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Tumor-Derived γδ Regulatory T Cells Suppress Innate and Adaptive Immunity through the Induction of Immunosenescence

Jian Ye,* Chunling Ma,*,† Eddy C. Hsueh,‡ Christopher S. Eickhoff,* Yanping Zhang,§ Mark A. Varvares,§ Daniel F. Hoft,*‡ and Guangyong Peng*§

Fundamentally understanding the suppressive mechanisms used by different subsets of tumor-infiltrating regulatory T (Treg) cells is critical for the development of effective strategies for antitumor immunotherapy. γδ Treg cells have recently been identified in human diseases including cancer. However, the suppressive mechanisms and functional regulations of this new subset of unconventional Treg cells are largely unknown. In the current studies, we explored the suppressive mechanism(s) used by breast tumor-derived γδ Treg cells on innate and adaptive immunity. We found that γδ Treg cells induced immunosenescence in the targeted naive and effector T cells, as well as dendritic cells (DCs). Furthermore, senescent T cells and DCs induced by γδ Treg cells had altered phenotypes and impaired functions and developed potent suppressive activities, further amplifying the immunosuppression mediated by γδ Treg cells. In addition, we demonstrated that manipulation of TLR8 signaling in γδ Treg cells can block γδ Treg-induced conversion of T cells and DCs into senescent cells in vitro and in vivo. Our studies identify the novel suppressive mechanism mediated by tumor-derived γδ Treg cells on innate and adaptive immunity, which should be critical for the development of strong and innovative approaches to reverse the tumor-suppressive microenvironment and improve effects of immunotherapy.

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Manipulating the immune system to recognize and eradicate cancer cells is an important and highly attractive alternative approach to treat patients with malignant tumors. However, the tumor-suppressive microenvironments created by tumor-associate regulatory T (Treg) cells are a major obstacle for effective antitumor immunity and successful tumor immunotherapy (1, 2). Recent studies have already identified several subsets of Treg cells, such as Tr1, Th3, and CD8+ Treg cells, in human cancers (3–5). Thus, a better understanding of the immunosuppressive mechanisms used by these tumor-derived Treg cells is critical for the success of immunotherapy for cancer.

Negative regulation mediated by conventional T cells in the tumor-suppressive microenvironment has been extensively studied. However, little is known about the potential roles of γδ T cells in antitumor immune responses. The negative regulation of γδ1 T cells in mouse models of induced mucosal tolerance, ocular tolerance, and self-tolerance has been well documented (6–9). Furthermore, studies from mouse tumor models have suggested that γδ T cells in the tumor microenvironment may be involved in the induction of tumor-specific immune tolerance (10–12). We discovered that enriched γδ1 Treg cell populations (7.2–75.7%; mean 33.2%) in the tumor-infiltrating lymphocytes (TILs) obtained from breast cancer patients can suppress naive and effector T cell responses and block the maturation and activities of dendritic cells (DCs) in vitro (13). We further showed that the high level of γδ T cells infiltrating in human breast cancer tissues was correlated with poor survival and high risk of relapse and could be used as a novel and independent prognostic factor in human breast cancer (14). These studies implicate the potential function of γδ Treg cells in the immunopathogenesis of human breast cancer. In addition, this new subset of Treg cells has also been identified in patients by more recent studies from other groups (15, 16).

Cellular senescence was initially described in human fibroblasts with limited passages in cell culture (17). There are two major categories of cellular senescence, as follows: replicative senescence, which occurs due to telomere shortening or dysfunction (18, 19), and premature senescence, which is induced by a variety of extrinsic forms of stress, such as oxidative stress, DNA damage, and activation of certain oncogenes (20–22). Recent studies suggest that replicative senescence also occurs within the human immune system. Accumulation of senescent CD8+ T cells has been found in persons during normal aging, younger persons with chronic viral infections, and patients with certain types of cancers (23–27). Furthermore, we more recently identified that naturally occurring human CD4+CD25+ Treg cells can induce responder T lymphocyte senescence (28). Senescent T cells develop significant phenotypic alterations, such as permanent loss of CD28 expression, cell cycle arrest, and upregulation of the cell cycle–related

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Abbreviations used in this article: DC, dendritic cell; LN, lymph node; PD-L1, program death ligand 1; PPD, purified protein derivative; SA-β-Gal, senescence-associated β-galactosidase; SP, spleen; TIL, tumor-infiltrating lymphocytes; Treg, regulatory T.

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genes p53, p21, and p16 (23, 28). In addition, senescent T cells have exhibited functional changes, including defective killing abilities and the development of potent negative regulatory functions (24, 27–31). However, the precise molecular mechanisms responsible for the induction of these senescent cells are still under investigation.

In the current studies, we further explored the suppressive mechanism(s) used by tumor-derived γδ Treg cells on innate and adaptive immunity. We found that γδ Treg cells can also induce both T cell and DC senescence, resulting in their impaired phenotypic and functional features. Importantly, these senescent T cells and DCs induced by γδ Treg cells became suppressive cells, further amplifying the immunosuppression mediated by Treg cells. In our efforts to identify the strategies to reverse γδ Treg cell suppression, we found that manipulation of TLR8 signaling in γδ Treg cells can block γδ Treg–induced conversion of T cells and DCs into senescent cells in vitro and in vivo in animal models. Our studies identify the novel suppressive mechanism mediated by tumor-derived γδ Treg cells on innate and adaptive immunity, which provides new insights relevant for the development of strong and innovative approaches for improved tumor immunotherapy.

**Materials and Methods**

**T cells and other cell lines**

 Buffy coats from healthy donors were obtained from the Gulf Coast Regional Blood Center (Houston, TX). These studies were approved by the Institutional Review Boards. PBMCs were purified from buffy coats using Ficoll-Paque. 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 >97%, as confirmed by flow cytometry. Human γδ Treg cells (primary or cell lines) were established from the primary breast cancer tissues in our laboratory and maintained in T cell medium containing 10% human AB serum and 50 U/ml IL-2 (13, 14).

