Enrichment of CCR6⁺Foxp3⁺ regulatory T cells in the tumor mass correlates with impaired CD8⁺ T cell function and poor prognosis of breast cancer

Lin Xu¹, Wei Xu, Shenglong Qiu², Sidong Xiong*  

Institute for Immunobiology and Department of Immunology, Shanghai Medical College of Fudan University, 138 Yixueyuan Road, Shanghai 200032, PR China

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Abstract  
CCR6⁺ subset of CD4⁺ regulatory T cells, a newly characterized subset of Tregs, has been reported to contribute to local immune inhibition. However, whether CCR6⁺ Tregs are present in tumor environment and their relation to the prognosis of tumor remain to be elucidated. In this study, we found that CCR6⁺CD4⁺CD25⁺Foxp3⁺ Tregs, expressing high levels of CD45RO, are dominantly enriched in tumor mass from patients with breast cancer. Furthermore, the frequency of CCR6⁺ Tregs, but not CCR6⁻ Tregs in tumor infiltrating lymphocytes (TILs), significantly increased in patients during tumor progression, which reversely correlated with decreased frequency of the IFN-γ⁺CD8⁺T cells in TILs. Most importantly, the frequency of CCR6⁺ Tregs, but not CCR6⁻ Tregs, reversely correlated to the survival of patients with breast cancer. This study suggested that a new subset of tumor-resident Tregs, CCR6⁺ Tregs, may be dominantly responsible for the immunosuppression in tumor immunity and a potential predictor of the poor prognosis of breast cancer.

Introduction

CD4⁺CD25⁺Foxp3⁺ regulatory T cells (Tregs), a subpopulation of CD4⁺ T cells constitutively expressing transcription factor forkhead box protein3 (Foxp3), effectively suppress the proliferation and activity of both CD4⁺CD25⁻ and CD8⁺ T cells in a contact-dependent manner through inhibition of interleukin 2 production [1,2]. Previous studies have consistently demonstrated the enrichment of CD4⁺ Tregs in the tumor infiltrating lymphocytes (TILs) in various tumor mass [3,4] and their critical roles in the immunosuppression in the tumor hosts [5–7]. Accumulating data have indicated that there are distinct subsets of Tregs which play different roles in diverse animal model, mediating immune suppression or immune tolerance [8–11]. However, whether a distinct subset of Tregs is present in the tumor environment during the progression of tumor and their contribution to the progression of tumor remains not fully understood.

* Corresponding author. Fax: +086 21 54237749.  
E-mail address: sdxiongfd@126.com (S. Xiong).

¹ Present work unit: Department of Immunology, Zunyi Medical College, Guizhou 563000, PR China.
² Present work unit: Department of General Surgery, Shanghai First People’s Hospital, Shanghai Jiaotong University, Shanghai 200032, PR China.

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Recent study demonstrated a new subset of Tregs, which express CC chemokine receptor type 6 (CCR6), displayed a memory effector like phenotype and played an important role in regulating the function of CD4+ effector T cells especially in the pathogenesis of experimental allergic encephalomyelitis (EAE) [12]. Our previous work has shown that CD4+ Tregs were enriched in the TILs of tumor mass in a murine model of breast cancer and predominantly inhibited the local Th1 immune response [13]. We further found that these local CD4+ Tregs expressed high level of CCR6 molecule. Interestingly, CCR6+CD4+ Treg cells were also found dominantly enriched in the tumor mass of murine breast cancer and potently inhibited the function of CD4+ T cells and CD8+ T cells (unpublished data). Similar results suggested that CCR6+ Tregs were enriched in the inflammatory sites in EAE and more powerfully proliferated in response to antigen stimulation [12]. Other reports further demonstrated that CCR6+ Tregs were attracted via CCL20 chemotaxis [14]. However, the distribution of CCR6+ Tregs and their correlation to the prognosis of tumor patients remain to be elucidated, which might be helpful for the understanding of the mechanism of Treg subsets contributing to immunosuppression and ultimate clinical diagnosis and therapy of tumor patients.

