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14. ABSTRACT

Immunosuppressive microenvironments induced by Treg cells is a major barrier for effective anti-tumor immunity. We recently discovered accumulated γδ1 Treg cells in the human breast tumor microenvironment, which use a novel suppressive mechanism of senescence induction on T cells and DCs in the tumor suppressive microenvironment. Importantly, these senescent T cells and DCs also develop strong suppressive activity. In this application, our subject and purpose is to explore the mechanisms responsible for DC senescence in the tumor microenvironment mediated by breast cancer-derived γδTreg cells, and then develop novel immunotherapeutic strategies against breast cancer.

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INTRODUCTION

Immunosuppressive microenvironments induced by Treg cells is a major barrier for effective anti-tumor immunity. We recently discovered accumulated γδ1 Treg cells in the human breast tumor microenvironment, which use a novel suppressive mechanism of senescence induction on T cells and DCs in the tumor suppressive microenvironment. Importantly, these senescent T cells and DCs also develop strong suppressive activity. In this application, our subject and purpose is to explore the mechanisms responsible for DC senescence in the tumor microenvironment mediated by breast cancer-derived γδTreg cells, and then develop novel immunotherapeutic strategies against breast cancer. We will determine whether the lipid metabolism alterations in DCs are induced by human tumor-derived γδ Treg cells responsible for DC senescence and tolerogenic functions. We will identify what unique signaling pathway(s) and molecules controlling DC senescence and imbalance of lipid metabolism mediated by breast cancer-derived γδ Treg cells. In addition, we further determine whether reprogramming of DC metabolism and functions can enhance anti-tumor immunity for breast tumor immunotherapy.

KEYWORDS

γδTreg cells, DC, senescence, lipid metabolism, STAT1/STAT3, anti-tumor immunity

ACCOMPLISHMENTS

A. Major goals of the project:

Goals	month	Complete %
Specific Aim 1: To determine whether the lipid metabolism alterations in DCs induced by human tumor-derived γδ Treg cells are responsible for DC senescence and tolerogenic functions		
Major Task 1: To study the differences in metabolites and lipid species between normal DCs and senescent DCs		
Subtask 1: Compare lipid species differences between mature and immature DCs; and functional mature DCs and senescent DCs with tolerogenic functions through a mass spectrometry-based lipidomic screen	1-3	100%
Subtask 2: Compare the lipidomic profiles between senescent DCs and breast tumor-derived DCs purified from cancer patient tumor tissues through a mass spectrometry-based lipidomic screen	4-6	100%
Milestone(s) Achieved: Obtain lipidomic profiles of different type DCs	6	
Major Task 2: To determine whether γδ Treg-induced altered DC lipid species/metabolism are causally linked to DC senescence and tolerogenic functions		
Subtask 1: Determine whether the reduced lipid species are critical for γδ Treg-induced senescence in DCs through the gain-of-function strategy with addition of the reduced lipid species	7-9	100%
Subtask 2: Identify whether the increased lipid species are also important for senescent DC development through the loss-of-function strategy with inhibition of the increased lipid species	10-12	100%
Milestone(s) Achieved: Identify specific lipid species responsible for DC senescence and dysfunctions mediated by Treg cells.	12	

Specific Aim 2: To identify unique signaling pathway(s) and molecules controlling DC senescence and imbalance of lipid metabolism mediated by breast cancer-derived γδ Treg cells		
Major Task 3: Determine whether transcription factors STAT1 and STAT3 are critical and important in regulating DC senescence and lipid metabolism inhibition induced by γδ Treg cells		
Subtask 1: Determine whether we can prevent senescence induction and lipid metabolism inhibition in DCs through functional blockage of STAT1/STAT3 signaling using STAT signaling inhibitors or shRNA in responder DCs	13-15	100%
Subtask 2: Identify the unique key adaptor molecules involved in STAT1/STAT3 signaling that functionally regulate DC senescence and lipid metabolism imbalance induced by γδ Treg cells	16-18	50%
Milestone(s) Achieved: Discover novel and unique downstream or upstream targets regulated by STAT1/STAT3 during the DC senescence.	18	
Major Task 4: Determine whether the PD-L1-dependent mechanism is responsible for the suppressed lipid metabolism in DCs induced by breast cancer-derived γδ Treg cells		
Subtask 1: Determine whether PD-L1 blockage with a PD-L1 neutralization antibody can prevent DC senescence induction, phenotypic and function changes induced by γδ Treg cells.	19-21	100%
Subtask 2: Determine whether PD-L1 blockage with a PD-L1 neutralization antibody can reverse DC lipid metabolism changes induced by γδ Treg cells.	22-24	50%
Milestone(s) Achieved: Identify PD-L1 are important in regulating DC senescence and lipid metabolism inhibition induced by γδ Treg cells.	24	
Specific Aim 3: To identify whether reprogramming of DC metabolism and functions can enhance anti-tumor immunity for breast tumor immunotherapy		
Major Task 5: Determine blockade of STAT1/3 and/or PD-L1 signaling in DCs can reprogram DC metabolism and synergistically enhance the efficacy of a breast cancer therapeutic vaccine for tumor immunotherapy. We propose to need totally 216 of NSG mice for this study.		
Subtask 1: Evaluate the effect of checkpoint blockade of PD-L1 or STAT1/STAT3 inhibition on anti-tumor immunity. We propose to need totally 216 of NSG mice for 3 subtasks. 60 NSG mice will be purchased from The Jackson Laboratory, and we will breed partial mice by our lab.	25-36	50%
Subtask 2: Evaluate the effect of checkpoint blockade of PD-L1 or STAT1/STAT3 inhibition on the suppression and senescence induction in DC and DC-induced HER-2-specific effector $CD4^+$ and $CD8^+$ T cells.	25-36	50%
Subtask 3: Investigate whether immune checkpoint blockade of PD-L1	25-36	50%

and STAT1/STAT3 inhibition have synergistic effects on the reversal of Treg suppression and senescence induction on DCs.		
Milestone(s) Achieved: Characterization of effects of checkpoint blockade of PD-L1 or STAT1/STAT3 inhibition on anti-tumor immunity; publication of 1-2 peer reviewed papers.	36	

B. Studies and Results: During the first year (Year 1) of grant-funded period, we have focused on the following directions of senescent DCs in the tumor microenvironment: **1)** Identify the differences in metabolites and lipid species between normal DCs and the γδ Treg-induced senescent DCs; **2)** Dissect the unique signaling pathway(s) and molecules controlling DC senescence and imbalance of lipid metabolism mediated by breast cancer-derived γδ Treg cells.

