Squamous Cell Tumors Recruit γδ T Cells Producing either IL17 or IFNγ Depending on the Tumor Stage

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Abstract

The identification of reciprocal interactions between tumor-infiltrating immune cells and the microenvironment may help us understand mechanisms of tumor growth inhibition or progression. We have assessed the frequencies of tumor-infiltrating and circulating γδ T cells and regulatory T cells (Treg) from 47 patients with squamous cell carcinoma (SCC) to determine if they correlated with progression or survival. Vδ1 T cells infiltrated SCC tissue to a greater extent than normal skin, but SCC patients and healthy subjects had similar amounts circulating. However, Vδ2 T cells were present at higher frequencies in circulation than in the tissue of either cancer patients or healthy donors. Tregs were decreased in the peripheral blood of SCC patients, but were significantly increased in the tumor compartment of these patients. Tumor-infiltrating γδ T cells preferentially showed an effector memory phenotype and made either IL17 or IFNγ depending on the tumor stage, whereas circulating γδ T cells of SCC patients preferentially made IFNγ. Different cell types in the tumor microenvironment produced chemokines that could recruit circulating γδ T cells to the tumor site and other cytokines that could reprogram γδ T cells to produce IL17. These findings suggest the possibility that γδ T cells in SCC are recruited from the periphery and their features are then affected by the tumor microenvironment. Elevated frequencies of infiltrating Vδ2 T cells and Tregs differently correlated with early and advanced tumor stages, respectively. Our results provide insights into the functions of tumor-infiltrating γδ T cells and define potential tools for tumor immunotherapy. Cancer Immunol Res, 5(5): 397–407. ©2017 AACR.

Introduction

Epithelial surfaces are selective barriers and, together with immune system, defend from tissue-damaging radiation, bacterial and viral invasion, toxins and mutagens. Skin cancers [melanoma, basal cell carcinoma (BCC) and squamous cell carcinoma (SCC)] are the most frequent cancer types in white populations (1), with SCC having an 18 to 20 times higher incidence than malignant melanoma (2, 3).

Tumor-infiltrating leukocytes have been found in different solid tumors (4) and the extent of their infiltration has been often associated with improved prognosis (5). A minor population of T cells, named γδ T cells, that is about 5% of CD3+ T cells in the peripheral blood, is present in other anatomic sites, such as the intestine or the skin (6). These invariant γδ T cells bind to different ligands, depending on which V genes are used. Those expressing the Vδ2 gene paired with the Vγ9 chain account for 50% to >90% of the γδ T-cell population in the peripheral blood of healthy subjects and recognize phosphoantigens (PAg) in association with butyrophilin 3A1 (BTN3A1; ref. 7), either exogenous PAgS derived from the microbial nonmevalonate isoprenoid byosynth-esis pathway or endogenous PAgS derived from the mevalonate pathway in human cells, which is highly active upon infection or tumor transformation (8, 9). In contrast, γδ T cells that express Vδ1 gene paired with different Vγ elements, abound in the intestine or the skin (10), recognize the MHC class I-related molecules MICA, MICB, and ULBP2 expressed on epithelial cells upon oxidative stress and heat shock and on many hematopoietic and epithelial tumoral cells (8, 11).

γδ T cells are among the TILs in many types of cancer, but it is unclear if they correlate positively or not with tumor growth or have any prognostic value. For instance, Vδ1-expressing and IL17-producing γδ TILs are negative prognosticators in colorectal and breast cancer (12–14), whereas the presence of the Vδ2 subset among TILs positively correlates with early-stage melanoma (15)
and patient’s survival. Taken together, these data show a dual role for γδ T cells in the tumor microenvironment that probably depends on the specific γδ T-cell subset/function recruited to the tumor site.

In contrast, regulatory T cells (Treg) inhibit T-cell activation and impair antitumor immune responses. Several human cancers, including SCC, have elevated frequencies of Tregs in peripheral blood and within tumors, which are associated with poor outcomes (16–18). In addition, human skin also contains a resident population of Tregs that account for 5% to 10% of the total skin-resident T cells (17, 19, 20). A reciprocal cross-regulation has been found between γδ T cells and Tregs during mycobacterial infection: Tregs inhibit γδ T-cell proliferation and IFNγ production (21, 22), but, on the other hand, Vγ9Vδ2 T cells antagonize expansion of Tregs and subsequent suppression of T-cell responses (23). However, the relationship between Tregs and γδ T cells in SCC and other cancers has not been studied thoroughly.

In this article, we have analyzed the frequency and the phenotype of γδ T cells (both Vδ1 and Vδ2 T cells) and Tregs among TILs and in the peripheral blood of patients with SCC to determine if a specific subset of γδ T cells contributes to the pathogenesis of SCC.

