products and subsequently provoke DC maturation, antigen presentation, and cytokine secretion.[15 16] Given that γδ Treg-induced senescent DCs had impaired phenotypes and functions, we next determined whether those senescent DCs had altered TLR expression levels. We observed that different TLRs were highly expressed in mature DCs. However, treatment with γδ Treg cells but not control CD4-C1 effector T cells significantly downregulated most TLR expressions except TLR8 in senescent DCs (figure 1E). Our results indicate that human breast tumor-derived γδ Treg cells induce DCs into senescent DCs that have tolerogenic phenotypes and impaired functions.

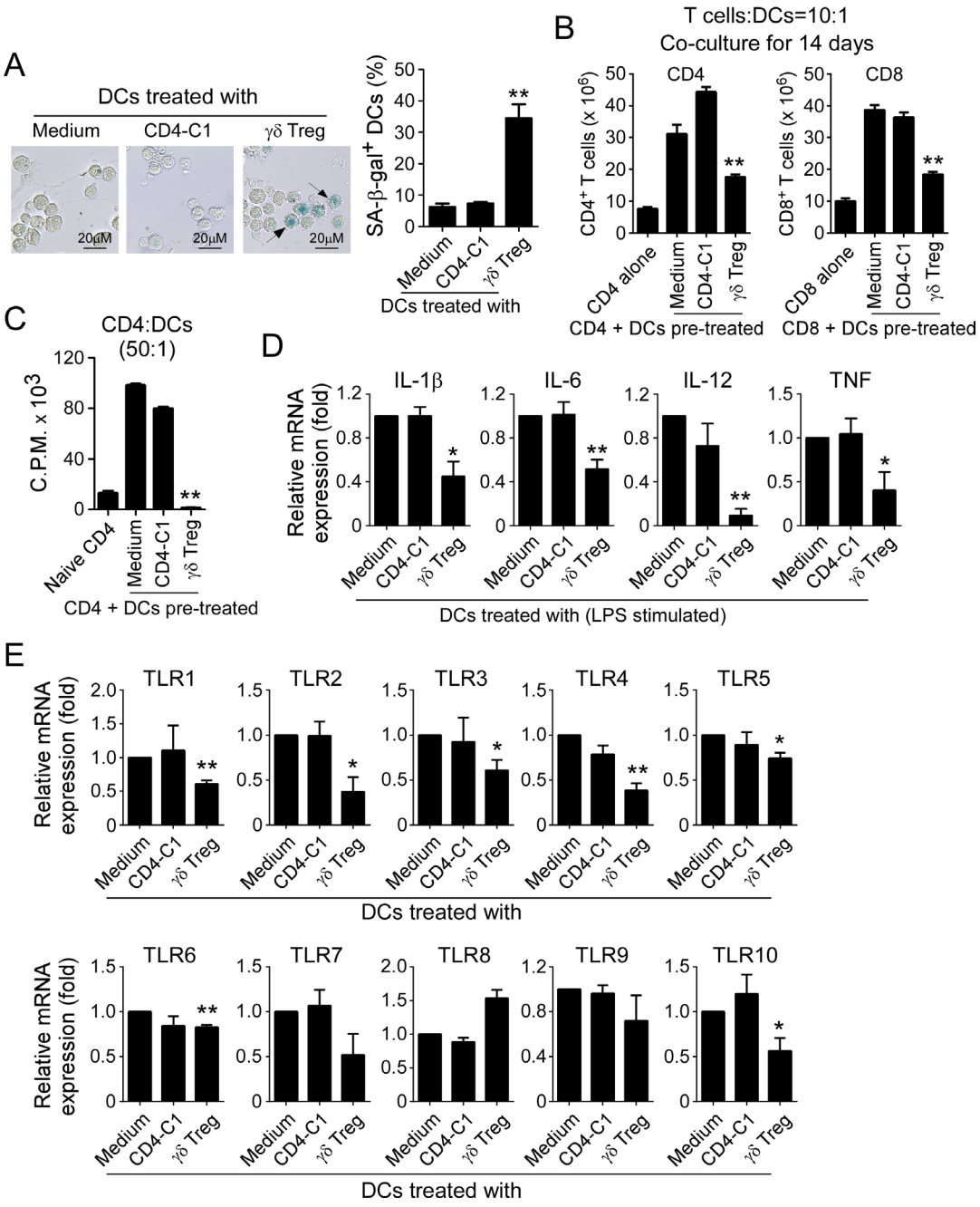

Figure 1 Senescent DCs induced by tumor-derived γδ Treg cells have tolerogenic phenotypes. (A) γδ Treg treatment markedly upregulated SA-β-Gal expression in DCs. Monocyte-derived human immature DCs were incubated alone or co-cultured with γδ Treg or CD4-C1 effector T cells at a ratio of 3:1 in the presence of GM-CSF, IL-4 and TNF-α for 2 days. The treated DCs were purified and stained for SA-β-Gal expression. Results shown in the right panel are mean±SD from three independent experiments. **p<0.01, compared with the medium only and CD4-C1 treatment groups. (B) Decreased stimulation capacity on T-cell growth mediated by senescent DCs. $CD4^+$ or $CD8^+$ T cells were co-cultured with DCs pretreated with γδ Treg cells or control CD4-C1 cells at a ratio of 10:1 for 14 days. The treated $CD4^+$ or $CD8^+$ T cells numbers were counted. **p<0.01, compared with the medium only and CD4-C1 treatment DC groups. (C) Senescent DCs induced by γδ Treg cells strongly inhibited the proliferation of responding $CD4^+$ T cells stimulated with an anti-CD3 antibody using [^{3}H]-thymidine incorporation assays after culture for 3 days. DC preparation and procedure were the same as in (A). **p<0.01, compared with the medium only and CD4-C1 treatment DC groups. (D) and (E) Significant decreases of gene expression of inflammatory cytokines and TLRs by γδ Treg-induced senescent DCs. Cell treatment and procedure were the same as in (A). The mRNA expression levels of each cytokine after LPS stimulation for 24 hours (in D) and TLR (in E) in DCs were determined by real-time PCR. The expression level was normalized to glyceraldehyde-3-phosphate dehydrogenase expression and adjusted to the levels in mature DCs in the medium. Data shown are as mean±SD from three independent experiments. **p<0.01, compared with the DCs in medium-only and CD4-C1 cell treatment groups. DCs, dendritic cells; GM-CSF, granulocyte macrophage colony-stimulating factor; IL, interleukin; LPS, lipopolysaccharides; mRNA, messenger RNA; SA-β-Gal, senescence-associated beta-galactosidase; TLR, toll-like receptor; TNF, tumor necrosis factor; Treg, regulatory T cells.

Senescent DCs induced by γδ Treg cells inhibit Th1, Th2, and Th17 cell differentiation, but promote the development of FoxP3$^+$ Treg cells

Senescent DCs induced by tumor-derived γδ Treg cells suppress the proliferation and growth of responder T cells. We thus confirmed that suppression of T cells mediated by γδ Treg-induced senescent DCs is not due to the promotion of apoptosis or cell death in CD4$^+$ T cells (online supplemental figure S2). γδ Treg-treated senescent DCs highly expressed PD-L1 and had the typically tolerogenic DC phenotypes. Therefore, we reasoned that senescent DCs might differentially suppress distinct T helper cell lineages. We co-cultured naive CD4$^+$ T cells with senescent DCs induced by γδ Treg cells under the T-cell culture medium for 6 days and the portions of Th1, Th2, Th17, and Treg cells were determined by flow cytometry analyses. We found that the senescent DCs induced by γδ Treg cells dramatically decreased IFN-γ, IL-4, and IL-17-producing T-cell populations in the co-cultured T cells, compared with T cells stimulated by mature DCs pretreated with or without control CD4-C1 effector T cells (figure 2A). However, senescent DCs induced by γδ Treg cells promoted FoxP3$^+$ T-cell populations in the co-cultured CD4$^+$ T cells, suggesting the development of Treg cells (figure 2A). This result was supported by the studies from other groups demonstrating that tolerogenic DCs have the ability to convert naive T cells into FoxP3$^+$ Treg cells with suppressive activity.[17 18] We next determined whether senescent DCs also affect effector CD4$^+$ T subset polarization. We co-cultured naive CD4$^+$ T cells with senescent DCs induced by γδ Treg cells or control DCs under Th1 or Th17 differentiation-biased conditions. γδ Treg-induced senescent DCs showed strong suppression of Th1 cell differentiation as indicated by reduced expression of IFN-γ. In contrast, mature DCs treated or untreated with control CD4-C1 effector T cells promoted Th1 differentiation (figure 2B). Similarly, senescent DCs induced by γδ Treg cells potently suppressed the differentiation and polarization of Th17 cells under Th17 cell-biased conditions (figure 2C). Collectively, these results suggest that γδ Treg-induced senescent DCs with tolerogenic phenotypes can inhibit effector Th1, Th2, and Th17 cell differentiation but drive FoxP3$^+$ Treg development.

STAT3 and PD-L1 are key molecules involved in the regulation of DC senescence induced by tumor-derived γδ Treg cells

Based on our transcriptional profiling of the Treg-induced senescent T cells, we have identified that STAT1 and STAT3 are the most significantly altered transcription factors involved in the regulation of T-cell senescence.[11 14] Furthermore, JAK/STAT3 signaling is also involved in fatty acid β-oxidation and regulation of cancer stem cell self-renewal and chemoresistance in breast cancer.[19] In addition, our studies have demonstrated that γδ Treg treatment significantly upregulates PD-L1 expression in treated senescent DCs (online supplemental figure 1). Therefore, we further investigated causative relationships between STAT signaling, PD-L1 expression, and DC senescence and tolerogenic functions induced by γδ Treg cells. We first confirmed that mature DCs treated with γδ Treg cells but not control effector CD4-C1 cells had significantly enhanced activation and phosphorylation of STAT3, as well as PD-L1 expression (figure 3A). Furthermore, pretreatment of mature monocyte-derived DCs with STAT3 inhibitor S3I-201, or an anti-PD-L1 neutralizing antibody, significantly blocked phosphorylation of STAT3 and expression of PD-L1, respectively, in senescent DCs induced by γδ Treg cells (figure 3B). Importantly, we found that pretreatment of DCs with S3I-201 or anti-PD-L1 neutralizing antibody dramatically prevented senescence induction in DCs mediated by γδ Treg cells (figure 3C). In addition, blockage of STAT3 or PD-L1 activities with the inhibitor and neutralizing antibody in DCs recovered the downregulated expression of CD83 and costimulatory molecules CD80, CD86, and HLA-DR, as well as decreased expression of PD-L1 in senescent DCs mediated by γδ Treg cell treatment (figure 3D). Notably, blockage of STAT3 or PD-L1 activities in DCs also prevented the decrease of proinflammatory cytokines IL-1β, IL-6, IL-12, and TNF-α after stimulation with LPS, as well as reversed the downregulation of different TLRs in senescent DCs induced by γδ Treg cells (figure 3E and online supplemental figure S3). These results suggest that transcription factor STAT3 and checkpoint inhibitory molecular PD-L1 directly control the molecular processes of senescence induction and dysfunction in DCs mediated by γδ Treg cells.

Senescent DCs induced by γδ Treg cells fail to process tumor antigens and activate antigen-specific T cells

We have shown that senescent DCs have an impaired ability to process and present purified protein derivative (PPD)-specific T-cell immune responses.[7] We next determined whether senescent DCs have the decreased capacity to process and present a real tumor antigen to induce tumor-specific T-cell immune responses. HER2/neu is a well-established tumor antigen in breast cancer and has been extensively evaluated in both murine and human models.[20 21] We selected HER2/neu 369–377 (KIFGSLAFL) and HER-2/neu 689–697 (RLLQETELV), two HLA-A2.1-restricted cytotoxic T lymphocyte (CTL) epitopes, for our current studies.[22–24] CD4$^+$ and CD8$^+$ T cells purified from healthy donors were co-cultured with HER2 protein-pulsed autologous DCs, or HLA-A2-restricted two peptide-pulsed DCs, respectively. Those DCs were pretreated with medium, control CD4-C1 effector T cells, or breast cancer-derived γδ Treg cells before loading with HER2 tumor antigens. We found that senescent DCs induced by γδ Treg cells pulsed with HER2 protein had a weak ability to stimulate autologous CD4$^+$ T-cell proliferation and growth. In contrast, HER2 protein-pulsed DCs pretreated with or without CD4-C1 effector T cells strongly stimulated autologous CD4$^+$ T-cell proliferation and growth (figure 4A). Similarly, γδ Treg-induced senescent DCs pulsed with HER2 HLA-A2 peptides had a decreased ability to stimulate autologous CD8$^+$ T-cell proliferation, compared with peptide-pulsed

