Specific recruitment of γδ regulatory T cells in human breast cancer

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Abstract

Understanding the role of different subtypes of tumor-infiltrating lymphocytes (TILs) in the immunosuppressive tumor microenvironment is essential to improving cancer treatment. Enriched γδ1 T cell populations in tumor-infiltrating lymphocytes (TILs) suppress T cell responses and dendritic cell maturation in breast cancer, where their presence is correlated negatively with clinical outcomes. However, mechanism(s) that explain the increase in this class of T regulatory cells (γδ Treg) in breast cancer patients have yet to be elucidated. In this study, we showed that IP-10 secreted by breast cancer cells attracted γδ Treg cells. Using neutralizing antibodies against chemokines secreted by breast cancer cells, we found that IP-10 was the only functional chemokine that causes γδ Treg cells to migrate toward breast cancer cells. In a humanized NSG mouse model, human breast cancer cells attracted γδ Treg cells as revealed by a live cell imaging system. IP-10 neutralization in vivo inhibited migration and trafficking of γδ Treg cells into breast tumor sites, enhancing tumor immunity mediated by tumor-specific T cells. Together, our studies show how γδ Treg accumulate in breast tumors, providing a rationale for their immunological targeting to relieve immunosuppression in the tumor microenvironment.

Precis

Findings rationalize the use of anti-IL-10 antibodies to block migration of a class of T regulatory cells into the breast cancer microenvironment, thereby de-repressing the activity of antitumor T cells.
Introduction

Malignant tumors may use different strategies to evade immune surveillance. Induction and expansion of Treg cells in the tumor microenvironment is one of those actions that mediate immune suppression and dysfunction in cancer patients and also becomes a major obstacle for successful immunotherapy (1, 2). This notion is established and supported by many recent studies. Increased proportion of Treg cells exist in the tumor-infiltrating lymphocytes (TILs), peripheral blood lymphocytes and regional lymph node lymphocytes of patients with different types of tumors, including breast cancer (3-7). Furthermore, the increased frequencies of Treg cells are clinically correlated with tumor progression and demonstrated as a prognostic factor for the prediction of outcomes in cancer patients with different tumors (3, 5, 8-11). Recently, several strategies, including depletion or blockage of Treg suppression through targeting CD25 or CTLA-4 molecules, have been utilized in animal models and human clinical trials, yielding promising results (12-14).

Besides the naturally occurring CD4+CD25+ Treg cells, Treg cells are heterogeneous, existing as other subsets including adaptively induced Treg cells of Tr1, Th3, CD8+ as well as γδ Treg cells, which have all been identified in human cancers (15-19). γδ T cells have been demonstrated to play a regulatory role in different forms of tolerance (20-23) and have broad regulatory effects on CD4+, CD8+, IL-17+ and Treg cells in different diseases (24-27). Furthermore, studies from mouse tumor models have shown that γδ T cells in the tumor microenvironment may be involved in the induction of tumor-specific immune tolerance (28-30). We previously observed that γδ1 Treg cells accumulated in breast cancer TILs have strong suppressive activities on responding T cells and block the maturation and activities of dendritic cells (19). In order to explore the potential functions of γδ Treg cells in the immunopathogenesis of human breast cancer, we more recently demonstrated that high level of γδ T cells infiltrating in human breast cancer tissue 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 (31). Furthermore, those breast tumor-derived γδ Treg cells suppress innate and adaptive immunity through...
the induction of immunosenescence (32). The involvement of human tumor immunity by this new subset of Treg cells was also reported by another group (33). Thus, a better understanding of the suppressive mechanism(s) and regulations of tumor-derived γδ Treg cells is critical for the development of strong and innovative approaches to reverse the tumor suppressive microenvironment and improve effects of immunotherapy against breast cancer.

Dissecting mechanisms responsible for the accumulation of different types of Treg cells in tumor sites will provide alternative strategies for anti-tumor immunotherapy. One potential mechanism is that the tumor microenvironment preferentially recruits Treg cells. It has been shown that the migration of Treg cells into tumor microenvironments may be facilitated by the expression of specific chemokines and their receptors (5, 34, 35). Studies of Hodgkin's lymphoma and ovarian cancer showed that tumor microenvironmental CCL22 derived from cancer cells specifically recruits the CCR4*CD4+ Treg cells to tumor sites (5, 36, 37). In addition, CCR5-dependent chemotaxis is involved in Treg migration into pancreatic adenocarcinoma which expresses CCR5 ligand, CCL5 (35). Although high percentages of γδ Treg cells exist in breast cancer TILs, the origin and mechanisms governing γδ Treg increase in cancer patients remains unknown.

In our efforts to explore the mechanisms responsible for the accumulation of γδ Treg cells in breast cancer patients, we identified that IP-10 secreted by breast cancer cells significantly attracted the migration of γδ Treg cells. Through both in vitro and in vivo studies, we further demonstrated that human breast cancer utilized the IP-10-mediated recruitment as an important mechanism for the attraction and accumulation of γδ Treg cells in the tumor suppressive microenvironment. These studies provide new insights relevant for the development of novel cancer immunotherapeutic approaches capable of preventing the trafficking of γδ Treg cells into the breast cancer tumor microenvironment and reversing Treg-induced immune suppression.
Material and methods

Human samples and cell lines.

Tumor samples were obtained from breast cancer patients treated at the Department of Surgery, Saint Louis University from 2004 to 2010 who have given informed consents for enrollment in a prospective tumor procurement protocol approved by the Saint Louis University Institutional Review Board. Paired fresh tumor tissues and normal breast tissues were obtained perioperatively and snap frozen in liquid nitrogen (N=46). In addition, fresh-frozen metastatic cutaneous melanoma and colon cancer tumor tissues were also collected as controls for this study.