**Senescence-associated β-galactosidase staining**

Senescence-associated β-galactosidase (SA-β-Gal) activity in senescent T cells was detected, as previously described (28, 32). Naive CD4+ T cells, CD8+ T cells, or DCs were labeled with CFSE (4.5 μM) and cocultured with or without γδ Treg or control T cells at different ratios of 10:1 to 1:1 in anti-CD3–coated 24-well plates for 3 or 5 d. In some experiments, naive T cells and DCs were cultured in T cell supernatants from γδ Treg or control T cells. Naive T cells or DCs were separated from cocultures using FACS sorting on γδ Treg-positive populations, and then washed in PBS (pH 7.2), fixed in 3% formaldehyde, and incubated overnight at 37˚C with freshly prepared SA-β-Gal staining solution (1 mg/ml X-gal, 5 mM K4Fe(CN)6, 5 mM K3Fe(CN)6, 2 mM MgCl2) in PBS at pH 6.0. The stained cells were washed with H2O and examined microscopically.

For some experiments, the cocultured naive T cells were determined for SA-β-Gal expression in the presence of the following TLR ligands: Pam3CSK4 (200 ng/ml), poly(I:C) (25 μg/ml), LPS (100 ng/ml), flagellin (10 μg/ml), loxoribine (500 μM), R837 (10 μg/ml), sRNA40 (10 μg/ml) (Invivogen, San Diego, CA), and oligonucleotides CpG-B (3 μg/ml), poly-T3 (3 μg/ml), and poly-G3 (3 μg/ml) (synthesized by Invitrogen, Carlsbad, CA).

**Cell cycle and apoptosis assays**

Naive CD4+ T cells were cocultured with CFSE-labeled γδ Treg cells in the presence of plate-bound anti-CD3 Ab (2 μg/ml). After 72 h of coculture, apoptotic naive CD4+ T cells was analyzed after staining with PE-labeled annexin V and 7-aminoactinomycin D (BD Biosciences, San Diego, CA) gating on CFSE-negative cell populations. For cell cycle analysis, cocultured naive T cells were fixed with 70% ethanol overnight, washed with PBS, and incubated with propidium iodide (10 μg/ml) and RNase A (100 μg/ml). Naive CD4+ or CD8+ T cells cocultured with or without the CD4-C1 effector T cells served as controls. All the stained cells were analyzed on a FACSCalibur (BD Biosciences), and data were analyzed with FlowJo software (Tree Star).

**Flow cytometry analysis**

The expression markers on T cells and DCs were determined by FACS analysis after surface or intracellular staining with anti-human–specific Abs conjugated with either PE or FITC. These human Abs included the following: anti-CD4, anti-CD8, anti-CD27, anti-CD28, anti-CD80, anti-CD83, anti-CD86, anti–HLA-DR, and anti–program death death ligand 1 (PD-L1), which were purchased from BD Biosciences. All stained cells were analyzed on a FACSCalibur flow cytometer (BD Biosciences), and data were analyzed with FlowJo software (Tree Star).

**Induction, phenotype, and suppressive function of senescent DCs**

Immature and mature DCs were derived from the monocytes of healthy donors in culture with IL-4 (100 ng/ml) and GM-CSF (100 ng/ml) with or without TNF-α (15 ng/ml) (R&D Systems). The immature or mature DCs were treated with γδ T cells, as we described previously (13). In brief, 5 × 105 γδ T cells or control CD4-C1 effector T cells were cocultured with 1 × 106 immature DCs in 24-well plates in medium containing IL-4 and GM-CSF with TNF-α. After 48 h, the treated and untreated DCs were purified and divided into different groups. For senescence induction assay, SA-β-Gal staining was performed on DCs. For phenotypic analysis, the surface markers of CD83, CD80, CD86, HLA-DR, and PD-L1 of DCs were analyzed by flow cytometry. For cytokine secretion detection, the secrete cytokine was determined by ELISA kits (R&D Systems). For cytokine secretion detection, the cytokines Cytokine secretion detection, the cytokines were measured by ELISA kits (R&D Systems). The stimulatory and suppressive activities of normal and senescent DCs on the responding T cell proliferation were determined using [3H]thymidine incorporation assays.

**Ag presentation function of DCs**

The capacity of DCs to process and present Mycobacterium tuberculosis purified protein derivative (PPD; Statens Serum Institute) to T cells was determined. Immature and mature DCs were derived from the monocytes of healthy donors in culture with IL-4 (100 ng/ml) and GM-CSF (100 ng/ml) with or without TNF-α (15 ng/ml) (R&D Systems). Immature DCs were treated with medium, γδ T cells, or CD4-C1 for 2 d, and then purified and pulsed with 20 μg/ml PPD for 90 min. PPD-pulsed DCs (1 × 105) were cultured with autologous CD4+ T cells (1 × 105) for 24 h in the presence of 50 μM IL-2 (at d 7, PPD-pulsed DCs were added into cocultures again). PPD-specific IFN-γ–producing CD4+ T cells were determined by flow cytometry after restimulation with PPD-pulsed autologous mature DCs at a ratio of 10:1.

**In vivo studies**

Rag1−/− mice (lacking T and B cells) were originally purchased from National Cancer Institute and maintained in the institutional animal facility. All animal studies have been approved by the Institutional Animal Care Committee. Naive CD4+ T cells (5 × 106/mouse), γδ Treg cells (2 × 107/mouse), and CD8-C1 effector T cells (2 × 107/mouse) were pre-activated with anti-CD3 (2 μg/ml) and adoptively cotransferred into Rag1−/− mice through i.v. injection into the following groups: naive CD4+ T cells alone, naive CD4+ T cells plus γδ Treg cells, or naive CD4+ T cells plus CD8-C1 effector T cells. Five to 10 mice were included in each group.
in a parallel experiment, γδ Treg cells were pretreated with TLR8 ligand (poly-G3, 3 μg/ml) or control poly-T3 (3 μg/ml) for 48 h before injection. Blood, lymph nodes (LN), and spleens (SP) were harvested at 12 d postinjection, and mononuclear cells were purified over Ficoll. The transferred human CD4+ and CD8+ T cells were isolated by FACS sorting for subsequent phenotypic and functional analyses in vitro. SA-β-Gal staining and [3H]thymidine incorporation assays were performed, as described above.