Materials and Methods

Characteristics of sample cohort

In this study, we enrolled 47 patients with histologically confirmed diagnosis of cutaneous SCC treated between 2012 and 2015 at the Plastic Surgery Unit of the University of Palermo. The cohort was composed by 9 females and 38 males. The median age was 71 years for male and 74 for female (range, 45–96). All patients were staged according to the international staging system for skin cancer as described in Supplementary Table S1. Patients were followed for a minimum of 1 year and data on disease-related mortality and relapse rates were collected.

Healthy subjects were volunteers in good and stable clinical condition and had laboratory parameters in the physiologic range; median age was 74 years (range, 45–96).

Blood was drawn before the surgical excision. The study was approved by the Ethical Committee of the University Hospital, Palermo, where the patients were recruited. The study was performed in accordance with the principles of the Helsinki declaration. Blood was drawn before the surgical excision. The study was approved by the Ethical Committee of the University Hospital, Palermo, where the patients were recruited. The study was performed in accordance with the principles of the Helsinki declaration.

Isolation of circulating and tumor-infiltrating immune cells and flow cytometry analysis

Peripheral blood mononuclear cells (PBMCs) were obtained by density gradient centrifugation using Ficoll–Hypaque (Pharmacia Biotech). Tissue specimens were obtained from 47 different individuals and were used to study the composition of the different subpopulations. The fluororescin isothiocyanate (FITC)-, phycoerythrin (PE), PE-Cy5-, allophycocyanin (APC)-, phycoerythrin-Cy7 (PECy7)-, allophycocyanin-Cy7 (APC-Cy7)-conjugated monoclonal antibodies (mAb) used to characterize the entire population were the following: anti-CD3 (Cat 45-0037 eBioscience, Cat 300412 and Cat 300420 BioLegend), anti-CD45 (Cat 560274 BD Biosciences), anti-pan-γδ TCR (Cat 555717 BD), anti-Vδ1 (Cat PG196007 Thermo Fisher), anti-Vδ2 (Cat 331408 BioLegend), anti-CD27 (Cat 17-0279 eBioscience), anti-CD45RA (Cat 25-0458 eBioscience), anti-CD14 (Cat 325632 BioLegend), anti-CD19 (Cat 302228 BioLegend). Anti-CXCR1 (Cat 555939), anti-CXCR2 (Cat 551127), anti-CXCR3 (Cat 560831), anti-CXCR4 (Cat 560669), anti-CCR2 (Cat 561744), anti-CCR3 (Cat 564189), and anti-CCR5 (Cat 555992) were all purchased from BD Biosciences. To evaluate Tregs, we used anti-CD3 (Cat 45-0037 eBioscience), anti-CD4 (Cat 130-080-501 Miltenyi Biotec), anti-CD45 (Cat 560274 BD Biosciences), anti-CD25 (Cat 130-101-426 Miltenyi Biotec), and FoxP3 (Cat 130-093-013 Miltenyi Biotec). Cells were fixed and permabilized according to the manufacturer’s instructions (Miltenyi Biotec GmbH, Treg detection kit). Expression of surface markers was determined by flow cytometry on a FACSCanto II Flow Cytometer with the use of the FlowJo software (BD Biosciences). The gating strategy involved progressively measuring total cells, viable cells only, and lymphomonocytes and specific cell types. For every sample, 100,000 nucleated cells were acquired and values are expressed as a percentage of viable lymphomonocytes, as gated by forward and side scatter.

Cytokine production

The cytokine production capacity of Vδ1 and Vδ2 T cells was determined after stimulation with PMA (BD Biosciences, 20 ng/mL, final concentration) and ionomycin (BD Biosciences, 1 μmol/L, final concentration) for 4 hours at a cell concentration of 106/mL. After 1-hour stimulation, Monensin (Sigma-Aldrich, 2 μmol/L, final concentration) for 4 hours at a cell concentration of 106/mL. After 1-hour stimulation, Monensin (Sigma-Aldrich, 2 μmol/L, final concentration) was added, and the cells were incubated at 37°C in 5% CO2 for remaining time. Staining of intracellular cytokines was performed by incubation of fixed permeabilized cells with FITC- or PE-labelled anti-IFNγ (Cat 554700 and 554701 BD Biosciences) and APC-labelled anti-IL17A mAbs (Cat 506916 BioLegend). After two more washes in PBS containing 1% FCS, the cells were analyzed by FACS as above described. Viable lymphocytes were gated by forward and side scatter, and analysis was performed on 100,000 acquired events for each sample by using FlowJo and the following gating strategy to detect lymphocytes: FSC/SIDE, live cells, single cells, double-positive CD3, and TCR Vδ1 and Vδ2 cells.