Buffy coats from healthy donors were obtained from the Gulf Coast Regional Blood Center at Houston. Peripheral blood mononuclear cells (PBMCs) were purified from buffy coats using Ficoll-Paque. Bulk CD4⁺ and γδ T cells were isolated by either positive or negative selection with microbeads (Miltenyi Biotec) according to manufacturer’s instructions. CD4⁺CD25⁺ Treg cells were further purified from CD4⁺ T cells by FACS sorting after staining with anti-CD25-PE (BD Bioscience). The purity of the T cells was >95%, as confirmed by flow cytometry. Human γδ Treg cells were established from the primary breast cancer tissues in our laboratory (19, 31, 32). Breast tumor cell lines MCF-7 and MDA-MB-453 were obtained from the American Tissue Culture Collection (ATCC). Melanoma MC135, MC586 and MC136 were established in our laboratory and maintained in RPMI 1640 medium containing 10% fetal calf serum (FCS). Melanoma 586mel and paired TIL586 were obtained from the Surgery Branch, NCI. Breast carcinoma cell lines (BC31, BC30, and BC20) were established in our laboratory and maintained in keratinocyte medium containing 25 mg/ml bovine pituitary extract, 5 ng/ml epidermal growth factor, and 2% heat-inactivated FBS, and penicillin-streptomycin (Invitrogen, Inc. San Diego, CA).

Generation of tissue-infiltrating lymphocytes

Tumor and normal tissue-infiltrating lymphocytes were generated from different tumor and normal tissues, as we previously described (19, 38, 39). Briefly, tissues were minced into small pieces followed by digestion with collagenase type IV, hyaluronidase, and deoxyribonuclease. After digestion,
cells were washed in RPMI1640, and then cultured in RPMI1640 containing 10% human AB serum supplemented with L-glutamine, 2-mercaptoethanol and 50 U/ml of IL-2 for the generation of T cells.

**Immunohistochemical and indirect immunofluorescence staining**

The γδ T cells and IP-10⁺ tumor cells in cancer and normal tissues were determined using immunohistochemical staining, as we described previously (31). The frozen sections were stained with a mouse anti-human γδ TCR (clone B1.1, eBioscience) monoclonal and rabbit anti-human IP-10 (R & D Systems) antibodies, and then followed the procedure of the Histostain®-Plus 3rd Gen IHC Detection Kit (Invitrogen, CA). Controls were performed by incubating slides with the isotype control antibody instead of primary antibodies, or second antibody alone. The positive cells in tissues were evaluated manually using a computerized image system composed of a Leica ICC50 camera system equipped on a Leica DM750 microscope (North Central Instruments, Minneapolis, MN). Photographs were obtained from 20 randomly selected areas within the tumor tissues of 10 cancer nest areas and 10 cancer stroma areas at a high-power magnification (400 ×). Ten fields (400 ×, magnification) of each tumor tissue section, including both cancer nest and stroma areas were counted and summed, and the means of positive cell numbers per field reported.

For indirect immunofluorescence staining of γδ T cells and IP-10⁺ tumor cells, frozen sections were incubated with a mixture of mouse anti-human TCRγδ and goat anti-human IP-10 (R & D Systems) primary antibodies, and then with a mixture of two secondary antibodies [CFL555-conjugated donkey against goat (Santa Cruz Biotechnology) and A488-conjugated rabbit against mouse (Cell Signaling Technology)]. Specimens were then counter stained with 4'-6-Diamidino-2-phenylindole (DAPI) (Invitrogen).

**Chemotaxis assay**

Chemotaxis assays were performed using 24-well Transwell chemotaxis plates (5-μm pore size; Corning Costar) as we described previously (19, 38). Culture supernatants from different tumor cell lines and freshly digested tumor tissues, or various concentrations of recombinant human IP-10 in
RPMI 1640 medium were added to the lower chamber of the Transwell plates. Human breast tumor derived γδ1 Treg cell lines or control T cells (1 × 10^5 cells) were transferred into upper chambers. After 150 min at 37°C, chemotaxis was quantified by detecting the numbers of cells that migrated into the lower chamber. The chemotaxis index was calculated by dividing the numbers of cells migrated in response to test supernatants or recombinant human IP-10 by the numbers of cells migrated in response to medium alone. For antibody blocking assays, various concentrations of neutralization anti-IP-10, anti-MCP-1, anti-IL-8, and/or anti-VEGF (R & D Systems) antibodies were added into culture supernatants and incubated at 37°C for 30 min before performing chemotaxis assays.

**Flow cytometry analysis**

The expression markers on T cells and tumor cells were determined by FACS analyses after surface staining or intracellular staining with anti-human specific antibodies conjugated with either PE or FITC. These human antibodies included: anti-IP-10, anti-CCR4, anti-CCR5, anti-CCR6, anti-CCR7, anti-CXCR3 and anti-TCRγδ, which were purchased from BD Biosciences or eBioscience. All stained cells were analyzed on a FACSCalibur flow cytometer (BD Bioscience) and data analyzed with FlowJo software (Tree Star).

**Cytokine antibody array**

Tumor cell lines (0.5 × 10^6/ml) were cultured in 24-well plates for 48 hours, and chemokines and cytokines released into culture supernatants were determined using human Bio-Plex Chemokine Assay Kits (Bio-Rad) according to manufacturer’s instructions.