Statistical analysis
Unless indicated otherwise, data are expressed as mean ± SD. The significance of difference between groups was determined by a two-tailed Student t test or the one-way ANOVA.

Results
Tumor-derived γδ Treg cells induce cell cycle arrest in responder T cells
We previously demonstrated that high percentages of γδ Treg cells exist in the TILs of breast cancer patients (13, 14). To further explore the suppressive mechanisms used by tumor-derived γδ Treg cells (13, 14, 33), we first investigated the suppressive capacity of γδ Treg cells using functional proliferation assays. We observed that tumor-derived γδ Treg cells strongly inhibited the proliferation of naive CD4+ and CD8+ T cells in the presence of anti-CD3 Ab using [3H]thymidine incorporation assays, whereas control CD4-C1 effector cells, a Th1 cell line, increased the proliferation of naive T cells (Fig. 1A). Recent studies have shown that CD4+ Treg cells suppress naive T cell proliferation through the induction of apoptosis or cytolysis in the Treg-treated cells (35). We then tested whether tumor-derived γδ Treg cells use the same mechanisms to inhibit T cell proliferation. Apoptosis and cell death in naive CD4+ T cell populations cocultured with γδ Treg cells were measured. As shown in Fig. 1B, naive CD4+ T cells in medium alone or treated with control CD4-C1 T cells contained 14–17% apoptotic T cells after stimulation with anti-CD3 Ab. However, γδ Treg cells did not induce increased apoptosis or cell death in CD4+ T cells, indicating that suppression in responder T cells induced by human γδ Treg cells is not through the induction of cell apoptosis and cytolysis. In parallel, we studied the cell cycle distribution of the naive CD4+ T cells treated with γδ Treg cells. We found that 55–60% of anti-CD3–activated naive CD4+ T cells remained in G0/G1 in the medium alone and the control CD4-C1 treatment groups. In contrast, >85% of naive CD4+ T cells treated with γδ Treg cells remained in G0/G1, indicating that γδ Treg cell treatment promotes the accumulation of naive T cells in cell cycle arrest (Fig. 1C). We thus determined whether cell cycle regulatory molecules p53, p21, and p16 are involved in γδ Treg–induced cell cycle arrest in responder T cells. As expected, significantly increased p53, p21, and p16 expressions were observed in naive CD4+ T cells after treatment with γδ Treg cells (Fig. 1D). These data clearly suggest that γδ Treg cells induce cell cycle arrest, but not apoptosis or cytolysis of responder T cells.

FIGURE 1. The suppression of responder T cells mediated by human tumor-derived γδ Treg cells is due to the induction of cell G0/G1 cycle arrest. (A) Suppression of naive T cell proliferation by γδ Treg cells. CD4-C1 effector T cells served as a negative control displaying no suppressive activity. Naive CD4+ or CD8+ T cells were cocultured with γδ Treg or control T cells at a ratio of 10:1. The proliferation of naive T cells in the presence of anti-CD3 Ab was determined by [3H]thymidine incorporation assays. (B) The suppression of naive CD4+ T cell proliferation mediated by γδ Treg cells is not due to the induction of apoptosis. (C) γδ Treg cells promoted the accumulation of naive CD4+ T cells in G0/G1 cell cycle arrest. Naive CD4+ T cells were cocultured with CFSE-labeled γδ Treg or CD4-C1 cells in the presence of plate-bound anti-CD3 Ab. Apoptosis in naive CD4+ T cells was analyzed after staining with PE-labeled annexin V and 7-aminoactinomycin D gating on CFSE-negative cell populations [in (B)]. Cell cycle distribution in naive CD4+ T cells was analyzed after incubation with 10 μg/ml propidium iodide and 100 μg/ml RNase A [in (C)]. Naive CD4+ T cells cocultured with or without CD4-C1 T cells served as controls. (D) Cell cycle regulatory molecules p21, p16, and p53 were involved in human γδ Treg–induced T cell senescence. Cell treatment and procedure were the same as in (B) and (C). Cocultured naive CD4+ T cells were purified by FACS, and then lysates were prepared for Western blot analyses. Data shown in (A)–(D) are representative of three independent experiments with similar results.
γδ Treg cells suppress naive and effector T cells through the induction of T cell senescence

We have recently shown that human naturally occurring CD4+ CD25+ Treg cells can induce responder T lymphocyte senescence (28). Given that our current studies showed that γδ Treg cell treatment resulted in the accumulation of naive T cells in G0/G1 cell cycle phases as well as cell cycle arrest, we reasoned that γδ Treg cells performing their suppressive function on naive T cells may use the same suppressive mechanism as that of CD4+CD25+ Treg cells. To test the possibility that γδ Treg cells may also induce responder T cell senescence, naive CD4+ and CD8+ T cells were labeled with CFSE and then cocultured with γδ Treg cells or control CD4-C1 effector T cells at different ratios in the presence of plate-bound anti-CD3 Ab (2 μg/ml) for different days. Cocultured naive T cells were then purified by FACS, and SA-β-Gal expression, the first biomarker used to identify senescent human cells, was determined (28, 32). As shown in Fig. 2A, naive CD4+ and CD8+ T cells treated with γδ Treg cells significantly induced cell senescence, resulting in SA-β-Gal expression. However, naive CD4+ and CD8+ T cells cultured in medium only or cocultured with CD4-C1 effector T cells did not induce SA-β-Gal expression. Furthermore, we observed that the percentages of the SA-β-Gal–