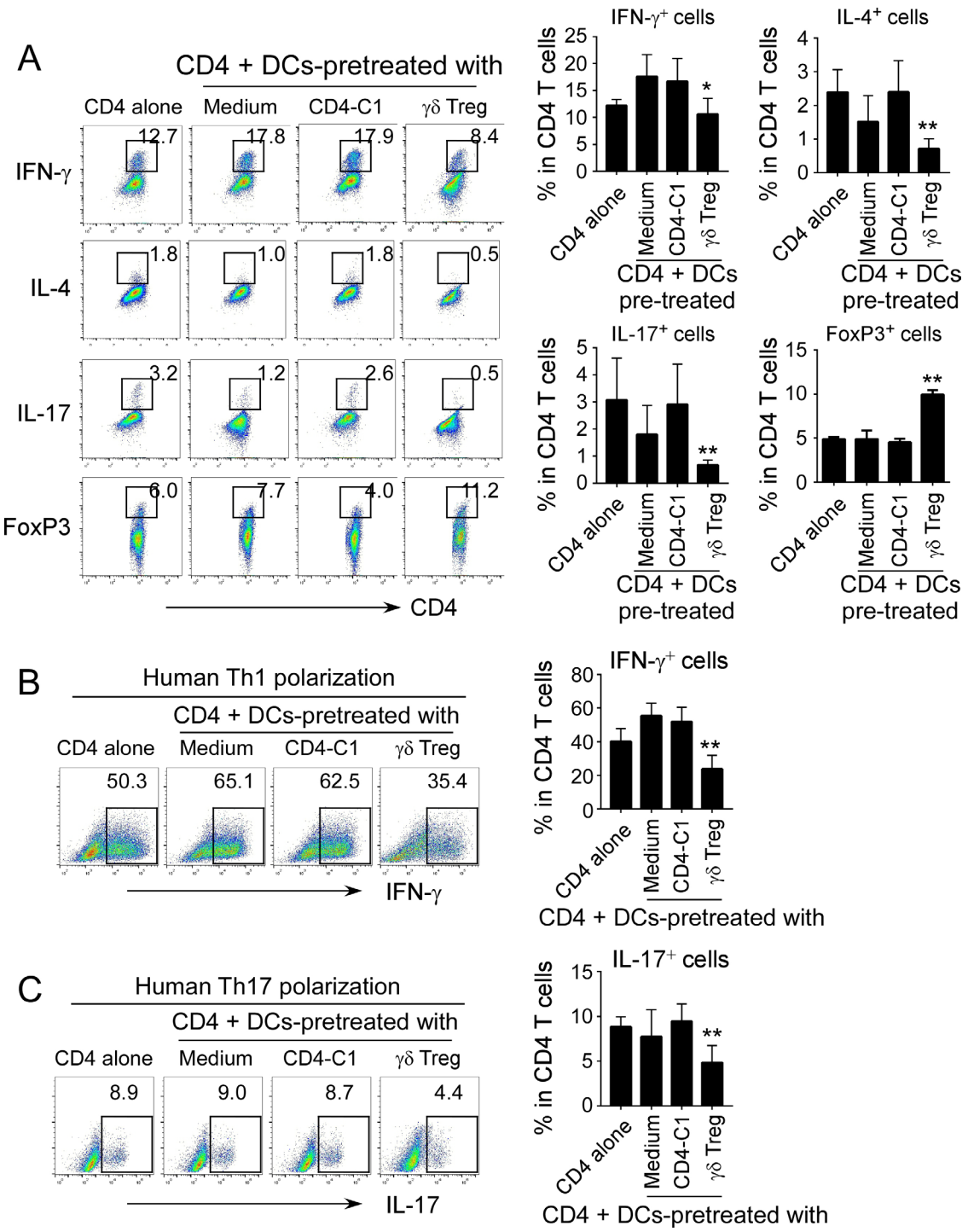

Figure 2 Effects on T-cell subset differentiation mediated by γδ Treg-induced senescent DCs. (A) Senescent DCs induced by γδ Treg cells had inhibitory effects on Th1, Th2 and Th17 cell development but promoted FoxP3$^+$ Treg cell generation. Immature DCs were incubated alone or co-cultured with γδ Treg or CD4-C1 T cells at a ratio of 3:1 in the presence of GM-CSF, IL-4, and TNF for 2 days, and then co-cultured with human naive CD4$^+$ T cells at a ratio of 1:10 in the plate-bound anti-CD3/CD28 for 6 days. Results are representative intracellular staining of CD4$^+$ T subsets with different DC treatments using the flow cytometry analyses (left panel). Data in the right panel are mean±SD from three independent experiments of different T subset cells with indicated DC treatments. *p<0.05 and **p<0.01, compared with the DCs in the medium-only group. (B) and (C) Senescent DCs induced by γδ Treg cells inhibited human Th1 and Th17 cell polarization from naive CD4$^+$ T cells in vitro. Treated DCs were co-cultured with human naive CD4$^+$ T cells at a ratio of 1:10 in the presence of plate-bound anti-CD3/CD28 under Th1 or Th17-polarization conditions for 6 days. IFN-γ or IL-17-producing T cells were determined using flow cytometry analyses after stimulation with PMA and ionomycin. Data in the right panel are mean±SD from three independent experiments of T-cell subsets with indicated DC treatments. **p<0.01, compared with the DCs in the medium-only group. DCs, dendritic cells; GM-CSF, granulocyte macrophage colony-stimulating factor; IFN, interferon; IL, interleukin; PMA, phorbol myristate acetate; TNF, tumor necrosis factor; Treg, regulatory T cells.

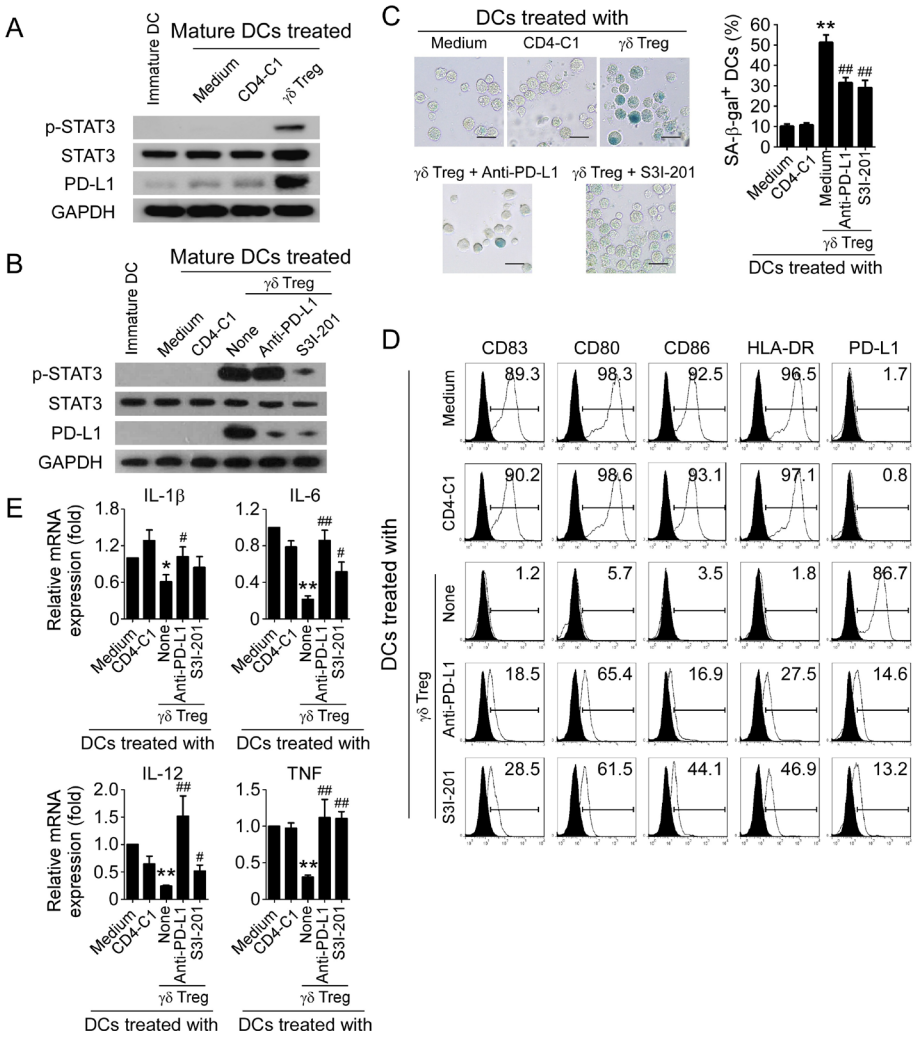

Figure 3 PD-L1 and STAT3 signaling are required for the induction of senescent DCs mediated by γδ Treg cells. (A) Upregulation of PD-L1 and phosphorylated STAT3 in senescent DCs induced by γδ Treg cells. Immature DCs were incubated alone or co-cultured with γδ Treg or CD4-C1 T cells at a ratio of 3:1 in the presence of GM-CSF, IL-4, and TNF-α for 2 days. Expressions of PD-L1 and phosphorylated STAT3 in DCs were determined by western blot analyses. (B) Inhibition of PD-L1 or STAT3 signaling by an anti-PD-L1 neutralizing antibody or specific STAT3 inhibitor significantly suppressed PD-L1 expression and STAT3 phosphorylation in senescent DCs using western blot analyses. Immature DCs were pretreated with or without an anti-PD-L1 antibody (5 µg/mL) for 2 hours or S3I-201 (10 µM) for 24 hours, and then incubated alone or co-cultured with γδ Treg or CD4-C1 effector T cells at a ratio of 3:1 in the presence of GM-CSF, IL-4, and TNF-α for 2 days. (C) Inhibition of PD-L1 or STAT3 signaling markedly decreased SA-β-Gal expression in DCs treated by γδ Treg cells. DC treatment and procedure were the same as in (B). The treated DCs were purified and stained for SA-β-Gal. Results shown in the right panel are mean±SD from three independent experiments. **p<0.01, compared with the medium only and CD4-C1 treatment groups. ##p<0.01, compared with the Treg-treated DC-only group. (D) and (E) Inhibition of PD-L1 or STAT3 signaling recovered the impaired phenotypes and cytokine secretions in senescent DCs induced by γδ Treg cells. DC treatment and procedure were the same as in (B). The treated DCs were purified and analyzed phenotypes by flow cytometry (in D), and mRNA expression levels of each cytokine were determined by the real-time qPCR analyses after stimulation with LPS for 24 hours (in E). Data are shown as mean±SD from three independent experiments. **p<0.01, compared with the medium-only and CD4-C1 treatment groups. ##p<0.01, compared with the Treg-treated DC group. DCs, dendritic cells; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GM-CSF, granulocyte macrophage colony-stimulating factor; IL, interleukin; LPS, lipopolysaccharides; mRNA, messenger RNA; PD-L1, programmed death-ligand 1; qPCR, quantitative PCR; SA-β-Gal, senescence-associated beta-galactosidase; TNF, tumor necrosis factor; Treg, regulatory T cells.

DCs alone or DCs pretreated with CD4-C1 effector T cells (figure 4A). We then determined whether blockage of PD-L1 and/or STAT3 signaling can recover the ability of γδ Treg-induced senescent DCs to stimulate T-cell proliferation. As expected, we found that treatment with a PD-L1 neutralizing antibody or STAT3 inhibitor S3I-201 dramatically reversed the ability of HER2-pulsed senescent DCs to stimulate autologous T-cell proliferation (figure 4B).

We further investigated HER2 antigen-specific immune responses induced by DCs with different treatments. $CD4^+$ or $CD8^+$ T cells were cocultured with HER2 protein-pulsed