Negative control (background) values were not subtracted, as the median backgrounds for isotype-matched mAbs was 0.0028% (range, 0.0006–0.0063%). Samples were considered positive if the number of cells was equal to or greater than 0.01% and at least 10 clustered events were apparent. This empiric cutoff value was predicted to be >90% different from background, at an α of 0.05 (24).

CAF, MSC, and SDAC conditioned medium; cytokine and migration analyses

Primary cancer-associated fibroblasts (CAF), skin squamous cancer stem cells (SCC), and sphere-derived adherent cells (SDAC) were obtained from surgical resection of skin SCC.
subjected to mechanical and enzymatic digestion with collagenase (0.6 mg/mL, Gibco) and hyaluronidase (10 μg/mL, Sigma). Cell suspension was cultured in 10% fetal bovine serum (FBS) Dulbecco’s Modified Eagle’s Medium (DMEM) in adhesion flasks, in order to obtain CAFs or in low-adhesion conditions and in serum-free medium supplemented with EGF and β-FGF, which allows the selective growth of skin squamous CSCs. Skin squamous CSCs were cultured in 10% FBS DMEM in adherent conditions to obtain SDACs. Cells were washed twice in PBS and incubated in their fresh CSC medium for 48 hours. CSCs medium was deprived of EGF and β-FGF. The medium was then collected and used for luminescent assay.

Forty-eight cytokines [IL1α, IL1β, IL1R antagonist, IL2, IL2Ra, IL3, IL4, IL5, IL6, IL7, IL9, IL10, IL12 (p40), IL13, IL15, IL16, IL17, IL18, TNF-α, TNF-β, IFNα2, IFNγ, G-CSF, GM-CSF, M-CSF, FGF-β, VEGF, PDGF, MIF, MIG, HGF, LIF, β-NGF, SCF, SCGF-β, SDF-1α, TRAIL, eotaxin, IP-10, IL8, MIP-1α, MIP-1β, MCP-1, RANTES, CTACK, GRO-α, and MCP-3] were analyzed in CAF, CSC, and SDAC-conditioned medium obtained from SCC by xMAP multiplex technology on the Luminex platform (Luminex), using Bio-Rad reagents (Bio-Plex Pro Human Cytokine 27-plex Assay #M500KCAF0Y and Bio-Plex Pro Human Cytokine 21-plex Assay #MFO005KMII, Bio-Rad) acquired and analyzed with the Bioplex Manager Software (Bio-Rad). Responses were scored positive if the value was 2-fold over the negative control. Briefly, 50 μl bead solution (containing assay buffer and 5,000 beads) was added to the appropriate wells in a 96-well Millipore plate (Millipore). Fifty microliters assay buffer was added to each standard well and 50 μl diluted standard serum pool, diluted 2-, 4-, 8-, 16-, 32-, 64-, 128-, 256-, 512-, and 1024-fold from 1:25 to 1:3,200 to each standard well and 50 μl diluted streptavidin–R–PE, diluted 1:250 in assay buffer, was added to each well. After 4 hours of incubation at 37°C, the bead solution was washed once with RPMI1640 medium, cell count readjusted by Trypan Blue, and 20 μl cell suspension was placed in the top chamber of the Transwell. As a negative control, 50 μl washed medium without FCS was added to the lower chamber. As a positive control with FCS, 50 μl cell suspension was added to the top chamber of the Transwell plate (Millipore). Fifty microliters sample, diluted to 1:25 and 1:400, respectively, was added to each sample well. Standard and positive controls were diluted in assay buffer, and samples were diluted in assay buffer with 10% sample blocking buffer. After 30 minutes of incubation at room temperature on a plate shaker and two washes, 50 μl biotinylated detection Ab, diluted 1:1000 in assay buffer with a final Ab concentration of 1.0 μg/mL, was added to each well. After further 30 minutes of incubation at room temperature on a plate shaker and two washes, 50 μl diluted streptavidin–R–PE, diluted 1:250 in assay buffer, was added to each well. After further 30 minutes of incubation at room temperature on a plate shaker and two washes, the samples were analyzed on the Luminex machinery (Luminex).

TGFβ was measured by ELISA according to the manufacturer’s instructions (R&D Systems).