**In vivo studies**

NOD-scid IL2Rgamma^null^ (NSG) and Rag1^-/-^ immunodeficient mice were purchased from The Jackson Laboratory and maintained in the institutional animal facility. All animal studies have been approved by the Institutional Animal Care Committee. Human MDA-MB-453 breast tumor cells (5 × 10^6) in 100 μl of buffered saline were subcutaneously injected into NSG mice. γδ1 Treg, γδ2 and CD4^+^ T cells
were incubated with 320 μg/ml of XenoLight DiR (Caliper Life Science) for 30 minutes. Stained T cells were washed twice in PBS and then injected tail intravenously (5 × 10^6/mouse in 200 μl of buffered saline) into MDA-bearing (tumor size about 10 × 10 mm) NSG mice. Five to ten mice were included in each group. Mice were imaged with an In Vivo Spectrum Imaging System (IVIS) (Caliper Life Science) at 60 min, and 1, 2, 3, 5, 7 and 10 days post injection. The appropriate filter set for DiR imaging 710 nm excitation and 760 nm emission was used. Mice were imaged dorsally, right laterally and ventrally at all time points. For IP-10 blocking experiments, mice were injected with a neutralizing antibody against human IP-10 (clone 33036, R & D Systems) in tumor sites at 2 hours before γδ Treg cell injection, and at days 3, 6 and 9 after γδ T cell injection. The changes of signal density were checked at day 2, 4, and 6 after γδ Treg cell transfer. Furthermore, tumor tissues were removed at 10 days post T cell adoptive transfer and human T cell infiltration determined using immunohistochemical staining as described above. For adoptive transfer immunotherapy experiments, mixtures of human 586mel tumor cells (4 × 10^6/mouse) and breast MDA-MB-453 cancer cells (3 × 10^6/mouse) were subcutaneously injected into NSG mice. Human 586mel-specific CD8+ TIL586 cells (5 × 10^6/mouse) combined with or without γδ Treg cells (3 × 10^6/mouse) were adoptively transferred through intravenous injection on day 3. Concurrently, some tumor-bearing mice were injected with either human IP-10 neutralizing antibody or isotype control antibody (40 μg/mouse) into tumor sites at 2 hours before T cell adoptive transfer and then every 5 days after their transfer. Tumor volumes were measured every 3 days. Five mice were included in each group.

**Statistical analysis**

Unless indicated otherwise, data are expressed as mean ± standard deviation (SD). The significance of difference between groups was determined by paired or unpaired two-tailed Student's t-test or the one-way analysis of variance (ANOVA). Differences were considered significant for p values less than 0.05.

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Results

High percentages of γδ Treg cells infiltrate in breast cancer tissues

To identify the suppressive mechanisms mediated by the breast cancer tumor microenvironment, we have demonstrated that γδ1 Treg cells were prevalent in breast tumor-infiltrating lymphocytes from breast cancer patients (19, 31, 32). These breast tumor-derived γδ1 Treg cells not only suppress the proliferation and effects of naïve and tumor-specific T cells, but also inhibit the maturation and function of dendritic cells (19, 32). However, the mechanism governing the γδ Treg cell accumulation in breast tumor sites remains unknown. To further investigate the role and regulation of γδ Treg cells in human breast cancer, we collected additional human breast cancer samples, generated TILs and characterized the tumor-infiltrating γδ1 T cells. Consistent with our previous results, we found that breast cancer-derived TILs contained high percentages of γδ1 T cells in the total TIL populations (4-77%, mean 29.1%), while in normal breast tissue-infiltrating lymphocytes and melanoma-derived TILs, the percentages of γδ T cells were low (2-4%, mean 2.8%; 3-8%, mean 5.8%, respectively) (Fig. 1A). Using [3H]-thymidine incorporation assays, we confirmed that these breast cancer-derived γδ T cell lines have strong suppressive activity on the responding CD4+ T cell proliferation (Supplementary Fig. 1A). These results prompted us to investigate whether γδ T cells were prevalent in situ in breast tumor sites (31). We further performed immunohistochemical staining to detect γδ T cells in 46-freshly frozen breast cancer sections and patient-paired normal breast tissues (Fig. 1B). Significantly increased numbers of γδ T cells were detected in breast tumor tissues (43 of 46 tumor samples), but not in normal breast tissues (2 of 46 breast tissues). Notably, tumor nodes were surrounded by these γδ1 T cells. In addition, higher amounts of γδ1 T cells existed in cancer tissues from the late stage breast cancer patients (III and IV) than those from patients with early stages of cancer progression (I and II) (Fig. 1B). These results combined with our previous studies clearly suggest the prevalence of γδ T cells in the breast tumor microenvironment which may play critical role in the immune pathogenesis of human breast cancer (31).
Breast cancer cells attract the migration of γδ Treg cells

Given that high amounts of γδ1 Treg cells in breast tumor but not in normal breast tissues, we thus reasoned that human breast cancer cells may use the recruitment strategy to attract the migration of γδ1 Treg cells into tumor sites. To test this possibility, we first investigated if culture supernatants from freshly digested human tumor tissues, including melanoma, breast and colon cancers, can attract γδ1 Treg cell migration. As shown in Fig. 2A, we found that supernatants from breast cancer tissues induced significant migration of γδ Treg cells compared with culture medium in Transwell chemotaxis assays. In contrast, the supernatants from the culture supernatants of melanoma and colon cancer tissues had no or little chemotactic activity for γδ Treg cells. This result was further confirmed in the culture supernatants obtained from the primary tumor cell lines. We showed that supernatants from primary breast tumor cell lines, but not from melanoma and colon cancer cells dramatically induced γδ Treg cell chemotaxis (Fig. 2B). These results indicate that breast cancer cells secrete molecules that selectively recruit γδ T cells to the tumor microenvironments.

Because estrogen receptor (ER) -negative breast cancer patients have a worse prognosis than ER-positive patients, we next determined whether ER expression status in breast cancer patients affects their capacity to attract the migration of γδ Treg cells into tumor sites. We found that the culture supernatants of digested breast tumor tissues collected from ER⁺ and ER⁻ breast cancer patients can both significantly attract the migration of γδ Treg cells to a similar level (Fig. 2C), indicating that ER expression on breast cancer cells does not affect their ability to attract γδ Treg cells. Epidermal growth factor receptor 2 (HER2) expression level in tumor cells is another important prognostic factor for breast cancer outcomes. Our recent studies have further demonstrated a significant positively correlation between intra-tumoral γδ T cell numbers and HER2 expression in breast cancer patients (31). However, we observed that culture supernatants from HER2⁺ and HER2⁻ human breast cancer tissues have a similar chemotactic activity on γδ Treg cells (Fig. 2D). These data suggest ER or HER2 expression in breast cancers does not affect their chemotactic activity for γδ Treg cells.
IP-10 secreted by breast tumor cells is responsible for the migration and trafficking of γδ Treg cells