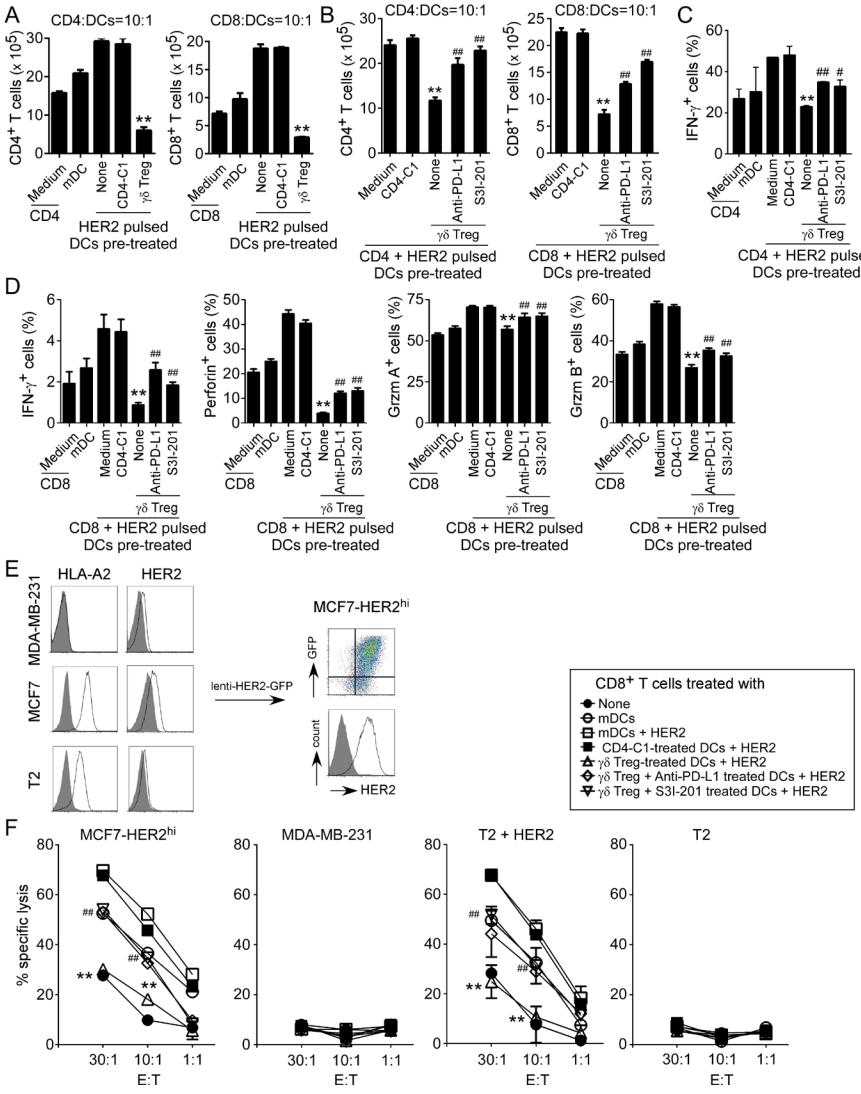

Figure 4 Senescent DCs induced by γδ Treg cells have impaired functions to present tumor antigens and activate T cells. (A) γδ Treg treatment inhibited the ability of DCs pulsed with HER2 antigen to stimulate autologous T-cell proliferation. Immature DCs were co-cultured with or without γδ Treg or CD4-C1 T cells at a ratio of 3:1 in the presence of GM-CSF, interleukin-4, and TNF-α for 2 days. Autologous $CD4^+$ or $CD8^+$ T cells were co-cultured with HER2 protein-pulsed (2 µg/mL) or peptides-pulsed (20 µg/mL) autologous DCs, respectively, for 14 days, and T-cell numbers were then counted. Data shown are mean±SD from two independent experiments. **p<0.01, compared with the medium only and CD4-C1 treatment DC groups. (B) Blockage of PD-L1 or STAT3 signaling recovered the ability of HER2-pulsed senescent DCs to stimulate autologous T-cell proliferation. Immature DCs were pretreated with or without an anti-PD-L1 antibody (5 µg/mL) or S3I-201 (10 µM), then co-cultured with Treg or control CD4-C1 T cells as described in (A). Data shown are mean±SD from three independent experiments. **p<0.01, compared with the medium-only and CD4-C1 treatment groups, ##p<0.01, compared with DCs treated with γδ Treg cells. (C) and (D) Blockage of PD-L1 or STAT3 signaling in HER2-pulsed senescent DCs recovered the IFN-γ production and effector molecule expression in treated HER2-specific T cells. Cell treatment and procedure were identical to (B). Cytokine and effector molecule expression in DC-treated T cells was determined by flow cytometry. Data shown are mean±SD from two to four independent experiments. **p<0.01, compared with the medium-only and CD4-C1 treatment DC groups. #p<0.05 and ##p<0.01, compared with DCs treated with γδ Treg cells. (E) MCF7 lines were infected with lentivirus containing HER2/neu-GFP, and then sorted by FACS to generate a stable HER2 overexpression cell line ($MCF7\text{-}HER2^{hi}$). (F) Blockage of PD-L1 or STAT3 signaling in DCs reversed the impaired cytotoxicity of HER2 antigen-specific $CD8^+$ T cells mediated by γδ Treg-induced senescent DCs. DCs treatment and procedure were the same as in (B). After 14 days of co-culture, the DC-primed autologous $CD8^+$ cells were harvested, and determined the HER2-specific cytotoxicity using a standard 4 hours Calcein-AM release assay. $MCF7\text{-}HER2^{hi}$ cells ($HLA\text{-}A2^+$, HER2 overexpression) and T2 cells pulsed with HER2 peptides were used as target cells. Control targets included MDA-MB-231 cells ($HLA\text{-}A2^-$, $HER2^-$) and T2 cells. Various E/T ratios were tested as indicated. Data shown are mean±SD from two independent experiments. **p<0.01, compared with the medium only and CD4-C1 treatment DC groups. ##p<0.01, compared with DCs treated with γδ Treg cells. DCs, dendritic cells; mDC: mature dendritics cells; E:T, effector:target; FACS, fluorescence-activated cell sorting; GM-CSF, granulocyte macrophage colony-stimulating factor; HLA, human leukocyte antigen; IFN, interferon; PD-L1, programmed death-ligand 1; TNF, tumor necrosis factor; Treg, regulatory T cells.

autologous DCs with the different treatments as described in figure 4A. After 14 days of co-culture, $CD4^+$ or $CD8^+$ T cells sensitized by DCs with different treatments were restimulated with HER2 protein-pulsed autologous peripheral blood mononuclear cells (PBMCs) or HER2 peptides-pulsed T2 cells, respectively, and HER2-specific cytokines were determined by flow cytometry. HER2 protein-pulsed DCs pretreated with or without CD4-C1 T cells significantly induced the increases of HER2-specific IFN-γ-producing and IL-17-producing T-cell populations in the co-cultured $CD4^+$ T cells (figure 4C and online supplemental figure S4A). In contrast, γδ Treg-induced senescent DCs as antigen-presenting cells only induced minor levels of HER2-specific IFN-γ-producing T-cell populations in co-cultured $CD4^+$ T cells (figure 4C). In addition, HER2 peptide-pulsed DCs pretreated with or without CD4-C1 T cells induced the increases of HER2-specific IFN-γ-producing $CD8^+$ T cells as well as expression of perforin, granzyme A, and granzyme B in $CD8^+$ T cells. However, γδ Treg-induced senescent DCs pulsed with HER2 peptide had an impaired ability to stimulate autologous $CD8^+$ T cell to produce IFN-γ, perforin, granzyme A, and granzyme B, compared with T-cell groups stimulated with DCs alone or DCs pretreated with CD4-C1 effector T cells (figure 4D and online supplemental figure S4B). Furthermore, we also demonstrated that treatment with an anti-PD-L1 antibody or STAT3 signaling inhibitor reversed the impaired function of senescent DCs induced by γδ Treg cells on T-cell effector molecule expressions (figure 4C,D and online supplemental figure S4A,B).

In addition to HER2-specific effector molecule production, we analyzed the cytotoxic activity of $CD8^+$ T cells primed by HER2 peptide-pulsed DCs with different treatments. We transfected MCF7 breast tumor cells with lentivirus expressing HER2 to establish a breast cancer cell line with high HER2 expression ($HER2^{hi}$, $HLA\text{-}A2^+$) (figure 4E). We used MCF7-$HER2^{hi}$ and T2 ($HLA\text{-}A2^+$) cells loaded with the relevant peptides and labeled with calcein AM as two target cells. HER2-specific T cells primed by the HER2-pulsed mature DCs or CD4-C1 pretreated DCs exhibited a strong cytotoxic activity towards T2 cells loaded with the relevant peptides, but not with T2 alone (figure 4F). In addition, the HER2-specific T cells could lyse MCF7-$HER2^{hi}$ ($HLA\text{-}A2^+HER2^+$) but not MDA-MB-231 ($HLA\text{-}A2^-HER2^-$) breast cancer tumor cells, demonstrating that the HLA-A2-restriction and antigen specificity of T-cell activity (figure 4F). However, T cells primed with senescent DCs induced by γδ Treg cells had a very weak ability to lyse the peptide-pulsed T2 cells and MCF7-$HER2^{hi}$ cells, while treatment with an anti-PD-L1 antibody or the inhibitor S3I-201 can significantly recover HER2-specific cytotoxicity of the T cells induced by senescent DCs (figure 4F). These results suggest that senescent DCs induced by γδ Treg cells lost the capacity to process and present HER2 tumor antigen to T cells and induce HER2-specific T-cell responses.

To identify the mechanism(s) responsible for impaired functions of T cells induced by senescent DCs, we found that both $CD4^+$ and $CD8^+$ T cells primed by senescent DCs but not control DCs expressed high levels of exhaustion markers, including programmed cell death protein (PD)-1, T-cell immunoglobulin and mucin domain (Tim)3, and the cytotoxic T-lymphocyte-associated antigen (CTLA)-4 (online supplemental figure S4C,D). Furthermore, blockage of PD-L1 or STAT3 signaling in DCs can decrease the expression of these exhaustion molecules in both $CD4^+$ and $CD8^+$ T cells mediated by senescent DCs (online supplemental figure S4C,D).

Blockages of PD-L1 and/or STAT3 signaling reverse the impaired APC functions for vaccination of senescent DCs induced by γδ Treg cells in vivo

We then addressed whether senescent DCs induced by γδ Treg also have an impaired ability to induce HER2-specific immune responses in vivo. Human PBMCs ($HLA\text{-}A2^+$) were intravenously injected into female immunodeficient NOD-scid $IL2Rg^{null}$ (NSG) mice to reconstitute humanized mice. One week after human PBMC engraftment, treated or untreated DCs (autologous) were pulsed with HER2 HLA-A2 peptides (369–377 and 689–697) for 2 hours. Humanized NSG mice were vaccinated with 1×10^6 donor-matched HER2 peptide-loaded DCs and were then boosted with the donor-matched HER2-pulsed DCs again at day 7 post first immunization. Concomitantly, mice were administrated with an anti-PD-L1 neutralizing antibody or STAT3 inhibitor S3I-201 through intraperitoneal injection on days 2, 5, 8, and 11 after the adoptive transfer of DCs. Blood and spleens (SP) were harvested at day 15 post DC vaccination, and human $CD4^+$ and $CD8^+$ T cells were isolated to determine the HER2-specific immune responses (figure 5A). We first determined the effector cytokine and molecule expression in purified $CD8^+$ T cells with different DC vaccinations and treatments. $CD8^+$ T cells purified from HER2 peptide-pulsed mature DCs (mDCs) dramatically induced the increases of HER2-specific IFN-γ-producing and T-bet^+ T-cell populations, as well as increased expression of perforin and granzyme B in $CD8^+$ T cells (figure 5B). In contrast, $CD8^+$ T cells purified from vaccinated mice with HER2 peptide-pulsed senescent DCs induced by γδ Treg cells had an impaired ability to secrete HER2-specific IFN-γ and express perforin, T-bet, and granzyme B (figure 5B). Furthermore, we also found that administration with an anti-PD-L1 antibody and the STAT3 signaling inhibitor SI-201 can reverse the decreased expressions of effector molecules on $CD8^+$ T cells purified from the mice with γδ Treg-induced senescent DC vaccination (figure 5B).

We then determined the cytotoxicity of T cells purified from vaccinated mice with HER2 peptide-pulsed DCs. $CD8^+$ T cells purified from the blood of vaccinated NSG mice were restimulated ex vivo by calcein AM-labeled T2 cells loaded with the same HER2 peptide or MCF7-$HER2^{hi}$ cells, as well as control MDA-MB-231 or T2 cells alone for 24 hours. $CD8^+$ T cells purified from HER2-pulsed mature DC-immunized mice can recognize and efficiently lyse the same HER2 peptide-pulsed T2 cells