To this end, in the present study, the distribution of CCR6+ Treg cells in 28 patients with breast cancer was analyzed. It was found that CCR6+CD4+CD25high Tregs were dominantly enriched in the tumor mass especially in patients at advanced stage and could more potently inhibit the proliferation of CD4+CD25- T cells than their CCR6− counterpart. Furthermore, CCR6+ Treg frequency in patients with metastasis and the correlation of prevalence of CD4+ CD25highCCR6+ Tregs to the survival of breast cancer patients were also analyzed.

Material and methods

Patients

Between June 2003 and March 2007, we collected PBMCs and tumor samples from patients with breast cancer in First People's Hospital, Shanghai, China. The study group (n=28) comprised chemotherapy and radiotherapy naive patients with breast cancer, classified as stages I to II (termed as early stage) and stages III to IV (termed as advanced stage). The control group (n=14) consisted of age- and sex-matched healthy volunteers. Subjects with autoimmune diseases (e.g. rheumatoid arthritis, systemic lupus erythematosus), chronic infections (e.g. human immunodeficiency virus infection, tuberculosis), bone marrow involvement, anti-coagulant and anti-thrombotic drug using, or those who had received immunosuppressive treatment were excluded. All patients gave informed consent approved by the local Ethics Committee. Review of pathology reports confirmed the diagnosis. Information regarding clinical pathological characters of patients was summarized in Table 1.

Cell isolation

The lymphocytes were harvested from tumors by a discontinuous density gradient method. Briefly, tumors were removed aseptically and minced with scissors into 1–2 mm3 pieces. The minced tumors were then stirred in 40 ml complete RPMI 1640 containing 40 mg collagenase, type IV (Sigma), 4 mg deoxyribonuclease (Sigma) and 100 U hyaluronidase (Sigma) for 3 h at room temperature. The tumor cell suspension was filtered through a nylon-mesh screen with pores of 50 μm to remove cell clumps, and the filtrate was then centrifuged (250×g, 10 min). The cell pellet was washed twice with serum-free RPMI 1640 and resuspended in complete RPMI 1640. A 4-ml aliquot of cell suspension of disaggregated tumor was placed on top of the gradient formed by overlapping a cushion of 100% Ficoll-Paque (Pharmacia Fine Chemicals, Piscataway, NJ) with an equal volume of 75% Ficoll-Paque (Pharmacia Fine Chemicals, Piscataway, NJ) with an equal volume of 75% Ficoll-Paque in RPMI 1640. Gradients (14 ml) were centrifuged at 800×g for 30 min at room temperature. The distinct band formed at the interface between 75% and 100% Ficoll-Paque was collected and washed three times in fresh medium.

Peripheral blood was obtained at the time of tumor collection. Blood was drawn into heparin containing vacutainer tubes (Becton Dickinson, Franklin Lakes, NJ), diluted 2:1 with Dulbecco's phosphate buffered salt solution 1× without calcium or magnesium (Mediatech) and then separated by centrifugation over a ficoll (Pharmacia Biotech AB) density gradient for 20 min at 1000×g at room temperature. PBMCs were collected, washed, and resuspended in RPMI 1640 (containing 10% FCS) for future use.

Flow cytometry

Flow cytometry was performed on a FACS Calibur (BD Biosciences) with CellQuest Pro software using directly conjugated mAbs against the following markers: CCR6-FITC, CD4-PerCP, CD25-allophycocyanin, CD45RO-PE, CTLA-4-PE, CD127-PE and CD62L-PE, with corresponding isotype-
matched controls (either BD Biosciences or eBioscience Systems). Foxp3 staining was conducted using the human Regulatory T cell staining kit (eBioscience) and run according to the manufacturer’s protocol. To determine the percentage of Tregs, lymphocytes were gated by plotting forward vs. side scatter followed by gating on CD4+ T cells. Gated cells were then analyzed for CD25 and CCR6 expression. To harvest CD4+CD25(high) CCR6+ Treg cells, FACS sorting was used to purify the CD4+CD25(high)CCR6+ Treg from TILs. CD4+CD25(high)CCR6+ Tregs were also purified.

Suppression assays

To test CD4+CD25(high) CCR6+ Treg cells suppressive activity, 5×10⁴ CD4+CD25− cells were treated with 2 μg/ml anti-CD3 (eBioscience) and anti-CD28 (eBioscience) for 12 h as effector cells, then incubated with or without CCR6+ Tregs at a ratio of 2:1 for 72 h in complete medium containing RPMI 1640 (Sigma, St. Louis, MO) supplemented with 5% FCS. [3H] thymidine (0.5 μCi/well) was added for the last 18 h of culture.