![Image of Fig. 2](http://www.jimmunol.org/)

**FIGURE 2.** Human γδ Treg cells induce senescence in naive CD4+ and CD8+ T cells. (A) γδ Treg cell treatment significantly increased SA-β-Gal–positive T cell populations in naive CD4+ and CD8+ T cells. Naive CD4+ or CD8+ T cells cultured in medium only or cocultured with CD4-C1 effector T cells had little or no SA-β-Gal expression. CFSE-labeled naive CD4+ or CD8+ T cells were incubated alone or cocultured with γδ Treg or CD4-C1 T cells at a ratio of 5:1 in the presence of plate-bound anti-CD3 (2 μg/ml) for 5 d. The treated naive CD4+ or CD8+ T cells were purified by FACs, and SA-β-Gal expression was determined. The SA-β-Gal–positive T cells were identified with dark blue granules, as indicated by the arrows. (B) Decreased expression of CD27 and CD28 in naive CD4+ and CD8+ T cells treated with γδ Treg cells. Cell treatment and procedure were the same as in (A). CD27 and CD28 expression in treated naive CD4+ and CD8+ T cells was analyzed by FACS gating on CFSE-positive populations. (C) Suppressive function of Treg-induced senescent T cells. Both senescent CD4+ and CD8+ T cells induced by γδ Treg cells strongly inhibited the proliferation of responding CD4+ T cells. In contrast, naive T cells treated or untreated with control CD4-C1 effector T cells did not affect the proliferation of responding CD4+ T cells. Cell treatment and procedure were the same as in (A). Treated CD4+ and CD8+ T cells were purified, and the suppressive activities on CD4+ T cell proliferation were evaluated using [3H]thymidine incorporation assays. (D) Senescent CD8+ T cells induced by γδ Treg cells dominantly existed in the CD8+CD28− cell populations. Cell treatment and procedure were the same as in (A). Treg-treated CD8+ T cells expressing CD28 were determined and sorted by FACs. SA-β-Gal expression in CD8+CD28− and CD8+CD28+ cell populations purified from Treg-treated CD8+ T cells was determined. Results shown in the right panel are mean ± SD from three independent experiments. (E) Suppressive function of CD8+CD28− and CD8+CD28+ cell populations purified from γδ Treg–treated CD8+ T cells. CD8+CD28− cell population strongly inhibited the proliferation of responding CD4+ T cells. In contrast, CD8+CD28+ cell population had minor suppressive activity on the proliferation of responding CD4+ T cells. Naive CD4+ T cells were cocultured with purified CD8+CD28− or CD8+CD28+ cell populations for 3 d. The suppressive activities of these two populations on CD4+ T cell proliferation were evaluated using [3H]thymidine incorporation assays. Results shown in (C)–(E) are mean ± SD from three independent experiments with similar results.
positive cell populations in naïve CD4+ and CD8+ T cells dramatically increased with longer times of coculture with γδ Treg cells (Supplemental Fig. 1). Accumulating evidence suggests that permanent loss of CD28 expression is the most consistent biological indicator of aging in senescent T cells in elderly people and in patients with chronic viral infections (23, 29). Thus, we examined whether human γδ Treg–induced senescent T cells can also downregulate the expression of the costimulatory molecules CD27 and CD28. We found that naïve CD4+ and CD8+ T cells cocultured with medium only or with CD4-C1 T cells expressed high levels of CD27 and CD28 costimulatory molecules. In contrast, naïve CD4+ and CD8+ T cells cocultured with γδ Treg cells dramatically downregulated costimulatory molecule CD27 and CD28 expression (Fig. 2B). In addition, we observed that freshly purified γδ T cells from breast tumor tissues also significantly increased SA-β-Gal–positive T cell populations and decreased CD27 and CD28 expression in the cocultured naïve CD4+ T cells (Supplemental Fig. 2A, 2B). To further identify the suppressive mechanisms mediated by tumor-derived γδ Treg cells, we found that the suppressive activity of tumor-derived γδ1 Treg cells relied on unknown soluble factors using Transwell assays (data not shown). In addition, culture supernatants from γδ1 Treg cells strongly inhibited the proliferation of naïve CD4+ T cells and induced responder T cell senescence expressing SA-β-Gal (Supplemental Fig. 2C, 2D). These results collectively suggest that both human naturally occurring CD4+CD25+ Treg cells and γδ Treg cells use the same mechanism to suppress naïve T cell proliferation through the induction of T cell senescence.

Because senescent T cells have exhibited functional changes, such as defective killing abilities and the development of potent negative regulatory functions (24, 27–31), we next investigated whether human γδ Treg–induced senescent T cells also have negative regulatory functions. We first evaluated cytokine profiles elaborated by the γδ Treg–induced senescent T cells stimulated with anti-CD3 Ab using an ELISA. Naïve CD4+ and CD8+ T cells treated with or without CD4-C1 effector T cells were included as controls. Both senescent CD4+ and CD8+ T cells secreted large amounts of proinflammatory cytokines IL-6, IFN-γ, and TNF-α, but not other cytokines, including IL-1β, IL-2, or IL-4. Furthermore, these Treg–induced senescent T cells secreted large amounts of IL-10 and moderate amounts of TGF-β1, whereas naïve CD4+ T cells treated with control CD4-C1 T cells did not secrete any IL-10 or TGF-β1 (Supplemental Fig. 3), suggesting that these senescent T cells may have a negative regulatory function. We then evaluated the suppressive activities of the senescent T cells on the proliferation of additional responding naïve CD4+ T cells. We found that both senescent CD4+ and CD8+ T cells induced by tumor-derived γδ Treg cells strongly inhibited the proliferation of responding CD4+ T cells. In contrast, naïve CD4+ and CD8+ T cells treated with or without control CD4-C1 T cells had no suppressive effects on the proliferation of responding CD4+ T cells (Fig. 2C). These results were consistent with our recent findings in the senescent T cells induced by CD4+CD25+ naturally occurring Treg cells (28), and further suggest that γδ Treg cells by inducing cell senescence in responder T cells can convert them into suppressive T cells. In addition, we purified CD28+ and CD28– populations from the γδ Treg–treated CD8+ T cells by FACS, and observed that senescent T cells (SA-β-Gal+) were mainly derived from CD28– responder T cell populations (Fig. 2D). Interestingly, we also observed that only the CD8+CD28– populations, but not

