Human CCR4$^+$CCR6$^+$Th17 Cells Suppress Autologous CD8$^+$ T Cell Responses

Fei Zhao, Bastian Hoechst, Jaba Gamrekelashvili, Lars A. Ormandy, Torsten Voigtländer, Heiner Wedemeyer, Kris Ylaya, Xin Wei Wang, Stephen M. Hewitt, Michael P. Manns, Firouzeh Korangy and Tim F. Greten

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The role of Th17 cells in cancer patients remains unclear and controversial. In this study, we have analyzed the phenotype of in vitro primed Th17 cells and further characterized their function on the basis of CCR4 and CCR6 expression. We show a novel function for a subset of IL-17-secreting CD4+ T cells, namely, CCR4+CCR6+Th17 cells. When cultured together, CCR4+CCR6+Th17 cells suppressed the lytic function, proliferation, and cytokine secretion of both Ag-specific and CD3/CD28/CD2-stimulated autologous CD8+ T cells. In contrast, CCR4+CCR6+CD4+ T cells, which also secrete IL-17, did not affect the CD8+ T cells. Suppression of CD8+ T cells by CCR4+CCR6+Th17 cells was partially dependent on TGF-β, because neutralization of TGF-β in cocultures reversed their suppressor function. In addition, we also found an increase in the frequency of CCR4+CCR6+, but not CCR4−CCR6+ Th17 cells in peripheral blood of hepatocellular carcinoma patients. Our study not only underlies the importance of analysis of subsets within Th17 cells to understand their function, but also suggests Th17 cells as yet another immune evasion mechanism in hepatocellular carcinoma. This has important implications when studying the mechanisms of carcinogenesis, as well as designing effective immunotherapy protocols for patients with cancer. The Journal of Immunology, 2012, 188: 000–000.

Th17 cells are a novel subset of CD4+ Th cells that play an important role in pathogenesis of several inflammatory and autoimmune diseases (1). These cells secrete IL-17, a cytokine that can recruit and expand neutrophils through secretion of chemokines (2). It has been shown that mice deficient in IL-17R are more susceptible to certain pathogens (3, 4). Recently, conflicting data have been reported on the role of Th17 cells in cancer (5–7). Increased frequencies of Th17 cells have been reported in patients with different types of tumors (8–11), although their exact role in cancer is still not clear.

The precise conditions for human Th17 cell development remain controversial. However, recent studies have demonstrated that TGF-β in combination with IL-1, IL-6, and IL-23 can induce the generation of Th17 cells from CD4+ memory T cells (1). Lack of a specific surface marker for Th17 cells causes difficulties in their isolation for direct ex vivo functional analysis. Human Th17 cells have been shown to express IL-17A, IL-17F, IL-22, IL-26, and IFN-γ, and their functions have been shown to be pivotal in the context of different autoimmune diseases such as psoriasis (12), asthma (13), rheumatoid arthritis (14), and inflammatory bowel disease (15).

Hepatocellular carcinoma (HCC) is the third leading cause of death from cancer and the fifth most common malignancy worldwide (16). HCC is often diagnosed at an advanced stage when it is no longer amenable to curative therapies (17). Highly active drug-metabolizing pathways and multidrug resistance transporter proteins in tumor cells further diminish the efficacy of current therapeutic regimens for this cancer type. Alternative approaches are therefore needed to overcome these barriers to successful therapy, and immunotherapy represents one possible option (18). A number of different immune-suppressor mechanisms and cells have been identified in HCC including CD4+ regulatory T cells, myeloid-derived suppressor cells, and IDO and programmed death 1 expression (19).