γδ T cells migration was evaluated using a 24-well Transwell plate (8.0-μm pore size; Corning); in brief, γδ T cells were washed once with RPMI1640 medium, cell count readjusted (105 cells/mL) in T-cell medium, and an aliquot (100 μL) of T-cell suspension was placed in the top chamber of the Transwell. The bottom chamber of the Transwell plate received conditioned medium obtained from SCC tissue upon culture in the absence of FCS at the indicated concentration prior to the addition of T cells in the top chamber. As a negative control, fresh medium without FCS was added to the lower chamber. After 4 hours of incubation at 37°C in a 5% CO2 atmosphere, the top chamber was removed, and the γδ T cells that had migrated into the bottom chamber were stained by the use of a modified Giemsa staining (DiffQuik). Cells in 3 or 4 randomly chosen fields (×400 magnification) were counted. For each experiment, 3 replicates were performed.

Figure 1.
Frequency of infiltrating γδ T cells expressing either Vδ1 or Vδ2 TCR-δ chains in HDs and SCC patients. A, Flow cytometry plot of representative primary data to define γδ T cells expressing either Vδ1 or Vδ2 TCR-δ chains in tissue and peripheral blood of HD subjects and SCC patients. Viable lymphocytes were gated by forward and side scatter, and the analysis was performed on 100,000 acquired events by using FlowJo. The following gating strategy was used to detect γδ T lymphocytes: FSC/SSC, live cells, single cells, CD3/Vδ1 or CD3/Vδ2 double-positive T cells. B, Histogram of cumulative data from 47 patients. Error bars indicate SEM (*, P < 0.01; **, P < 0.05). Data are from three independent experiments.
Statistical analysis

Statistical analysis was performed using GraphPad software (Prism Software). Data from different groups were compared with the nonparametric two-tailed Mann–Whitney test and Student t test. A two-tailed nonparametric Spearman test was used to compare the effect of cancer stage on the recovery rate and percentage of γδ T cells. Differences between group of patients with a probability of <0.05 were regarded as significant. All P values are two-tailed, and all statistical analyses were performed using the GraphPad software.

SPADE analysis. Spanning-tree progression analysis of density-normalized events (SPADE; ref. 25) clustering algorithm on the Cytobank.org platform was done to visualize single cells, among the live CD45+ lymphocytes from six subjects. The nodes of the tree reproduce clusters of cells that are similar in marker expression. SPADE uses the size and color of each node to signify the number of cells and median marker expression, respectively.

Results

γδ T cells were increased in TILs of SCC patients

In order to study the prevalence of γδ T cells among TILs in SCC, a single-cell suspension of tumor tissue or normal skin from healthy donors was prepared by enzyme digestion and Vδ1 or Vδ2 cell composition assessed by immunophenotyping. The results were compared with the frequencies of γδ T cells in peripheral blood mononuclear cells (PBMC) isolated from the same subjects. Figure 1A describes representative data of infiltrating and circulating γδ T cells expressing Vδ1 or Vδ2 TCR δ chains in SCC patients and in healthy donors (HD).

The γδ T cells expressing Vδ1 or Vδ2 were slightly increased in PBMCs of SCC patients compared with HDs, but only Vδ2 differences were statistically significant (Fig. 1B). The frequency of Vδ1 T cells was significantly elevated in tumor compartments, but not in the peripheral blood of SCC patients, and the relative percentage of Vδ1 T cells was significantly higher in TILs of SCC patients compared with that of normal skin obtained from HDs.

Similarly, the percentage of Vδ2 T cells was faint and often undetectable in normal skin of HDs, but significantly increased in tissue of SCC patients. Finally, and as expected, Vδ2 T cells were significantly elevated in the peripheral blood of SCC patients compared with healthy donor peripheral blood. Altogether, these results indicate that γδ T cells are an important component of TILs in SCC patients.

Phenotypic analysis of γδ T cells in SCC patients

Human γδ T cells include those with naïve or central-memory phenotypes (Tnaive, CD45RA+CD27+; TCm, CD45RA-CD27+) that are abundant in secondary lymphoid organs, without immediate effector function, and those with effector-memory (TEM, CD45RA-CD27-) and terminally differentiated (TEMRA, CD45RA+CD27-) phenotypes that migrate to inflammatory sites where they exert effector functions as cytokine production and cytotoxic activity (26). We studied the memory subset distribution of circulating and tumor-infiltrating γδ T cells, assessing the expression of CD27 and CD45RA on Vδ1 and Vδ2 cells. Analysis showed that the majority of Vδ1 and Vδ2 T cells in TILs of SCC

![Figure 2](image_url)

**Figure 2.**

Memory phenotype of Vδ1 and Vδ2 T cells in TILs and PBMCs of SCC patients. Phenotypical analysis of (A) infiltrating and (B) circulating Vδ1 and Vδ2 T cells from SCC patients and HDs upon staining with mAbs to CD45RA and CD27, after gating on CD3+ Vδ1+ or CD3+ Vδ2+ T cells. Data are from three independent experiments. C, Flow cytometry panels of a representative experiment, using the gating strategy described in the legend to Fig. 1. Isotype-matched mAbs were used as controls.
patients had a TEM (50%) phenotype, whereas the vast majority of V61 and V62 T cells in normal skin from HDs had a TCM (80%) phenotype (Fig. 2A).