![FIGURE 3. Human γδ Treg cells induce senescence in Th1 and Th17 cells. (A) Suppression of Th1 and Th17 cell proliferation by γδ Treg cells. CD4-C1 effector T cells displayed no suppressive activity and served as a negative control. Th1 or Th17 cells established from TILs were cocultured with γδ Treg or control T cells at a ratio of 10:1. The proliferation of Th1 or Th17 cells in the presence of anti-CD3 Ab was determined by [3H]thymidine incorporation assays. (B) and (C) γδ Treg cell treatment significantly increased SA-β-Gal–positive T cell populations in Th1 or Th17 cells. Th1 or Th17 cells cultured in medium only or cocultured with CD4-C1 effector T cells had little or no SA-β-Gal expression. CFSE-labeled Th1 or Th17 cells were incubated alone or cocultured with γδ Treg or CD4-C1 T cells at a ratio of 5:1 in the presence of plate-bound anti-CD3 (2 μg/ml) for 3 or 5 d. The treated Th1 or Th17 cells were purified by FACS and stained with SA-β-Gal staining reagents after an additional 3-d culture. The SA-β-Gal–positive T cells (5-d coculture) were identified with dark blue granules, as the arrows indicate in (B). (D) Decreased expression of CD27 and CD28 in Th1 and Th17 cells treated by γδ Treg cells. Cell treatment and procedure were the same as in (B). CD28 and CD28 expression in untreated Th1 and Th17 cells (5-d treatment) was analyzed by FACS.](http://www.jimmunol.org/)

CD8^+ CD28^+ populations, had potent suppressive effects on responding CD4^+ T cell proliferation (Fig. 2E). Collectively, these studies further confirm that tumor-derived γδ Treg cells use the induction of T cell senescence as the novel suppressive mechanism to inhibit T cell proliferation.

We next determined whether γδ Treg cells can also inhibit the proliferation of Th1 and Th17 effector T cell subsets, utilizing this novel suppressive mechanism of senescence induction. We included tumor-infiltrating Th1 and Th17 cell lines derived from breast cancer patients, as representative human Th1 and Th17 cells for our studies (36). We observed that γδ Treg cells also strongly inhibited the proliferation of Th1 and Th17 effector T cells (Fig. 3A). Furthermore, Th1 and Th17 cells cocultured with γδ Treg cells dramatically upregulated SA-β-Gal expression and markedly downregulated CD27 and CD28 costimulatory molecule expression (Fig. 3B–D). These results clearly indicate that the suppression of both naïve and effector T cells mediated by tumor-derived γδ Treg cells is due to the same suppressive mechanism that induces responder T cell senescence.

**γδ Treg cells induce senescence in DCs, resulting in their impaired phenotypic and functional features**

Our previous studies have shown that breast tumor–derived γδ Treg cells can block the maturation and function of DCs (13). We next investigated whether γδ Treg cells also used this novel suppressive mechanism of senescence induction to inhibit DC functions. Immature DCs were cocultured with γδ Treg cells or control CD4-C1 effector T cells for 48 h in medium containing IL-4, GM-CSF, and TNF-α. Treated and untreated DCs were purified, and SA-β-Gal expression was determined. Surprisingly, significantly increased senescent DC populations (~40%) were observed after coculture with γδ Treg cells, whereas coculture with CD4-C1 effector T cells or medium only induced minor SA-β-Gal expression in DCs (Fig. 4A). In addition, we demonstrated that both freshly purified γδ T cells from breast tumor tissues and cell culture supernatants markedly upregulated SA-β-Gal expression on DCs (Supplemental Fig. 4A, 4B). We further investigated whether senescent DCs induced by γδ Treg cells changed their maturation and functional markers. We observed that γδ Treg cell treatment not only induced senescence, but also dramatically downregulated CD83 expression in the treated DCs, suggesting that senescent DCs were deficient in maturation (Fig. 4B). In addition, senescent DCs induced by γδ Treg cells markedly downregulated costimulatory molecules CD80, CD86, and HLA-DR expression, consistent with our previous finding (13). Notably, we found that γδ Treg–induced senescent DCs significantly upregulated PD-L1 expression, a critical inhibitor molecule for the induction and maintenance of immune suppression (Fig. 4B) (37). However, CD4-C1 effector T cell treatment did not affect DC maturation and costimulatory molecule expression (Fig. 4B). These results suggest that γδ Treg–induced senescent DCs have a distinct deficiency in their maturation and costimulatory func-