We have initially analyzed different cytokines in primary tumors from patients with HCC and observed high concentrations of IL-17 and IL-23 in primary tumor supernatants. Based on these findings, we further analyzed T cell responses and noticed a significantly increased frequency of IL-17-secreting CD4+ T cells in peripheral blood of HCC patients, which led us to investigate the biological function of these cells and particularly their effect on CD8+ T cells. In vitro generated Th17 cells suppressed proliferation and cytokine secretion of CD3/CD28/CD2-stimulated CD8+ T cells in vitro. Further separation of Th17 cells into CCR4−CCR6+ and CCR4+CCR6+ cell populations indicated that only IL-17-secreting CCR4+CCR6+Th17, but not CCR4−CCR6+ CD4+ T cells suppressed proliferation and cytokine secretion of CD3/CD28/CD2 or Ag-stimulated CD8+ T cells in vitro. Finally, we were able to demonstrate an increase in the frequency of CCR4+CCR6+Th17, but not of CCR4−CCR6+ CD4+ T cell population in patients with HCC. Taken together, all our data suggest that CCR4+CCR6+Th17 cells represent another mechanism of
immune evasion in HCC patients. These findings might have important implications in designing immunotherapy protocols.

Materials and Methods

Patients and healthy donors

Blood samples were collected from patients with HCC seen at the Department of Gastroenterology, Hepatology and Endocrinology, Hannover Medical School (Hannover, Germany). HCC was diagnosed according to the guidelines of the European Association for the Study of the Liver. No patient included in this study received a tumor-specific therapy (including surgery, transarterial chemoembolization, ablation, or chemotherapy) at least 6 wk before analysis of blood samples. Written consent was obtained from all patients before blood and tumor sampling, and the Ethics Committee of Hannover Medical School approved the study protocol. Clinical information on HCC patients is shown as Supplemental data.

Preparation of tumor supernatant

Single-cell suspensions of tumors obtained from surgery were prepared by mechanical dissociation and collagenase/dispase treatment for 45–60 min at 37°C (Roche Diagnostics). After digestion, the cells were cultured at 37°C overnight. The supernatants were harvested and tested for the indicated cytokines by commercially available ELISA: IL-6 (ImmunoTools), IL-10 (ImmunoTools), IL-12p70 (eBiosciences), IL-17A (eBiosciences), IL-23 (eBiosciences), VEGF (PeproTech), TNF-α (ImmunoTools), IFN-γ (ImmunoTools), and TGF-β (eBiosciences).

Immunohistochemistry

Immunohistochemistry double staining for CD4 (catalog no. M3710; mouse mAb, clone, 4B12 at 1:50 titer detected with DAB; Dako, Carpinetia, CA) and IL-17 (catalog no. AF-317-NA, affinity-purified goat, at 1:50 titer detected with Fast Red; R&D Systems) was performed on formalin-fixed, paraffin-embedded tissue sections using Dako Env+ and Link+alkaline phosphatase detection systems. Slides were deparaffinized with graded alcohols and xylene, then subject to Ag retrieval for 30 min in a steamer with pH 9 Citrate retrieval buffer (Dako) blocked with 1.5% H2O2 for 20 min, treated with protein block (Dako) for 20 min, then hybridized with the anti-CD4 Ab for 60 min at room temperature, followed by secondary Ab reagent (Envision+; Dako) for 20 min, and DAB for 10 min, then double block (Dako) for 10 min, and anti–IL-17 Ab for 60 min at room temperature, followed by secondary Ab reagent (Link+LSAB+; Dako) for 15 min, with the addition of AP polymer for 15 min, and detection with Fast Red for 30 min. Slides were then dehydrated in graded alcohols and xylene, and coverslipped. Ags were detected singularly using the same protocol, as well as appropriate negative controls. Tonsil tissue was used as positive controls. Photomicrographs were with a Zeiss Axiosplan 2ie microscope, 40× Plan Apochromat objective with a color AxiosCam HRC camera and AxioVision 4.7 software.