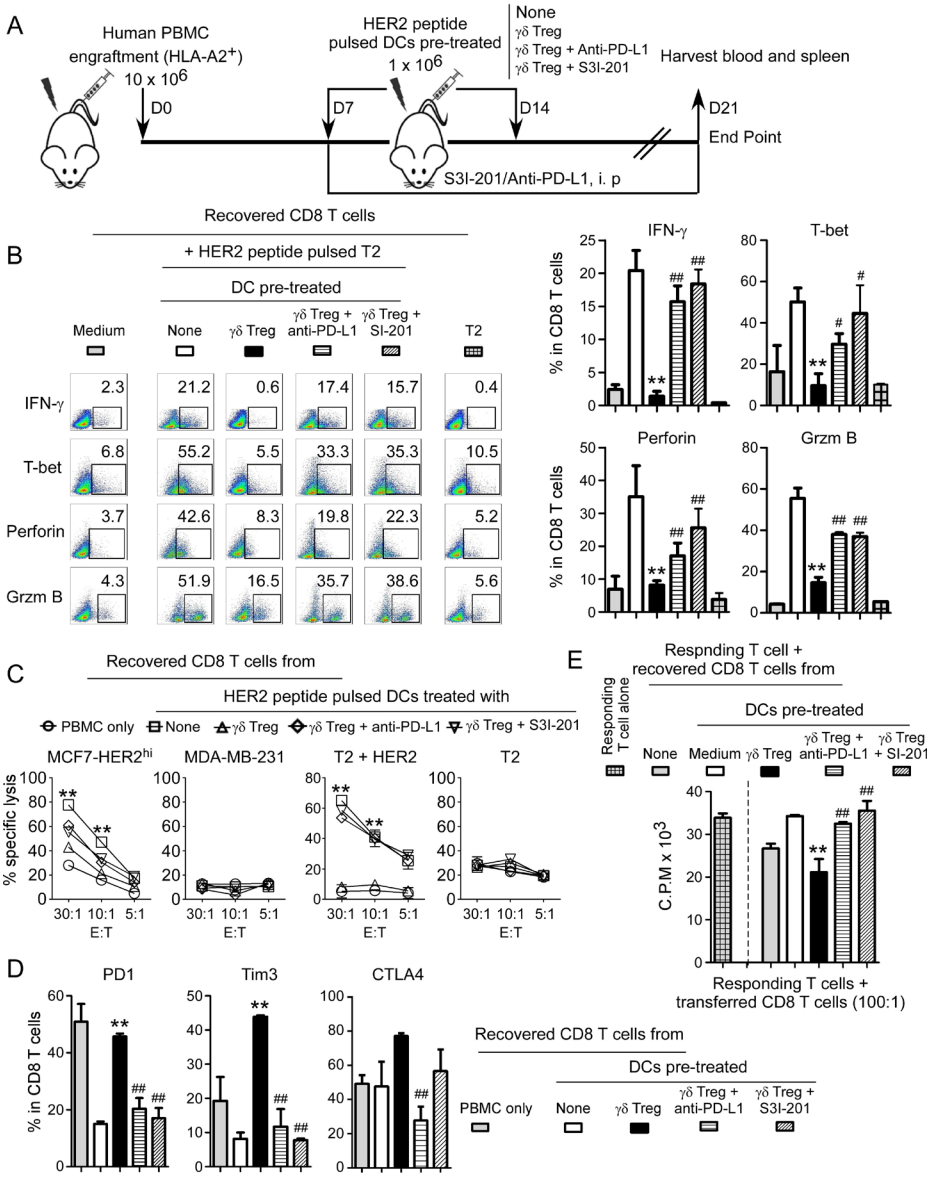

Figure 5 Blockage of PD-L1 and STAT3 signaling reverses the impaired APC functions of Treg-induced senescent DCs in vivo. (A) Schematic of the experimental workflow. NSG mice were engrafted with human PBMCs to reconstitute the immune system. Seven days and 14 days later, donor-matched HER2 peptide-pulsed mature DCs or γδ Treg-induced senescent DCs (1×10^6/mouse) were administrated through iv injection. The anti-PD-L1 neutralization antibody and S3I-201 were injected intraperitoneally three times a week. After 15 days of immunization, $CD8^+$ T cells from different organs were purified for the functional assays. (B) Blockage of PD-L1 or STAT3 signaling reversed impaired ability of senescent DCs to polarize and activate HER2-specific T cells. $CD8^+$ T cells purified from the blood of vaccinated mice were co-cultured with CFSE-labeled T2 cells loaded with HER2 peptides at a ratio of 1:1 for 24 hours. The indicated effector molecule expression in purified $CD8^+$ T cells was determined by flow cytometry. Data shown are mean±SD from two to four mice. **p<0.01, compared with the medium-only group. $^{\#\#}$p<0.01, compared with DCs treated with γδ Treg cells. (C) Blockage of PD-L1 or STAT3 signaling recovered impaired cytotoxicity of HER2-specific $CD8^+$ T cells purified from the blood of the vaccinated mice with senescent DCs using a standard calcein-AM release assay. Calcein-AM-labeled MCF7-$HER2^{hi}$, MDA-MB-231, or HER2 peptides-loaded or unloaded T2 cells were incubated with $CD8^+$ T cells at various ratios at 37°C for 4 hours. Data shown are mean±SD from three mice. **p<0.01, compared with the medium-only group. $^{\#\#}$p<0.01, compared with DCs treated with γδ Treg cells. (D) and (E) Blockage of PD-L1 and STAT3 signaling reversed T-cell exhaustion and suppression mediated by γδ Treg-induced senescent DC vaccination. Cell treatment and procedures were identical to (B). PD-1, Tim3, and CTLA-4 expression in purified $CD8^+$ T cells were determined by flow cytometry (in D). Suppression of purified $CD8^+$ T cells on the proliferation of other T cells was determined by $[^3H]$-thymidine incorporation assays after culture for 3 days (in E). Data shown are mean±SD from two to five mice. **p<0.01, compared with the medium-only group. $^{\#\#}$p<0.01, compared with DCs treated with γδ Treg cells. APC, antigen presenting cell; CTLA-4, the cytotoxic T-lymphocyte-associated antigen 4; CFSE, arboxyfluorescein succinimidyl ester; DCs, dendritic cells; E:T, effector:target; HLA, human leukocyte antigen; IFN, interferon; PBMCs, peripheral blood mononuclear cells; PD-1, programmed cell death protein 1; PD-L1, programmed death-ligand 1; Tim3, T-cell immunoglobulin and mucin domain 3; Treg, regulatory T cells.

and MCF7-HER2^{hi} tumor cells, but not T2 cells alone or HER2 negative MDA-MB-231 cells (figure 5C). However, CD8$^+$ T cells purified from immunized mice with HER2 peptide-pulsed γδ Treg-induced senescent DC vaccination had little ability to kill the same HER2 peptide-pulsed T2 cells and MCF7-HER2^{hi} tumor cells (figure 5C). While administration with the anti-PD-L1 antibody and STAT3 signaling inhibitor can recover cytotoxicity of CD8$^+$ T cells from immunized mice vaccinated with peptides-pulsed senescent DCs pre-induced by γδ Treg cells (figure 5C).

Consistent with our in vitro data, we found that CD8$^+$ T cells purified from immunized mice with HER2-pulsed Treg-induced senescent DC vaccination expressed high levels of exhaustion markers, including PD-1, Tim3 and CTLA-4, as well as had potent suppressive activity on the other T-cell proliferation (figure 5D,E). In addition, blockage of PD-L1 and STAT3 signaling in vivo can significantly decrease the expression of these exhaustion molecules and reverse their suppressive activity in CD8$^+$ T cells purified from vaccinated mice with HER2 peptides-pulsed γδ Treg-induced senescent DCs (figure 5D,E).

To further determine that senescent DCs induced by Treg cells have the tolerogenic functions for vaccination and induction of antigen-specific immune responses, we extended the above experiments to use HER2 protein-pulsed DCs as vaccines to explore HER2-specific immunity (online supplemental figure S5A). We therefore can evaluate both CD4$^+$ and CD8$^+$ T-cell functions from the immunized mice with different DC vaccinations. As shown in online supplemental figure S5, Treg-induced senescent DCs pulsed with HER2 protein cannot effectively induce HER2-specific immune responses in both CD4$^+$ and CD8$^+$ T cells, showing decreased expression of effector cytokine IFN-γ and molecules, promoted exhaustion marker expression and suppressive activity, as well as decreased HER2-specific cytotoxicity of CD8$^+$ T cells. However, blockage of PD-L1 or STAT3 signaling can recover T-cell effector functions via increasing effector molecular expression and HER2-specific cytotoxic activity, as well as decreasing exhaustion molecules in both CD4$^+$ and CD8$^+$ T cells mediated by senescent DC vaccination. Taken together, these results collectively indicate that γδ Treg-induced senescent DCs have significantly impaired antigen-presenting cell (APC) functions to induce tumor antigen-specific immune responses, and that PD-L1 and STAT3 signaling pathways are important for controlling their tolerogenic functions.

Prevention of senescence development in DCs induced by γδ Treg cells enhances tumor-specific immunity for tumor immunotherapy in vivo

We next investigated whether blockage of DC senescence can enhance antitumor immunity and immunotherapy in breast cancer. Human PBMCs (HLA-A2$^+$) were intravenously injected into female NSG mice to reconstitute humanized mice as described above. One week after human PBMC engraftment, autologous DCs pretreated with γδ Treg cells, or γδ Treg plus anti-PD-L1 neutralizing antibody or STAT3 inhibitor, were pulsed with HER2 peptides (HLA-A2-restricted 369–377 and 689–697) for 2 hours and then adoptively transferred through intravenous into reconstituted NSG mice. Seven days later, mice were boosted with the donor-matched DCs with the same treatments above. In addition, human breast cancer MCF7-HER2^{hi} tumor cells were implanted subcutaneously (s.c.) into the right flank of the PBMC-reconstituted NSG mice 3 days before the first DC vaccinations. Concomitantly, mice were administrated with an anti-PD-L1 neutralizing antibody or STAT3 inhibitor S3I-201 through intraperitoneal injection on day 7 when the mice received their first DC vaccinations (figure 6A).

We found that breast cancer MCF-7-HER2^{hi} cells grew progressively in NSG mice but slowly in PBMC-reconstituted mice. While the adoptive transfer of HER2 peptide-pulsed mature DCs, tumor growth in PBMC-reconstituted mice was significantly inhibited. However, transfer of HER2 peptide-pulsed senescent DCs induced by γδ Treg cells, tumor growth was markedly promoted in PBMC-reconstituted mice, suggesting that senescent DCs failed to initiate tumor-specific antitumor immunity (figure 6B). Importantly, treatments with the anti-PD-L1 antibody or STAT3 signaling inhibitor S3I-201 markedly reversed the inhibitory ability on tumor growth mediated by tumor-specific T cells primed with γδ Treg-pretreated DCs (figure 6B). To confirm the molecular changes of T cells responsible for their antitumor effects mediated by respective DC therapeutic vaccinations, we isolated CD8$^+$ T cells from blood and tumor tissues from tumor-bearing mice at 15 days after DC vaccinations and evaluated their phenotypes and functions. As expected, CD8$^+$ T cells purified from blood and tumor tissues of HER2-loaded mature DC-vaccinated and immune reconstituted mice had a strong cytotoxicity against MCF7-HER2^{hi} tumor cells and HER2 peptide-pulsed T2 cells (figure 6C,D). However, CD8$^+$ T cells purified from blood and tumor tissues from the vaccinated mice with HER2 peptide-loaded senescent DCs lost the ability to lyse target cells. Furthermore, CD8$^+$ T cells purified from blood and tumor tissues from the vaccinated mice with HER2 peptide-loaded senescent DCs but treatment with the anti-PD-L1 antibody or inhibitor S3I-201, showed recovered cytotoxic activity to lyse target MCF7-HER2^{hi} tumor cells and HER2 peptide-pulsed T2 cells (figure 6C,D).

We then determined the effector molecule expression by CD8$^+$ T cells purified from vaccinated mice after stimulation of T2 cells pulsed with HER2 HLA-A2 peptides. We found that CD8$^+$ T cells purified from blood and tumor tissues of HER2 peptides-loaded mature DC-vaccinated immune reconstituted mice expressed certain levels of IFN-γ, perforin, granzyme A and B after stimulation with HER2 peptide-pulsed T2 cells. However, CD8$^+$ T cells purified from blood and tumor tissues from the vaccinated mice with HER2 peptide-loaded senescent DCs significantly decreased the expression of all those effector molecules after HER2 antigen-specific stimulation (figure 6E). In addition, administration of anti-PD-L1 antibody or

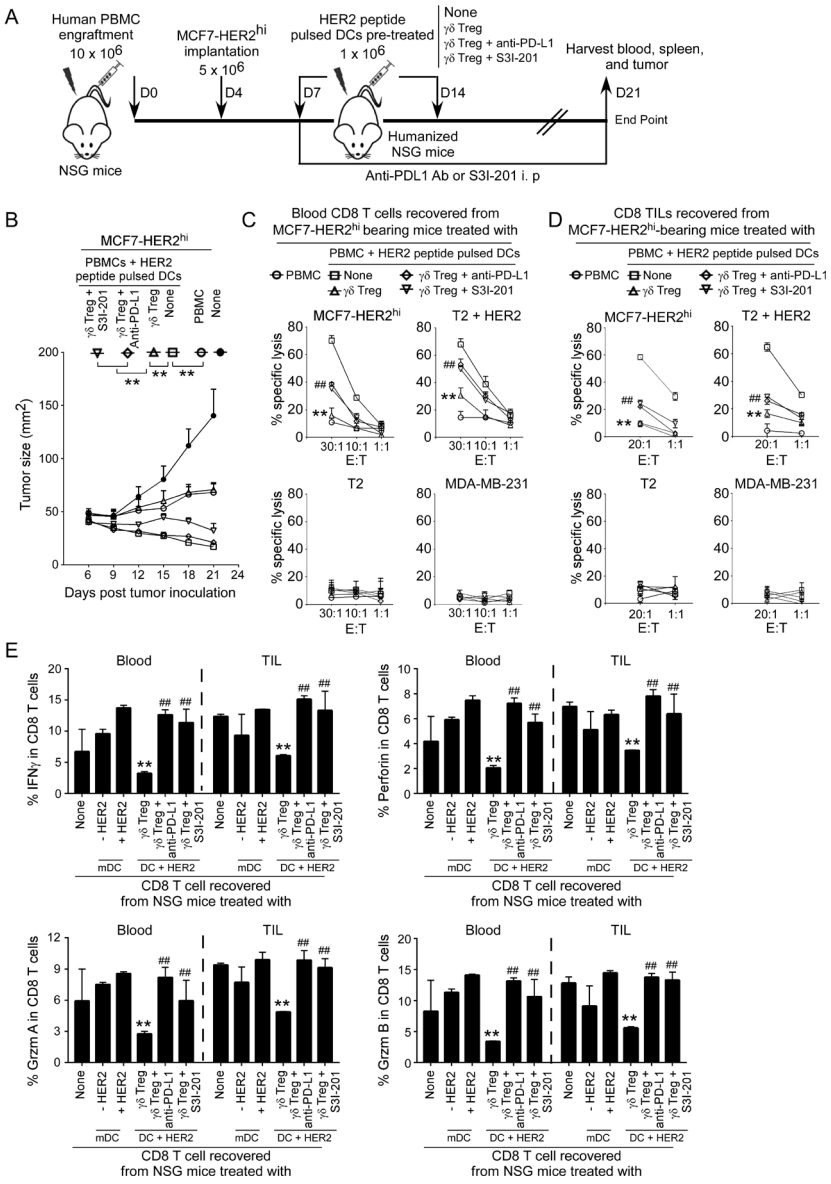

Figure 6 Prevention of senescence induction in DCs mediated by γδ Treg cells enhances tumor-specific immunity for immunotherapy in vivo. (A) Schematic of the experimental workflow. NSG mice were engrafted with human PBMCs to reconstitute the immune system. Human MCF7-HER2^{hi} tumor cells were subcutaneously injected into NSG mice on day 4 after engraftment of human PBMCs. On day 7 and day 14, donor-matched HER2 peptide-pulsed mature DCs with indicated pretreatments were adoptively transferred into tumor-bearing mice through intravenous injection. At day 21, CD8$^+$ T cells from different organs and tumors were purified for the functional assays. (B) Blockage of PD-L1 or STAT3 signaling in senescent DCs induced by γδ Treg cells recovered antitumor immunity against MCF7-HER2^{hi} tumor in senescent DC-vaccinated mice. The experimental procedure and cell treatment were described in (A). Tumor volumes were measured and presented at indicated time points as mean±SD (n=6 mice per group). **p<0.01. (C) and (D) Blockage of PD-L1 or STAT3 signaling recovered the impaired cytotoxicity of HER2-specific CD8$^+$ T cells purified from blood (in C) or tumor tissues (in D) of tumor-bearing and senescent DC-vaccinated mice with a calcein-AM release assay. Calcein-AM-labeled MCF7-HER2^{hi} (HLA A2$^+$, HER2$^+$), MDA-MB-231 (HLA A2$^-$, HER2$^-$), or HER2 peptide-loaded or unloaded T2 cells were incubated with CD8$^+$ T cells at various ratios at 37°C for 4 hours, and the release of calcein supernatants was determined. Data shown are mean±SD from two to six mice/group. **p<0.01, compared with the medium-only group. $^{\#\#}$p<0.01, compared with DCs treated with γδ Treg cells. (E) Blockage of PD-L1 or STAT3 signaling reversed the T-cell dysfunction mediated by γδ Treg-induced senescent DC vaccination. CD8$^+$ T cells purified from the blood and tumor tissues of tumor-bearing and DC-vaccinated mice were co-cultured with CFSE-labeled T2 cells loaded with the same peptides at a ratio of 1:1 for 24 hours. Effector molecule expression in purified CD8$^+$ T cells was determined by flow cytometry. Data shown are mean±SD from two mice/group. **p<0.01, compared with the medium-only group. $^{\#\#}$p<0.01, compared with DCs treated with γδ Treg cells. APC, antigen presenting cell; CTLA-4, the cytotoxic T-lymphocyte-associated antigen 4; CFSE, arboxyfluorescein succinimidyl ester; DCs, dendritic cells; mDCs, mature dendritic cells; E:T, effector:target; HLA, human leukocyte antigen; IFN, interferon; i.p., intraperitoneal injection; PBMCs, peripheral blood mononuclear cells; PD-1, programmed cell death protein 1; PD-L1, programmed death-ligand 1; Tim3, T-cell immunoglobulin and mucin domain 3; TIL, tumor-infiltrating T cells; Treg, regulatory T cells.