**FIGURE 4.** Human γδ Treg cells induce DC senescence. (A) γδ Treg cell treatment markedly upregulated SA-β-Gal expression on DCs. DCs cultured in medium only or cocultured with CD4-C1 effector T cells had little SA-β-Gal expression. Immature DCs were incubated alone or cocultured with γδ Treg or CD4-C1 T cells at a ratio of 5:1 in the presence of GM-CSF, IL-4, and TNF-α for 2 d. The treated DCs were purified, and SA-β-Gal expression was determined. 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 expression of CD83, CD80, CD86, and HLA-DR and upregulation of PD-L1 in DCs treated with γδ Treg cells. Cell treatment and procedure were the same as in (A). All these markers were analyzed by FACS. (C) Senescent DCs induced by γδ Treg cells dominantly existed in the CD80^low^ cell populations. Furthermore, CD80^low^ cell populations purified from Treg-treated DCs showed increased PD-L1 expression. Cell treatment and procedure were the same as in (A). γδ Treg–treated DCs expressing CD80 were sorted by FACS. SA-β-Gal and PD-L1 expression in CD80^high^ and CD80^low^ cell populations purified from γδ Treg–treated DCs was determined. Results shown in the right panel are mean ± SD from three independent experiments. **p < 0.01 compared with the CD80^high^ group.
tions. Given that permanent loss of CD28 expression is the most consistent biological indicator of senescent T cells (23, 28, 29), and that senescent T cells (SA-β-Gal) induced by Treg cells were mainly derived from CD28 responder T cell populations (Fig. 2D), we thus reasoned that loss of costimulatory molecules, such as CD80 and CD86, might also be used as a biomarker for the senescent DCs induced by γδ Treg cells. CD80low and CD80high populations in the γδ Treg–treated DCs were purified by FACS. As expected, >80% of DCs in CD80low populations were SA-β-Gal+ DCs, whereas CD80high DCs only had a minor senescent population, similar to those in CD4-C1–treated DCs. Furthermore, CD80low DCs also highly expressed PD-L1 (Fig. 4C). In addition, similar results as shown in CD80low DCs were found in CD86low DCs treated with γδ Treg cells (data not shown). These data suggested that γδ Treg–induced senescent DCs were mainly derived from CD80low and CD86low populations, and that the loss of costimulatory molecules CD80 and CD86 was also a significant biomarker for the senescent DCs.

We then evaluated cytokine profiles elaborated by the γδ Treg–induced senescent DCs after stimulation with or without LPS. The untreated mature DCs secreted large amounts of proinflammatory cytokines IL-1β, IL-6, IL-12, and TNF-α. However, senescent DCs induced by γδ Treg cells dramatically decreased the release of these cytokines, whereas coculture with control CD4-C1 T cells did not affect the cytokine secretion by DCs (Fig. 5A). Furthermore, γδ Treg treatment did not promote the secretion of suppressive cytokines IL-10 and TGF-β by DCs (data not shown). In addition to the identification of altered phenotypes and cytokine profile in Treg-induced senescent DCs, we also investigated whether senescent DCs induced by γδ Treg cells had impaired functions. We first tested whether γδ Treg–induced senescent DCs have a deficiency in the ability to stimulate the proliferation of naive T cells. As shown in Fig. 5B and Supplemental Fig. 4C, senescent DCs induced by γδ Treg cells cannot stimulate both autologous and allogeneic naive CD4+ T cell proliferation. In contrast, DCs treated with or without CD4-C1 effector T cells strongly stimulated naive CD4+ T cell proliferation. We then determined whether senescent DCs induced by γδ Treg cells also had negative regulatory function. We evaluated the suppressive activity of senescent DCs on the proliferation of responding CD4+ T cells stimulated by anti-CD3 Ab. We found that senescent DCs strongly inhibited the proliferation of responding CD4+ T cells. In contrast, mature DCs induced an increased proliferation of responding CD4+ T cells (Fig. 5C). Furthermore, we found that the
CD80\textsuperscript{low} DC population in γδ Treg–treated DCs had more potent suppressive activity, whereas CD80\textsuperscript{high} population had no suppressive effect on the proliferation of responding T cells (Fig. 5D). In addition, we showed that the suppressive activity of these senescent DCs induced by γδ Treg cells was mediated through soluble factor(s) using Transwell assays (Supplemental Fig. 4D). Taken together, our results indicate that human tumor–derived γδ Treg cells not only induce DCs into senescent DCs that have immature phenotypes, suppressed secretion of proinflammatory cytokines, and impaired functions, but also convert them into suppressive DCs.

**Senescent DCs induced by γδ Treg cells have impaired APC function to process and present Ag to T cells**

Given that senescent DCs induced by γδ Treg cells have impaired functions to stimulate T cell proliferation, we next determined whether those senescent DCs still have the capacity to process and present a true Ag and induce Ag-specific T cell immune responses. To address this critical question, we used *M. tuberculosis* PPD as a model Ag. CD4\textsuperscript{+} T cells purified from two healthy donors were cocultured with PPD-pulsed autologous DCs, which were pretreated with medium, CD4-C1 T, or γδ Treg cells. As shown in Fig. 6A, γδ Treg–induced senescent DCs pulsed with PPD had a weak ability to stimulate autologous T cell proliferation. However, PPD-pulsed DCs pretreated with or without CD4-C1 effector T cells strongly stimulated autologous T cell proliferation, resulting in 40- to 60-fold number increases. In addition, PPD-pulsed DCs pretreated with or without CD4-C1 T cells dramatically induced the increases of PPD-specific IFN-γ–producing T cell populations in the cocultured T cells. In contrast, γδ Treg–induced senescent DCs as APCs only induced minor levels of PPD-specific IFN-γ–producing T cells (Fig. 6B). These results suggested that senescent DCs induced by γδ Treg cells lost the capacity to process and present an Ag to T cells and induce Ag-specific T cell proliferation.