Intracellular staining for IFN-γ

Lymphocytes were isolated from tumor mass. After staining of surface markers (CD8), cells were fixed and permeabilized using Cytofix/Cytoperm and Perm/Wash buffer from BD Biosciences according to the manufacturer’s instructions. Antibodies to cytokines (IFN-γ) including the corresponding isotype controls were obtained from BD Biosciences. Cells were stained with antibody against IFN-γ (1:100) at 25 °C for 20 min and washed twice in Perm/Wash before analysis.

Statistical analyses

Statistical analyses of the data were performed with the SPSS12.0 software. Data were analyzed using a one-way analysis of variance (ANOVA) or Kruskal–Wallis test with PRISM 4.0 (GraphPad Soft-ware Inc, San Diego, CA, USA). Actuarial overall survival rates were analyzed by the Kaplan–Meier method and survival was measured in weeks from diagnosis to death or the last review. The log-rank test was applied to compare among the groups. *p<0.05 was considered statistically significant in all comparisons.

Results

CCR6+ CD4+CD25(high) Tregs were dominantly enriched in the tumor mass

To determine whether CCR6+ Tregs are present in the tumor mass in patients with breast cancer, PBMCs and TILs were isolated from 28 breast cancer patients and the CD4+CD25(high) Treg frequency in PBMCs and TILs was firstly analyzed. As shown in Figures 1A and B, the CD4+CD25(high) T cells which expressed high level of Foxp3 were demonstrated as Tregs. Treg frequency in TILs (12.1%) was significantly higher than that in PBMCs (3.2%) of breast cancer patients and healthy donors (2.5%) (p<0.05, Figs. 1A & B). Furthermore, the frequency of CCR6+ subset of Tregs was analyzed and found significantly higher in TILs (7.4%) than that in PBMCs (1.23%) of breast cancer patients. Strikingly, compared to CCR6+ Tregs, the CCR6+ Treg frequency was significantly elevated in TILs (7.4% versus 4.2%) (Figs. 1C and D, p<0.05). These data suggested that CCR6+ Tregs were not only present but also dominantly enriched in the tumor mass of clinical breast cancer patients.

CCR6+ Tregs displayed memory effector like phenotype and potently inhibited the proliferation of CD4+CD25− T cells

To further investigate the characterization of CCR6+CD4+CD25(high) Tregs enriched in the tumor mass, the expression of surface molecules and the inhibitory activity of this Treg subset were determined. As shown in Figures 2A and B, as similar as their CCR6− counterpart, CCR6+ Tregs expressed high levels of Foxp3, CTLA-4 and low level of CD127, which were the typical phenotypes of Treg. However, CCR6+ Tregs expressed higher level of CD45RO and lower level of CD62L than CCR6− Tregs did (Fig. 2B). And regarding the inhibitory function, CCR6+ Tregs could more potently inhibit the proliferation of CD4+CD25− T cell than CCR6− Tregs (Fig. 2D, 55% vs. 36%, p<0.05). Moreover, when CCR6+ Tregs were separated from responder T cells by the semi-permeable trans-well membrane, the inhibitory effect of CCR6+ Tregs on CD4+CD25− T cells was abrogated indicating the inhibitory effect of CCR6+ Tregs is cell contact-dependent. (Fig. 2C, p<0.05). Similar phenotype and inhibitory function of CCR6+ Tregs isolated from PBMCs were also demonstrated (data not shown). These data suggested that CCR6+ Tregs displayed memory effector like phenotype and more powerful inhibitory activity than CCR6− Tregs.