To understand if the predominance of tumor-infiltrating V61 and V62 T cells with a TEM phenotype was due to the tumor microenvironment or simply reflected an overall bias in SCC patients, we studied the phenotype of γδ T cells in the PBMCs of HDs and SCC patients, and compared them with the phenotype of the infiltrating γδ T cells. V61 and V62 T cells obtained from PBMCs of HDs showed a predominant TCM phenotype, but V61 and V62 T cells obtained from PBMCs of SCC patients showed a homogeneous distribution of all memory subsets, with a slight and nonsignificant predominance of the TEMRA phenotype (Fig. 2B). In any event, both V61 and V62 γδ T cells with a TEM phenotype were poorly represented among circulating γδ T cells of HDs and SCC patients (usually less than 10%), while being the predominant population (>50%) in the tumor-infiltrating γδ T cells.

Thus, the γδ T cells committed to effector activities at the tumor site are phenotypically different with respect to circulating and/or resident γδ T cells. A typical FACS analysis of one representative sample illustrates the different phenotypes of V61 and V62 γδ T cells in each tested group (Fig. 2C).

Cytokine production by γδ T cells in SCC patients

In order to study cytokine production by tumor-infiltrating and circulating γδ T cells in SCC patients, PBMCs were isolated from SCC patients (n = 28) and HDs (n = 10). Similarly, single-cell...
suspension of tumor tissue (n = 45) or normal skin from HDs (n = 10) was prepared. Cells were stimulated with PMA and fomofycin in the presence of Brefeldin A followed by immunophenotyping. Figure 3A describes representative gating strategy to define Vδ1 and Vδ2 γδ T cells making IL17 or IFNγ, Vδ1 T cells from the PMBCs of both HDs and SCC patients, and normal skin of HD, preferentially produced IFNγ, whereas IL17 production was very low, if any (Fig. 3B). Conversely, Vδ1 T cells from TILs of SCC patients preferentially produced IL17, whereas IFNγ production was decreased compared with that of Vδ1 T cells from the PMBCs of both HDs and SCC patients, and normal skin of HDs.

Vδ2 T cells from PMBCs of HDs and SCC patients preferentially produced IFNγ, but not IL17; the intratumoral Vδ2 T cells from SCC patients behaved likewise. Production of IL17 was low in Vδ2 T cells from TILs of SCC patients, but significantly higher compared with that of Vδ2 T cells from the PMBCs of both HDs and SCC patients. The yield of primary Vδ2 T cells isolated from the normal skin from HDs was always too low to allow analysis of cytokine production. Altogether, these results indicated that γδ T cells (both Vδ1 and Vδ2), making IL17 (IL17-producing γδ T cells) were increased at the tumor site, but not in the circulation, of SCC patients.

To better define the IL17 or IFNγ profiles of tumor-infiltrating γδ T cells of SCC patients and HD, we used the SPADE (Spanning-Tree Progression Analysis of Density-normalized Events) algorithm, which distinguishes cell subsets by clustering, based on surface antigen expression denoted by a colour gradient (Fig. 3C). IL17 had homogeneous maps with highest intensity of Vδ1 expression, whereas IFNγ had diverse maps compared with that of IL17 and the highest intensity of Vδ2 surface marker expression.

Possible γδ T cell recruitment and polarization in the SCC tumor microenvironment

The increased frequency of γδ T cells observed in the tumor environment of SCC patients suggested that they would be migrating to the tumor from the circulation. To test this hypothesis, we analyzed expression of chemokine receptors on circulating γδ T cells of HDs and SCC patients. The γδ T cells had elevated expression of a variety of chemokine receptors, among which were CXCR1, CXCR2, CXCR3, CXCR4, CCR2, CCR3, and CCR5 (Fig. 4A). Of note, CCR5 was preferentially expressed on Vδ2, but not on Vδ1 T cells, whereas Vδ1 T cells expressed more CXCR1 than Vδ2 T cells. Other chemokine receptors were equally expressed by Vδ1 and Vδ2 T cells. Next, we evaluated chemokine production in the context of the tumor microenvironment. To this end, we...
cultured allogeneic CAFs, SDACs, and CSCs from tumor tissues of SCC patients (n = 5), and chemokine concentrations were measured in culture supernatant collected after 48 hours; as a control, we also analyzed fibroblasts isolated for the skin of HDs. CSCs produced large amounts of CXCL1, CXCL8, and CCL2, and intermediate concentrations of CXCL9, CXCL10, CXCL12, CCL5, CCL7, and CCL11 (Fig. 4B). Conversely, both CAFs and SDACs had a more limited chemokine-producing capacity and secreted high amounts of CXCL1 and CXCL8, and intermediate concentrations of CXCL12 and CCL2. Thus, several cell types in the tumor microenvironment of SCC patients produce chemokines with the potential to recruit circulating γδ T cells that express the relevant chemokine receptors.