**TLR8 signaling reverses γδ Treg cell–induced senescence in responder T cells and DCs**

We have previously demonstrated that TLR8 signaling reversed suppressive functions mediated by different subsets of human Treg cells, including CD4\textsuperscript{+}, CD8\textsuperscript{+}, and γδ Treg cells (4, 13, 33). We further showed that TLR8 signaling can also prevent the induction of T cell senescence mediated by naturally occurring CD4\textsuperscript{+} Treg cells (28). We next tested whether TLR8 signaling can also reverse the process of γδ Treg–induced T cell senescence. We cocultured naive CD4\textsuperscript{+} T cells with γδ Treg cells in the presence or absence of a panel of TLR ligands and control poly-T3, and tested for their ability to block the induction of T cell senescence. These TLR ligands included the following: Pam\textsubscript{3}CSK\textsubscript{4} (TLR2), poly(I:C) (TLR3), LPS (TLR4), flagellin (TLR5), loxoribine (TLR7), imiquimod-R837 (TLR7), ssRNA40 (TLR8), poly-G3 (TLR8), and CpG-B (TLR9) oligonucleotides. We found that only the TLR8 ligands poly-G3 and ssRNA40 significantly blocked the induction of responder T cell senescence induced by γδ Treg cells identified by SA-β-Gal expression (Fig. 7A). To exclude the possibility that TLR8 ligands prevented γδ Treg–induced senescence through direct effects on responder T cells, we pretreated γδ Treg cells or responder naive CD4\textsuperscript{+} T cells with TLR ligands. After extensive washes, these pretreated cells were then cocultured with untreated naive CD4\textsuperscript{+} T cells or Treg cells, respectively, and senescent T cell populations in cocultured naive CD4\textsuperscript{+} T cells were determined. We observed that pretreatment of γδ Treg cells with poly-G3...
drastically decreased the senescence induction in naive CD4+ T cell populations, whereas pretreatment of naive CD4+ T cells with TLR8 ligand poly-G3 did not prevent Treg-induced T cell senescence (Fig. 7B). Furthermore, we also tested whether TLR8 signaling can prevent the loss of costimulatory molecules, CD27 and CD28, in naive CD4+ T cells induced by γδ Treg cells. As expected, we found that the expression of CD27 and CD28 in naive CD4+ T cells treated with TLR8 ligands for 2 d, followed by coculture with untreated γδ Treg cells or naive CD4+ T cells, respectively, for 5 d. The numbers of SA-β-Gal-positive cells in treated CD4+ T cells were then determined. **p < 0.01 compared with the group not treated with TLR8 ligand. (C) Poly-G3 restored the expression of CD27 and CD28 in naive CD4+ T cells induced by human γδ Treg cells. Naive CD4+ T cells were cultured with γδ Treg at a ratio of 1:1 in the presence of poly-G3 or control poly-T3 for 5 d. Treated naive CD4+ T cells were separated, and CD27 or CD28 expression was analyzed by FACS.

**Prevention of T cell senescence induced by γδ Treg cells in vivo via TLR8 signaling**

We next investigated whether tumor-derived γδ Treg cells can convert naive T cells into senescent T cells with potent suppressive activity in vivo (13, 28, 33). Naive CD4+ T cells, γδ Treg cells, and CD8-C1 effector T cells (a control) were preactivated with anti-CD3 Ab and adoptively cotransferred into Rag1−/− mice in different combinations, including preactivated naive CD4+ T cells alone, preactivated CD4+ T cells plus γδ Treg cells, and preactivated CD4+ T cells plus CD8-C1 effector T cells. Transferred human CD4+ T cells were isolated from blood, LN, and SP in Rag1−/− mice to determine their senescence and suppressive activity. As shown in Fig. 8A, ~10–15% of adoptively transferred preactivated CD4+ T cells became senescent T cells in Rag1−/− mice at 12 d postinjection. However, significantly increased senescent T cell populations were induced in preactivated CD4+ T cells when cotransferred with γδ Treg cells (40%). In contrast, cotransfer with CD8-C1 T cells did not promote preactivated T cell senescence. These results clearly indicate that human γδ Treg cells can induce responder T cell senescence in vivo. We then determined the suppressive activity of the recovered CD4+ T cells on the proliferation of responding T cells using [3H]thymidine incorporation assays (28). As expected, we found that the purified CD4+ T cells from different organs previously cotransferred with γδ Treg cells potentely suppressed the proliferation of responding CD4+ T cells. However, purified CD4+ T cells previously cotransferred with or without control CD8-C1 effector T cells did not have any suppressive activity (Fig. 8B).

Because we have shown that TLR8 signaling in Treg cells can control Treg-induced senescence in vitro, we next investigated whether we can prevent the induction of senescent T cells mediated by γδ Treg cells in vivo by the manipulation of TLR8 signaling in...
this adoptive transfer model. γδ Treg cells were pretreated with TLR8 ligand (poly-G3) or control poly-T3 for 24 h. The treated or untreated γδ Treg cells were co-transfered with anti-CD3-pre-activated naive CD4+ T cells into Rag1−/− mice following the same procedures described as above. The senescent cell populations and suppressive activity of the recovered CD4+ T cells were then investigated at 12 d postinjection. As shown in Fig. 8C, pretreatment of γδ Treg cells with poly-G3 significantly blocked the induction of senescence in the transferred CD4+ T cells. γδ Treg cells were pretreated with poly-G3 or poly-T3 (control) for 2 d before co-transfer. The transferred human CD4+ T cells in different organs were isolated at 12 d postinjection for subsequent SA-β-Gal staining (C) and [3H]thymidine incorporation assays (D). *p < 0.05, **p < 0.01 compared with the medium-only and poly-T3 groups.

Discussion
Immunosuppressive microenvironments induced by different types of Treg cells present major barriers to successful antitumor immunotherapy. It is now widely acknowledged that the success of immunotherapy against cancer ultimately may depend on how well we understand the immunosuppressive mechanisms used by Treg cells, and whether we can modulate Treg function in the tumor microenvironment (1, 2). We recently discovered high percentages of γδ1 Treg cells existing in the human breast tumor microenvironment that suppressed CD4+ and CD8+ T cells and blocked the maturation and activity of DCs (13). In the current studies, we further explored the suppressive mechanisms used by tumor-derived γδ Treg cells on innate and adaptive immunity. We identified that human γδ Treg cells strongly suppressed naive and effecter T cell proliferation, as well as impaired DC functions through the induction of senescence in responder immune cells. In addition, we demonstrated that manipulation of TLR8 signaling in γδ Treg cells can prevent γδ Treg–induced conversion of T cells and DCs into senescent cells in vitro and in vivo. We have recently identified that human naturally occurring Treg cells also induce targeted T cell senescence (28). Therefore, our studies strongly suggest that although different types of Treg cells inhibit immune responses using different suppressive mediators at different levels, they may direct a similar fate in suppressed responder T cells. These studies should be critical for the development of strong and innovative approaches for improved tumor immunotherapy.

