Beneficial effect of T follicular helper cells on antibody class switching of B cells in prostate cancer

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Abstract. Prostate cancer is the most common malignancy in males and easily develops to be aggressive which is closely related to the chronic inflammatory tumor microenvironment *in situ*. This study aimed to assess the immunoglobulin G (IgG) subclass of B cells and explore their interactions with T follicular helper (Tfh) subsets in prostate cancer patients. The percentages of peripheral blood naïve B cells, memory B cells and mature B cells, as well as Tfh1, Tfh2 and Tfh17 cells were analyzed or sorted by FACSAria. The ratios of the different IgG subclasses (IgG1, IgG2, IgG3 and IgG4) were detected by ELISA, and the expression levels of CXCR3 and CCR6 were measured using RT-PCR and western blot analysis. Meanwhile a co-culture system of B and Tfh cells was to assess the effect of each Tfh subset on the antibody subclass switching of B cells *in vitro*. We observed higher percentages of 3 Tfh subsets and IgG4$^+$ B cells in the patients with prostate cancer than that in the health controls and proved a positive correlation between Tfh2 and IgG4$^+$ B cells. Then we verified that IL-4, IL-6, IL-10 and prostaglandin E2 (PGE2) effectively promoted antibody class switching of B cells, which may be mediated by inducing Tfh2 cells, yet the study was not completely dependent on Tfh cells. The results provide evidence of the B cell response to an immune suppressive environment by evaluating IgG4 antibodies, and established a relationship between IgG4$^+$ B cells and Tfh2 cells. Clarification of lymphocyte functions in the inflammatory microenvironment of tumors will be of potential therapeutic value.

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Abbreviations: DCs, regulatory dentritic cells; DRE, digital rectal examination; GCs, germinal centers; IgG, immunoglobulin G; MDSCs, myeloid-derived suppressor cells; NKT cells, nature killer T cells; NO, nitric oxide; PGE2, prostaglandin E2; PSA, prostate-specific antigen; TAMs, tumor-associated macrophages; Tfh cells, T follicular helper cells; Tregs, regulatory T cells

Key words: prostate cancer, B lymphocytes, T follicular helper cells, inflammatory factors

Introduction

Prostate cancer is the most commonly diagnosed malignancy in males and frequently metastasizes to the bone marrow (1) and easily develops resistance to endocrinotherapy and chemotherapy (2). The mechanisms attributed to tumor metastasis and resistance are mostly considered to be induced by the chronic inflammatory microenvironment in prostate tumors, which is characterized by high levels of IL-2, IL-4, IL-10, TNF-α and TGF-β (3-7). In addition, the tumor milieu also contains multiple increased inflammatory mediators such as chemokines, cytokines, reactive oxygen species and prostaglandin E2 (PGE2), resulting in tumor growth by elevating the expression of anti-apoptotic proteins such as Bcl2, and by activating transcription factors including cAMP, NF-κB and STAT3 (8-10).

Despite numerous studies investigating the clinical significance of local and peripheral blood T lymphocytes (11,12), the nature of B cell responses in the circulation and in tumor lesions and functional contributions of antibodies produced in cancer are still not well explained. Recently, B cells were reported to mediate immune modulating functions through modulating the balance of 4 subclasses of immunoglobulin G (IgG) (IgG1, IgG2, IgG3 and IgG4), that have different physiological functions in the formation of the immune complex and regulation of the immune process (13,14). IgG4$^+$ B cells were found to be infiltrating in lesions of patients with extrahepatic cholangiocarcinomas and pancreatic cancers (15,16). Previous studies also indicated abnormalities in the serum level of IgG4 in patients with melanoma (17,18). The molecular predilection for antibody class switching to secrete IgG4 have been regarded to be associated with IL-4 and IL-10 (19,20), but the exact function of B cells and the relative interaction with inflammatory cytokines in prostate cancer have not been explored explicitly.

As a Th subset characterized by positive CD4 and CXCR5, T follicular helper (Tfh) cells engage in promoting the growth, differentiation and class switching of B cells by secreting larger amounts of IL-21 in germinal centers (GCs) or outside GCs (21-24). Recently, Morita *et al* (25) distinguished 3 subclasses (Tfh1, Tfh2 and Tfh17) defined according to the

Table I. Characteristics of the study subjects.