In order to investigate γδ T-cell migration to the tumor microenvironment, we performed a migration assay in the presence of bulk SCC supernatant. The γδ T cells were attracted by SCC-conditioned medium compared with medium, indicating that chemokines present in the tumor microenvironment may cause recruitment of γδ T cells at the tumor site (Fig. 4C).

We next addressed the possibility that the tumor microenvironment of SCC patients also contained cytokines capable of polarizing the differentiation of IL17-producing γδ T cells; in fact, we previously demonstrated that γδ T cells may be induced to produce IL17 by combinations of IL6, TGFβ, IL1β, and IL23 (27). CSCs, CAFs, and SDACs produced significantly elevated concentrations of IL6. CSCs produced intermediate amounts of IL23 and TGFβ, and less IL1β, and CAFs produced much TGFβ, and little IL1β and IL23 (Fig. 4D). Finally, SDACs produced little TGFβ, IL1β, and IL23. Altogether, these results show that cytokines with the potential to polarize IL17-producing γδ T cells are produced by different cell types in the tumor environment of SCC patients.

Correlation between cytokine production, clinical stage of SCC, and clinical outcome
To investigate the clinical significance of γδ T cells, frequencies of these cells in TILs were analyzed according to the clinical stage and the clinical outcome of SCC patients. Because no reference value for these populations was available, patients were divided in two groups based on mean percent values of respective cells (data not shown and Fig. 5).

We observed no associations between the percentage of intratumoral Vδ1 and Vδ2 T cells and any of the well-established...
prognostic factors for SCC (clinical stage, recurrence/not recurrence, lymph node invasion, and overall survival; data not shown).

However, a correlation was found between the capability of \( gd \) T cells in TILs to produce IL17 or IFN-\( \gamma \), and the clinical stage of SCC patients (Fig. 5A). Significantly more IL17-producing V\( \delta 1 \) and V\( \delta 2 \) T cells were higher in SCC patients at stages I and II, but significantly decreased in patients with advanced disease (stages III and IV). As a control, percentages of \( gd \) T cells among TILs of SCC patients did not change with disease stage (Fig. 5B). This clearly indicated that the immune response was skewed toward IL17-producing cells in SCC patients.

We also assessed whether any correlation existed between IL17- or IFN-\( \gamma \)-producing intratumoral V\( \delta 1 \) and V\( \delta 2 \) T cells and clinicopathologic staging and follow-up data of SCC patients (Fig. 5C). The frequency of IL17-producing V\( \delta 1 \) and V\( \delta 2 \) T cells was higher in patients with high relapse, lymph node metastasis, and mortality rates, whereas the frequency of IFN-\( \gamma \)-producing V\( \delta 2 \) T cells was higher in patients with favorable outcome (no relapse, no lymph nodes invasion, and alive).

CD4\( ^+ \)CD25\( ^+ \)Foxp3\(^+ \) Tregs are increased in TILs of SCC patients

The frequency of Tregs in PBMCs of SCC patients and HDs was analyzed by surface staining for CD4 and CD25, followed by intracellular staining for Foxp3. We assessed infiltrating and circulating Tregs either in SCC patients or in HDs (Fig. 6A). Tregs were slightly decreased in PBMCs of SCC patients compared with HDs, but differences did not attain statistical significance. However, the percentage of Tregs in TILs was significantly higher than in PBMCs of SCC patients and HDs. The percentage of Tregs was significantly higher in TILs from SCC patients compared with the normal skin of HDs (Fig. 6B). Therefore, Tregs were decreased in the peripheral blood of SCC patients, but were significantly increased in the tumor compartment of these patients.

The frequency of infiltrating Tregs was significantly higher in SCC patients with advanced disease (stages III and IV) who had lymph node metastasis and a high relapse rate, and Treg frequency correlate with overall survival (Fig. 6C and 6D).