STAT3 inhibitor S3I-201 rescued the expression of those effector molecules in $CD8^+$ T cells purified from blood and tumor tissues from the vaccinated mice with HER2 peptide-loaded senescent DCs (figure 6E).

In addition to stimulation with HER2 antigen, we also investigated the non-specific functions of T cells purified from blood and tumor tissues in the MCF7-bearing mice vaccinated with HER2 peptide-loaded different DCs. We obtained consistent results as shown in HER2 antigen stimulation. We found that T cells purified from vaccinated mice with HER2 peptide-loaded senescent DCs induced by γδ Treg cells also dramatically decreased IFN-γ and perforin production after stimulation with phorbol myristate acetate (PMA) and ionomycin, while administration of the anti-PD-L1 antibody or inhibitor S3I-201 recovered the expression of IFN-γ and perforin in the purified T cells in the tumor-bearing mice vaccinated with HER2 peptide-pulsed senescent DCs (online supplemental figure S6A). Furthermore, we also explored the exhaustion status of T cells purified from vaccinated mice with HER2 peptides-pulsed different DCs. Consistent with the results shown in in vitro studies, $CD8^+$ T cells purified from both blood and tumor tissues in vaccinated mice with HER2 peptide-loaded senescent DCs induced by γδ Treg cells highly expressed checkpoint inhibitory molecules PD-1, Tim3 and CTLA-4. However, treatment with the anti-PD-L1 antibody or STAT3 inhibitor S3I-201 also significantly decreased the expression of those inhibitory molecules in the purified T cells (online supplemental figure S6B). These results collectively indicate that senescent DCs induced by γδ Treg cells induce exhausion in tumor-specific effector T cells with impaired antitumor activity in vivo and that blockages of PD-L1 and STAT3 signaling in DCs can prevent these suppressive effects on tumor-specific T cells for tumor immunotherapy in breast cancer.

DISCUSSION

Immunosuppressive tumor microenvironments mediated by tumor-infiltrating Treg cells that prevent effective antitumor immunity are a major obstacle to the success of immunotherapy.[2 3 25] We further identified that γδ Treg cells are accumulated in the breast cancer tumor microenvironment, which strongly suppresses both innate and adaptive immune cells and is negatively associated with clinical outcomes of patients with breast cancer.[4 7] Therefore, exploring the suppressive mechanisms mediated by tumor-associated γδ Treg cells is urgently needed to develop effective tumor immunotherapy for breast cancer. In the present study, we showed that breast tumor-derived γδ Treg cells promote the development of senescent DCs with tolerogenic functions, which can suppress T-cell proliferation, Th1 and Th17 cell differentiation, as well as promote the development of Treg cells. Molecularly, γδ Treg cells induced upregulation of PD-L1 and activation of STAT3 signaling pathway in DCs, leading to senescence development in DCs. Furthermore, blockages of PD-L1 and STAT3 signaling can prevent DC senescence induced by γδ Treg cells and enhance HER2 tumor-specific immune responses for tumor immunotherapy in breast cancer models. Our studies provide critical insights for the development of novel therapeutic strategies targeting a new subset of Treg cells and tolerogenic DCs for cancer treatment.

Understanding the cross-talk and interaction between Treg cells and DCs within the tumor microenvironment is critical for reconstituting an effector tumor microenvironment and enhancing tumor immunotherapy. Our studies have identified that breast cancer tumor-derived γδ Treg cells can induce senescence in both T cells and innate DCs.[7] We have further characterized senescent T cells and demonstrated that Treg-induced senescence in effector T cells is due to the glucose competition between Treg and responder T cells during their cross-talk, which triggers the DNA damage response in responder T cells.[6 11–14] These senescent T cells are functionally suppressive and molecularly distinct from anergic and exhausted T cells.[13 26] These cells express senescence-associated maker SA-β-Gal, downregulate CD27 and CD28 expression, and promote cell cycle arrest.[6 7 11] In addition, senescent T cells are metabolically active and possess a unique senescence-associated secretory phenotype, producing high amounts of cytokines, lipids, and metabolites.[6 7 11–13 26 27] However, the molecular features and tolerogenic functions of senescent DCs induced by Treg cells in the tumor microenvironment are unknown. In the current study, we further explored the phenotypic and functional changes of senescent DCs induced by breast cancer-derived γδ Treg cells. Besides high expression of senescence marker SA-β-Gal, senescent DCs show immature DC phenotypes with downregulation of costimulatory molecules, and reduced effector cytokine secretion. Furthermore, senescent DCs exhibit impaired abilities to sense and respond to pathogen-associate molecular stimulus evidenced by decreased expression of a panel of TLRs, show deficient to stimulate naïve cell proliferation and differentiation into effector T-cell subsets Th1 and Th17, as well as inhibit activation and priming of tumor antigen-specific effector T cells. In addition to direct suppression of effector T-cell functions, senescent DCs can promote Treg cell differentiation from naïve T cells and highly express checkpoint inhibitory molecule PD-L1, which indirectly suppresses the immune response of effector T cells for tumor immunity. These studies not only facilitate a better understanding of the novel mechanism responsible for the development of tolerogenic DCs within the suppressive microenvironment, but also provide an emerging concept that blockage of the development of senescent DCs with tolerogenic functions induced by Treg cells is critical for improving effective antitumor immunity and immunotherapy for patients with cancer.

PD-L1 is a key negative regulatory molecule, and checkpoint blockade therapy targeting PD-1/PD-L1 has become a first-line immunotherapeutic strategy in certain types of patients with cancer.[28 29] Furthermore,

the expression of PD-L1 in the tumor microenvironment is directly corrected with the clinical response of anti-PD-L1 immunotherapy in patients with cancer.[30–32] In addition to being highly expressed in tumor cells, PD-L1 is also expressed in different types of suppressive immune components such as myeloid-derived suppressor cells (MDSCs) and DCs in the tumor microenvironment.[33–35] However, the mechanism responsible for the expression of PD-L1 in tumor-infiltrating myeloid cells is unknown. Our current studies clearly demonstrated that interaction with tumor-associated Treg cells can promote PD-L1 expression in immature and mature DCs and induce senescence of DCs with tolerogenic functions. This may explain the causative relationship between Treg-mediated immune suppression and accumulated tolerogenic DCs in the tumor microenvironment of patients with cancer. The PD-L1/PD-1 pathway is a key inhibitory signaling pathway for tolerogenic DCs, regulating T-cell activation and tolerance.[36–38] However, how the expressed PD-L1 in senescent DCs contributes to the impaired function and immune suppression of DCs for tumor immunity is unknown. Through both in vitro and in vivo experiments in our current study, we have shown that blockage of PD-L1/PD-1 signaling in DCs can reverse the tolerogenic function and prevent DC senescence induced by γδ Treg cells. These results suggest that PD-L1 expression is critical for senescence induction and dysfunction of DCs in the tumor microenvironment. Further studies will continue to dissect how PD-L1/PD-1 signaling molecularly reprograms senescence development in DCs induced by those Treg cells in tumors, which should provide more innovative approaches to reverse senescence and tolerogenic functions of DCs and improve the effects of immunotherapy against cancer.

STAT signaling plays a critical role in the differentiation and activation of DCs.[39 40] Different STAT signaling may have distinct functions for DC maturation and function.[41 42] In both humans and mice, STAT3 signaling activation is linked to impaired maturation and effector functions of DCs to prime Th1 immune responses.[39 43 44] Furthermore, tumor-derived DCs showed hyperactivated STAT3 signaling associated with their tolerogenic functions in the tumor microenvironment.[40 45–49] In the current study, we identified that breast tumor-derived γδ Treg cells can induce STAT3 activation and phosphorylation in DCs, leading to senescence development, tolerogenic phenotypes, and impaired functions in DCs. These studies provide the potential mechanism responsible for the activation of STAT3 in the tumor microenvironment and further support the notion that STAT3 signaling is a potentially important target for cancer prevention and therapy.

Our studies clearly indicate that the development of senescence in immune cells, such as T cells and DCs is an important strategy for malignant tumors to escape immune surveillance during the tumor progression.[7] Accumulated senescent immune cells in the suppressive tumor microenvironment are also an obstacle to effective tumor immunity and immunotherapy.[12 13 27 50 51] Therefore, the development of effective strategies to prevent senescence in T cells and DCs is urgently needed for enhancing immunotherapy against cancer. Our recent studies have made progress in understanding T-cell senescence induced by both tumor cells and Treg cells.[11 12 14 27] Importantly, we have also explored novel strategies to control Treg and tumor cell suppression and prevent T-cell senescence to enhance antitumor efficacy for tumor immunotherapy.[12 14 52] We identified that activation of TLR8 signaling can reverse Treg and tumor-induced T-cell senescence and improve tumor-specific T-cell activity and function for tumor immunity. In addition, reprogramming of effector T-cell lipid metabolism with cPLA2α inhibition can prevent tumor-specific T-cell senescence and enhance antitumor immunity in adoptive transfer immunotherapy breast cancer and melanoma models.[14] Our current studies further demonstrated that either anti-PD-L1 treatment or blockage of STAT3 signaling can prevent DC senescence mediated by γδ Treg cells and enhance HER2 tumor-specific immune responses for tumor immunotherapy in breast cancer models. The current study not only identifies molecular targets and checkpoint signaling to control DC fate and dysfunction to enhance the efficacy of immunotherapy but also provides the rationale and foundation for the combinations of current checkpoint immunotherapy targeting anti-PD-L1 with other strategies to control immune suppression for tumor immunotherapy.

In summary, our current study provides critical evidence that human tumor-derived γδ Treg cells directly suppress DCs through the induction of immunosenescence, leading to tolerogenic DC characteristics and functions. The development of senescent DCs is molecularly controlled by the PD-L1 signaling and transcription factor STAT3. Furthermore, inhibitions of PD-L1 or/and STAT3 signaling in DCs can prevent γδ Treg-induced DC senescence and recover their effector functions for improved tumor vaccine and immunotherapy in vivo in breast cancer models. Our studies provide novel potential strategies for the prevention of senescence and dysfunction in innate immune cells in the tumor microenvironment for clinical immunotherapy against cancer.

MATERIALS AND METHODS

T cells and tumor cell lines

Buffy coats from healthy donors were obtained from the Gulf Coast Regional Blood Center (Houston, Texas, USA). PBMCs were purified from buffy coats using Ficoll-Paque and further performed the HLA class I tissue typing. Human naive $CD4^+$ and $CD8^+$ T cells were purified from PBMCs of healthy donors by EasySep enrichment kits (STEMCELL Technologies). The purity of naive T cells was >96%, as confirmed by flow cytometry. Human γδ Treg cells (primary or cell lines) were established from the primary breast cancer tissues from different patients with cancer in our laboratory and

maintained in T-cell medium containing 10% (vol/vol) human AB serum and 50 U/mL IL-2.[3 4 7] CD4-C1 effector T cells used for the studies were anti-CD3/CD28 activated $CD4^+$ T cells purified from the PBMCs of healthy donors. The human breast cancer cell lines MCF-7 and MDA-MB-231, and the T2 lymphoblastoid cell line were purchased from the American Type Culture Collection (Manassas, Virginia, USA). The stable HER2 overexpression MCF7 line (MCF7-$HER2^{Hi}$) was generated with infection of lentivirus carrying HER2/neu and confirmed by flow cytometry analysis with a PE-conjugated anti-HER2/neu monoclonal antibody (BioLegend, San Diego, California, USA). The tumor cell lines were routinely maintained in Roswell Park Memorial Institute (RPMI)-1640 media with 10% (vol/vol) heat-inactivated fetal bovine serum, 2 mM L-glutamine, 25 mM 4-(2-hydroxyethyl)-1-piperazineet hanesulfonic acid (HEPES), 100 µg/mL penicillin, and 100 µg/mL streptomycin.

HER2 protein or peptides and loading of target cells

HER2 HLA-A*0201 binding peptides HER2/neu 369–377 (KIFGSLAFL) and HER2/neu 689–697 (RLLQETELV) were synthesized by the 21st Century Biochemicals, Marlboro, Massachusetts, USA.[22–24] Recombinant human ErbB2/Her2 protein was purchased from R&D Systems (1129-ER). The powder of these lyophilized peptides and protein was dissolved in dimethyl sulfoxide and stored at −80°C until use according to the manufacturer's instructions.

The $HLA\text{-}A2^+$ DCs and T2 cells were incubated with HER2 HLA-A2 binding peptides 369–377 and/or 689–697 at the final concentration of 20 µg/mL at 37°C for 2 hours. In some experiments, the $HLA\text{-}A2^-$ DCs and autologous PBMCs were incubated with HER2 protein at a final concentration of 2 µg/mL at 37°C for 2 hours. HER2 peptides/protein-pulsed cells were harvested and used for the experiments.