Clinical indices	Group A (n=10)	Group B (n=10)	Group C (n=10)	Healthy controls (n=10)
Age (years)	65 (63-69)	68 (66-71)	70 (65-75)	27 (23-31)
Clinical stage	≤T1	T2	≥T3	-
PSA (ng/ml)	13.84[a] (5.31-30.05)	198.72[a] (8.35-825.41)	361.48[a] (64.24-1247.25)	0.85 (0.73-1.24)

Data are shown as median (range) for each group of subjects; [a]P<0.05 vs. the healthy controls; PSA, prostate-specific antigen.

expression of the CCR6 and CXCR3 chemokine receptors; Tfh1 cells are $CXCR3^+CCR6^-$ cells, Tfh2 cells are $CXCR3^-CCR6^-$ cells, whereas Tfh17 cells are $CXCR3^-CCR6^+$ cells. Tfh2 and Tfh17 cells could provide help to B cells via IL-21 production, resulting in immunoglobulin (Ig) secretion of various isotypes (IgM, IgA, IgG and IgE for Tfh2 cells). However the functions of different Tfh subsets in prostate cancer are largely unexplored. Here we found higher percentages of 3 Tfh subsets and $IgG4^+$ B cells in patients with prostate cancer and proved a positive correlation between Tfh2 and $IgG4^+$ B cells by a co-culture system. Then we verified that IL-4, IL-6, IL-10 and PGE2 could effectively mediate antibody class switching of B cells, but was not completely dependent on Tfh cells. These results may be helpful to understand the interaction between B and T lymphocytes.

Materials and methods

Patients. Thirty new onset patients (male, age ranging from 63 to 75 years) were diagnosed with prostate cancer by digital rectal examination (DRE), transrectal ultrasound guided automatic biopsy and detection of prostate-specific antigen (PSA). These patients were classified into 3 groups according to the prostate cancer stage and serum levels of PSA (Table I). Ten healthy volunteers (male, age ranging from 23 to 31 years) were recruited as controls in this study. All of the participants suffering no systemic disorders or viral infections and were enrolled at the Department of Urology, China-Japan Union Hospital of Jilin University from 2011 to 2013. Written informed consent was obtained from the guardians on behalf of all participants, and this study was reviewed and approved by the Ethics Committee of China-Japan Union Hospital of Jilin University, China.

Lymphocyte stimulation and isolation. Venous blood samples (10 μl) were collected from individual subjects, and Ficoll-Paque Plus (Amersham Biosciences, Piscataway, NJ, USA) was used to sort peripheral blood mononuclear cells (PBMCs) by density-gradient centrifugation. For the isolation of B-cell subsets, PBMCs were stained in duplicate with anti-CD19 PerCP (BD Biosciences, San Diego, CA, USA), anti-CD27 FITC (eBioscience, San Diego, CA, USA), anti-CD38 APC (eBioscience) and anti-IgM PE (BD Biosciences). The frequencies of the different B cell subsets-naïve B cells ($CD19^+IgM^+$), memory B cells ($CD19^+CD27^+$) and mature B cells ($CD19^+CD38^+$) were analyzed by flow cytometry after stimulation for 5 days in Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL, Gaithersburg, MD, USA) combined with 10% fetal bovine serum (FBS; Gibco), 2.5 ng/ml TLR9 ligand CpG 2006 ODN (Hycult Biotech, The Netherlands) and 30% supernatant of Epstein Barr Virus (EBV)-producing B95-8 cells *in vitro* as previously described (18,26).

To purified the Tfh cells, PBMCs were stained with anti-CD4 APC and anti-CXCR5 PerCP (both from BD Biosciences). The different Tfh populations were sorted with FACSAria (BD Biosciences) according to the expression of CXCR3 and CCL6 within the $CD4^+CXCR5^+$ cell population, and defined as Tfh1 ($CXCR3^+CCR6^-$), Tfh2 ($CXCR3^-CCR6^-$) and Tfh17 ($CXCR3^-CCR6^+$) (25). Sorted Tfh populations were stimulated for 5 days with plate-bound CD3 (1 μg/ml) and CD28 (10 μg/ml) mAbs (both from Invitrogen Life Technologies, Carlsbad, CA, USA) *in vitro*. Data were collected using a FACSAria analytical instrument, and analysis was performed by FlowJo software (v7.6).