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**Figure 6.**
Frequency of infiltrating and circulating Tregs in HDs and SCC patients. **A,** Flow cytometry plot of representative primary data to define Tregs in tissue and peripheral blood of HD subjects and SCC patients. Analysis was performed as described in the legend to Fig. 1. The following gating strategy was used to detect Tregs: FSC/SSC, live cells, single cells, CD3/CD4 double-positive T cells, and CD25 “Foxp3”. Isotype-matched mAbs were used as controls. **B,** Histogram of cumulative data from 47 patients. Error bars indicate SEM (**, \( P < 0.01 \); *, \( P < 0.05 \)). Data are from three independent experiments. **C,** Cumulative data of the frequency of Tregs in SCC patients at early or late clinical stage (I-II vs. III-IV) and in HD subjects. Error bars indicate SEM (**, \( P < 0.05 \)). **D,** Cumulative data of the frequency of Tregs in SCC patients related with relapse rate and overall survival; error bars indicate SEM (**, \( P < 0.05 \)).
Correlations between Tregs, V61 and V62 T cells, clinical stage, and outcome

A significant inverse correlation was found between V62 T cells and Tregs at early clinical stages (I and II) of SCC, which flipped to a statistically significant direct correlation in stages III and IV. Conversely, no correlation was found between Tregs and V61 T cells at different tumor stages (data not shown). This analysis suggests that a reciprocal relationship may exist between Tregs and V62 T cells, which is largely influenced by the specific tumor microenvironment.

Related to this, we further evaluated the relationship between V62 T cells and Tregs through ROC curve analysis of the Normalized Euclidian Distance (NED) of infiltrating Tregs and V62 T cells, calculated according to the following formula:

\[
\text{NED} = \sqrt{\left(\frac{\% \text{Treg}}{(\% \text{Treg} - \text{mean} \% \text{Treg})} \right)^2 - \left(\frac{\% \text{V62}}{(\% \text{V62} - \text{mean} \% \text{V62})} \right)^2}
\]

NED will have low values when normalized Tregs and V62 vary in similar ranges of values, and higher values when normalized Treg and V62 vary in different ranges of values.

The area under the ROC curve (AUC) obtained comparing the NED of percentage of infiltrating Tregs and V62 T cells at early (I and II) versus advanced (III and IV) stage of SCC was 0.89 [95% confidence interval (CI), 0.768–1.04] with \( P = 0.0016 \) (Fig. 7). According to the Youden Index, using a cutoff value of NED <2.33, chosen for scoring purposes to maximize the sum of sensitivity and specificity, we found that the sensitivity was 78.57% and specificity was 100%. Therefore, based on the NED values with the selected cutoff, 78.57% of subjects at the advanced stage of the tumor were correctly identified with a positive test, whereas 100% of subjects at the early stage of the tumor were correctly identified with a negative test. The ROC curve analysis further confirms that the mutual relationships between infiltrating Tregs and V62 T cells in early and advanced stage of SCC are different.

Discussion

The infiltration of tumors by certain subsets of T lymphocytes has largely been linked to favorable outcome in different types of cancer (4, 5), whereas other leukocyte subsets, such as Tregs and N2 macrophages, can confer a poor prognosis (28, 29).

γδ T cells have unique features that make them good candidates for effective antitumor immunotherapy (30), because they are not MHC restricted and do not rely on costimulatory signals. γδ T cells display potent cytotoxic and antitumor activities \textit{in vitro} (31–35) and in xenograft models \textit{in vivo} (36, 37), leading to the exploration of their therapeutic potential.

Several studies have shown that γδ T cells are present among tumor-infiltrating lymphocytes (TIL) obtained from different types of cancers, but their clinical relevance is still obscure, because some studies showed a correlation of γδ T cells and tumor remission, whereas others showed a correlation with tumor progression. Other papers found no correlation of these cells with any prognostic feature.

However, an analysis of expression signatures from \~18,000 human tumors with overall survival outcomes across 39 malignancies identified tumor-infiltrating γδ T cells as the most significant favorable cancer-wide prognostic signature (38), including also 74 SCC patients (head and neck carcinoma). These disparate findings strongly suggest that γδ T cells in the tumor microenvironment may play substantially different functions, depending on the specific γδ T-cell subset/function recruited to the tumor site. Furthermore, the net positive or negative biologic effect of γδ T cells may depend on the histologic tumor type and on the tumor site, reflecting microenvironmental differences.

Results reported here show that V61 T-cell numbers were significantly elevated in the tumor compartment compared with peripheral blood in SCC patients, whereas T cells obtained from HDs. Similarly, the percentage of V62 T cells was low and often undetectable in normal skin from HDs, but significantly increased in TILs of SCC patients. Tregs were significantly increased in the tumor compartment, but were decreased in the peripheral blood of SCC patients.