1. **Identify the differences in metabolites and lipid species between normal DC and breast/meanoma tumor-derived DCs purified from tumor-bearing mice** (Results for Specific Aim 1).

Based on results from the year 1 report, we continued to compare the lipidomic profiles between senescent DCs and breast tumor-derived DCs purified from cancer patient tumor tissues. We did not collect any tumor samples from breast cancer patients during the past year due to the covid-19 pandemic situation. We performed mouse DCs experiments instead of human DCs. We compared the lipidomic profiles between normal mouse DCs and tumor-derived DCs purified from tumor tissue of mouse breast tumor E0771/menaloma B16F10-bearing mice through a mass spectrometry-based lipidomic screen. Based on the preliminary studies of this project, we first determined whether DCs in the tumor microenvironments are senescent. We utilized murine mammary cancer cell line E0771 and melanoma cell line B16F0 to establish breast cancer and melanoma cancer models, respectively. $CD11c^+$ cells from different organs and tumor sites in the tumor-bearing mice were then purified and analyzed. We observed markedly elevated SA-β-gal-positive$CD11c^+$ cells existing in blood, lymph nodes, and tumors derived from tumor-bearing mice as compared to in control tumor-free mice (**Figure 1A**). We further observed increased LD formation in $CD11c^+$ cells in blood, lymph nodes, and tumors derived from tumor-bearing mice as compared to in control tumor-free mice using the oil red O staining (**Fig. 1B**). Then, we determined the lipid species differences between normal DCs and tumor-derived. We are particularly interested in the following 2 lipid subclasses, glycerophospholipids (GP) and sphingolipids (SP). Tumor-derived $CD11c^+$ cells have more all kinds of lipids compared with blood, lymph nodes and spleens. Especially, tumor-derived $CD11c^+$ cells have more 10 folds of cholesteryl ester (CE) and ceramide compared with blood, lymph nodes and spleens. And total levels of CE in tumor-derived $CD11c^+$ cells were increased with the progression of tumors. However, total levels of phosphatidylcholine (PC), free fatty acids (FFA), phosphatidylserine (PS), and phosphatidylethanolamine (PE) in tumor-derived $CD11c^+$ cells are not too high or decreased with the progression of tumors (**Fig. 1C and D**). *Collectively, these data strongly indicate that unbalanced lipid metabolism plays a crucial role in regulation of senescence development in DCs in the tumor microenvironment.*

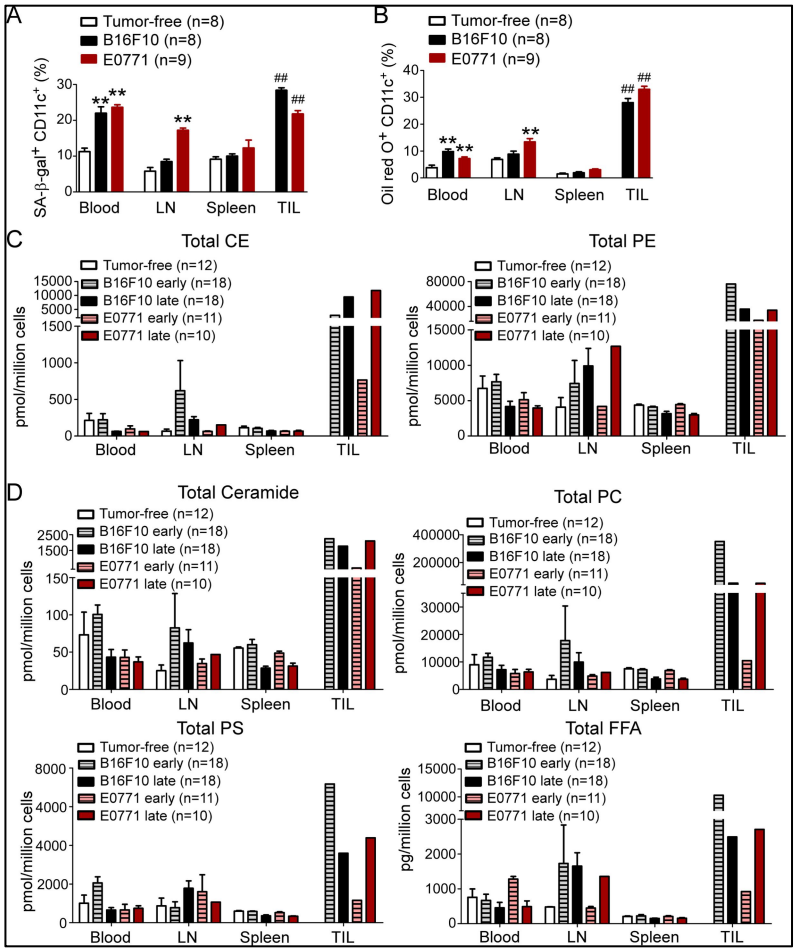

Fig. 1. Tumor-derived DCs are senescent and have unbalanced lipid metabolism. (A) CD11c$^+$ cells from different organs in E0771 and B16F10 bearing mice express high levels of SA-b-gal. **(B)** CD11c$^+$ cells from different organs in E0771 and B16F10 bearing mice have increased expression of oil red O. **(C)** and **(D)** Changes of the levels of total CE, Ceramide, PE, PC, PS, and FFA were observed in tumor-derived CD11c$^+$ cells compared with blood, lymph nodes and spleens. Lipid extracts from different groups were subjected to ESI-MS/MS analysis. Methods: Murine mammary cancer cell line E0771 and melanoma cell line B16F0 were injected to establish breast cancer and melanoma cancer models, respectively. CD11c$^+$ cells from different organs and tumor sites in the tumor-bearing mice and tumor-free mice were then purified and analyzed after tumor diameters reached 5-10 mm (early stage) or 15-20 mm (late stage). The purified CD11c$^+$ cells were subjected to SA-β-gal staining, oil red O staining, and ESI-MS/MS analysis. ** p<0.01, compared with tumor-free mice, ## p<0.01, compared with blood from tumor-free mice.