Cell co-culture. Sorted Tfh populations ($2.5x10^5$ cells/well) were co-cultured with B cells ($5x10^4$ cells/well each for 5 days for Ig measurements) in DMEM supplemented with 10% FBS in the presence of EBV and CpG 2006 ODN in 24-well plates (Corning Inc., Corning, NY, USA) precoated with CD3/CD28 mAbs. The concentrations of total IgG and its subclasses (IgG1, IgG2, IgG3 and IgG4) were determined by ELISA (Uscn Life Science, Wuhan, China) as described previously (17,18).

Analysis of mRNA levels by RT-PCR. Total RNA was extracted from Tfh ($2.5x10^5$ cells) populations co-cultured with B cells ($5x10^4$ cells/well) before or after IL-4, IL-6, IL-10 and PGE2 treatment, using TRIzol (Invitrogen) according to the manufacturer's instructions. RNA pellets were stored in sterile ribonuclease-free water. Reverse transcription was carried out using 1 μg total RNA, 0.5 μg oligo(dT) and Superscript II enzyme (Invitrogen). The gene-specific primers for RT-PCR were listed as follows: CXCR3 forward, 5'-ACACCTTCCTG CTCCACCTA-3' and reverse, 5'-GTTCAGGTAGCGGTCAA AGC-3'; CCR6 forward, 5'-ACAAAGCCATCCGTGTA ATC-3' and reverse, 5'-TTCTGAACTTCTGCCCAATAA-3'; GAPDH forward, 5'-GAGTCAACGGATTTGGTCGT-3' and reverse, 5'-TTGATTTTGGAGGGATCTCG-3'.

Western blot analysis. Total proteins were extracted from Tfh populations using lysis buffer (Beyourtime, Wuhan, China) supplemented with 1% protease inhibitor mixture (Sigma-Aldrich, St. Louis, MO, USA). Equal amounts of protein per sample were separated by 10% SDS-PAGE