Cells with a T gly phenotype were the predominant population among tumor-infiltrating V61 and V62 T cells, highly suggestive of cells that are home to sites of inflammation and that display immediate effector functions such as cytokine production. Accordingly, tumor-infiltrating V61 and V62 T cells preferentially produced IL17, but not IFNγ, whereas circulating V61 and V62 T cells had a reciprocal cytokine production pattern. The capability of γδ T cells in TILs to produce IL17 or IFNγ correlated with the clinical stage of SCC patients; IL17-producing γδ T cells were significantly more plentiful in patients with advanced disease (stages III and IV), whereas SCC patients at stages I and II had more IFNγ-producing γδ T cells.

Five different studies, three in mouse models (12, 39, 40) and two in human cancer (13, 14), show that IL17-producing T cells are key mediators of tumor-associated immunosuppression and promote tumor progression by several different mechanisms. Typically, murine γδ T cells are an innate source of IL17, a potential they acquire in the neonatal thymus independently upon encountering their specific antigen (41–44). Conversely, human γδ T cells default toward type 1 cytokine production and predominantly produce IFNγ upon activation. However, γδ T cells can divert from this typical Th1-like phenotype upon appropriate culture condition and polarize to different cytokine-producing subsets; for example, the addition of IL1β, IL6, IL23, and TGFβ together with TCR triggering promotes expression of the transcription factor RORC and polarization to IL17 γδ T cells (27). In other words, we reasoned that at early stages of tumor growth,
IFNγ-producing γδ T cells either expand locally (Vδ1) or are recruited to the tumor site from the peripheral blood (Vδ2) and may possibly exert antitumor activity; however, with a progressing tumor, cytokines present in the tumor microenvironment might reprogram γδ T cells to produce IL17, which instead promotes tumor growth. In agreement with this hypothesis, CSCs and partly SDACs and CAFs produced large amounts of CXCL1, CXCL8, and CXCL2, and intermediate concentrations of CXCL9, CXCL10, CXCL12, CCL5, CCL7, and CCL11, and circulating γδ cells expressed counter-receptors for many of these chemokines, suggesting that γδ cells may migrate from the circulation to the tumor site. IL6, IL1β, IL23, and TGFβ were also produced by CSCs, SDACs, and CAFs, thus making the environment conducive for differentiation of IL17-producing γδ T cells.

However, concerning IL23, we would like to point out that only p40 was tested, and p40 is also a constituent of IL12. Consistent with this, IL12 p70 was highly produced by any cell type in the SCC environment, and this balance between IL12 and IL23 might be therefore critical in order to determine whether the environment is oriented toward IFNγ polarization (in the case of IL12) or IL17 (in the case of IL23). Concerning IL12, CSCs and SDACs produced high amounts either of p40 (which is common between IL12 and IL23) and p70, conditioning the polarization toward IFNγ-producing cells. Additional studies are thus required to clarify the role of IL12/IL23 produced in the tumor microenvironment as a major determinant of γδ T-cell polarization.

We have no direct evidence supporting our contention that cytokines produced by tumor cells polarize γδ T cells toward IL17 production. We speculate this may occur on grounds that (i) γδ T cells (both Vδ1 and Vδ2) that produce IL17 are more highly represented in tumor tissue compared with peripheral blood of the same patients, indicating that this IL17 phenotype is largely shaped by the tumor microenvironment, (ii) tumor cells produce cytokines that are known to mediate differentiation of IL17-producing γδ cells in humans, and (iii) in human colorectal cancer, DC-derived IL23 was proposed to polarize γδ T cells toward IL17 production (13). Therefore, it is conceivable that γδ T cells are recruited into the tumor from draining lymph nodes or peripheral blood and then undergo functional changes in response to the local microenvironment, although we cannot rule out the possibility that tissue-resident γδ T cells are functionally affected locally.

We observed a correlation between cytokine-producing intra mural Vδ1 and Vδ2 T cells and clinicopathologic staging and follow-up data on SCC patients. Moreover, a significant inverse correlation was found between Vδ2 T cells and Tregs at early stages, which turned to a significant direct correlation at late stages. Moreover, the Vδ2/Treg ratio was prognostically accurate, suggesting that a reciprocal relationship may exist between Tregs and Vδ2 T cells in vivo, which is largely influenced by the specific tumor microenvironment.

In conclusions, results here reported show that γδ T cells (both Vδ1 and Vδ2) infiltrate the tumor tissue in SCC patients and their phenotype and functions are largely influenced by the tumor microenvironment, thus differentially affecting patients’ prognoses.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Squamous Cell Tumors Recruit γδ T Cells Producing either IL17 or IFN γ Depending on the Tumor Stage

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