2. Senescent DCs induced by γδ Treg cells inhibit Th1 and Th17 cell differentiation, but promote development of Foxp3$^+$ Treg cells (Results for Specific Aim 1).

Our studies showed that γδ Treg-treated senescent DCs produced high amounts of PDL1 and had the typical 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 γδ Treg-induced senescent DCs under T cell culture medium for 6 day, and the portions of Th1, Th2, Th17 and Treg cells were determined by flow cytometry analyses after stimulation with PMA and ionomycin. We found that the senescent DCs induced by γδ Treg cells dramatically decreased IFN-γ and IL-17-producing T cell populations in the co-cultured T cells, compared with T cells stimulated by mature DCs or DCs treated with control CD4-C1 effector T cells (**Fig. 2A**).In contrast, senescent DCs induced by Treg cells have no obvious effect on IL-4-producing T cell populations. In addition, senescent DCs induced by γδ Treg cells can promote Foxp3$^+$ T cell populations in the co-cultured CD4$^+$ T cells, suggesting development of Treg cells. We next determined whether senescent DCs can also affect 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 conditions. γδ Treg cells-induced senescent DCs showed suppression in Th1 cell differentiation compared to mature DCs treated or untreated with control CD4-C1 effector T cells as indicated by reduced expression of IFN-γ (**Fig. 2B**). Similarly, senescent DCs potently suppressed the differentiation of Th17 cells (**Fig. 2C**). *Collectively, these data suggest that γδ Treg-induced senescent DCs with tolerogenic phenotypes can inhibit both Th1 and Th17 cell differentiation and drive Foxp3$^+$ Treg development.*

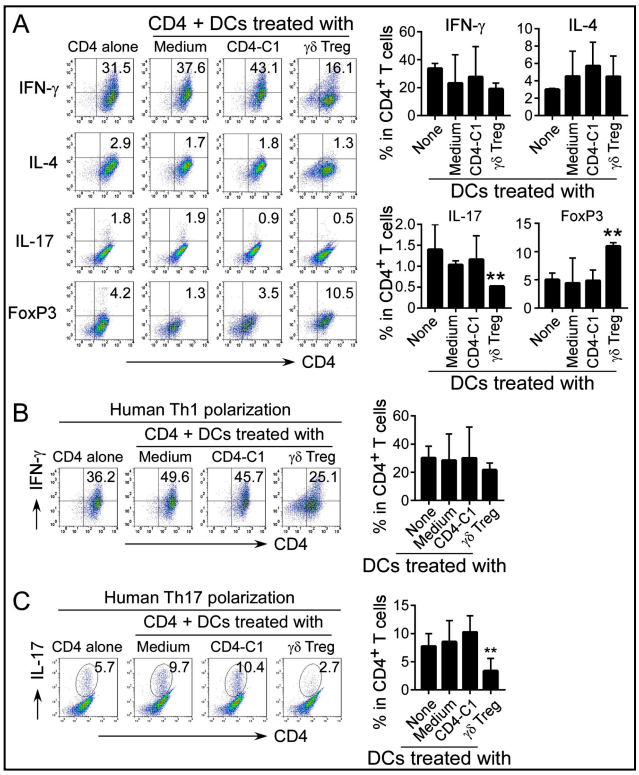

Fig. 2. Effects of γδ Treg-induced senescent DCs on T cell subset differentiation. (A) Senescent DCs induced by γδ Tregs had inhibitory effects on Th1 and Th17 cell development but promoted FoxP3$^+$ Treg generation. Immature DCs were incubated alone or co-cultured with γδ Treg or CD4-C1 T cells at a ratio of 3:1 in the presence of GM-CSF, IL-4, and TNF for 2 days. 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. Results are representative intracellular staining of CD4$^+$ T subsets with different DCs treatments using flow cytometry analyses after stimulation with PMA and ionomycin (left panel). Data in right panel are mean ± SD from three independent experiments of different T subset cells with indicated DC treatments. (**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 plate-bound anti-CD3/CD28 under Th1 or Th17-polarization condition for 6 days. Results shown in left panel are representative intracellular staining of IFN-γ or IL-17-producing T cells using flow cytometry analyses after stimulation with PMA and ionomycin. Data in right panel are mean ± SD from three independent experiments of T cell subsets with indicated DC treatments

3. Senescent DCs induced by γδ Treg cells have impaired APC functions, and PD1/PDL1 and STAT3 signaling are involved in the regulation of the impaired functions of senescent DCs. (Results for Specific Aim 1 and 2).