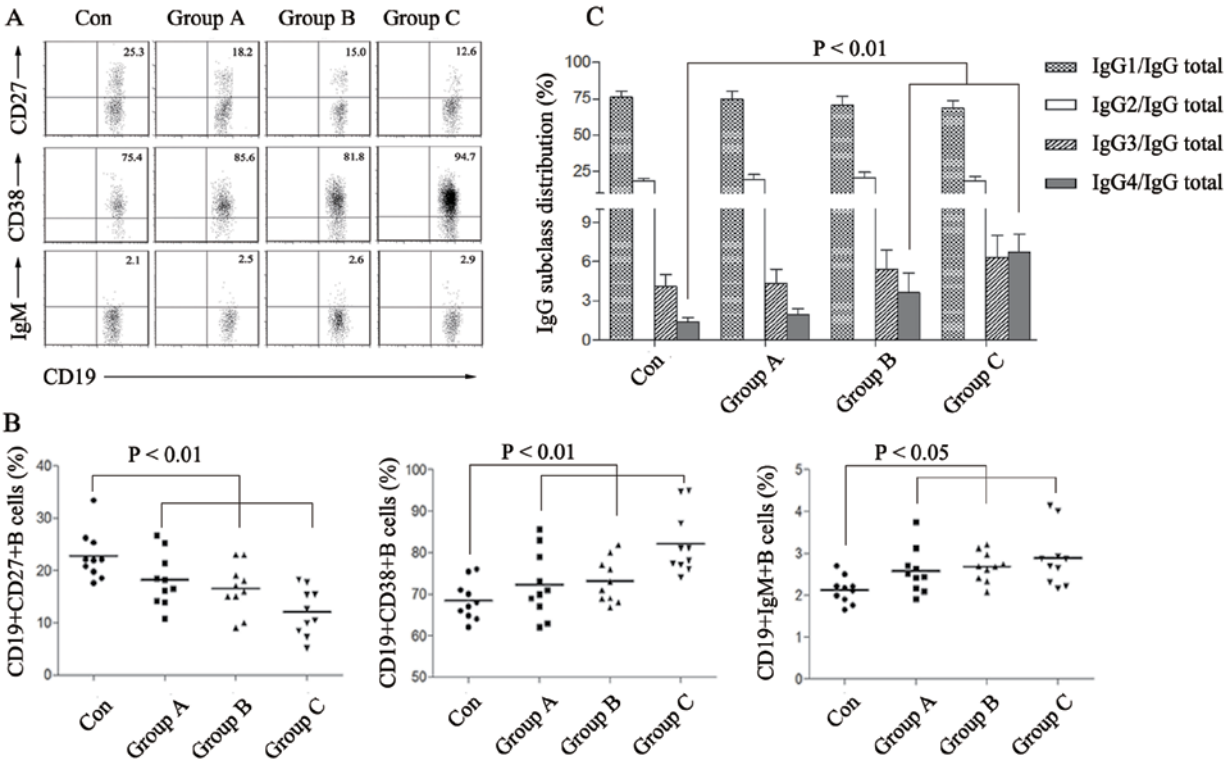

Figure 1. The distribution of different subsets of B cells and IgG subclasses. PBMCs ($5x10^5$/tube) were stained in duplicate with PerCP-anti-CD19, APC-anti-CD38, FITC-anti-CD27 and PE-anti-IgM. The frequency of naïve B cells, memory B cells and mature B cells were characterized by flow cytometric analysis. At least ~50,000 events were analyzed for each sample. Data are expressed as the mean values of individual participants from 2 separate experiments. (A) Flow cytometric analysis of naïve B cells ($CD19^+IgM^+$), memory B cells ($CD19^+CD27^+$) and mature B cells ($CD19^+CD38^+$). (B) Data of the B cell subsets are expressed as the mean values of individual participants from 3 separate experiments. Data were analyzed by the Kruskal-Wallis test. The horizontal lines indicate the median values for each group at the indicated time-points. (C) The variable ratios of IgG1/IgG, IgG2/IgG, IgG3/IgG and IgG4/IgG in the different patient groups and the healthy control as tested by ELISA.

gel electrophoresis and transferred to PVDF membranes (Invitrogen). Membranes were incubated with Abs against CXCR3 and CCR6 (Abcam, Cambridge, MA, USA) followed by an animal-matched horseradish peroxidase-conjugated secondary antibody respectively (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The densitometry score was determined with Quantity One software (V4.6).

Statistical analysis. All data are expressed as mean ± SD, and the Student's t-test was performed to analyze the results of the gene expression profiling assays. P-value <0.05 was considered to indicate a statistically significant result.

Results

Distribution of IgG secreted by B cells is switched toward the IgG4 subclass in the process of prostate cancer. It has been found that the level of IgG4 antibodies is obviously higher in melanoma lesions and is closely correlated with tumor severity (27,28). However, the distribution of IgG subclasses in prostate cancer is still unknown. To examine the proportions of IgG subclasses produced in prostate cancer, B cells from patient peripheral blood were cultured with CpG 2006 ODN and EBV for 5-day stimulation, and then IgG1, IgG2, IgG3, IgG4 and nonspecific IgG titers in the supernatants were measured by ELISA. We found a significantly increased frequency of mature $CD19^+CD38^+$ B cells and a decreased level of memory $CD19^+CD27^+$ B cells in the prostate cancer groups compared with these frequencies in the healthy controls, while the $CD19^+IgM^+$ naïve B cells were not obviously changed (Fig. 1A and B). As assessed using ELISA analysis, each IgG subset/IgG total ratio was IgG1: 76.2±4.1%; IgG2: 18.3±2.1%; IgG3: 4.1±0.9%; IgG4: 1.4±0.3% in the healthy controls; IgG1: 74.7±5.5%; IgG2: 19.1±3.9%; IgG3: 4.3±1.1%; IgG4: 1.9±0.5% in group A; IgG1: 70.5±6.0%; IgG2: 20.5±3.7%; IgG3: 5.4±1.5%; IgG4: 3.6±1.5% in group B and IgG1: 68.6±5.5%; IgG2: 18.4±3.4%; IgG3: 6.3±1.7%; IgG4: 6.7±1.4% in group C respectively (Fig. 1C). The results showed an obvious increase in mature B cells in peripheral circulating blood and the majority of them mainly tended to secret IgG4 antibodies. The high level of $IgG4^+$ B cells may participate in tumor invasion and metastasis.