Th17 cell culture in vitro

CD4+ memory T cells were isolated from freshly obtained PBMC using Memory CD4+ T Cell Isolation Kit (Miltenyi Biotech) according to the manufacturer's instructions. The enriched CD4+ memory T cells were cultured in human T cell medium (RPMI 1640 medium supplemented with 10% human serum). CD4+ memory T cells were stimulated with anti-CD3/CD28/CD2 beads (Miltenyi Biotech) for 7 d in the presence of recombinant IL-1β (10 ng/ml), IL-6 (40 ng/ml), IL-23 (50 ng/ml), neutralizing anti-TGF-β (0.5 ng/ml), and anti–IFN-γ (5 mg/ml; R&D Systems).

Cell isolation and sorting

Human PBMCs were isolated from freshly obtained healthy donor blood by Ficoll density gradient centrifugation (Biochrom AG). CD8+ T cells were sorted from the autologous PBMC as CD3+CD8+ cells. The purity of the cells was ≥95% after sorting. CD4+IL-17+ were isolated from Th17 cell cultures from CD4+ memory T cells and staining reagents from the IL-17 secretion Assay-Detection Kit (Miltenyi Biotech). In brief, in vitro cultured Th17 cells were stimulated with CytoStim. After 4 h, cells were first labeled with IL-17 catch reagent followed by another 45-min incubation period at 37°C. Finally, cells were stained with an IL-17 detection Ab together with anti-CD4 (and anti-CCR4 and anti-CCR6 Abs where indicated). CCR4+CCR6+ Th17 and CCR4+CCR6+ CD4+ T cells were sorted as CCR4+CCR6+ and CCR4+CCR6+ cells from CD4+IL-17+ populations using a BD FACS Aria cell sorter system (Becton Dickinson). CD4+IL-17+ cells were sorted in parallel as controls. For analysis of the effect of Th17 cells on Ag-specific T cell responses, CCR4+CCR6+ and CCR4+CCR6+ CD4+ T cells were sorted from 7-d in vitro cultured CD4+ memory T cells using BD FACS Aria cell sorting system (Becton Dickinson).

Flow cytometry analysis

FACS acquisition was performed on LSR-II, and results were analyzed with FlowJo version 9.3.1.2 software (TreeStar). FACS staining was done with the following Abs: anti-CD4 (Miltenyi Biotech); anti–IL-17 (eBiosciences); anti-CD45RA, anti-CD45RO (ImmunoTools); anti-CCR4, anti-CCR5, anti–CCR6, anti–IL-23R (R&D Systems); anti–CXCR3, anti-CD161, anti–IFN-γ, and anti–Foxp3 (BD Biosciences). Anti–TGF-β (BioLegend) was used to stain membrane-bound TGF-β. Isotype-matched Ab controls were used as indicated. For intracellular cytokine analysis, PBMCs were stimulated with PMA/ionomycin in the presence of GolgiPlug (BD Biosciences), and intracellular cytokine staining was performed according to the manufacturers' instructions.

Coculture assays

Sorted CD3+CD8+ T cells were cocultured with gamma-irradiated autologous CCR4+CCR6+Th17, CCR4+CCR6+CD4+, or CD4+IL-17+ T cells at different ratio in the presence of CD3/CD28/CD2 beads. Supernatants were collected after 48 h, and IFN-γ secretion was tested by IFN-γ ELISA (eBiosciences). At 72 h after coculture, H+ labeled thyminide was added and cells were incubated for an additional 16 h. CD8+ T cell proliferation was determined by measuring [H] incorporation after 16 h. For analysis of Ag-specific T cell responses, M1 peptide-specific T cells were generated as previously described (20). Ag-specific lysis was determined at an E:T ratio of 5:1 using a standard 4-h [3H] release assay in the presence or absence of autologous CCR4+CCR6+CD4+ and CCR4+CCR6+CD4+ T cells. In some experiments, indicated concentrations of anti–TGF-β (R&D Systems) were used to neutralize TGF-β; the corresponding isotype control was also used as indicated.

Statistical analysis

All the statistical analyses were based on two-tailed Student's t test. All p values <0.05 were considered to be significant.