Given that senescent DCs induced by γδ Treg cells have impaired functions to stimulate T cell proliferation, and have the impaired capacity to process and present antigen-specific T cell immune responses. We next determined whether those senescent DCs have the decreased capacity to process and present a real tumor antigen to induce tumor-specific T cell immune responses. We selected HER-2/neu, a well-established tumor antigen in breast cancer, as the target antigen for our studies. CD4$^+$ and CD8$^+$ T cells purified from healthy donors were cocultured with HER2 protein-pulsed autologous DCs, which were pretreated with medium, control CD4-C1 effector T cells, or γδ Treg cells. 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 (**Fig. 3A**). Similarly, γδ Treg-induced senescent DCs pulsed with HER2 peptide (HLA-A2 restrict) had a decreased ability to stimulate autologous CD8$^+$ T cell proliferation, compared with DCs alone or pretreated with CD4-C1 effector T cells (**Fig. 3A**). We then determined whether blockage of PDL1 and STAT3 signaling can recover the ability of γδ Treg-induced senescent DCs to stimulate T cell proliferation. As expected, we found that treatments with a PDL1 neutralizing antibody or STAT3 inhibitor S3I-201 dramatically reversed the ability of HER2-pulsed senescent DCs to stimulate autologous T cell proliferation (**Fig. 3B**). We further investigated HER2 antigen-specific immune responses induced by DCs with different treatment. CD4$^+$ or CD8$^+$ T cells were cocultured with HER2 protein-pulsed autologous DCs with the different treatments as described in Fig. 3A. After 14 days of co-culture, CD4$^+$ or CD8$^+$ T cells sensitized by DCs with different treatments were re-stimulated with HER2 protein-pulsed autologous PBMC or HER2 peptides-pulsed T2 cells, and HER2-specific cytokines were determined by flow cytometry. HER2 protein-pulsed DCs pretreated with or without CD4-C1 T cells dramatically induced the increases of HER2-specific IFN-γ-producing T cell populations in the

cocultured both CD4⁺ and CD8⁺ T cells (**Fig. 3C-3D**). In contrast, γδ Treg-induced senescent DCs as APCs only induced minor levels of HER2-specific IFN-γ-producing T cell populations in co-cultured T cells. In addition, γδ Treg-induced senescent DCs pulsed with HER2 peptide had an impaired ability to stimulate autologous CD8⁺ T cell to produce perforin, granzyme A and granzyme B, compared with T cell groups stimulated with DCs alone or DCs pretreated with CD4-C1 effector T cells (**Fig. 3D** and **3E**). Furthermore, we also demonstrated that treatments with anti-PDL1 antibody and STAT3 signaling inhibitor can reverse the impaired functions of senescent DCs induced by γδ Treg cells on T cell proliferation and effector molecule expression (**Fig. 3C-3E**).

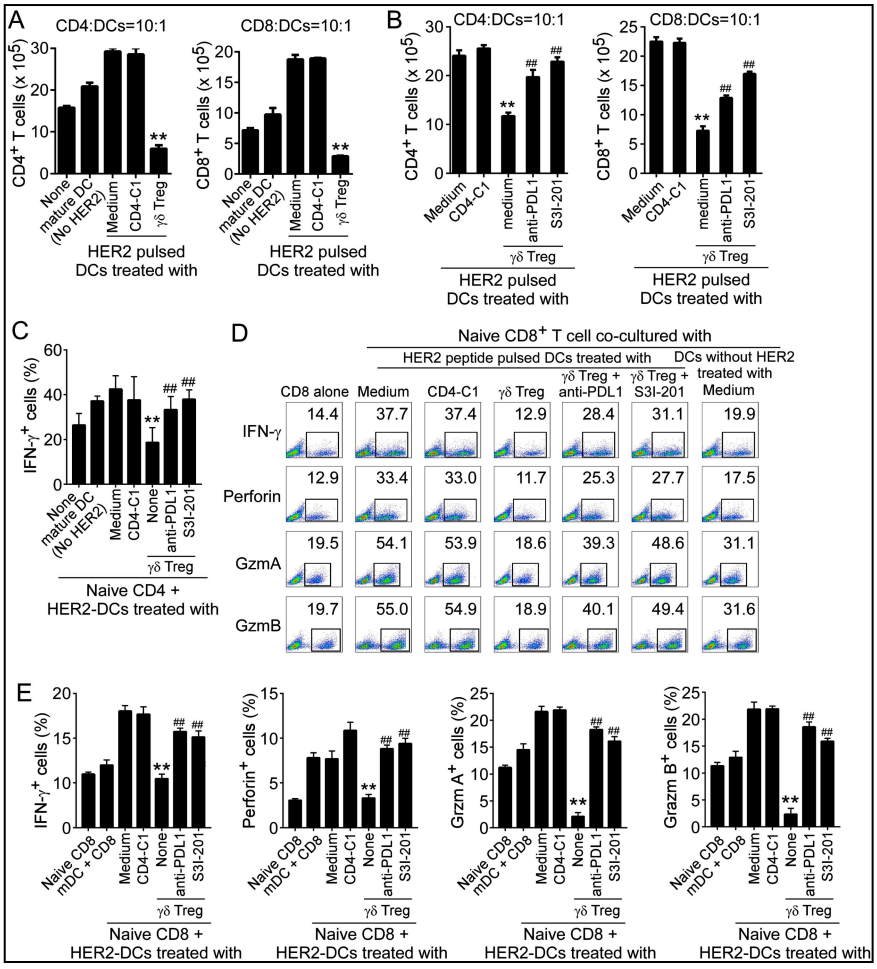

Fig. 3. Senescent DCs induced by γδ Treg cells have impaired APC functions, and PD1/PDL1 and STAT3 signaling are involved in the regulation of the impaired functions of senescent DCs. (A) γδ Treg treatment inhibited the ability of HER2 antigen pulsed DCs to stimulate autologous T cell proliferation. 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, IL-4, and TNF for 2 days. The treated DCs were purified and loaded with 20 μg/ml HER2 peptides or 2 μg/ml HER2 protein. Autologous CD4⁺ or CD8⁺ T cells were cocultured with HER2 protein-pulsed or peptides-pulsed autologous DCs for 14 days. The CD4⁺ or CD8⁺ T cells numbers were counted. ** p<0.01, compared with the medium-only and CD4-C1 treatment DC groups **(B)** Blockage of PD1/PDL1 or STAT3 signaling obviously reversed the decreased ability of HER2-pulsed senescent DCs to stimulate autologous T cell proliferation. Immature DCs were pretreated with or without anti-PDL1 antibody (5 μg/ml) or S3I-201 (10 μM), then co-cultured with γδ Treg or CD4-C1 T cells. T cells and DCs treatment and procedure were the same as in (**A**). **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)-(E)**. Blockage of PD1/PDL1 or STAT3 signaling recovered the IFN-γ production and effector molecule expression in HER2-specific T cells mediated by HER2-pulsed senescent DCs. Cell treatment and procedure were identical to (B). Cytokine and effector molecule expression in DC-treated T cells were determined by FACS, **p<0.01, compared with the medium only and CD4-C1 treatment groups, and ##p<0.01, compared with DCs treated with γδ Treg cells. Data shown in A, B, C, E are mean ± SD from three independent experiments of T cells/DCs sets.