Induction of mature and senescent DCs

$CD14^+$ monocyte precursor cells were purified from freshly isolated PBMCs by EasySep enrichment kits (STEMCELL Technologies) under the manufacturer's protocol. Immature and mature DCs were induced from $CD14^+$ cells in the presence of IL-4 (20 ng/mL) and GM-CSF (100 ng/mL) with or without TNF-α (20 ng/mL, R&D Systems), respectively.[3 7] The senescent DCs were induced after culture with breast tumor-derived γδ Treg cells, as we previously described.[7] In brief, immature DCs were cultured with γδ Treg cells at a ratio of 3:1 in the presence of GM-CSF, IL-4, and TNF-α for 2 days, and the treated DCs (senescent DCs) were then purified after depletion of co-cultured T cells based on CD3 expression for different experiments.

T-cell subset differentiation

The differentiation of human T-cell subsets from naive $CD4^+$ T cells was performed as previously described.[52 53] Briefly, the treated DCs were purified and co-cultured with human naive $CD4^+$ T cells at a ratio of 1:10 in plate-bounded anti-CD3/CD28 (2 µg/mL, Bio X Cell) in normal T-cell culture medium for 6 days. For Th1 differentiation, DCs and naive T cells were co-cultured at a ratio of 1:10 in the presence of anti-IL-4-neutralizing antibody (10 µg/mL, 11B11; Bio X Cell) and recombinant human (rh)IL-12 (10 ng/mL; R&D Systems). For Th17 differentiation, DCs and naive T cells were co-cultured at a ratio of 1:10 in the presence of rhIL-1β (10 ng/mL), rhIL-6 (20 ng/mL), rhIL-23 (10 ng/mL) and recombinant human transforming growth factor-beta (rhTGF-β) (3 ng/mL) (R&D System) for 6 days.

In vitro generation of HER2-specific T cells

Treated or untreated DCs were pulsed with HER2 HLA-A2-binding peptides 369–377 and 689–697, or HER2 protein for 2 hours. Purified autologous $CD8^+$ T cells were co-cultured with HER2-loaded DCs at a ratio of 10:1 in the RPMI 1640 medium supplemented with 10% AB serum and human IL-2 (50 IU/mL). The T cells were restimulated with HER2 peptide or protein-pulsed autologous DCs at day 7. After 14 days of sensitization, the stimulated $CD8^+$ cells were collected for analyses of function and antigen specificity.

Senescence-associated beta-galactosidase staining

SA-β-Gal activity in DCs was detected as we described previously.[6 7 11] Immature or mature DCs were co-cultured with or without breast tumor-derived γδ Treg cells or control T cells at a ratio of 3:1 in 24-well plates for 2 days. Co-cultured DCs were separated and stained for SA-β-Gal. For some experiments, DCs were co-cultured with Treg cells in the presence of an anti-PD-L1 antibody (5 µg/mL) (BioLegend, clone: 29E.2A3) or STAT3 signaling inhibitor S3I-201 (10 µM, Sigma-Aldrich).

Flow cytometry analysis

The expression markers on T cells and DCs were determined by flow cytometry analysis after surface staining or intracellular staining with specific anti-human antibodies conjugated with fluorescein isothiocyanate (FITC) or phycoerythrin (PE). The human antibodies included: anti-CD4, anti-CD8, anti-CTLA-4, anti-PD-1, anti-Tim3, anti-CD80, anti-CD83, anti-CD86, anti-HLA-DR, anti-PD-L1, anti-IL-4, anti-IL-17A, anti-IFN-γ, anti-perforin, anti-granzyme A, anti-granzyme B, anti-FoxP3, and anti-T-bet, which were purchased from BD Biosciences, BioLegend, or Cell Signaling Technology. Intracellular staining of cytokines was performed on T cells after stimulation with PMA and ionomycin in the presence of GolgiStop for 5.5 hours, then permeabilized and fixed using the Cytofix/Cytoperm (BD Pharmingen) according to the manufacturer's instructions.

For antigen-specific cytokine production and transcription factor expression, $CD4^+$ or $CD8^+$ T cells pretreated with different DCs were co-cultured with arboxyfluorescein succinimidyl ester (CFSE)-labeled T2 cells or autologous PBMCs loaded with HER2 peptides or protein at

a 1:1 ratio in the presence of 1 µL/mL GolgiStop (BD Pharmingen). After 20–24 hours, HER2-specific cytokine production and transcription factor expression were analyzed by flow cytometry based on the CFSE-negative cell populations.

For T-cell apoptosis assay, naive $CD4^+$ T cells were co-cultured with CFSE-labeled DCs pretreated with medium, CD4-C1, or γδ Treg cells in the presence of plate-bound anti-CD3/CD28 antibodies (2 µg/mL). After 6 days of co-culture, apoptosis in $CD4^+$ T cells was analyzed after staining with PE-labeled annexin V and 7-aminoactinomycin D (BD Biosciences, San Diego, California, USA) and gating on CFSE-negative cell populations.

All stained cells were analyzed on an LSR II cytometer (BD Bioscience) and data was analyzed with FlowJo software (Tree Star).

Functional proliferation assay

T-cell proliferation was performed by a [^{3}H]-thymidine incorporation assay, as we previously described.[1 3 6 11 12] Briefly, naïve $CD4^+$ T cells (1×10^5/well) purified from healthy donors were co-cultured with DCs at different ratios in a 96-well plate with T-cell assay medium containing 2% human AB. After 56 hours of culture, [^{3}H]-thymidine was added at a final concentration of 1 µCi/well, followed by an additional 16 hours of culture. The incorporation of [^{3}H]-thymidine was measured with a liquid scintillation counter.

Reverse-transcription quantitative PCR analysis

Total RNA was extracted from the cells using TRIzol reagent (Invitrogen), and complementary DNA was transcribed using a SuperScript II RT Kit (Invitrogen), both according to manufacturers' instructions. Expression levels of each gene were determined by reverse-transcription PCR using specific primers, and messenger RNA levels in each sample were normalized to the relative quantity of the housekeeping glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene expression. All experiments were performed in triplicate. The specific primers used for assays are listed in online supplemental table S1.

Western blotting analysis

Immature DCs were cultured with or without γδ Treg or CD4-C1 T cells at a ratio of 3:1 in the presence of GM-CSF, IL-4, and TNF-α for 2 days. For some experiments, immature DCs were pretreated with an anti-PD-L1 neutralization antibody (5 µg/mL) for 2 hours or STAT3 signaling inhibitor S3I-201 (10 µM) for 24 hours. The whole cell lysates of the purified DCs were prepared for western blot analyses. Western blots were developed with the Chemiluminescent Substrate (KPL, Maryland, USA). The rabbit polyclonal antibodies used in western blot analyses are as follows: anti-STAT3, anti-phospho-STAT3 (Tyr705), anti-phospho-STAT3 (S727), anti-PD-L1 and anti-GAPDH. All antibodies were purchased from Cell Signaling Technology. The dilution for primary antibody was 1:1,000 for the studies.

In vitro cytotoxic assay

The in vitro cytotoxic assay was performed as previously described with some modifications.[54 55]

DC-sensitized human $CD8^+$ T cells from in vitro or in vivo were used as effector cells, and peptide-pulsed T2 or protein-pulsed autologous PBMCs, as well as $HER2^+$ or $HER2^-$ breast tumor cell lines were used as target cells, which were prestained with 10 µM fluorescent dye calcein-AM (1:1,000 dilution). Effector T cells were mixed with 10^5 different calcein-AM-labeled target cells at variable effector:target ratios with a final volume of 200 µL in 96-well round-bottom plates. The cell mixtures were centrifuged at 300×g for 5 min and then incubated at 37°C in 5% CO_2 for 4 hours. The spontaneous release of calcein-AM in target cells was determined by incubating target cells in a medium only. The maximal release of calcein-AM in target cells was determined by adding 0.1% Triton X (Sigma-Aldrich) to lyse all the target cells. After 4-hour incubation, plates were centrifuged at 300×g for 5 min, and 100 µL of supernatant from each sample was transferred to a 96-well plate (OptiPlate 96F; Perkin-Elmer) and fluorescence was measured using a fluorometer (SpectraMax M3 Microplate Reader; Molecular Devices) at an excitation wavelength of 480 nm and emission wavelength of 538 nm. The mean value from the triplicate of each sample was used for the calculation of cytotoxicity. Cytotoxicity was measured as per cent specific release of calcein-AM and calculated using the following formula: %specific release = (experimental release – spontaneous release)/(maximal release – spontaneous release) × 100.

In vivo studies

NOD-scid IL2Rγ^{null} mice (NSG, Stock No. 005557, strain NOD.Cg-Prkdc^{scid}Il2rg^{tm1Wjl}/SzJ) were purchased from The Jackson Laboratory and maintained in the institutional animal BSL2 facility. Female NSG mice with 6–8 weeks were used for the studies. All animal studies have been approved by the Institutional Animal Care Committee in Saint Louis University (Protocol No. 2411).

For the DC-based vaccine and immunization model: Human PBMCs (1×10^7/mouse) were intravenously injected into the NSG mice to reconstitute the immune system with human T cells. Seven days later, mice were vaccinated by the intravenous injection with the donor-matched HER2 (peptides or protein)-pulsed different types of pretreated DCs (1×10^6/mouse) (mature DCs, γδ Treg-treated DCs, γδ Treg and anti-PD-L1 antibody-treated DCs, and γδ Treg and inhibitor S3I-201-treated DCs). Seven days after the first DC immunization, the immunized mice were boosted with the donor-matched HER2 (protein or peptides)-pulsed respective DCs again. After the first immunization of DCs into the mice, an anti-PD-L1 neutralizing antibody (100 µg/mouse) and STAT3 inhibitor S3I-201 (10 mg/kg/mouse) were intraperitoneally injected into mice vaccinated with anti-PD-L1

neutralizing antibody or S3I-201 pretreated DCs, respectively, for a total of three doses with 3-day intervals. Two mice were included in each group in one round of the experiment. Blood and spleens were harvested at 21 days post PBMC injection. The transferred human $CD4^+$ and $CD8^+$ T cells were isolated and recovered by antibody-coated microbeads (STEMCELL Technologies) for subsequent phenotypic and functional analyses in vitro.

For tumor growth and antitumor immunity studies: Human HLA-$A2^+$ PBMCs (1×10^7/mouse) were injected through intravenously into the NSG mice at day 0. At day 4, MCF7-$HER2^{hi}$ cells (5×10^6/mouse) were implanted s.c. into the mammary fat pad of the recipient mice. At day 7, tumor-bearing mice were vaccinated by the intravenous injection with the donor-matched HER2 HLA-A2 peptide-pulsed different types of pretreated DCs (1×10^6/mouse) (mature DCs, γδ Treg-treated DCs, γδ Treg and anti-PD-L1 antibody-treated DCs, and γδ Treg and inhibitor S3I-201-treated DCs). The DC reimmunization and intraperitoneal injection of an anti-PD-L1 neutralizing antibody (100 µg/mouse) or STAT3 inhibitor S3I-201 (10 mg/kg/mouse) were identical to the procedures described in the DC-based vaccine and immunization model above. Two mice were included in each group in one round of the experiment. Tumor size was measured from day 6 after tumor inoculation with calipers every 3 days. Tumor size was calculated on the basis of two-dimension measurements. Blood, spleens, and tumors were harvested at 21 days post-injection. The transferred human $CD4^+$ and $CD8^+$ T cells were isolated and recovered for subsequent phenotypic and functional analyses in vitro.

Statistical analysis

Statistical analysis was performed with GraphPad Prism V.7.0 software (GraphPad Software). Unless indicated otherwise, data are expressed as mean±SD. For multiple-group comparison studies, the one-way analysis of variance was used, followed by Dunnett's test for comparing experimental groups against a single control. For a single comparison between two groups, paired Student's t-test was used. A non-parametric t-test was chosen if the sample size was too small and did not fit the Gaussian distribution.

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Patient consent for publication Not applicable.

Ethics approval Not applicable.

Provenance and peer review Not commissioned; externally peer reviewed.

Data availability statement Data sharing not applicable as no data sets generated and/or analyzed for this study. Data are available upon reasonable request. All data relevant to the study are included in the article or uploaded as supplementary information.