To investigate which cytokines are secreted by breast cancer cell lines and involved in the specific recruitment of γδ1 Treg cells, we collected cell culture supernatants from breast cancer cell lines and determined the chemokines released into culture supernatants using a human chemokine antibody array. As shown in Fig. 3A, we found that primary breast tumor cell line BC31 secreted large amounts of IL-8, IP-10 and VEGF, and mid-level amounts of G-CSF, MCP-1 and RANTES, as well as some FGF and TGF-β, but not other cytokines and chemokines. We obtained very similar cytokine profiles from other breast tumor cell lines (data not shown). We further examined which cytokines/chemokines secreted by breast cancer cells are responsible for γδ Treg cell recruitment. We used specific neutralizing antibodies against the identified chemokines/cytokines and found that only IP-10 neutralizing antibody abolished the chemotactic activity of the breast cancer supernatant to attract γδ Treg cells (Fig. 3B). In addition, we found that recombinant IP-10 had the same chemotactic activity for various γδ Treg cell lines, and the activity displayed a dose-dependent chemotaxis (Supplementary Fig. 1B). These results suggest that breast cancer cells secrete IP-10 that recruits γδ T cells into the tumor microenvironment.

We next investigated whether IP-10 is the only functional chemokine that attracts the migration of γδ1 Treg cells by breast cancer cells. We first confirmed that breast cancer cell lines MCF7, MDA, BC30 and BC31, but not melanoma cell lines MC586 and MC136, highly expressed IP-10 using flow cytometry analyses with intracellular staining of IP-10 expression (Fig. 3C). We then checked whether γδ Treg cells solely expressed IP-10 receptor, CXCR3, compared with the other T cell subsets. As expected, we found that γδ Treg cells expressed high level of CXCR3. Furthermore, those γδ Treg cells also expressed CCR5, CCR6 and CCR7, but not CCR4. Surprisingly, CD4+CD25+ Treg and naïve CD4+ T cells had a similar chemokine receptor pattern that also expressed CXCR3, CCR5,
CCR6 and CCR7 (Fig. 3D). We eliminated the function of IP-10 in the breast cancer cells using a neutralizing antibody and then tested whether IP-10-blocked breast cancer cells still had chemotactic activity for γδ Treg cells. We also included other types of T cells as controls, including γδ2 T, CD4+ Th1, CD4+CD25+ Treg and Th17 cells. Consistent with our studies in Fig. 3B, we found that neutralizing antibody against IP-10 can significantly abolish the chemotaxis of γδ Treg cells induced by breast cancer cells. However, supernatants from breast cancer cells did not have any chemotactic activity on γδ2, CD4+CD25+ Treg and CD4+ Th1 cells no matter whether IP-10 was blocked or not, although these cells also expressed CXCR3 (Fig. 3E). In addition, we found that breast tumor cells dramatically attracted Th17 cell migration, but neutralization of IP-10 cannot abolish the breast tumor-mediated chemotaxis of Th17 cells (Fig. 3E). These results were consistent with our previous finding that breast tumor cells attracted human Th17 cells to tumor sites through RANTES and MCP-1, but not through IP-10 (38). These data may also suggest that tissue or cell type specificity is required for the interactions among chemokine/chemokine ligand-expressing cells (40).

Co-localization of γδ Treg cells and IP-10-expressing tumor cells in situ in human breast tumor tissues.

To further investigate the functional effect of IP-10 involved in the recruitment of γδ Treg cells by breast cancer cells, we determined whether IP-10-expressing tumor cells were co-localized with γδ Treg cells in breast tumor tissues. IP-10-expressing tumor cells and γδ T cells were visualized and analyzed in the frozen serial tissue sections from normal breast and breast tumor tissues (31, 41). As shown in Fig. 4A, large amounts of γδ T cells infiltrated in the breast tumor tissue but not in normal breast tissue. Furthermore, high percentages of IP-10-expressing tumor cells were observed in situ in breast tumor tissues. In contrast, very few IP-10 positive cells were found in normal breast tissues. In addition, serial tissue sections and staining analyses suggested that IP-10-expressing tumor cells were surrounded by γδ T cells (data not shown). To better visualize the interaction between IP-10-expressing breast tumor cells and γδ T cells in situ in tumor sites, immunofluorescence double staining
with anti-IP-10 and anti-TCRγδ antibodies in the same sections from breast tumor tissues were also performed. As shown in Fig. 4B, IP-10-expressing breast tumor cells were co-localized with γδ T cells in tumor tissues. These results collectively demonstrated that breast tumor cells can selectively recruit γδ Treg cells through the tumor-derived IP-10. Given that the numbers of intra-tumoral γδ T cells are varied among the breast cancer patients, we determined the correlation between tumor-infiltrating γδ T cells and tumor IP-10 expression levels in breast cancer patients. Unexpectedly, correlation analyses showed that no significant difference of γδ Treg cell infiltration was found among human breast tumor tissues with different IP-10-expressing levels (Supplementary Fig. 2).

**IP-10 controls γδ Treg cell trafficking and migration in vivo in mouse tumor models.**

These *in vitro* studies provided us important information regarding the specific recruitment of γδ Treg cells by IP-10 produced by human breast tumor cells. However, a key unanswered question that arises from these experiments was how breast cancer-derived IP-10 controls γδ Treg cell trafficking and migration to tumor sites *in vivo*. To address this question, we performed complementary *in vivo* studies, using the adoptive transfer of human γδ Treg cells into human breast tumor-bearing NOD-Scid IL2Rgamma<sup>−/−</sup> (NSG, lacking T and B cells) mouse models through the *in vivo* live imaging systems (IVIS). Human MDA-MB-453 breast cancer cells were subcutaneously injected into NSG mice to establish tumor-bearing mice. After tumor sizes reached to about 10 x 10 mm, XenoLight DiR stained γδ<sup>1</sup> Treg cells were adoptively transferred into MDA-MB-453-bearing NSG mice. In addition, γδ<sup>2</sup> T and CD4<sup>+</sup> T cells purified from healthy donors were included as cell controls. T cell trafficking and distribution in tumor-bearing mice were imaged at dorsal, right lateral and ventral positions with IVIS at various time points post T cell adoptive transfer.