To identified the mechanisms responsible for impaired functions of T cells induced by senescent DCs, we found that T cells primed by senescent DCs but not control DCs expressed high levels of exhaustion markers including PD1, Tim3 and CTLA4 (**Fig. 4A**). Furthermore, blockage of PDL1 and STAT3 signaling can significantly decrease expression of these exhaustion molecules in T cells mediated by senescent DCs (**Fig. 4A**). In addition to HER2-specific effector molecule production, we analyzed the cytotoxic activity of T cells primed by HER2-pulsed DCs with different treatments. We utilized MCF7 breast cancer cells with high HER2 expression and T2 loaded with the relevant peptides as two target cells (**Fig. 4B**). 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 peptide as well as MCF7 cells (HLA-A2⁺ HER2⁺), but not with T2 alone or

MDA-MB-231(HLA-A2$^+$ HER2$^-$) breast cancer tumor cells, demonstrating the HLA-A2-restriction and antigen specificity of this activity (**Fig. 4C**). However, T cells primed with senescent DCs induced by Treg cells have very weak ability to lyse the peptide-pulsed T2 cells and MCF7 cells, while treatments with an anti-PDL1 antibody or inhibitor S3I-201 can significantly recover HER2-specific cytotoxicity of the T cells induced by senescent DCs (**Fig. 4C**). *These results suggest that senescent DCs induced by γδ Treg cells lost the capacity to process and present HER2 antigen to T cells and induce HER2-specific T cell responses*.

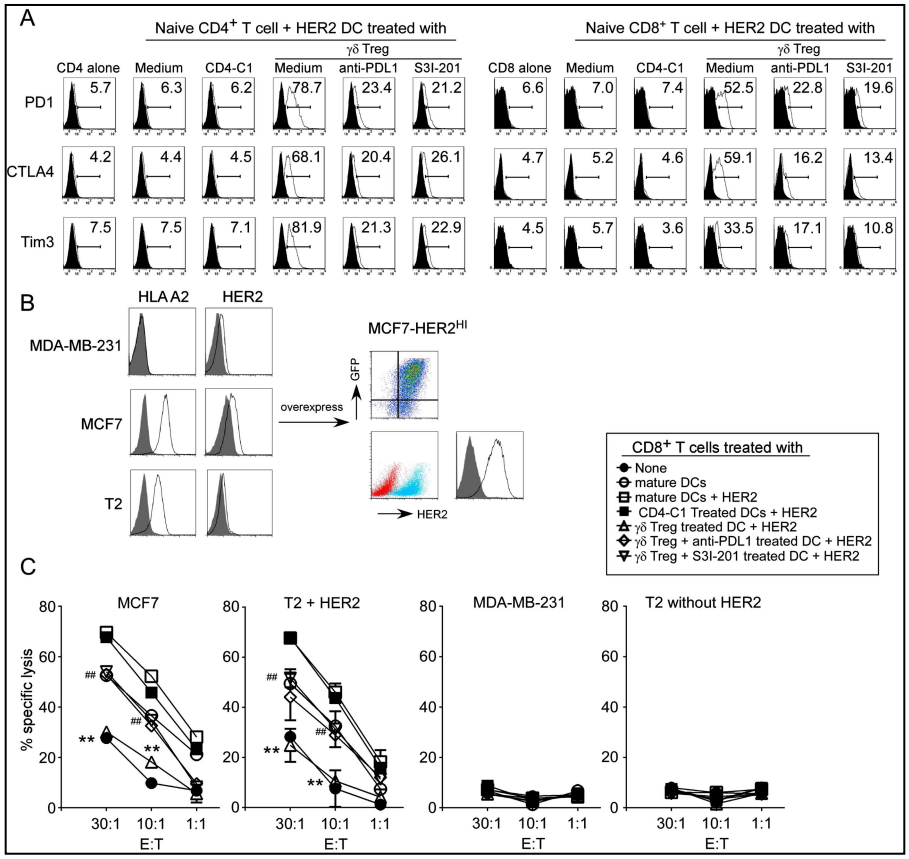

Fig. 4. Senescent DCs induced by γδ Treg cells have impaired APC functions to induce antigen-specific T cell responses. **(A)** Blockage of PD1/PDL1 or STAT3 signaling prevented the enhanced expression of exhaustion markers in T cells stimulated by HER2-pulsed senescent DCs. Cell treatment and procedure were identical to Fig 3. PD1, Tim3, and CTLA4 expression on CD4$^+$ and CD8$^+$ T cells after sensitized with DCs pulsed with the recombinant HER2 protein or HER2 peptides were determined by the flow cytometry. **(B)** HER2 overexpression in MCF7 lines. MCF7 lines with stable HER2 overexpression (MCF7HER2) were generated through infection with lentivirus containing HER2/neu-GFP and then were sorted by FACS gating on GFP$^+$ populations. **(C)** Blocking PD1/PDL1 or STAT3 signaling reversed the impaired HER2 antigen-specific cytotoxicity of CD8$^+$ T cells mediated by γδ Treg-induced senescent DCs. DCs treatment and procedure were the same as in Fig. 4. After 14 days of sensitization, the DC-primed human CD8$^+$ cells were harvested and determined the HER2-specific CTL responses by a standard 4-h Calcein-AM release assay. MCF7 cells (HLA-A2$^+$, HER2 over-expression) and T2 cells pulsed with HER2 peptides were used as target cells (T). Control targets included MDA-MB-231 cells (HLA-A2$^-$, HER2$^-$) and T2 cells. Various E/T ratios were tested as indicated. **p<0.01, compared with the medium only and CD4-C1 treatment groups, ##*p*<0.01, compared with DCs treated with γδ Tregs.