CCR6+ Treg prevalence was correlated to the clinical stage of breast cancer patients

To explore the relationship between CCR6+ Treg prevalence and clinical prognosis of breast cancer, 28 breast cancer patients were further divided into two groups according to their metastasis status. As shown in Table 2, the frequency of CCR6+ Tregs in TILs from patients with metastasis was similar to that without metastasis (p>0.05). However, the frequency of CCR6+ Tregs in TILs from patients with metastasis was significantly higher than that without metastasis (p<0.05).
To further investigate the relationship between CCR6+ Treg frequency and clinical prognosis of breast cancer, breast cancer patients were divided into two groups termed as early stage (stage I+II, 12 patients) and advanced stage (stage III+IV, 16 patients) and the CD4+CD25high Treg frequency in TILs at early stage and advanced stage was analyzed by FACS. As shown in Figure 3, in contrast to Tregs in PBMC population, the CD4+CD25high Treg frequency in TILs at advanced stage (16.34%) was significantly higher than that at early stage (6.87%, \( p < 0.05 \)). Regarding to CCR6+ subset of Tregs in TILs,

![Graphs showing the relationship between Treg frequency and clinical prognosis of breast cancer.](image-url)
it was shown that the frequency of CCR6+ Tregs in TILs (12.11%) at advanced stage was significantly higher than that at early stage (3.02%, Figures 4A, B, D, \( p \leq 0.05 \)). While the frequency of CCR6− subset of Tregs in TILs at advanced stage did not change significantly, compared to that at early stage (Figs. 4B and C, \( p > 0.05 \)). To further confirm these results, the absolute number of CCR6+ CD4+CD25high Tregs per gram tumor tissue was calculated and similar results were obtained.

**Figure 2**  
CCR6+ Tregs displayed memory effector like phenotype and more effectively inhibited the proliferation of CD4+CD25− T cells than CCR6− counterpart. TILs were isolated from patient as described previously. (A) CD4+CD25highCCR6+ regulatory T cells were gated and purified by FACS sorting. (B) The expression of Foxp3, CTLA-4, CD45RO, CD127 and CD62L on CD4+CD25highCCR6+ Tregs were analyzed by FACS. (C, D) The inhibitory function of CD4+CD25highCCR6+ regulatory T cells on CD4+CD25− T cells was performed by [3H]-incorporation assay. Representative data referring to the mean of data obtained from all the patients included in each group were shown. *\( p < 0.05 \).
CCR6+ Treg prevalence was reversely correlated to IFN-γ production of CD8+ T cells

It is known that IFN-γ secreting CD8+ T cells within TIL population are essential for the protective immunity against tumor. Therefore, the correlation of CCR6+ Treg prevalence to the CD8+ T cell activity was then investigated. As shown in Figures 5A and B, compared to the early stage, the CD8+ T cell and CD4+ T cell frequency in TILs at advanced stage did not change significantly. However, the IFN-γ+CD8+ T cell frequency (30.5%) in TILs at advanced stage significantly decreased compared to that at early stage (23.4%) (Fig. 5C, p < 0.05) indicating the compromised anti-tumor immune response. In constant with that, a close correlation was

**Table 2** The frequency of CCR6+CD4+CD25high Tregs in patients with breast cancer.

<table>
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<th>Without metastasis</th>
<th>With metastasis</th>
<th>p value</th>
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<tbody>
<tr>
<td>CCR6+ Treg(%)</td>
<td>3.85 ± 0.67</td>
<td>4.23 ± 0.88</td>
<td>0.256</td>
</tr>
<tr>
<td>CCR6+ Treg(%)</td>
<td>3.66 ± 0.77</td>
<td>9.33 ± 1.45</td>
<td>&lt;0.001</td>
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Arithmetic mean ± standard error of the mean.  
<sup>a</sup> Mann–Whitney test.

(Fig. 4E, p < 0.05). These data suggested that CCR6+ Treg prevalence was correlated to the clinical stage of breast cancer patients.

**Figure 3** CD4+CD25high Tregs were enriched in the tumor mass at advanced stage. PBMCs and TILs were isolated from breast cancer patients. (A) CD4+CD25high T cells were gated and purified by FACS sorting. (B) The expression of Foxp3 was determined. (C) The frequency of CD4+CD25high Tregs in PBMCs and TILs was analyzed by FACS in clinical breast cancer patients at early-stage (n = 12) and advanced-stage (n = 16) or in healthy donors (n = 14), respectively. Representative data referring to the mean of data obtained from all the patients included in each group were shown. (D) The mean frequency of CD4+CD25high Tregs in PBMCs and TILs was shown. *p < 0.05.
found between IFN-γ+CD8+ T cell frequency and CCR6+ Treg frequency (Fig. 5E, p < 0.05) but not CCR6− Treg frequency (Fig. 5G, p N 0.05). There was no correlation between CD8+ T cell frequency and CCR6+/CCR6− Tregs in TILs (Figs. 5D, F, p N 0.05).

