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 (TIL) in the immunosuppressive tumor microenvironment is essential for improving cancer treatment. Enriched γδ T-cell populations in 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 regulatory T cells (γδ Treg) in patients with breast cancer have yet to be elucidated. In this study, we show that IP-10 secreted by breast cancer cells attracted γδ Tregs. Using neutralizing antibodies against chemokines secreted by breast cancer cells, we found that IP-10 was the only functional chemokine that causes γδ Tregs to migrate toward breast cancer cells. In a humanized NOD-scid IL-2Rγnull (NSG) mouse model, human breast cancer cells attracted γδ Tregs as revealed by a live cell imaging system. IP-10 neutralization in vivo inhibited migration and trafficking of γδ Tregs into breast tumor sites, enhancing tumor immunity mediated by tumor-specific T cells. Together, our studies show how γδ Tregs accumulate in breast tumors, providing a rationale for their immunologic targeting to relieve immunosuppression in the tumor microenvironment.

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Introduction

Malignant tumors may use different strategies to evade immune surveillance. Induction and expansion of regulatory T cells (Treg) in the tumor microenvironment is one of those actions that mediate immune suppression and dysfunction in patients with cancer and also becomes a major obstacle for successful immunotherapy (1, 2). This notion is established and supported by many recent studies. Increased proportion of Tregs exist in the tumor-infiltrating lymphocytes (TIL), 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 Tregs are clinically correlated with tumor progression and shown as a prognostic factor for the prediction of outcomes in patients with cancer with different tumors (3, 5, 8–11). Recent-ly, several strategies, including depletion or blockage of Treg suppression through targeting CD25 or CTLA-4 molecules, have been used in animal models and human clinical trials, yielding promising results (12–14).

Besides the naturally occurring CD4+CD25+ Tregs, Tregs are heterogeneous, existing as other subsets including adaptively induced Tregs of Tr1, Th3, CD8+ as well as γδ Tregs, which have all been identified in human cancers (15–19). γδ T cells have been shown to play a regulatory role in different forms of tolerance (20–23) and have broad regulatory effects on CD4+, CD8+, interleukin (IL)-17+, and Tregs 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 γδ Tregs accumulated in breast cancer TILs have strong suppressive activities on responding T cells and block the maturation and activities of dendritic cells (19). To explore the potential functions of γδ Tregs in the immunopathogenesis of human breast cancer, we more recently showed 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 Tregs suppress innate and adaptive immunity through the induction of immunosenescence (32). The involvement of human tumor immunity by this new subset of Tregs was also reported by another group (33). Thus, a better understanding of the suppressive mechanism(s) and regulations of tumor-derived γδ Tregs 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 Tregs in tumor sites will provide alternative strategies for antitumor immunotherapy. One potential mechanism is that the tumor microenvironment preferentially recruits Tregs. It has been shown that the migration of Tregs 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+ Tregs 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 Tregs exist in breast cancer TILs, the origin and mechanisms governing γδ Treg increase in patients with cancer remains unknown.

In our efforts to explore the mechanisms responsible for the accumulation of γδ Tregs in patients with breast cancer, we identified that IP-10 secreted by breast cancer cells significantly attracted the migration of γδ Tregs. Through both in vitro and in vivo studies, we further showed that human breast cancer used the IP-10–mediated recruitment as an important mechanism for the attraction and accumulation of γδ Tregs 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 Tregs into the breast cancer tumor microenvironment and reversing Treg-induced immune suppression.