As shown in Fig. 5A, in early time points (before 3 days) post T cell injection, γδ<sup>1</sup> Treg cells randomly migrated into different organs, including spleen, cervical and peripheral lymph nodes (LN's), as well as tumor sites. However, the signal density of γδ Treg cells significantly increased in tumor sites in the
late time points (after 3 days) post T cell transfer; and this T cell accumulation continued to persist through the whole observation period (10 days), indicating accumulation of $\gamma\delta$1 Treg cells into the tumor sites. Similar to $\gamma\delta$1 Treg cells, human $\gamma\delta$2 and CD4$^+$ T cells also migrated and distributed into spleen, cervical, peripheral LNs and tumor sites at the early times. However, unlike $\gamma\delta$1 Treg cells, $\gamma\delta$2 T and CD4$^+$ cells did not accumulate in tumor sites and even decreased at the late times (after 3 days). Notably, adoptively transferred human $\gamma\delta$1 Treg, $\gamma\delta$2 T and CD4$^+$ T cells markedly proliferated within NSG mice, and the proliferation capacity of $\gamma\delta$1 Treg cells was lower than that of the latter two control T cells. In addition, human T cell infiltration into tumor tissues from different groups was verified using the immunohistochemistry staining of human CD3$^+$ T cells. As expected, we observed that large amount of human CD3$^+$ T cells infiltrated into breast tumor tissues from the $\gamma\delta$1 Treg cell adoptive transfer group. However, there was significantly lower numbers of human CD3$^+$ T cells infiltrating into breast tumor tissues from the groups injected with CD4$^+$ and $\gamma\delta$2 T cells (Fig. 5B). Collectively, these results clearly indicate that human breast tumor cells can selectively attract $\gamma\delta$1 Treg cells into tumor sites in vivo.

We then determined if the human breast tumor-induced trafficking and migration of $\gamma\delta$ Treg cells into the tumor sites is controlled by the tumor-derived IP-10 in vivo, using this adoptive transfer tumor model. $\gamma\delta$1 T cells were intravenously injected into MDA-MB-453-bearing NSG mice. Concurrently, mice were injected with human IP-10 neutralizing antibody into tumor sites before and after $\gamma\delta$1 Treg cell adoptive transfer, and the $\gamma\delta$1 T cell trafficking and accumulation into tumor sites were checked by the IVIS. As expected, treatment with neutralizing antibody against human IP-10, but not isotype control antibody, significantly blocked the trafficking and accumulation of $\gamma\delta$1 Treg cells into tumor sites (Fig. 6A). Furthermore, we confirmed using the immunohistochemical staining of $\gamma\delta$ T cells in tumor sections that high amount of $\gamma\delta$ Treg cells infiltrated into breast tumor tissues obtained from isotype antibody treatment group, but not from the IP-10 neutralizing antibody treatment group (Fig.
6B). These data further suggest that tumor-derived IP-10 is critical and controls the recruitment of $\gamma\delta$ Treg cells into breast tumor sites in vivo.

**Blockage of $\gamma\delta$ Treg cell trafficking into the tumor microenvironment via IP-10 neutralization enhances anti-tumor immunity in vivo in an adoptive transfer therapy model.**

We next explored whether blockage of $\gamma\delta$ Treg cell trafficking and migration into the tumor sites can enhance anti-tumor immunity, using our previously established adoptive transfer immunotherapy model (19, 42, 43). Given that human 586mel tumor cells don’t express IP-10 and IP-10-expressing melanoma cells cannot survive in vivo (44), we thus utilized a mixture of human 586mel tumor cells (as a target for tumor-specific T cells) and human breast MDA-MB-453 cancer cells (as a source of IP-10 for $\gamma\delta$ Treg attraction) in our following studies. Human 586mel tumor cells and MDA-MB-453 breast cancer cells were subcutaneously injected into NSG mice. DiR-stained 586mel-specific CD8$^+$ TIL586 cells combined with or without breast cancer-derived $\gamma\delta$ Treg cells were adoptively transferred through intravenous injection on day 3. Concurrently, mice were injected with human IP-10 neutralizing antibody into tumor sites before and after T cell adoptive transfer, and the CD8$^+$ TIL586 cells cell trafficking and accumulation into tumor sites were checked by the IVIS. In addition, tumor growth was evaluated. We first determined whether neutralizing antibody against human IP-10 affects CD8$^+$ TIL586 cell migration and trafficking induced by 586mel cells. As expected, IP-10 neutralizing antibody treatment didn’t inhibit the TIL586 cell trafficking into tumor tissues or distribution both in 586mel-bearing Rag1$^{-/-}$ or in MDA-MB-453 and mel586 tumor-bearing NSG mice (Supplementary Fig. 3). Furthermore, we observed that 586 tumor cells grew progressively in NSG mice. When tumor-specific CD8$^+$ TIL586 T cells, which can kill the 586mel, were adoptively transferred, tumor growth was significantly inhibited. However, adoptive transfer of CD8$^+$ TIL586 T cells plus $\gamma\delta$ Treg cells did not inhibit tumor growth in NSG mice. Importantly, intratumoral injection of human IP-10 neutralizing antibody, but not the isotype control antibody, dramatically suppressed tumor growth, suggesting that IP-10 neutralization prevents $\gamma\delta$ Treg cell infiltration into tumor sites and enhances the anti-tumor
ability mediated by TIL586 CD8\(^+\) T cells (**Fig. 6C**). Taken together, our studies clearly indicate that blockage of \(\gamma\delta\) Treg cell trafficking into the tumor microenvironment via IP-10 neutralization can mediate enhanced anti-tumor immunity *in vivo*, and could be a promising strategy for breast cancer immunotherapy.
Discussion

It is well established that recruitment of Treg cells into tumor microenvironments is one of major strategies utilized by tumor cells to induce immune suppression and evade immune surveillance (1, 45). Breast cancer can recruit FoxP3⁺ Treg cells into tumor sites, leading to impaired anti-tumor immune responses and promoting tumor metastases (10, 46). Besides FoxP3⁺ Treg cells, we have further shown that high proportion of γδ Treg cells existed in human breast cancer patients (19, 31). These breast tumor-derived γδ Treg cells have potent suppressive activities on CD4⁺ and CD8⁺ T cell proliferation and effector functions, as well as on DC maturation and activities (19, 31, 32). However, the origin and mechanisms governing γδ Treg increase in breast cancer patients remains unknown. In the current study, we performed both in vitro and in vivo studies showing that breast cancer cells secrete IP-10 which mediates the trafficking and migration of γδ Treg cells into tumor sites. Our studies provide new insights relevant for the development of an alternative cancer immunotherapeutic approach other than depletion capable of controlling γδ Treg-mediated immune suppression for human breast cancer immunotherapy.