Elevated CCR6+ Treg prevalence predicted poor survival of breast cancer patients

To further address whether CCR6+ Treg prevalence was associated with the prognosis of breast cancer, 12 out of 28 breast cancer patients who did not receive any antitumor therapy since the time of diagnosis to death were divided into 2 groups according to the mean value of CCR6+ Treg frequency as High CCR6+ Treg group (n=8, average CCR6+ Treg frequency: 11.8%±3.2%) and Low CCR6+ Treg group (n=4, average CCR6+ Treg frequency: 5.6%±1.7%). The patients who failed to return for follow-up evaluation and died from known tumor-unrelated causes were excluded from the death record. The results showed that patients with high CCR6+ Treg frequency had significantly shorter survival time course (115 days) in comparison with those with low CCR6+ Treg frequency (168 days) (Fig. 6A, p < 0.05). When patients were divided into 2 groups according to the CCR6− Treg frequency as High CCR6− Treg (n=7, average CCR6− Treg frequency: 5.4%±1.3%) and Low CCR6− Treg (n=5, average CCR6− Treg frequency: 2.7%±0.9%), no correlation between CCR6− Treg prevalence and survival could be found (Fig. 6B, p > 0.05). These data suggested that the prevalence of CCR6+ Treg subset, but not CCR6− Treg, was closely correlated to the prognosis of breast cancer patients.

Discussion

In the present study, a distinct subset of CD4+CD25high Tregs, CCR6+ Tregs, was found dominantly enriched in the tumor
CCR6⁺ Tregs enrichment correlates with prognosis of breast cancer
mass in patients with breast cancer. Moreover, these CCR6+ Tregs could more effectively inhibit the proliferation of CD4+CD25− T cells than their CCR6− counterpart in vitro. Most importantly, the increased frequency of CCR6+ Tregs, but not CCR6− Tregs, was closely correlated to the poor survival of breast cancer patients, which has been demonstrated to be correlated with impaired function of CD8+ T cells during tumor progression [18].

We further demonstrated that the frequency of IFN-γ secreting CD8+ T cell was significantly reduced during tumor progression, which was reversely correlated to the increased infiltration of CCR6+ Tregs in the tumor mass. Other group also reported the impaired proliferation and IFN-γ secretion of CD8+ T cells during tumor progression [18]. Regarding the mechanism, it was demonstrated that suppression of IFN-γ secreting CD8+ T cells by Tregs was mediated by direct T cell–T cell interactions and CD137 might be critical for the suppression of CD4+ Tregs on CD8+ T cells [19,20]. In addition, it is suggested that GITR might be involved in the suppression of IFN-γ production by CD8+ T cells [21]. The exact mechanism through which CCR6+ Tregs inhibited IFN-γ production by CD8+ T cells need further experiment. However, our preliminary data suggested that the cell–cell contact mechanism may contribute to this inhibition.

It has been well accepted that increased CD4+ Tregs was closely related to poor prognosis of patients with various kinds of tumor [1,22–24]. On the basis of that, our study found that the increased frequency of a new subset of Tregs, CCR6+ Tregs (but not CCR6− Treg) has positive correlation to the impaired function of CD8+ TILs as well as the poor diagnosis of patient. It suggested that the CCR6+ subset of Tregs might be mainly responsible for long-term immunosuppression in the tumor environment.

In all, our study found the enrichment of CCR6+CD4+CD25high Tregs in the tumor mass in patients with breast cancer and a positive correlation of increased CCR6+ Treg frequency to the impaired function of CD8+ TILs as well as the poor prognosis of patient. However, successive broad screening approaches on the role of CCR6+ Tregs in other tumor hosts will be worthwhile to further substantiate these initial results, which might throw a novel insight on the role of resident unique subset of regulatory T cells in the tumor mass and provide helpful thoughts for the designing of Treg-based immunotherapy strategy against tumor in the future.

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