Materials and Methods

Human samples and cell lines

Tumor samples were obtained from patients with breast cancer treated at the Department of Surgery, Saint Louis University (Saint Louis, MO) 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 (Houston, TX). Peripheral blood mononuclear cells 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 the manufacturer’s instructions. CD4+CD25− Tregs were further purified from CD4+ T cells by fluorescence-activated cell sorting (FACS) analysis after staining with anti-CD25-PE (BD Biosciences). The purity of the T cells was more than 95%, as confirmed by flow cytometry. Human γδ Tregs 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. Melanoma MC133, MC586, and MC136 were established in our laboratory and maintained in RPMI-1640 medium containing 10% fetal calf serum. Melanoma 586mel and paired TILs were obtained from the Surgery Branch, National Cancer Institute (Bethesda, MD). Breast carcinoma cell lines (BC51, BC30, and BC20) were established in our laboratory and maintained in keratinocyte medium containing 25 mg/mL bovine pituitary extract, 5 ng/mL EGF, 2% heat-inactivated FBS, and penicillin–streptomycin (Invitrogen, Inc.).

Generation of TILs

Tumor and normal TILs 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 RPMI-1640, and then cultured in RPMI-1640 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 T cell receptor (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, Inc.). Controls were carried out 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). 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 were 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 [CruzFluor (CFL) 555–conjugated donkey against goat (Santa Cruz Biototechnology) and Alexa Fluor 488–conjugated rabbit against mouse (Cell Signaling Technology)]. Specimens were then counterstained with 4′,6-diamidino-2-phenylindole (DAPI; Invitrogen, Inc.).

Chemotaxis assay

Chemotaxis assays were conducted 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 γδT Treg lines or control T cells (1 × 10^6 cells) were transferred into upper chambers. After 150 minutes 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-monocyte chemotactant protein (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 minutes before conducting 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 phycoerythrin (PE) or fluorescein isothiocyanate (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 Biosciences) and data were analyzed with FlowJo software (TreeStar).

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 the manufacturer’s instructions.

In vivo studies
NOD-scid IL-2R^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 × 10^5) in 100 μL of buffered saline were subcutaneously injected into NSG mice. γδT Treg, γδT and CD4^+ T cells were incubated with 320 μg/mL of XenoLight DiR (Caliper Life Sciences) 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 mm × 10 mm) NSG mice. Five to 10 mice were included in each group. Mice were imaged with an In Vivo Spectrum Imaging System (IVIS; Caliper Life Science) at 60 minutes, and 1, 2, 3, 5, 7, and 10 days after 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 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 transfer. Furthermore, tumor tissues were removed at 10 days after T-cell adoptive transfer and human T-cell infiltration determined using immunohistochemical staining as described earlier. 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^+ TILs were (5 × 10^6/mouse) combined with or without γδ Tregs (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 ± SD. The significance of difference between groups was determined by paired or unpaired two-tailed Student t test or the one-way ANOVA. Differences were considered significant for P values less than 0.05.

Results
High percentages of γδ Tregs infiltrate in breast cancer tissues
To identify the suppressive mechanisms mediated by the breast cancer tumor microenvironment, we have shown that γδT Tregs were prevalent in breast TILs from patients with breast cancer (19, 31, 32). These breast tumor–derived γδT Tregs 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 accumulation in breast tumor sites remains unknown. To further investigate the role and regulation of γδ Tregs 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%), whereas 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. S1A). These results prompted us to investigate whether γδ T cells were prevalent in situ in breast tumor sites (31). We further conducted 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 γδ T1 cells. In addition, higher amounts of γδ1 T cells existed in cancer tissues from the patients with late-stage breast cancer (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 Tregs**

Given the high amounts of γδ1 Tregs 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 Tregs into tumor sites. To test this possibility, we first investigated whether culture supernatants from freshly digested human tumor tissues, including melanoma, breast, and colon cancers, can attract γδ1 Treg migration. As shown in Fig. 2A, we found that supernatants from breast cancer tissues induced significant migration of γδ Tregs 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 Tregs. 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 chemotaxis (Fig. 2B). These results indicate that breast cancer cells secrete molecules that selectively recruit γδ T cells to the tumor microenvironments.