Results

Supernatants from HCC tumor samples contain IL-17, IL-17, IL-6, and VEGF

Cytokines, which can induce both systemic and local immune suppression, can be secreted by tumors and tumor surrounding cells. We analyzed fresh viable tumor samples from six random HCC patients. No differences in the ratio of immune versus stroma cells were observed in the tissues analyzed. We tested HCC tumor supernatants for VEGF, TNF-α, TGF-β, IFN-γ, IL-6, IL-10, IL-12, IL-17A, IL-23. The predominant cytokines detected in the majority of HCC tumor supernatants were IL-17A, IL-23, IL-6, and VEGF (Fig. 1A). This prompted us to look for IL-17–secreting CD4+ T cells in peripheral blood of HCC patients and healthy controls stimulated with PMA/ionomycin. The frequency of IL-17+CD4+ T cells was significantly increased in peripheral blood of HCC patients as compared with healthy donors and hepatitis C virus patients (Fig. 1B). Immunohistochemical analysis was performed to examine tumor-infiltrating CD4+ T cells and revealed that the majority of CD4+ T cells coexpressed IL-17 with no preference for a specific location (intrahepatic/peritumoral; Fig. 1C).

IL-17-producing CD4+ T cells suppress CD8+ T cell function

To obtain enough cells for functional experiments, we stimulated sorted CD4+ memory T cells with anti-CD3/CD28/CD2 beads in the presence of IL-1β, IL-6, and IL-23 cytokines, and anti–IL-4 and anti–IFN-γ Abs. As shown in Fig. 2A, IL-17–secreting CD4+ T cells were expanded to >25% on in vitro culture. Next, the phenotype of in vitro primed and ex vivo isolated IL-17+CD4+ T cells were compared (Fig. 2B). Both groups of cells were CD45RA and CD45RO+, expressed CCR4, CCR6, low levels of IL-23R, and CX3CR3. They also expressed CD161, which has been suggested as an additional marker for Th17 cells (21, 22). To
examine the possible function of these cells, we next isolated IL-17+ CD4+ T cells from in vitro cultures (Fig. 2C) and coincubated them with CD3/CD28/CD2-stimulated autologous CD8+ T cells at different cell ratios.

As shown in Fig. 2D, CD4+IL-17+ cells inhibited the proliferation of autologous CD8+ T cells in a dose-dependent manner. There was a 14.2 ± 6.15, 35.9 ± 8.72, and 51.1 ± 5.82% inhibition of T cell proliferation at CD8/Th17 cell ratios of 1:0.5, 1:1, and 1:5, respectively. In contrast, CD4+ IL-17- T cells did not suppress the proliferation at any ratio tested. CD4+IL-17+ cells also reduced IFN-γ secretion by autologous CD8+ T cells, which was not observed when CD8+ T cells were coincubated with CD4+ IL-17- T cells (Fig. 2E).

CCR4+CCR6+Th17 cells suppress proliferation and IFN-γ production of autologous CD8+ T cells

Two distinct subpopulations of IL-17-producing T cells based on the expression of CCR4 and CCR6 have been recently identified (23). Therefore, we also analyzed the expression of CCR4 and CCR6 simultaneously on CD4+IL-17+ cells directly ex vivo and after in vitro priming. In vitro priming of Th17 cells increased the frequency of CCR4+CCR6+CD4+ T cells from 6.9 to 34.4% and from 54.2 to 85% when CD4+IL-17+ cells were analyzed (Fig. 3A). In parallel, the frequency of CCR4+CCR6- cells decreased on in vitro priming. Based on these findings, we decided to sort CCR4+CCR6+Th17 and CCR4-CCR6- CD4+ and CD4-IL-17- T cells from in vitro primed T cell cultures for our functional studies. For this, in vitro primed Th17 cultures were first stained with a bispecific IL-17 capture Ab, and IL-17+ cells were further separated into CCR4+CCR6+ and CCR4-CCR6- populations (Fig. 3B). Using this sorting strategy, we were able to obtain >96% pure T cell populations regarding IL-17 analysis and 74% pure cell populations regarding CCR4/CCR6 staining. Finally, we tested IL-17 and IFN-γ secretion from CCR4+CCR6+ and CCR4+CCR6-Th17, as well as CD4+IL-17- T cells. Although neither CCR4+CCR6+ nor CCR4+CCR6-Th17 stained positive for intracellular IFN-γ, 50% of CD4+ T cells isolated from IL-17+ cultures were IFN-γ+ (Fig. 3B).