Emerging studies are showing that elevated levels of CD4⁺CD25⁺ and CD8⁺ Treg cells exist in peripheral blood, tumor draining lymph nodes, and TILs in patients with different types of cancers (3-5). Importantly, the increased frequencies of Treg cells were clinically correlated with tumor pathogenesis (5, 8, 47, 48). We have recently shown high percentages of γδ Treg cells with potent suppressive activity among TILs from breast cancer patients (19). We further analyzed in situ the quantity of γδ T cells in tumor tissues with different cancer stages using immunohistochemical staining, and then performed retrospective and multivariate analyses of the correlations between γδ T cell levels with other prognostic factors and clinical outcomes (31). We observed that γδ T cell numbers were significantly increased in breast cancer tissues but not in normal breast tissues, especially in the late stages (III and IV) of cancer patients (Fig. 1). Furthermore, we found that patients with a high proportion of γδ T cells have advanced cancer stages, HER2 expression status...
and high lymph node metastasis, and that high numbers of γδ T cells in breast cancer tissues identified poor survival rate and high risk of relapse patients (31). These studies clearly suggest that the development of effective strategies targeting γδ Treg cells is essential for breast cancer immunotherapy.

The accumulation of Treg cells in the tumor microenvironment may due to different potential mechanisms, including trafficking, expansion or conversion (1). Our current studies strongly suggest that breast tumor cells may favor the prevalence of γδ1 Treg cells in tumor microenvironments through the recruitment of γδ Treg cells from periphery into tumor sites. Our in vitro studies showed that culture supernatants from breast cancer cells can significantly induce chemotaxis of γδ Treg cells which was dependent of cancer cell-secreted IP-10. We further demonstrated that IP-10 controlled breast tumor-derived γδ1 Treg cell migration and trafficking into tumor sites in vivo in animal models using an In Vivo Imaging System. Aside from the IP-10-mediated recruitment of γδ Treg cells into tumor sites, it is still possible that tumor microenvironment factors, tumor cells, as well as tumor-derived stromal cells, may also contribute to the γδ Treg cell expansion and conversion. Studies from conventional Treg cells have shown that tumor microenvironment factors, including IL-10 and TGFβ, vascular endothelial growth factor (VEGF) as well as some types of DCs and regulatory B cells, can convert naïve and/or effector T cells into suppressive Treg cells (49, 50). Given that our data have shown that breast cancer cells also secrete VEGF and TGFβ (Fig. 3A), and no significant correlation between numbers of γδ Treg cell infiltration and IP-10 expression levels among human breast tumor tissues, as well as that we observed non-suppressive γδ1 T cells existing in the breast cancer TILs (19), there still exists a possibility that conversion in situ may contribute to the accumulation of γδ Treg cells in the breast cancer tumor microenvironment. We will continue our efforts to explore other potential mechanisms responsible for the accumulation of γδ Treg cells in human breast cancer in the future studies.
Treg cell migration into tumor sites is dependent on the interactions between chemokines secreted by the tumor microenvironment and the chemokine receptors expressed on Treg cells (5, 10). Our current studies have shown that IP-10 was intensely expressed in human breast cancer tissues in situ and in breast cancer cells, consistent with other reports (51). Furthermore, IP-10 receptor, CXCR3, was found to express in \( \gamma\delta \) Treg cells. In addition, our in vitro and in vivo studies clearly showed that interaction between IP-10 (tumor cells) and CXCR3 (\( \gamma\delta \) Treg cells) is the main mechanism responsible for \( \gamma\delta \) Treg trafficking and migration into tumor sites. Interestingly, CXCR3 is expressed on almost all different types of T cells, including Th1, Th17, CTLs, and Treg cells (Fig. 3 and (38)). However, we observed that IP-10 secreted by breast cancer cells only selectively attracted the migration of \( \gamma\delta \) Treg cells, but not other CXR3-expressing T cell subsets. These studies may suggest that tissue or cell type specificity is required for the interactions among chemokine/chemokine ligand-expressing cells (40). In support of this notion, our previous studies have shown that that breast cancer microenvironment also can recruit Th17 cells through chemokines RANTES and MCP-1, but not via IP-10 (38). In addition, one study from other group showed that CXCR3 ligand expression in regulatory DC subset induced the recruitment of CD4\(^+\) Th1 cells responses (52).

Several strategies, including depletion or blockage of CD4\(^+\)CD25\(^+\) Treg suppressive activities through targeting CD25 or CTLA-4 molecules, have recently been utilized in clinical trials for tumor immunotherapy (12-14). However, our identification of \( \gamma\delta \) Treg cells in the breast cancer patients further suggests that control of CD4\(^+\)CD25\(^+\) Treg cells is not enough for the reversal of immune suppression. We have demonstrated that human TLR8 signaling reversed the suppressive functions of tumor-derived CD4\(^+\), CD8\(^+\) and \( \gamma\delta \) Treg cells (17, 19, 42). Furthermore, our current in vitro and in vivo studies clearly suggest that IP-10 secreted by human breast cancer cells controls the recruitment of \( \gamma\delta \) Treg into tumor sites, and that blockage of \( \gamma\delta \) Treg cell trafficking into the tumor microenvironment significantly inhibits their suppression and enhances anti-tumor immunity. These studies provide promising alternative strategies that would augment the anti-tumor immune responses.
for human breast cancer immunotherapy. One way that we can functionally inactivate Treg cell suppression without changing the Treg repertoire or effector T cell functions via TLR8 signaling. In addition, we may block the recruitment and generation of γδ Treg cells through IP-10/CXCR3 by the breast tumor microenvironment.