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

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

To investigate which cytokines are secreted by breast cancer cell lines and are involved in the specific recruitment of γδ1 Tregs, 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 granulocyte colony-stimulating factor (G-CSF), MCP-1, and RANTES (regulated upon activation, normal T-cell expressed, and secreted), as well as some fibroblast growth factor (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 recruitment. We used specific neutralizing antibodies against the identified chemokines/cytokines and found that only IP-10-neutralizing
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antibody abolished the chemotactic activity of the breast cancer supernatant to attract γδ Tregs (Fig. 3B). In addition, we found that recombinant IP-10 had the same chemotactic activity for various γδ Treg lines, and the activity displayed a dose-dependent chemotaxis (Supplementary Fig. S1B). 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 γδ Tregs 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 cyto-

metry analyses with intracellular staining of IP-10 expression (Fig. 3C). We then checked whether γδ Tregs solely expressed IP-10 receptor, CXCR3, compared with the other T-cell subsets. As expected, we found that γδ Tregs expressed high level of CXCR3. Furthermore, those γδ Tregs also expressed CCR5, CCR6, and CCR7, but not CCR4. Surprisingly, CD4+CD25+ Treg and naive 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 Tregs. 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 Tregs 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).

Colocalization of γδ Tregs 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 Tregs by breast cancer cells, we determined whether IP-10–expressing tumor cells were colocalized with γδ Tregs 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 conducted. As shown in Fig. 4B, IP-10–expressing breast tumor cells were colocalized with γδ T cells in tumor tissues. These results collectively showed that breast tumor cells can selectively recruit γδ Tregs through the tumor–derived IP-10. Given that the numbers of intratumoral γδ T cells are varied among the patients with breast cancer, we determined the correlation between tumor-infiltrating γδ T cells and tumor IP-10 expression levels in patients with breast cancer. Unexpectedly,
correlation analyses showed that no significant difference of γδ Treg infiltration was found among human breast tumor tissues with different IP-10–expressing levels (Supplementary Fig. S2).

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

These *in vitro* studies provided us important information about the specific recruitment of γδ Tregs 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 carried out complementary *in vivo* studies, using the adoptive transfer of human γδ Tregs into human breast tumor–bearing NSG (lacking T and B cells) mouse models through the IVIS. Human MDA-MB-435 breast cancer cells were subcutaneously...
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Injected into NSG mice to establish tumor-bearing mice. After tumor sizes reached to about 10 mm × 10 mm, Xenolight DiR-stained γδ1 Tregs were adoptively transferred into MDA-MB-453–bearing NSG mice. In addition, γδ2 T and CD4+ 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 after T-cell adoptive transfer.

As shown in Fig. 5A, in early time points (before 3 days) after T-cell injection, γδ1 Tregs randomly migrated into different organs, including spleen, cervical, and peripheral lymph nodes, as well as tumor sites. However, the signal density of γδ Tregs significantly increased in tumor sites in the late time points (after 3 days) after T-cell transfer, and this T-cell accumulation continued to persist through the whole observation period (10 days), indicating accumulation of γδ1 Tregs into the tumor sites. Similar to γδ1 Tregs, human γδ2 and CD4+ T cells also migrated and distributed into spleen, cervical, peripheral lymph nodes, and tumor sites at the early times. However, unlike γδ1 Tregs, γδ2 T and CD4+ cells did not accumulate in tumor sites and even decreased at the late times (after 3 days).

Notably, adoptively transferred human γδ1 Treg, γδ2 T, and CD4+ T cells markedly proliferated within NSG mice, and the proliferation capacity of γδ1 Tregs 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 γδ1 Treg 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 γδ2 T cells (Fig. 5B). Collectively, these results clearly indicate that human breast tumor cells can selectively attract γδ1 Tregs into tumor sites in vivo.

We then determined whether the human breast tumor–induced trafficking and migration of γδ Tregs into the tumor sites is controlled by the tumor-derived IP-10 in vivo, using this adoptive transfer tumor model. γδ1 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 γδ1 Treg adoptive transfer, and the γδ1 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 γδ1 Tregs into tumor sites (Fig. 6A). Furthermore, we confirmed using the immunohistochemical staining of γδ T cells in tumor sections that high amount of γδ Tregs 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 γδ1 Tregs into breast tumor sites in vivo.