To investigate the potential effects of Th17 cells on CD8+ T cell responses, we coincubated irradiated CCR4+CCR6+Th17 and CCR4+CCR6+CD4+ T cells at different ratios with CD3/CD28/CD2-stimulated autologous CD8+ T cells and analyzed IFN-γ release as well as proliferation of CD8+ T cells. As shown in Fig. 3C, CCR4+CCR6+Th17 cells inhibited the proliferation of autologous CD8+ T cells in a dose-dependent manner. There was a 26.9 ± 6.23.5, 30.9 ± 1.03, and 48.0 ± 15.11% inhibition of T cell proliferation at CD8/Th17 cell ratios of 1:1, 1:2, and 1:4, respectively. In contrast, CCR4+CCR6+CD4+ T cells, as well as CD4+IL-17- T cells, did not suppress the proliferation at any ratio tested. CCR4+CCR6+Th17 cells also reduced IFN-γ secretion by autologous CD8+ T cells, which was not observed when CD8+ T cells were coincubated with CD4+ IL-17- T cells (Fig. 3E).

FIGURE 1. Evaluation of different cytokines and IL-17–secreting CD4+ T cells in HCC patients. (A) Tumor supernatant from HCC samples contains significant amounts of IL-17, IL-23, IL-6, and VEGF. Single-cell suspensions of tumors were prepared as described in Materials and Methods. Supernatants were harvested and tested for different cytokines by ELISA. (B) Frequency of IL-17–secreting CD4+ T cells is increased in the peripheral blood of HCC patients. PBMCs from healthy donors (n = 15), HCC patients (n = 46), or HCV (n = 18) patients were analyzed for IL-17–secreting CD4+ T cells. ***p < 0.001. (C) CD4+ T cells in HCC coexpress IL-17. Immunohistochemical detection of CD4 (brown, DAB) and IL-17 (red, Fast Red) in different HCC samples was performed. Shown are the results of four of five different tumors analyzed. Scale bar, 20 μm.
Suppression of CD8⁺ T cell responses by CCR4⁺CCR6⁺Th17 cells is partly mediated by TGF-β

To understand how CCR4⁺CCR6⁺Th17 cells suppress CD8⁺ T cell function, we repeated the coculture experiment using a 0.4-μm permeable transwell plate. In the presence of transwell, CCR4⁺CCR6⁺Th17 cells failed to inhibit CD8⁺ T cell function, suggesting that cell contact was needed (Fig. 4A). TGF-β is a cytokine with profound inhibitory function, which can also exert its function when cell membrane bound (25). Therefore, we analyzed surface expression of TGF-β, which was higher on CCR4⁺CCR6⁺Th17 cells than on CCR4⁻CCR6⁺ CD4⁺ cells (Fig. 4B, 4C). Next, we coincubated CD8⁺ T cells with CCR4⁺CCR6⁺Th17 cells in the presence of anti–TGF-β and found that CCR4⁺CCR6⁺Th17 cells were less potent in inhibition of CD8⁺ T cell proliferation (Fig. 4D). Similar results were observed for IFN-γ release by CD8⁺ T cells in the presence of anti–TGF-β (data not shown).