4. **Blockage of PD1/PDL1 and STAT3 signaling reversed the impaired APC functions of senescent DCs induced by γδ Treg cells in vivo** (Results for Specific Aims 2 and 3)

We then addressed whether senescent DCs induced by γδ Treg can also have impaired ability to induce HER2-specific immune responses in vivo. Human PBMCs were intravenously (i.v.) injected into female immunodeficient NOD-scid IL2Rg^{null} (NSG) mice to reconstitute humanized mice. One week after human PBMC engraftment, treated or untreated DCs were pulsed with HER2 peptides (HLA-A2-restricted 369-377 and 689-697) for 2 hours. Humanized NSG mice were then vaccinated with 5 × 10^5 donor matched HER2 peptides-loaded DCs. Immunized mice were boosted with the donor matched HER2-pulsed DCs again at day 7 post first immunization. Concomitantly, mice were administrated with an anti-PDL1 neutralizing antibody or STAT3 inhibitor S3I-201 through intraperitoneal injection on days 2, 5, 8, and 11 after adoptive transfer of DCs. Blood, lymph nodes (LN) 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 (**Fig.**

5A). We first determined the effector cytokine and molecule expression in purified CD8$^+$ with different DC vaccinations and treatments. CD8$^+$ T cells purified from HER2 peptides-pulsed mDCs dramatically induced the increases of HER2-specific IFN-γ-producing and T-bet$^+$ T cell populations, and expression of perforin and granzyme B (**Fig. 5B**). In contrast, CD8$^+$ T cells purified from vaccinated mice with HER2 peptides-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 (**Fig. 5B**). Furthermore, we also found that administration with anti-PDL1 antibody and STAT3 signaling inhibitor can reverse the impaired functions of γδ Treg-induced senescent DC vaccination on expression of effector molecules on T cells (**Fig. 5B**). We then determined the cytotoxicity of T cells purified from HER2 peptide-pulsed DC vaccinated mice. CD8$^+$ T cells purified from blood of humanized mice were re-stimulated ex vivo by Calcein AM-labeled T2 cells loaded with the same HER2 peptide for 24 hours. CD8$^+$ CTLs from HER2-pulsed mature DC-immunized mice can recognize and efficiently lyse the same HER2 peptide-pulsed T2 cells and HER2$^+$ MCF7 cells, but not T2 cells alone or HER2 negative MDA-MB-231 cells (**Fig. 5C**). However, CTLs from immunized mice with HER2-pulsed Treg-induced senescent DC vaccination have little ability to kill the same HER2 peptide-pulsed T2 cells and HER2$^+$ MCF7 (**Fig. 5C**). While administration with anti-PDL1 antibody and STAT3 signaling inhibitor can recover cytotoxicity of CD8+ T cells from immunized mice with γδ Treg-induced senescent DC vaccination (**Fig. 5C**).