Blockage of γδ Treg cell trafficking into the tumor microenvironment via IP-10 neutralization enhances antitumor immunity in vivo in an adoptive transfer therapy model

We next explored whether blockage of γδ Treg cell trafficking and migration into the tumor sites can enhance antitumor immunity, using our previously established adoptive transfer immunotherapy model (19, 42, 43). Given that human 586mel tumor cells do not express IP-10 and IP-10–expressing melanoma cells cannot survive in vivo (44), we thus used a mixture of human 586mel tumor cells (as a target for tumor-specific T cells) and human breast MDA-MB-453 breast cancer cells (as a source of IP-10 for γδ 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+ TILs/586 cells combined with or without breast cancer–derived γδ Tregs 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 did not 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. S3). 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 γ6 Tregs 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 γ6 Treg infiltration into tumor sites and enhances the antitumor ability mediated by TIL586 CD8\(^+\) T cells (Fig. 6C). Taken together, our studies clearly indicate that blockade of γ6 Treg cell trafficking into the tumor microenvironment via IP-10 neutralization can mediate enhanced antitumor immunity in vivo, and could be a promising strategy for breast cancer immunotherapy.

Figure 5. Visualization of breast tumor–induced γ6 Treg cell trafficking and migration in vivo with live imaging analyses. A, γ6 Tregs specifically accumulated in tumor sites in human breast tumor MDA-MB-453–bearing NSG mice. In contrast, γ62 and CD4\(^+\) T cells, served as controls, randomly migrated and distributed in the MDA-MB-453–bearing NSG mice. XenLight 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 indicate 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 γ6 T cells, but not γ62 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 after T-cell adoptive transfer using immunohistochemical staining with anti-human CD3 antibody in the frozen sections. Left, photomicrographs of CD3\(^+\) T cells in tumor tissues from different adoptive transfer groups. Right, a summary of γ6 Treg, γ62, 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.
Discussion

It is well established that recruitment of Tregs into tumor microenvironments is one of the major strategies used by tumor cells to induce immune suppression and evade immune surveillance (1, 45). Breast cancer can recruit FoxP3⁺ Tregs into tumor sites, leading to impaired antitumor immune responses. One approach to overcoming this immune suppression is to block chemokines that recruit Tregs into the tumor microenvironment. In this study, we investigated the role of IP-10 in recruiting Tregs into breast cancer. Neutralization of IP-10 inhibited the migration and trafficking of γδ Tregs and enhanced antitumor immunity in vivo. This was demonstrated by blocking the breast tumor-induced trafficking of γδ Tregs in vivo in human breast tumor MDA-MB-453-bearing NSG mice. XenoLight Dil-stained γδ Tregs 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 μg/mouse) into tumor sites at 2 hours before γδ Treg 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 after injection. The circles indicate 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 γδ T cells into the breast tumor tissues. Human γδ T cells in frozen sections of breast tumors obtained from MDA-MB-453-bearing NSG mice were analyzed at 10 days after T-cell adoptive transfer using immunohistochemical staining with anti-human TCR γδ antibody. Left, photomicrographs of γδ T cells in tumor tissues from the antibody treatment groups. Right, γδ Treg 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 γδ Treg cell trafficking into the tumor microenvironment via IP-10 neutralization enhances antitumor immunity in vivo. Mixtures of human 586mel tumor cells (4 × 10⁵/mouse) and breast MDA-MB-453 cancer cells (3 × 10⁶/mouse) were subcutaneously injected into NSG mice. Human 586mel-specific CD8⁺ TIL586 cells (5 × 10⁶/mouse) combined with or without γδ Tregs (3 × 10⁶/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 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 ANOVA.
responses and promoting tumor metastases (10, 46). Besides FoxP3+ Tregs, we have further shown that high proportion of γδ Tregs existed in human patients with breast cancer (19, 31). These breast tumor-derived γδ Tregs have potent suppressive activities on CD4+ and CD8+ T-cell proliferation and effector functions, as well as on denritic cell maturation and activities (19, 31, 32). However, the origin and mechanisms governing γδ Treg increase in patients with breast cancer remains unknown. In the current study, we carried out both in vitro and in vivo studies showing that breast cancer cells secrete IP-10, which mediates the trafficking and migration of γδ Tregs 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+ Tregs exist in peripheral blood, tumor draining lymph nodes, and TILs in patients with different types of cancers (3–5). Importantly, the increased frequencies of Tregs were clinically correlated with tumor pathogenesis (5, 8, 47, 48). We have recently shown high percentages of γδ Tregs with potent suppressive activity among TILs from patients with breast cancer (19). We further analyzed in situ the quantity of γδ T cells in tumor tissues with different cancer stages using immunohistochemical staining, and then conducted 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 patients with cancer (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 Tregs is essential for breast cancer immunotherapy.