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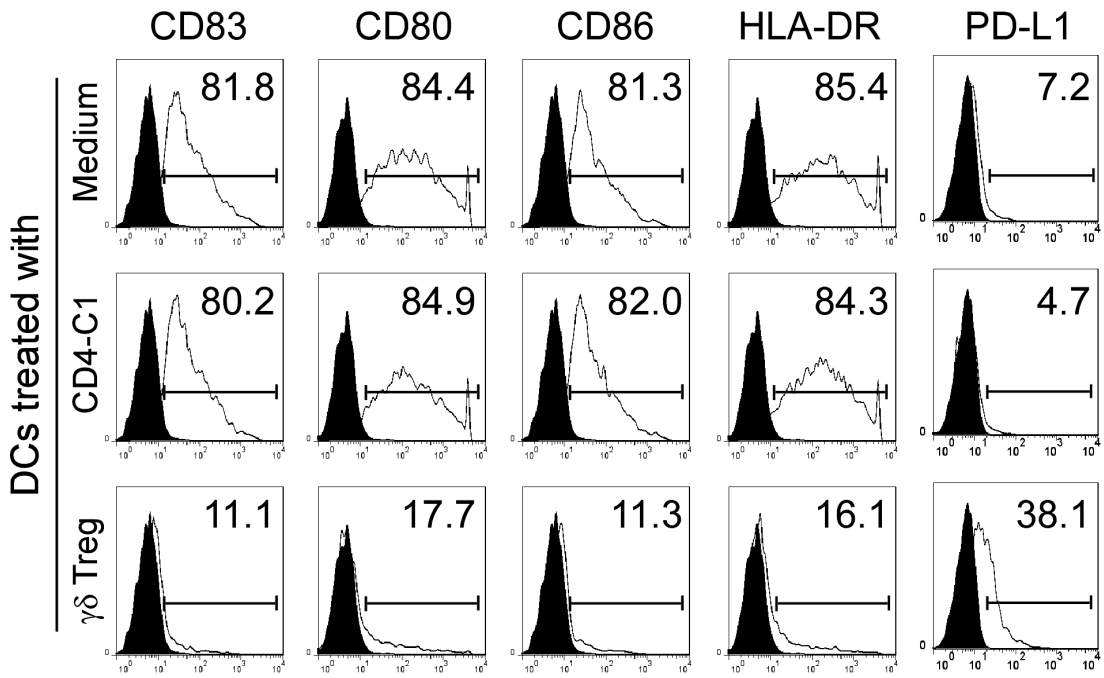
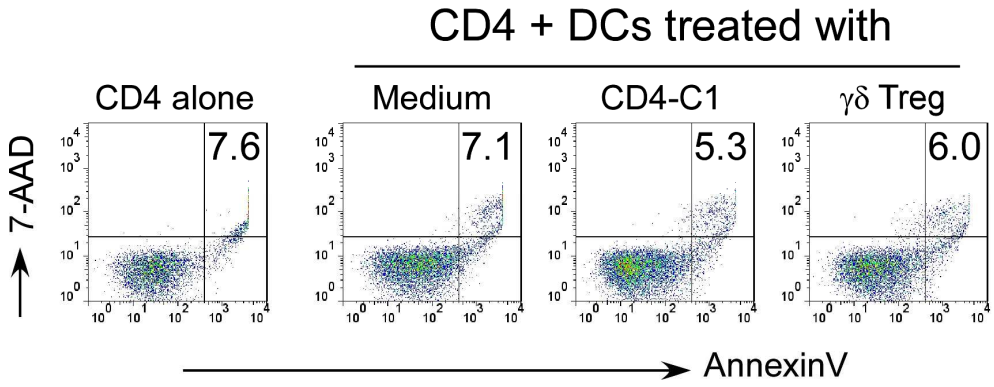


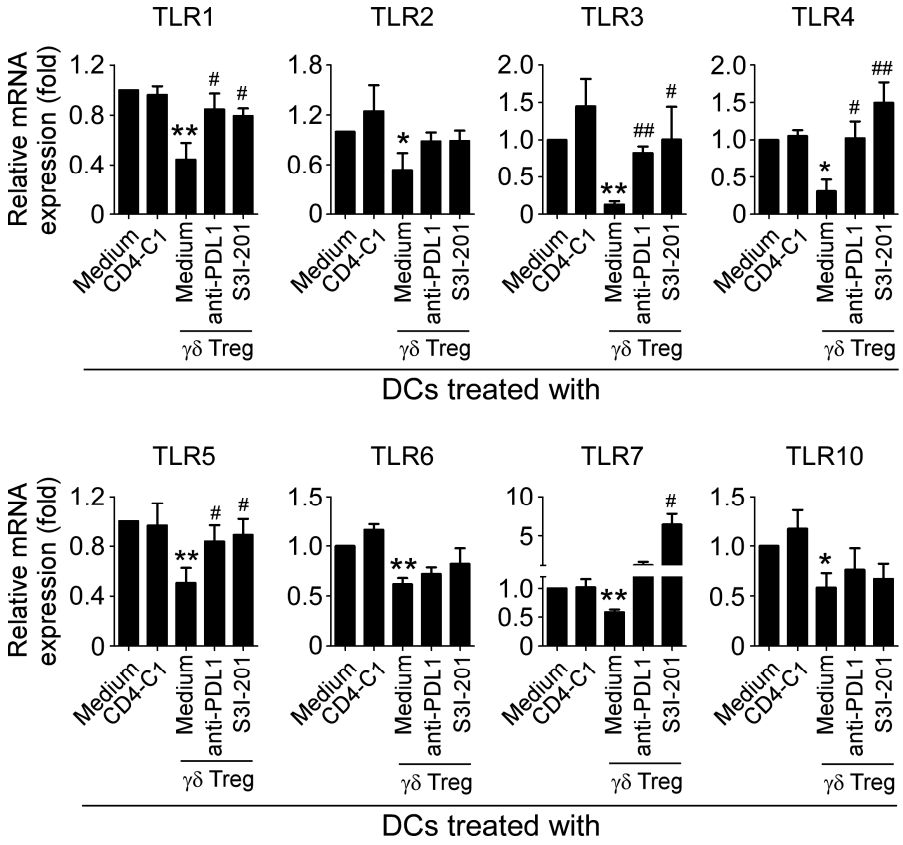
Supplementary Figure S1. Downregulation of costimulatory molecules but upregulation of PD-L1 in DCs treated with γδ Treg cells.

Monocyte-derived human immature DCs were incubated alone or co-cultured with γδ Treg or CD4-C1 effector T cells at a ratio of 3:1 in the presence of GM-CSF, IL-4, and TNF-α for 2 days. The treated DCs were purified and cell surface markers were determined with flow cytometry.



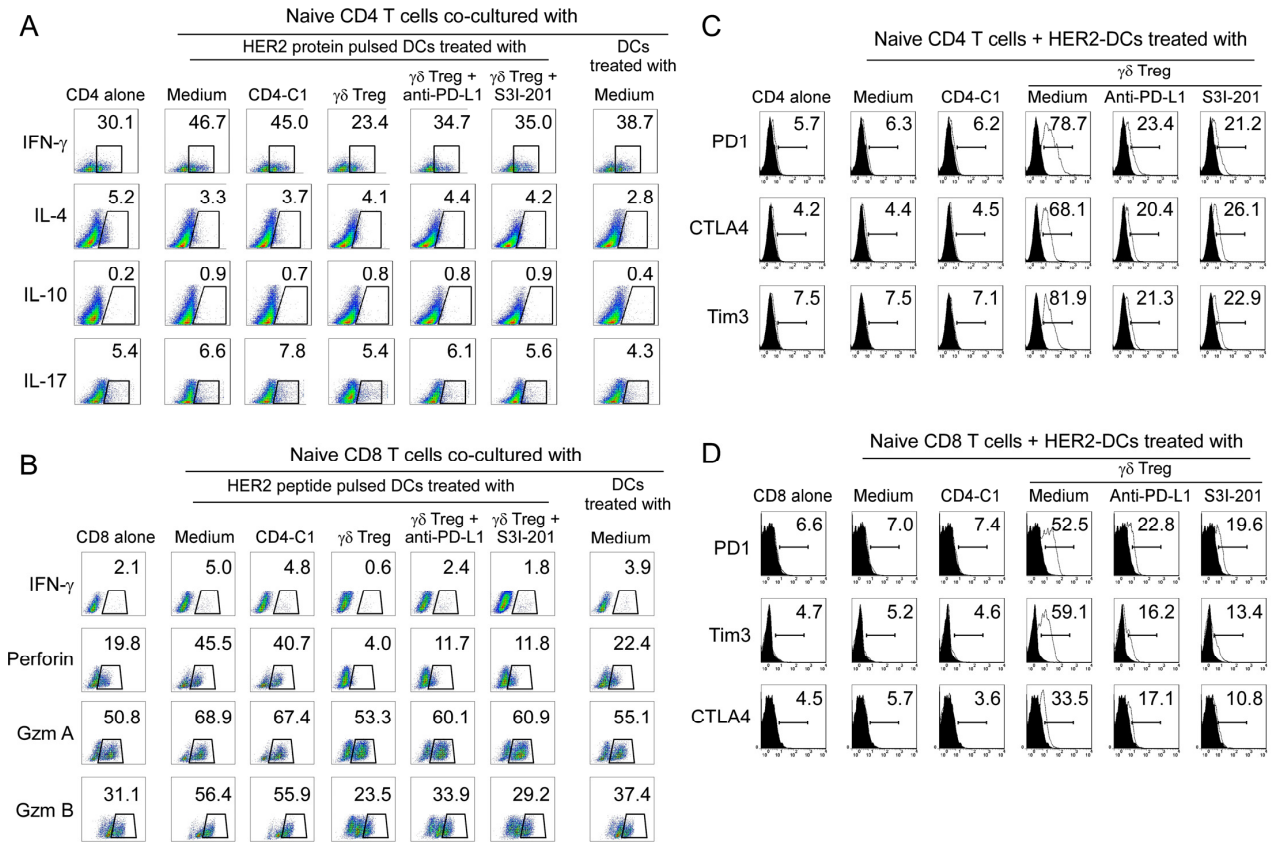
Supplementary Figure S2. Senescent DCs do not induce apoptosis of T cells.

Immature DCs were cultured alone or co-cultured with γδ Treg or CD4-C1 T cells at a ratio of 3:1 in the presence of GM-CSF, IL-4, and TNF-α for 2 days. The treated DCs were purified and co-cultured with human naive $CD4^+$ T cells at a ratio of 1:10 in the plate-bound anti-CD3/CD28 for 6 days. Apoptosis in $CD4^+$ T cells was analyzed by flow cytometry after staining with annexin V and 7-AAD.



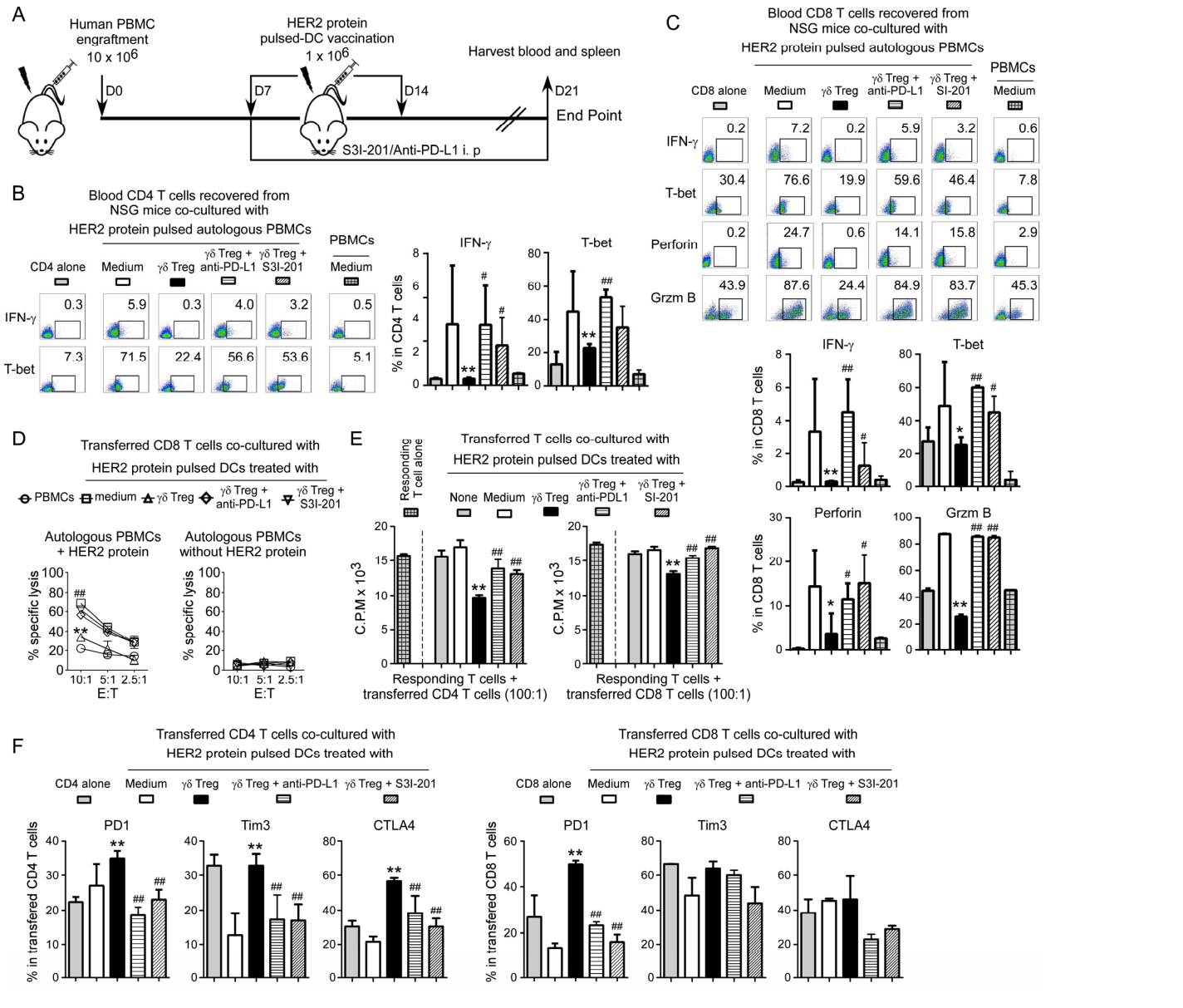
Supplementary Figure S3. Inhibition of PD-L1 or STAT3 signaling recovers the impaired TLR expression in senescent DCs induced by γδ Treg cells.

Immature DCs were pretreated with or without an anti-PD-L1 antibody (5 μg/ml) for 2 hours or S3I-201 (10 μM) for 24 hours. The PD-L1 and STAT3 activity-blocked or -unblocked DCs were then incubated alone or co-cultured with γδ Treg or CD4-C1 T cells at a ratio of 3:1 in the presence of GM-CSF, IL-4, and TNF-α for 2 days. The treated DCs were purified and mRNA expression levels of each TLR were determined by the real-time qPCR analyses. The expression level was normalized to GAPDH expression and adjusted to the levels in DCs with the medium-only group. Data are shown as mean ± SD from two-six independent experiments. *p<0.05 and **p<0.01, compared with the medium only and CD4-C1 treatment groups. #p<0.05 and ##p<0.01, compared with the Treg-treated DC-only group.