γδ T cells serve not only as sentinels in the innate immune system, but also act as a bridge between innate and adaptive immune responses, performing multiple functions (38–40). The roles of human Vγ9Vδ2 T cells in mediating immunity against microbial pathogens and tumors have been well described (38, 41). Several clinical trials focusing on the activation of Vγ9Vδ2 T cells as a cancer treatment in patients with renal cell carcinoma, non-Hodgkin lymphoma, or multiple myeloma and prostate cancer have shown promising results (42–44). Besides the important roles of γδ T cells in antimicrobial and antitumor immunity, recent studies in mice and humans suggested that γδ T cells may also have negative regulatory functions. The negative regulation of γδ T cells in mouse models of induced mucosal tolerance, ocular tolerance, and self-tolerance, as well as prevention of food allergy, has been well documented (6–9, 45). Furthermore, the immunomodulation role of IELs in epithelia tissues has been established (6, 46). In addition, studies from mouse tumor models have suggested that γδ T cells in the tumor microenvironment may be involved in the induction of tumor-specific immune tolerance (10–12). A high frequency of γδ1 T cells has been shown among TILs or circulating PBMCs from cancer patients (47, 48). However, little is known about negative regulatory function of these γδ T cells in antitumor immunity in cancer patients. We recently analyzed cell populations in TILs isolated from human breast tumors and identified high percentages of γδ1 Treg cells with
potent suppressive function existing in the tumor microenvironment (13). We further explored the potential functions of γδ Treg cells in the immunopathogenesis of human breast cancer. We observed that patients with a high proportion of γδ T cells have advanced cancer stages and high LN metastasis. Importantly, high numbers of γδ T cells in breast cancer tissues identified poor survival rate and high risk of relapse patients (14). Dissecting the functional role of different subsets of regulatory TILs in the tumor-suppressive microenvironment is critical for the development of effective strategies for antitumor immunotherapy.

Although significant progress has been made in delineating the molecules and mechanisms that Treg cells use to mediate suppression, the majority of these studies and mechanisms are performed and obtained from conventional Treg cells. However, the suppressive mechanisms induced by γδ Treg cells are still unclear. Recent studies suggested that the possible suppressive mechanisms used by mouse γδ T cells were through Fas and Fas ligand pathway, and/or secretion of suppressive cytokines TGF-β and IL-10 (7, 46). In our efforts to identify the suppressive mechanisms of human breast tumor–derived γδ Treg cells, we found that the suppressive effects mediated by human γδ Treg cells were through unknown soluble factors (data not shown). Furthermore, our current studies clearly showed that human γδ Treg cells induced responder T cells and DCs into senescent immune cells, but not induction of their apoptosis or cytolyis. In addition, we characterized the Treg-induced senescent T cells and DCs, and showed that these cells had significant phenotypic and functional changes. γδ Treg–induced senescent T cells dramatically downregulated expression of costimulatory molecules CD27 and CD28, indicating their dysfunction. Senescent DCs also had immature phenotypes, suppressed secretion of effector cytokines, and upregulated suppressive molecule PD-L1, as well as impaired costimulation and APC functions. More importantly, besides the altered phenotypes, both senescent T cells and DCs converted into suppressive immune cells that had negative regulatory effects on immune responses. Future studies should be focused on the dissection of the suppressive molecules produced by tumor-derived γδ Treg cells responsible for suppressive effects and induction of immune cell senescence.

Given that senescent T cells and DCs induced by human breast tumor–derived γδ Treg cells possess potent suppressive function, the possibility of prevention of senescence induction and restoring the effector function of Treg-induced senescent T cells and DCs is also critical for antitumor immunity. TLRs have been recognized as critical components of the innate immune system and as very important for regulating Treg function (49, 50). We have demonstrated that human TLR8 signaling can reverse the suppressive functions of tumor-derived CD4⁺, CD8⁺, and γδ Treg cells (4, 13, 33). In the more recent and current studies, we further showed that manipulation of TLR8 signaling in naturally occurring CD4⁺ Treg and γδ Treg cells can also prevent senescence induction in responder immune cells by abrogating human Treg cell–suppressive activity in vitro and in vivo (28). These studies provide a novel strategy capable of preventing human Treg cell–suppressive function and augmenting immune responses directed against infectious diseases and cancer. Another challenge for the success of immunotherapy against cancer is how to identify the origin and mechanisms governing the increase of different types of Treg cells in cancer patients. Recent studies suggest that there are several potential sources of Treg cells that exist in tumor sites. One key mechanism responsible for accumulation of Treg cells within the tumor microenvironment is preferential recruitment of these Treg cells. Studies of Hodgkin’s lymphoma and ovarian cancer have shown that tumor microenvironmental CCL22 derived from cancer cells specifically recruits CCR4-positive CD4⁺ Treg cells to tumor sites (51, 52). Our current and previous studies have shown that increased numbers of γδ T cells were only observed in breast tumor tissues, but not in normal breast tissues, suggesting the recruitment and expansion of γδ T cells by the tumor microenvironment (13). Our future studies will also focus on the identification of mechanisms responsible for the accumulation of γδ T cells in the tumor microenvironments.

In summary, our current study provides the critical evidence that human tumor–derived γδ Treg cells directly suppress both innate (DCs) and adaptive immune cells (naive and effector T cells) through the induction of senescence in the responder immune cells. Importantly, γδ Treg–induced senescent T cells and DCs altered their phenotypes and functions. Both senescent DCs and T cells had potent suppressive activities that may result in an amplified immune suppression induced indirectly by γδ Treg cells in the tumor-suppressive microenvironment. Furthermore, we demonstrated that γδ Treg–induced T cell senescence can be prevented by the manipulation of TLR8 signaling in γδ Treg cells. These studies provide new insights relevant for the development of novel strategies capable of augmenting antitumor immune responses by eliminating or reversing the suppressive functions of γδ Treg cells.

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References