Percentage of circulating Tfh2 are upregulated in patients with prostate cancer. Tfh cells are a subset of T cells characterized by increased expression of molecules including CXCR5, PD-1, ICOS, CD40L and IL-21, specialized in facilitating B cell growth, differentiation and class switching. Tfh cells generally localize in GCs, however they have been proven to exist in human peripheral blood to regulate the immune system as well (22,29). Here, we identified $CD4^+CXCR5^+$ T cells as Tfh cells and divided Tfh cells into 3 different subsets: $CXCR3^+CCR6^-$ Tfh1 cells, $CXCR3^-CCR6^-$ Tfh2 cells and $CXCR3^-CCR6^+$ Tfh17 cells and then carried out analysis by flow cytometry. We observed that the percentage of Tfh2 cells was positively increased along with prostate cancer

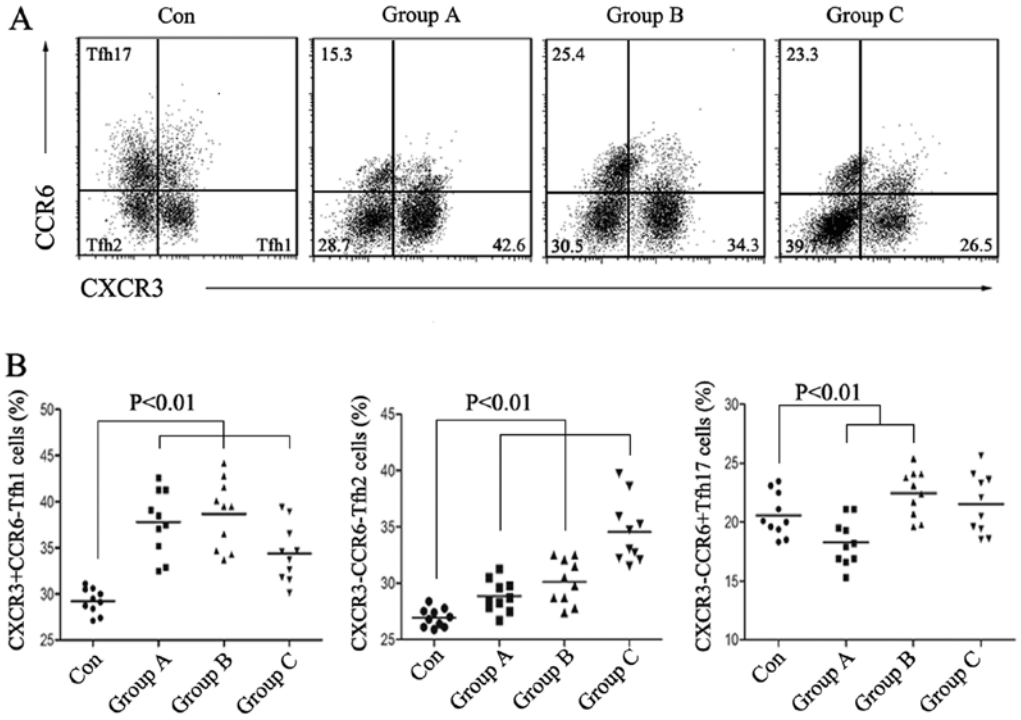

Figure 2. FACS analysis of the numbers of different subsets of Tfh cells in the individual patient groups. PBMCs ($5x10^5$/tube) were isolated from the subjects and were stained in duplicate with anti-CD4, anti-CXCR5, anti-CXCR3 and anti-CCR6. The percentages of Tfh1, Tfh2 and Tfh17 cells were characterized by flow cytometric analysis. At least ~50,000 events were analyzed for each sample. Data are expressed as the mean values of individual participants from 2 separate experiments. (A) Flow cytometric analysis of $CXCR3^+CCR6^-$ Tfh1 cells, $CXCR3^-CCR6^-$ Tfh2 cells and $CXCR3^-CCR6^+$ Tfh17 cells. (B) Data of the different Tfh subsets are expressed as the mean values of individual participants from 3 separate experiments. Data were analyzed by the Kruskal-Wallis test. The horizontal lines indicate the median values for each group.