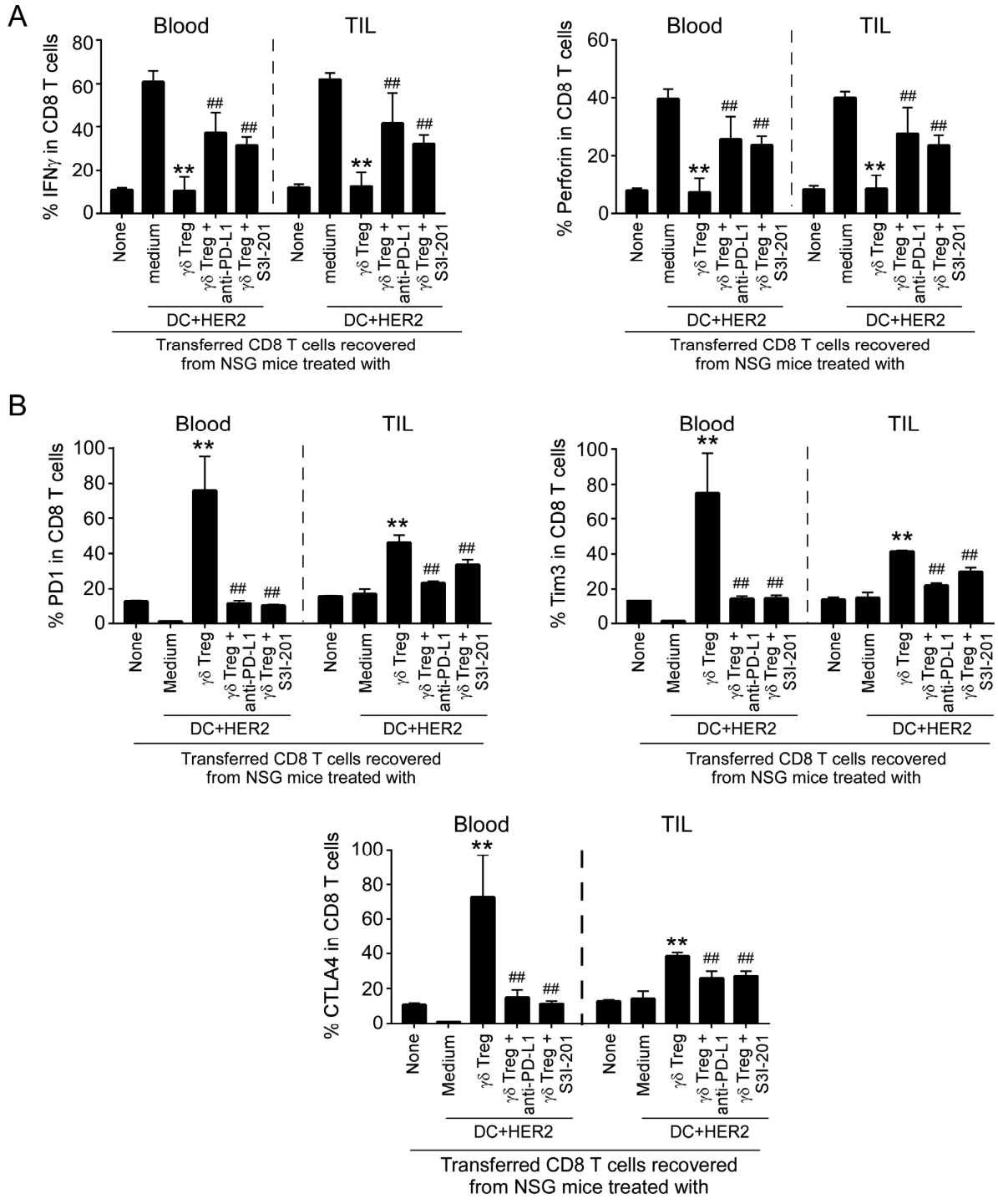
Supplementary Figure S4. Inhibition of PD-L1 or STAT3 signaling in DCs recovers the impaired effector T cell functions induced by senescent DCs.

(A) and **(B)** Blockage of PD-L1 or STAT3 signaling recovered the IFN-γ production and effector molecule expression in HER2-specific T cells mediated by HER2-pulsed senescent DCs. Immature DCs were pretreated with or without an anti-PD-L1 antibody (5 μg/ml) for 2 hours or S3I-201 (10 μM) for 24 hours, and then were co-cultured with or without γδ Treg or CD4-C1 T cells at a ratio of 3:1 in the presence of GM-CSF, IL-4, and TNF-α for 2 days. Autologous $CD4^+$ or $CD8^+$ T cells were then cocultured with HER2 protein-pulsed (2 μg/ml, in A) or peptides-pulsed (20 μg/ml, in B) above treated autologous DCs for 14 days. Cytokine and effector molecule expression in DC-treated T cells were determined by the flow cytometry after re-stimulation with DCs pulsed with the recombinant HER2 protein and HER2 peptides, respectively. **(C)** and **(D)** Blockage of PD-L1 or STAT3 signaling prevented the expression of exhaustion markers in T cells induced by HER2-pulsed senescent DCs. Cell treatment and procedure were identical to (A) and (B). PD1, Tim3, and CTLA4 expression on $CD4^+$ (in C) and $CD8^+$ (in D) T cells were determined by flow cytometry after re-stimulation with DCs pulsed with the respective HER2 protein or HER2 peptides.


A
Human PBMC engraftment 10 x 10^6
HER2 protein pulsed-DC vaccination 1 x 10^6
Harvest blood and spleen
D0
D7
D14
D21
End Point
S3I-201/Anti-PD-L1 i. p
B
Blood CD4 T cells recovered from NSG mice co-cultured with HER2 protein pulsed autologous PBMCs
CD4 alone
Medium
γδ Treg
γδ Treg + anti-PD-L1
γδ Treg + S3I-201
PBMCs
IFN-γ
T-bet
% in CD4 T cells
C
Blood CD8 T cells recovered from NSG mice co-cultured with HER2 protein pulsed autologous PBMCs
CD8 alone
Perforin
Grzm B
% in CD8 T cells
D
Transferred CD8 T cells co-cultured with HER2 protein pulsed DCs treated with
PBMCs
medium
γδ Treg
γδ Treg + anti-PD-L1
γδ Treg + S3I-201
Autologous PBMCs + HER2 protein
Autologous PBMCs without HER2 protein
% specific lysis
E:T
E
Transferred T cells co-cultured with HER2 protein pulsed DCs treated with
Responding T cell alone
None
Medium
γδ Treg
γδ Treg + anti-PDL1
γδ Treg + SI-201
C.P.M x 10^3
Responding T cells + transferred CD4 T cells (100:1)
Responding T cells + transferred CD8 T cells (100:1)
F
Transferred CD4 T cells co-cultured with HER2 protein pulsed DCs treated with
Transferred CD8 T cells co-cultured with HER2 protein pulsed DCs treated with
PD1
Tim3
CTLA4
% in transfered CD4 T cells
% in transfered CD8 T cells

Supplementary Figure S5. Blockage of PD-L1 and STAT3 signaling reverses tolerogenic functions of Treg-induced senescent DCs to induce HER2 antigen-specific immune responses in vivo.

(A) Schematic of the experimental workflow. NSG mice were engrafted with human PBMCs. Seven days and 14 days later, donor-matched HER2 protein-pulsed mature DCs or γδ Treg-induced senescent DCs (1×10^6 /mouse) were vaccinated through i.v injection. On day 7, an anti-PD-L1 neutralization antibody or inhibitor S3I-201 was injected intraperitoneally into mice three times a week. After 15 days of DC immunizations, $CD4^+$ and $CD8^+$ T cells were purified from the blood and spleens for the functional assays. **(B)** and **(C)** Blockage of PD-L1 or STAT3 signaling reversed the impaired functions of senescent DCs to activate HER2-specific T cells. $CD8^+$ T cells purified from the blood of DC-vaccinated mice were co-cultured with CFSE-labeled autologous PBMCs loaded with the HER2 protein at a ratio of 1:1 in a 24-well plate for 24 h. The indicated effector molecule expression in purified $CD4^+$ (in B) and $CD8^+$ T cells (in C) was determined by flow cytometry. *p<0.05 and **p<0.01, compared with the medium-only group. $^{\#}$p<0.05 and $^{\#\#}$p<0.01, compared with DCs treated with γδ Treg cells. **(D)** Blockage of PD-L1 or STAT3 signaling recovered impaired cytotoxicity of HER2-specific $CD8^+$ T cells purified from the blood of HER2 protein-pulsed senescent DC-immunized mice with a standard Calcein-AM release assay. Calcein-AM-labeled autologous PBMCs loaded with/without HER2 protein were incubated with $CD8^+$ T cells at various ratios at 37°C for 4 h, and the release of calcein-AM in the supernatants was determined. **p<0.01, compared with the medium-only group. $^{\#\#}$p<0.01, compared with DCs treated with γδ Treg cells. **(E)** and **(F)** Blockage of PD-L1 or STAT3 signaling reversed T cell exhaustion and suppression mediated by HER2 protein pulsed and Treg-induced senescent DC vaccination. Cell treatment and procedures were identical to (B). PD-1, Tim3, and CTLA4 expression in purified $CD4^+$ and $CD8^+$ T cells were determined by flow cytometry (in E). Suppression of the purified $CD4^+$ and $CD8^+$ T cells on the proliferation of other T cells was determined by [^{3}H] thymidine incorporation assays (in F). **p<0.01, compared with the medium-only group. $^{\#\#}$p<0.01, compared with DCs treated with γδ Treg cells.


A
Blood
TIL
% IFNγ in CD8 T cells
% Perforin in CD8 T cells
None
medium
γδ Treg
γδ Treg + anti-PD-L1
γδ Treg + S3I-201
DC+HER2
Transferred CD8 T cells recovered from NSG mice treated with
B
% PD1 in CD8 T cells
% Tim3 in CD8 T cells
% CTLA4 in CD8 T cells
Medium

Supplementary Figure S6. Prevention of senescence induction in DCs mediated by γδ Treg cells enhances effector functions of tumor-specific T cells in vivo.

(A) Blockage of PD-L1 or STAT3 signaling in senescent DCs induced by γδ Treg cells recovered IFN-γ production and perforin expression in tumor-specific $CD8^+$ T cells. The experimental procedures and injections were described in Figure 6. $CD8^+$ T cells were purified from the blood and tumor tissues, and indicated molecule expression in $CD8^+$ T cells was determined by flow cytometry. Data shown are mean ± SD from 3 mice. **p<0.01, compared with the medium only group. ##p<0.01, compared with DCs treated with γδ Treg cells. **(B)** Blockage of PD-L1 and STAT3 signaling prevented exhaustion in tumor-specific T cells purified from DC-vaccinated and tumor-bearing mice. The experimental procedures and injections were described in Figure 6. $CD8^+$ T cells were purified from the blood and tumor tissues, and the indicated exhaustion marker expression in $CD8^+$ T cells was determined by flow cytometry. Data shown are mean ± SD from 2 mice. **p<0.01, compared with the medium-only group. ##p<0.01, compared with DCs treated with γδ Treg cells.

Supplementary Table 1. Primers used for Real-time quantitative RT-PCR

Genes	Primers
IL-1β Forward	ACAGATGAAGTGCTCCTTCCA
IL-1β Reverse	GTCGGAGATTCGTAGCTGGAT
IL-6 Forward	GTAGCCGCCCCACACAGA
IL-6 Reverse	CATGTCTCCTTTCTCAGGGCT
IL-12 Forward	GCCGCGCCTCGGGACAATTA
IL-12 Reverse	GCCACAAGGAGGAGGCTGCG
TNF Forward	GGAGAAGGGTGACCGACTCA
TNF Reverse	CTGCCCAGACTCGGCAA
TLR1 Forward	CAGTGTCTGGTACACGCATGGT
TLR1 Reverse	TTTCAAAAACCGTGTCTGTTAAGAGA
TLR2 Forward	CGTTCTCTCAGGTGACTGCTC
TLR2 Reverse	GGCATTGTCCAGTGCTTCAACCCA
TLR3 Forward	AGTTGTCATCGAATCAAATTAAAGAG
TLR3 Reverse	TGGGACCCAGCTGGACATTGTT
TLR4 Forward	CAGAGTTTCCTGCAATGGATCA
TLR4 Reverse	GCTTATCTGAAGGTGTTGCACAT
TLR5 Forward	TCTCCACAGTCACCAAACCA
TLR5 Reverse	AAGCTGGGCAACTATAAGGTCA
TLR6 Forward	GAAGAAGAACAACCCTTTAGGATAGC
TLR6 Reverse	AGGCAAACAAAATGGAAGCTT
TLR7 Forward	TTACCTGGATGGAAACCAGCTACT
TLR7 Reverse	TCAAGGCTGAGAAGCTGTAAAGCTA
TLR8 Forward	CAGAATAGCAGGCGTAACACATCA
TLR8 Reverse	TGTCAAGGCGATTGCCACTGA
TLR9 Forward	TGTGAAGCATCCTTCCCTGT
TLR9 Reverse	GAGAGACAGCGGGTGCAG
TLR10 Forward	GCCCAAGGATAGGCGTAATG
TLR10 Reverse	TGTGGTCCCCAACTTCCCAAGGA
H-GAPDH Forward	AGCCGCATCTTCTTTTGCGTCG
H-GAPDH Reverse	GACCAGGCGCCCAATACG