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Conflict-of interest disclosure
The authors declare no financial or commercial conflict of interest.
References


Figure legends

Figure 1

High percentages of $\gamma\delta$1 Treg cells exist in TILs of breast cancer patients.

(A) Human breast TILs contained high percentages of $\gamma\delta$1 T cells (4-77%, mean 29.1%), while in normal breast tissue-infiltrating T cells and melanoma TILs only have few $\gamma\delta$1 T cell populations (2-4%, mean 2.8%; 3-8%, mean 5.8%, respectively). TILs and normal tissue-infiltrating lymphocytes were isolated from freshly digested tissues and $\gamma\delta$1 T cell populations analyzed by flow cytometry. (B) Significantly increased numbers of $\gamma\delta$ T cells existed in breast cancer tissues with early stages (I + II) and late stages (III + IV), compared with those in normal breast tissues. Frozen sections from breast tumor samples and controls of paired normal breast tissues (n=46) were immunohistochemically stained to detect $\gamma\delta$ T cells. Number of $\gamma\delta$ T cells shown is the average numbers per high field (400 x) in each tissue sample. The mean number of $\gamma\delta$ T cells in each group is shown as a horizontal line. Significance was determined by paired t test (breast cancer vs normal breast tissues), or unpaired t test (early stages vs late stage of breast cancer tissues). **p< 0.01.

Figure 2

Breast cancer cells enhance the migration of $\gamma\delta$ Treg cells.

(A) Culture supernatants from freshly digested breast cancer tissues induced $\gamma\delta$1 Treg cell chemotaxis. However, supernatants from digested melanoma or colon cancer tissues had low activity on the migration of $\gamma\delta$1 Treg cells. Cultured supernatants from 3 of different digested tumor tissues were collected and chemotactic activity for $\gamma\delta$1 Treg cells determined. Chemotaxis assay was performed using 24-well transwell chemotaxis plates (5 $\mu$m pore-size, Costar). (B) Culture supernatants from primary breast tumor cell lines (BC20, BC30 and BC31) also induced $\gamma\delta$1 Treg cell chemotaxis. By contrast, supernatants from melanoma cell lines (MC586 and MC135) and colon cancer cell lines (CC5 and CC12) do not elicit appreciable chemotactic activity for $\gamma\delta$ Treg cells. Culture supernatant collection and chemotaxis assay were identical as in (A). Results in (A) and (B)
are representative of three independent experiments with four independent γδ Treg cell lines derived from 3 patients with similar results. *p< 0.05 and **p< 0.01 compared with medium only group. (C) and (D) ER or HER2 expression in breast cancer tissues does not affect their chemotactic activity for γδ Treg cells. Culture supernatants from digested human breast cancer tissues with different ER and HER2 expression statuses have a similar chemotactic activity for γδ Treg cells. Culture supernatant collection and chemotaxis assay were identical as in (A). Results are representative of four independent γδ Treg cell lines derived from 3 patients with similar results.

**Figure 3**

**IP-10 secreted by breast tumor cells is responsible for the migration and trafficking of γδ Treg cell.**

(A) Cytokines were released by BC31 breast tumor cells. BC31 breast tumor cells secreted large amounts of IL-8, IP-10 and VEGF, and mid-level amounts of G-CSF, MCP-1 and RANTES, as well as some FGF and TGFβ. BC31 tumor cells were cultured for two days, and culture supernatants were collected and tested for cytokine secretion using a Bio-Plex Cytokine Assay Kit. (B) Neutralizing antibody against IP-10 abolished the chemotactic activity of the breast cancer supernatants for γδ1 Treg cells, but the neutralizing antibodies against other cytokines failed to block the chemotactic activity. Culture supernatants from breast tumor cells were pre-incubated with 10 μg/ml of neutralizing antibodies against IL-8, IP-10, MCP-1, RANTES and VEGF, and then tested for the ability to attract γδ1 Treg cells by chemotaxis assays. **p< 0.01 compared with the antibody non-treatment group. Data are representative of four independent γδ Treg cell lines derived from 3 patients with similar results. (C) Breast tumor cell lines MCF7, MDA, BC30 and BC31 expressed higher level of IP-10, while melanoma cell lines MC586 and MC1363 had no or low levels of IP-10 expression. IP-10 expression on different tumor cell lines was analyzed by flow cytometry. (D) Chemokine receptor expression on γδ Treg, CD4⁺CD25⁺ Treg, and naïve CD4⁺ T cells. Different subsets of T cells were stained with specific antibodies conjugated with either PE or FITC against the indicated chemokine.
receptors and analyzed by flow cytometry. CD4⁺CD25⁺ Treg and naïve CD4⁺ T cells were included as controls. Data are representative of three independent cell lines with similar results. (E) Culture supernatants from breast cancer cells induced significantly migration of γδ¹ Treg cells and Th17 cells, but not other T cell subsets (γδ² T, CD4⁺ Th1 and CD4⁺CD25⁺ Treg cells). In addition, neutralizing antibody against IP-10 specifically blocked the chemotactic activity of breast cancer tumor cells for γδ Treg cells (BTIL31 and BTIL11) but not Th17 cells. Culture supernatants from breast tumor cells were pre-incubated with or without 10 μg/ml of the IP-10 neutralizing antibody, and then tested for the ability to attract different subsets of T cell lines by chemotaxis assays.

Figure 4

Co-localization of γδ Treg cells and IP-10-expressing tumor cells in human breast tumor tissues.