The accumulation of Tregs in the tumor microenvironment may be due to different potential mechanisms, including trafficking, expansion, or conversion (1). Our current study strongly suggests that breast tumor cells may favor the prevalence of γδ Tregs in tumor microenvironments through the recruitment of γδ Tregs from periphery into tumor sites. Our in vitro studies showed that culture supernatants from breast cancer cells can significantly induce chemotaxis of γδ Tregs that was dependent of cancer cell-secreted IP-10. We further showed that IP-10 controlled breast tumor–derived γδ Treg migration and trafficking into tumor sites in vivo in animal models using an IVIS. Besides the IP-10–mediated recruitment of γδ Tregs 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 expansion and conversion. Studies from conventional Tregs have shown that tumor microenvironment factors, including IL-10 and TGF-β, VEGF as well as some types of denritic cells and regulatory B cells, can convert naïve and/or effector T cells into suppressive Tregs (49, 50). Our data have shown that breast cancer cells also secrete VEGF and TGF-β (Fig. 3A), and there is no significant correlation between numbers of γδ Treg infiltration and IP-10 expression levels among human breast tumor tissues. Furthermore, we observed nonsuppressive γδ T cells existing in the breast cancer TILs (19). Thus, there still exists a possibility that conversion in situ may contribute to the accumulation of γδ Tregs in the breast cancer tumor microenvironment. We will continue our efforts to explore other potential mechanisms responsible for the accumulation of γδ Tregs in human breast cancer in the future studies.

Treg migration into tumor sites is dependent on the interactions between chemokines secreted by the tumor microenvironment and the chemokine receptors expressed on Tregs (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 γδ Tregs. In addition, our in vitro and in vivo studies clearly showed that interaction between IP-10 (tumor cells) and CXCR3 (γδ Tregs) is the main mechanism responsible for γδ Treg trafficking and migration into tumor sites. Interestingly, CXCR3 is expressed on almost all different types of T cells, including Th1, Th17, CTLs, and Tregs (Fig. 3; ref. 38). However, we observed that IP-10 secreted by breast cancer cells only selectively attracted the migration of γδ Tregs, but not other CXCR3-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 denritic cell 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 used in clinical trials for tumor immunotherapy (12–14). However, our identification of γδ Tregs in the patients with breast cancer further suggests that control of CD4+CD25+ Tregs is not enough for the reversal of immune suppression. We have shown that human TLR8 signaling reversed the suppressive functions of tumor-derived CD4+, CD8+, and γδ Tregs (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 γδ Treg into tumor sites, and that blockage of γδ Treg trafficking into the tumor microenvironment significantly inhibits their suppression and enhances antitumor immunity. These studies provide promising alternative strategies that would augment the antitumor immune responses for human breast cancer immunotherapy. One way that we can functionally inactivate Treg suppression without changing the Treg repertoire or effector T-cell functions is via TLR8 signaling. In addition, we may block the recruitment and generation of γδ Tregs through IP-10/CXCR3 by the breast tumor microenvironment.
Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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