CCR4⁺CCR6⁺Th17 cells suppress Ag-specific CD8⁺ T cell responses

Because Ag-specific lysis is one major feature of CD8⁺ T cells, we wanted to test the effect of CCR4⁺CCR6⁺Th17 cells on peptide-specific CD8⁺ T cell responses. An influenza matrix peptide (M1)-specific CD8⁺ T cell line was established as previously described (20), and M1-peptide–specific T cells were incubated with gamma-irradiated CCR4⁺CCR6⁺ or CCR4⁻CCR6⁺ CD4⁺ T cells from autologous Th17 in vitro culture. Peptide-specific lysis was analyzed in a standard 4-h cytotoxicity assay. As shown in Fig. 5A, CCR4⁺CCR6⁺, but not CCR4⁻CCR6⁺ CD4⁺, T cells suppressed Ag-specific lysis by CD8⁺ T cells in a dose-dependent manner. Next, Ag-specific cytokine secretion by CD8⁺ T cells was analyzed. Again, CCR4⁺CCR6⁺Th17, but not CCR4⁻CCR6⁺ CD4⁺, T cells suppressed IFN-γ secretion of M1-specific CD8⁺ T cells (Fig. 5B, 5C).

CCR4⁺CCR6⁺Th17 cell analysis in peripheral blood of HCC patients

Our studies so far clearly indicated that only CCR4⁺CCR6⁺Th17 cells, but not CCR4⁻CCR6⁺IL-17⁺ CD4⁺ T cells, suppress the function of CD8⁺ T cells. Therefore, we analyzed the frequency of both CCR4⁺CCR6⁺IL-17⁺CD4⁺ and CCR4⁻CCR6⁺IL-17⁺ CD4⁺ T cells in peripheral blood of patients with HCC, healthy control subjects, and patients with chronic HCV infection. The highest
frequency of CCR4\(^+\)CCR6\(^+\)IL-17\(^+\) CD4\(^+\) T cells was detected in peripheral blood from patients with HCC (0.45 \(\pm\) 0.09\% in PBMCs and 1.14 \(\pm\) 0.22\% in CD4\(^+\) T cells) followed by patients with chronic HCV infection (0.25 \(\pm\) 0.06\% in PBMCs and 0.42 \(\pm\) 0.09\% in CD4\(^+\) T cells), who were used as a tumor-free patient control with chronic liver disease (Fig. 6A, 6B). Next, we examined the phenotype of CCR4\(^+\)CCR6\(^+\)Th17 cells from patients with HCC and compared them with CCR4\(^{-}\)CCR6\(^+\)IL-17\(^+\) CD4\(^+\) T cells. Relative CXCR3 and CD161 mean fluorescence intensity values were slightly greater on CCR4\(^+\)CCR6\(^+\)Th17 cells compared with CCR4\(^{-}\)CCR6\(^+\)IL-17\(^+\) CD4\(^+\) T cells (Fig. 6C).

**Discussion**

Th17 cells are a novel subset of Th cells that play a major role in tissue inflammation, protection against pathogens, and autoimmune diseases. IL-17 elicits the production of a variety of pro-inflammatory cytokines including GM-CSF, TNF-\(\alpha\), IL-1\(\beta\), IL-6, and IL-23, which is also important for the differentiation, expansion, and survival of Th17 cells (1). In this study, we have examined a subset of Th17 cells in more detail and demonstrate that CCR4\(^+\)CCR6\(^+\)Th17 cells impair the function of CD8\(^+\) T cells. CCR4\(^+\)CCR6\(^+\)Th17 cells, but not CCR4\(^{-}\)CCR6\(^+\)CD4\(^+\) T cells, inhibit the lytic function, cytokine secretion, and proliferation of autologous Ag-specific CD8\(^+\) T cells. Interestingly, analysis of CD4\(^+\) T cells and Th17-related/dependent cytokines revealed an increase of Th17 subset in peripheral blood from patients with HCC, as well as increased levels of IL-17 and IL-23 in primary tumors, suggesting a novel immune evasion mechanism in this patient population.