(A) Co-localization of IP-10-expressing tumor cells and γδ Treg cells was analyzed in serial tissue sections using immunohistochemical staining. Large amounts of γδ T cells (breast tumor No. 1) infiltrated in the breast tumor tissues but not in normal breast tissues. Breast tumor No. 2, served as a control, shows less amounts of γδ T cell infiltration in the tumor tissue. Furthermore, high percentages of IP-10-expressing tumor cells were observed in situ in serial breast tumor tissues. Frozen tissue sections from normal breast and cancer tissues were immunohistochemically stained with anti-TCRγδ (left panel) and anti-IP-10 (right panel) antibodies. (B) Co-localization of IP-10-expressing tumor cells and γδ Treg cells was analyzed in the same sections of human breast tumor tissues using immunofluorescent analyses. IP-10-exressing breast tumor cells were co-localized with γδ T cells in the tumor tissues. Blue, red and green indicated the cell nuclear, IP-10 expression, and TCRγδ expression, respectively. Breast tumor No. 1 shows large amounts of γδ T cell infiltration, and tumor No. 2 shows less amounts of γδ T cells infiltrated in the tumor tissue.
Figure 5

Visualization of breast tumor-induced $\gamma \delta^1$ Treg cell trafficking and migration in vivo with live imaging analyses.

(A) $\gamma \delta^1$ Treg cells specifically accumulated in tumor sites in human breast tumor MDA-MB-453-bearing NSG mice. In contrast, $\gamma \delta^2$ and CD4$^+$ T cells, served as controls, randomly migrated and distributed in the MDA-MB-453-bearing NSG mice. XenoLight DiR stained T cells were imaged with IVIS Spectrum at different time points following their adoptive transfer into MDA-MB-453-bearing NSG mice. The circles indicated the tumor growth sites. Data show the dorsal, ventral, and right lateral images of a single mouse as a representative of 5 mice per group. Color bars represent signal intensity scale over whole body. (B) Large amounts of adoptively transferred $\gamma \delta$ T cells, but not $\gamma \delta^2$ and CD4$^+$ T cells, infiltrated and accumulated into the breast tumor tissues in MDA-MB-453-bearing NSG mice. Human T cell accumulations into breast tumor tissues were analyzed at 10 days post T cell adoptive transfer using immunohistochemical staining with anti-human CD3 antibody in the frozen sections. Left panels are photomicrographs of CD3$^+$ T cells in tumor tissues from different adoptive transfer groups. Right panel is a summary of $\gamma \delta^1$ Treg, $\gamma \delta^2$ or CD4$^+$ T cell numbers per high microscope field in the tumor tissues from 5 mice of each group. Significance was determined by paired t test. Results shown in (A) and (B) are representative of three independent experiments with similar results.

Figure 6

Neutralization of IP-10 inhibits the migration and trafficking of $\gamma \delta$ Treg cells and enhances anti-tumor immunity in vivo.

(A) Neutralization of IP-10 significantly blocked the breast tumor-induced trafficking of $\gamma \delta^1$ Treg cells in vivo in human breast tumor MDA-MB-453-bearing NSG mice. XenoLight DiR stained $\gamma \delta^1$ Treg cells were imaged at different time points following their adoptive transfer into MDA-MB-453-bearing NSG mice. Concurrently, some tumor-bearing mice were injected with either human IP-10 neutralizing antibody or isotype control antibody (40 $\mu$g/mouse) into tumor sites at 2 hours before $\gamma \delta^1$ Treg cell
adoptive transfer and then at 2, 4, and 6 days after their transfer. Mice were imaged with IVIS spectrum at 3, 5, and 10 days post injection. The circles indicated the tumor growth sites. Data show the images of a single animal as a representative of 5 animals per group. Results are representative of three independent experiments. (B) Treatment with human IP-10 neutralizing antibody dramatically decreased the numbers of adoptively transferred $\gamma\delta$ T cells into the breast tumor tissues. Human $\gamma\delta$ T cells in frozen sections of breast tumors obtained from MDA-MB-453-bearing NSG mice were analyzed at 10 days post T cell adoptive transfer, using immunohistochemical staining with anti-human TCR$\gamma\delta$ antibody. Left panels are photomicrographs of $\gamma\delta$ T cells in tumor tissues from the antibody treatment groups. Right panel shows $\gamma\delta$1 Treg cell numbers per high microscope field in tumor tissues from two treatment groups (n=5 mice/group). Significance was determined by paired t test. (C) Blockage of $\gamma\delta$ Treg cell trafficking into the tumor microenvironment via IP-10 neutralization enhances anti-tumor immunity in vivo. Mixtures of human 586mel tumor cells ($4 \times 10^6$/ mouse) and breast MDA-MB-453 cancer cells ($3 \times 10^6$/ mouse) were subcutaneously injected into NSG mice. Human 586mel-specific CD8$^+$ TIL586 cells ($5 \times 10^6$/ mouse) combined with or without $\gamma\delta$ Treg cells ($3 \times 10^6$/ mouse) were adoptively transferred through intravenous injection on day 3. Concurrently, some tumor-bearing mice were injected with either human IP-10 neutralizing antibody or isotype control antibody (40 $\mu$g/mouse) into tumor sites at 2 hours before T cell adoptive transfer and then every 5 days after their transfer. Tumor volumes were measured every 3 days and presented as mean ± SD (n = 5 mice per group). The significance of difference between groups was determined by paired t-test or the one-way analysis of variance.
Fig. 2  Ye, et al.

A

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p=0.74

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Fig. 4  Ye, et al.

A  

\(\gamma\delta\) T staining

Normal breast No.1

| 100 | 400 |

Breast tumor No.1

| 100 | 400 |

Breast tumor No.2

| 100 | 400 |

IP-10 staining

| 100 | 400 |

| 100 | 400 |

B

| DAPI | Anti-IP-10 | Anti-TCR\(\gamma\delta\) | Merged |

Breast tumor No.1

|  |  |  | |

Breast tumor No.2

|  |  |  | (× 200) |
Fig. 6 Ye, et al.

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Tumor size (mm²) vs. Days post tumor inoculation
Specific recruitment of $\gamma\delta$ regulatory T cells in human breast cancer

Jian Ye, Chunling Ma, Fang Wang, et al.

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