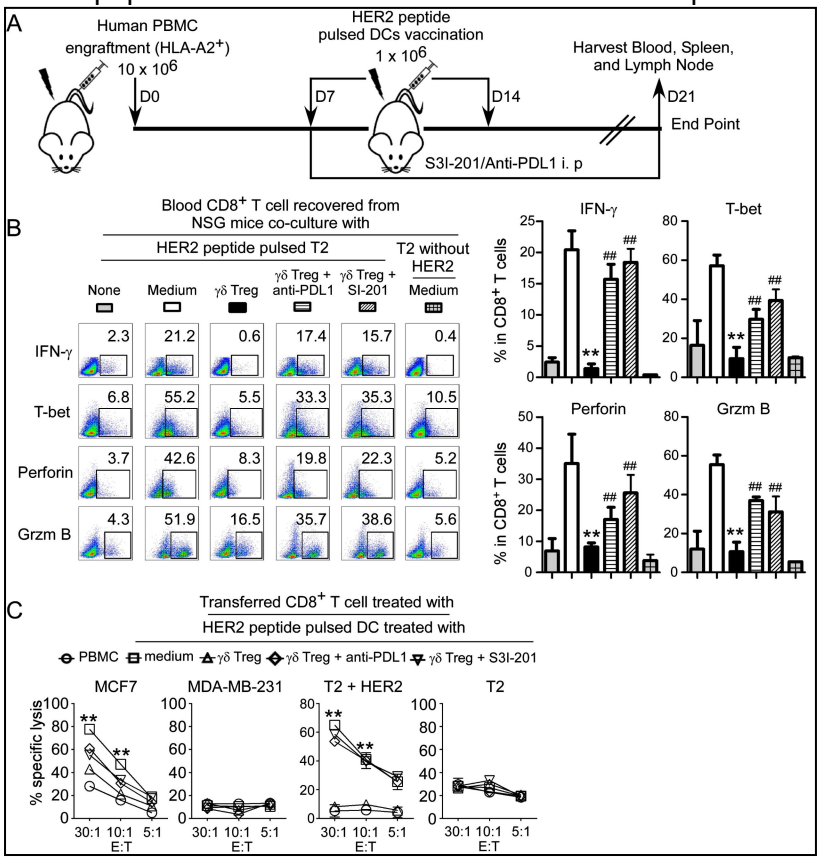

Fig. 5. Blockage of PD1/PDL1 and STAT3 signaling reversed the impaired APC functions of Treg-induced senescent DCs in vivo. (A) Schematic of the experimental workflow. NSG mice were engrafted with human PBMCs. 7 days and 14 days later, donor matched HER2 peptide-pulsed mature DCs and γδ Treg-induced senescent DCs (1 × 10^6 /mouse) were vaccinated through iv injection. Anti-PDL1 neutralization antibody and S3I-201 were injected intraperitoneally three times a week. After 15 days of immunization, CD8$^+$ T cells from blood were purified for the functional assays. **(B)** Blockage of PD1/PDL1 or STAT3 signaling reversed the impaired ability of senescent DCs to activate HER2 specific T cells. CD8$^+$ CTLs purified from blood of vaccinated mice were co-cultured with CFSE labeled T2 cells loaded with HER2 peptide at a ratio of 1:1 in a 24-well plate for 24 h. The indicated effector molecule expression in purified CD8$^+$ T cells were determined by FACS. **<0.01, compared with the medium only groups and ## p<0.01, compared with DCs treated with γδ Treg cells. **(C)** Blockage of PD1/PDL1 or STAT3 signaling recovered impaired HER2-specific cytotoxicity of CD8+ T cells purified from senescent DC vaccination mice. CD8$^+$ CTLs purified from blood of vaccinated mice were tested for their cytotoxic activity in a standard Calcein-AM release assay. Calcein-AM-labeled MCF7 (HLA A2$^+$, HER2$^+$), MDA-MB-231 (HLA A2$^-$, HER2$^-$), or HER2 peptides loaded or unloaded T2 cells were used as targets. Target cells were incubated with effector cells at various ratios at 37°C for 4 h. Supernatants were harvested and release of calcein was determined. **<0.01, compared with the medium-only groups, and ##p<0.01, compared with DCs treated with γδ Treg cells.

Consistence with our in vitro data, we found that T cells purified from immunized mice with HER2-pulsed Treg-induced senescent DC vaccination expressed high levels of exhaustion markers, including PD1, Tim3

and CTLA4, as well as had potent suppressive activity on the other T proliferation (**Fig. 6A and 6B**). In addition, blockage of PDL1 and STAT3 signaling in vivo can significantly decrease expression of these exhaustion molecules and reverse their suppressive activity in T cells purified from vaccinated mice with HER2 peptides-pulsed Treg-induced senescent DCs (**Fig. 7A**).

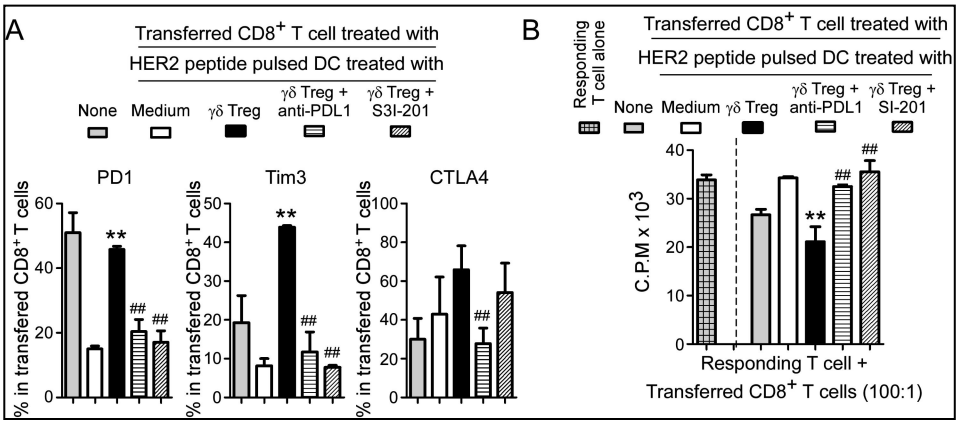

Fig. 6. Blockage of PD1/PDL1 and STAT3 signaling reversed the T cell exhaustion and suppression mediated by Treg-induced senescent DCs vaccination. Cell treatment and procedures were identical to Fig. 6B. PD1, Tim3, and CTLA4 expression in purified CD8⁺ T cells were determined by the flow cytometry (in A). Suppression of purified CD8⁺ T cells on the proliferation of other T cells was determined by [³H]- thymidine incorporation assays (in B). **<0.01, compared with the medium-only groups, and ## p<0.01, compared with DCs treated with γδ Treg cells.

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 for the studies (**Fig. 7A**). We therefore can evaluate both CD4⁺ and CD8⁺ T cell functions from the immunized mice with different DC vaccinations. As shown in **Fig. 7**, Treg-induced senescent DCs cannot effectively induce HER2-specific immune responses in CD4⁺ and CD8⁺ T cells, showing decreased expression of effector cytokine and molecules, promoted exhaustion marker expression and suppressive activity, as well as decreased cytotoxicity of CD8⁺ T cells. In addition, blockage of PDL1 and STAT3 signaling can reverse those molecular processes in T cells mediated by senescent DC vaccination. *Taken together, these results collectively indicate that γδ Treg-induced senescent DCs have significantly impaired APC functions to induce tumor antigen specific immune responses, and that PDL1 and STAT3 signaling pathways are important for their tolerogenic functions.*