Immune responses have been shown to be of pivotal importance for the outcome of patients with HCC. We have previously demonstrated that significant gene expression changes occur in the liver microenvironment of patients with HCC and accompanying venous metastases. Mainly inflammation/immune responses contributed to this expression signature (26). Recently, high neutrophil infiltration of HCC has been shown to be a powerful predictor of
disease progression and poor overall survival after tumor resection (27). Finally, increased intratumoral IL-17–producing cells have been found to correlate with poor survival in HCC patients (10). However, none of these studies has been able to demonstrate a mechanistic link as to how Th17 cells potentially contribute to a worse outcome. Therefore, we decided to analyze Th17 cells in HCC patients in more detail with the aim to elucidate their possible function.

Th17 cells have recently been the focus of numerous studies, but only limited information is available on their role in cancer (5). The majority of studies have been performed in murine tumor models (6, 7, 28, 29). Several studies have investigated Th17 cells in cancer patients; however, the function of these cells remains to be shown (8, 9, 11). Animal studies have revealed contradictory and puzzling data as to the role of Th17 cells in tumor immunity. In some studies, a beneficial effect of Th17 cells was shown (8, 30, 31).

**FIGURE 4.** CCR4CCR6Th17 suppress autologous CD8+ T cells in a cell-contact–dependent manner, and suppression is partly mediated by membrane-bound TGF-β. (A) CCR4CCR6Th17 or CCR4CCR6 CD4+ T cells were cocultured with autologous CD3CD8+ T cells at a ratio of 5:1 with or without transwell and stimulated as described. IFN-γ secretion of CD8+ T cells was measured. Data shown are the cumulative result of four independent experiments. (No suppression was seen when CCR4CCR6Th17 cells and CD8+ T cells were separated by transwell.) (B and C) CCR4CCR6Th17 and CCR4CCR6 CD4+ T cells were sorted from in vitro cultured Th17 cells and stained for surface TGF-β. A representative FACS histogram (B) and the cumulative mean fluorescence intensity for TGF-β (C) of seven independent samples are shown. (D) Sorted and gamma-irradiated CCR4CCR6Th17 were cocultured with autologous CD3CD8+ T cells at a ratio of 5:1 as described. TGF-β neutralizing Ab or corresponding isotype control Ab was added at the indicated concentrations. Data shown are the cumulative result of five independent experiments. *p < 0.05.

**FIGURE 5.** CCR4CCR6Th17 cells suppress lytic function and IFN-γ secretion of Ag-specific CD8+ T cells. (A) Peptide-specific T cells were cultured in the absence or presence of CCR4CCR6 Th17 or CCR4CCR6 CD4+ T cells in different ratios as indicated. After 1 h, 51Cr-labeled and peptide-pulsed T2 cells were added at a ratio of 5:1 (E:T), and lysis was determined by standard 51Cr-release assay. Data shown are cumulative results from four independent experiments. (B and C) CCR4CCR6Th17 or CCR4CCR6 CD4+ T cells were incubated with peptide-stimulated, Ag-specific T cells at a ratio of 5:1, and IFN-γ production was analyzed by intracellular staining. Data shown are one representative dot plot (B) and the cumulative results of five independent experiments (C). *p < 0.05.
31), whereas others have reported opposite results (6, 32). Recently, it has been suggested that depending on the setting, the TGF-β/IL-6/IL-23 and IL-17 axis might or might not trigger tumor progression (33).

Previously, different human CD4+IL-17+ T cell subpopulations have been described (23, 34). CCR4+CCR6+ CD4+ T cells are a more homogenous population secreting mainly IL-17 and expressing the Th17-specific transcription factor RORc-2, whereas CCR4−CCR6+ CD4+ T cells express mainly IFN-γ and low amounts of IL-17. This population is also referred in other studies as Th1/Th17 cells (23, 35). We decided to examine both IL-17–producing cell populations in parallel. Only the individual/separate functional analysis of CCR4+CCR6+Th17 cells and CCR4−CCR6− CD4+ T cells enabled us to unmask the novel function of Th17 cells.

Based on our in vitro results demonstrating that only CCR4+CCR6+Th17 cells suppressed CD8+ T cell responses, we analyzed both subpopulations in peripheral blood from patients with HCC. Interestingly, we observed an increase in frequency of CCR4+CCR6+Th17 cells in peripheral blood of HCC patients. Although it was not possible to test the function of CCR4+CCR6+Th17 cells in vitro because of their low frequency in peripheral blood, it should be noted that CCR4+CCR6+CD127+CD25low CD4+ T cells from HCC patients, which contain ~15% IL-17+ cells, were more potent suppressors of autologous CD8+ T cells than CCR4−CCR6− CD4+ T cells (data not shown).

Interestingly, a different mechanism of Th17-mediated immune suppression has recently been described in a murine model of encephalomyelitis infection (36). In this study, murine Th17 cells upregulated antiapoptotic molecules in infected cells, thereby protecting them from lytic activity of cytotoxic T cells. In contrast, the inhibition mediated through human CCR4+CCR6+Th17 cells was a direct, cell-to-cell contact-mediated event, which neutralized effector CD8+ T cell function. We tested a number of different cytokine-blocking Abs (data not shown); however, only neutralization of TGF-β partially reversed the suppressor function of CCR4+CCR6+Th17 cells on CD8+ T cells. TGF-β, which can be produced by both lymphoid and nonlymphoid cells, is known to have direct and T cell-mediated effects on tumor growth. Although it can directly affect tumor growth, metastasis, and angiogenesis, it has been shown that in patients with melanoma, Ag-specific CD8+ T cell effector function in vitro is inhibited by the addition of TGF-β (37). Furthermore, CD4+ regulatory T cells also suppress tumor-specific CD8+ T cell cytotoxicity via a TGF-β–dependent mechanism (38), which makes this cytokine an interesting target for cancer immunotherapy.

Until today, Th17 cells have been mainly implicated to be important in the context of inflammation. Interestingly, inflammation-associated cytokines such as IL-1β, IL-6, and TGF-β, and IL-23 impair the generation of Th1 responses and promote the induction of Th17 cells. Cytokines secreted by Th17 cells such as IL-17, IL-21, and IL-22 will then support their proliferation, activation, and maintenance, which according to the findings presented in this article, impair CD8+ T cell function, in part, by TGF-β expression. Our data suggest that Th17 cells can downregulate important mechanisms critical for immune surveillance and antitumor immunity, thereby facilitating tumor growth. Therefore, Th17 cells could not only be one of the major players in modulation of immune response, but they could serve as potential targets for immune-based therapies in inflammation-induced cancers.

**FIGURE 6.** Increased frequency of CCR4+CCR6+Th17 cells in peripheral blood from patients with HCC. (A and B) Frequency of CCR4+CCR6+Th17 and CCR4−CCR6− CD4+ T cells. PBMCs from HCC patients (n = 22), healthy donors (n = 13), and nontumor chronic HCV-infected patients (n = 14) were analyzed. The frequency of CCR4−CCR6− and CCR4+CCR6+ cells within the CD4+Foxp3+ (A) and within the Foxp3−PBMC gate (B) is shown. *p < 0.05. (C) CCR4−CCR6−Th17 and CCR4+CCR6+ CD4+ T cells from HCC patients were stained for CD45RA, CCR5, IL-23R, CD161, and CXCR3. Shown are representative dot plots of eight different donors.
Disclosures
The authors have no financial conflicts of interest.

References
Table 1:

Patient Characteristics

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</tr>
<tr>
<td>BCLC B</td>
<td>13</td>
</tr>
<tr>
<td>BCLC C</td>
<td>29</td>
</tr>
</tbody>
</table>

HBC, hepatitis B virus; HCV, hepatitis C virus; BCLC, Barcelona Clinic Liver Cancer