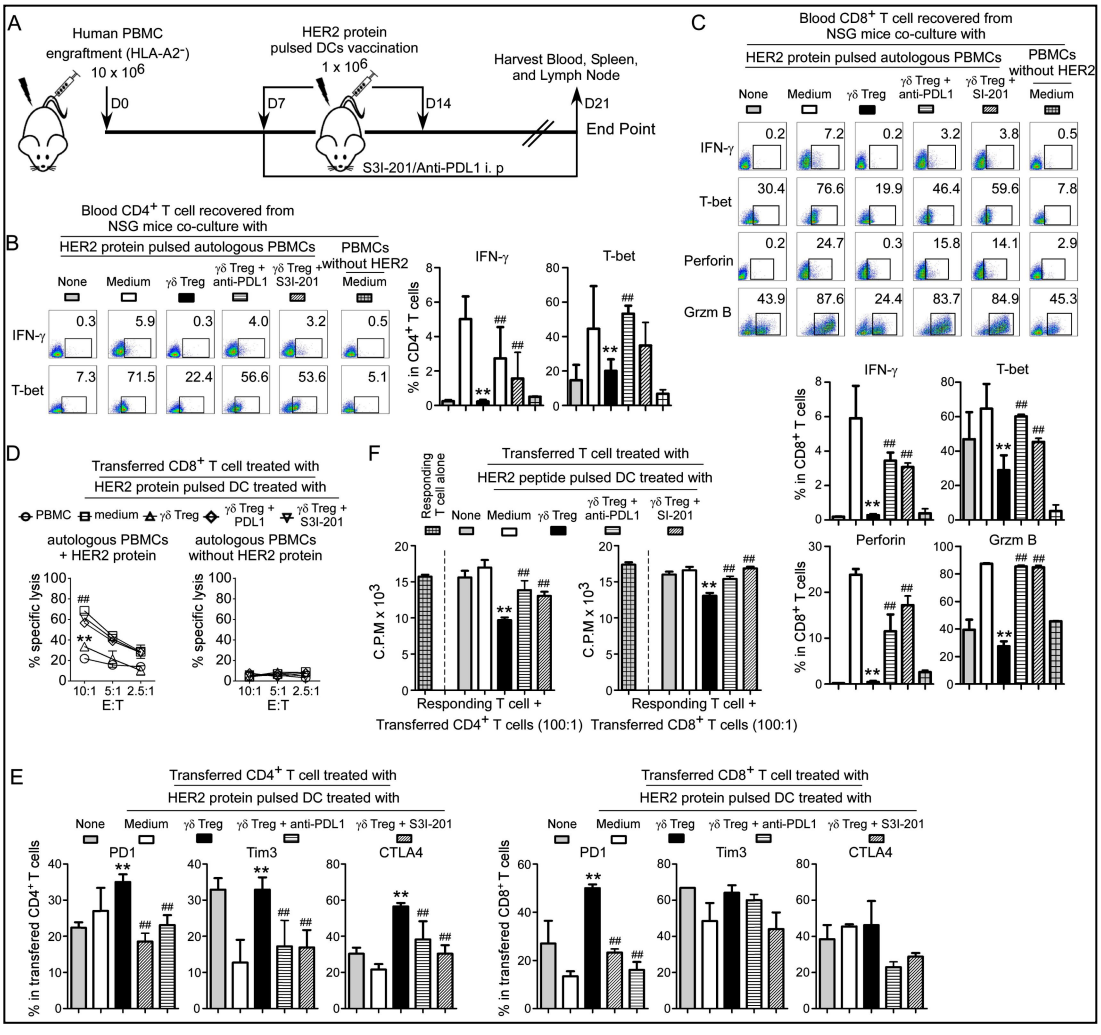

Fig. 7. Blockage of PD1/PDL1 and STAT3 signaling reversed the tolerogenic functions of Treg-induced senescent DCs to induce HER2 antigen-specific immune responses in vivo. (A) Schematic of the experimental workflow. NSG mice were engrafted with human PBMCs. Seven days and 14 days later, donor matched HER2 protein- pulsed mature DCs

or γδ Treg-induced senescent DCs (1 × 10^6 /mouse) were vaccinated through iv injection. Anti-PDL1 neutralization antibody and S3I-201 were injected intraperitoneally three times a week. After 15 days of immunization, $CD4^+$ and $CD8^+$ T cells from blood were purified for the functional assays. **(B)** and **(C)** Blockage of PDL1 or STAT3 signaling reversed the impaired functions of senescent DCs to activate HER2-specific T cells. $CD8^+$ CTLs purified from blood of vaccinated mice were co-cultured with CFSE-labeled autologous PBMCs loaded with the same 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) were determined by FACS. **<0.01, compared with the medium only group, and ##p<0.01, compared with DCs treated with γδ Treg cells. **(D)** Blockage of PD1/PDL1 or STAT3 signaling recovered impaired HER2-specific cytotoxicity of $CD8^+$ T cells purified from HER2 protein-pulsed senescent DC immunized mice. $CD8^+$ CTLs purified from blood of vaccinated mice were tested for their cytotoxic activity in a standard Calcein-AM release assay. Calcein-AM-labeled autologous PBMCs loaded with/without HER2 protein were used as targets. Target cells were incubated with effector T cells at various ratios at 37°C for 4 h. Supernatants were harvested and release of calcein was determined. **<0.01, compared with the medium only group, and ##p<0.01, compared with DCs treated with γδ Treg cells. **(E)** and **(F**) Blockage of PD1/PDL1 and STAT3 signaling reversed the T cell exhaustion and suppression mediated by HER2 protein pulsed Treg-induced senescent DC vaccination. Cell treatment and procedures were identical to B. PD1, Tim3, and CTLA4 expression in purified $CD4^+$ and $CD8^+$ T cells were determined by the flow cytometry (in E). Suppression of purified $CD4^+$ and $CD8^+$ T cells on the proliferation of other T cells was determined by [^{3}H] thymidine incorporation assays (in F). **<0.01, compared with the medium only group, and ## p<0.01, compared with DCs treated with γδ Treg cells.

C. Opportunities for training and professional
Nothing to Report.

D. Disseminated to communities of interest
Nothing to Report.

E. Plans for future studies in year 3: We will continue our efforts for the following studies: **1)** we will further identify potential mechanisms responsible for the changed metabolism and function in senescent DCs induced by breast cancer-derived γδ Treg cells *in vitro* and *in vivo* in animal models (Aim 2 and 3); and **2)** we will identify whether reprogramming of DC metabolism and functions can enhance anti-tumor immunity for breast tumor immunotherapy *in vivo* in animal models (Aim 3).

IMPACT

A. Impact on the development of the principal disciplines of the project
Nothing to Report.

B. Impact on other disciplines
Nothing to Report.

C. Impact on technology transfer
Nothing to Report.

D. Impact on society beyond science and technology
Nothing to Report.

CHANGES/PROBLEMS

A. Changes in approach
Nothing to Report.

B. Problems or delays
Nothing to Report.

C. Changes on expenditures
Nothing to Report.

D. Changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents
Nothing to Report.

PRODUCTS

Nothing to Report.

PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

A. Individuals have worked on the project

Collaborate with Dr. Ford as listed in the proposal.

B. The change in the active other support of the PD/PI(s) or senior/key personnel

Nothing to Report.

C. Involved other organizations as partners

Nothing to Report.

SPECIAL REPORTING REQUIREMENTS

Nothing to Report.

APPENDICES