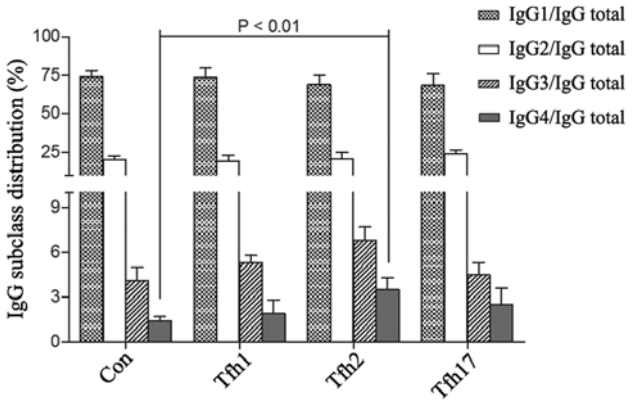

Figure 3. The interaction between Tfh2 cells and B cells. Perpheral blood B cells ($5x10^4$/well) from healthy volunteers were cultured with the corresponding purified Tfh subsets ($2.5x10^5$/well) for 5 days respectively on CD3/CD28 mAb precoated 24-well plates. B cells cultured with no Tfh cells were set as the negative control. CpG 2006 ODN and EBV were used to stimulate B cells *in vitro*. IgG4/IgG total ratio was increased in the B cells cultured with Tfh2 compared with that in the negative control (P<0.05), but was not changed obviously in B cells cultured with Tfh1 or Tfh17.

progression, while that of Tfh17 changed irregularly and Tfh1 showed a sharply decrease in group C (Fig. 2A and B). The results imply that Tfh2 cells participate in the development of prostate cancer.

Tfh2 promotes the production of $IgG4^+$ B cells. Considerating the effect of Tfh cells on promoting B cell maturation, we next investigated the different functions of the 3 Tfh subtypes. We co-cultured peripheral blood B cells from healthy volunteers with correspondingly purified Tfh1, Tfh2 and Tfh17 cells on 24-well plates precoated with CD3 and CD28 mAbs. In contrast, B cells cultured with no stimulus were set to be the negative control. After stimulating B cells with CpG 2006 ODN and EBV for 5 days, we detected an increased IgG4/IgG total ratio in blood B cells cultured with Tfh2 (IgG1: 69.2±6.0%; IgG2: 20.5±4.7%; IgG3: 6.8±0.9%; IgG4: 3.5±0.8%), while the IgG4/IgG total ratio remained low when B cells were cultured with Tfh1 (IgG1: 73.7±6.5%; IgG2: 19.1±3.9%; IgG3: 5.3±0.5%; IgG4: 1.9±0.9%) and Tfh17 (IgG1: 68.6±7.5%; IgG2: 24.0±2.8%; IgG3: 4.5±0.8%; IgG4: 2.5±1.1%) (Fig. 3). Thus in Tfh cells, Tfh2 may act as a regulator to improve polarized expression of IgG4 secreted by B cells.

Inflammatory cytokines significantly improve B cell polarization, inducing IgG4 secretion. Prostate cancer easily develops to become immune tolerant, which is related to the cancer microenvironment characterized by high levels of cytokines such as IL-4, IL-6, IL-10, nitric oxide (NO), TGF-β and PGE2. Taking into account the upregulated $IgG4^+$ B cells in patients with malignant prostate cancer, we explored the above soluble factors that may contribute to the polarization of B cells for producing IgG4 antibodies and investigated whether the effect of these cytokines was mediated by Tfh2. We cultured B cells with $CD4^+CXCR5^+$ Tfh cells from volunteers in DMEM supplemented with CpG 2006 ODN and EBV on a 24-well plate procoated with CD3/CD28 mAbs, and then added

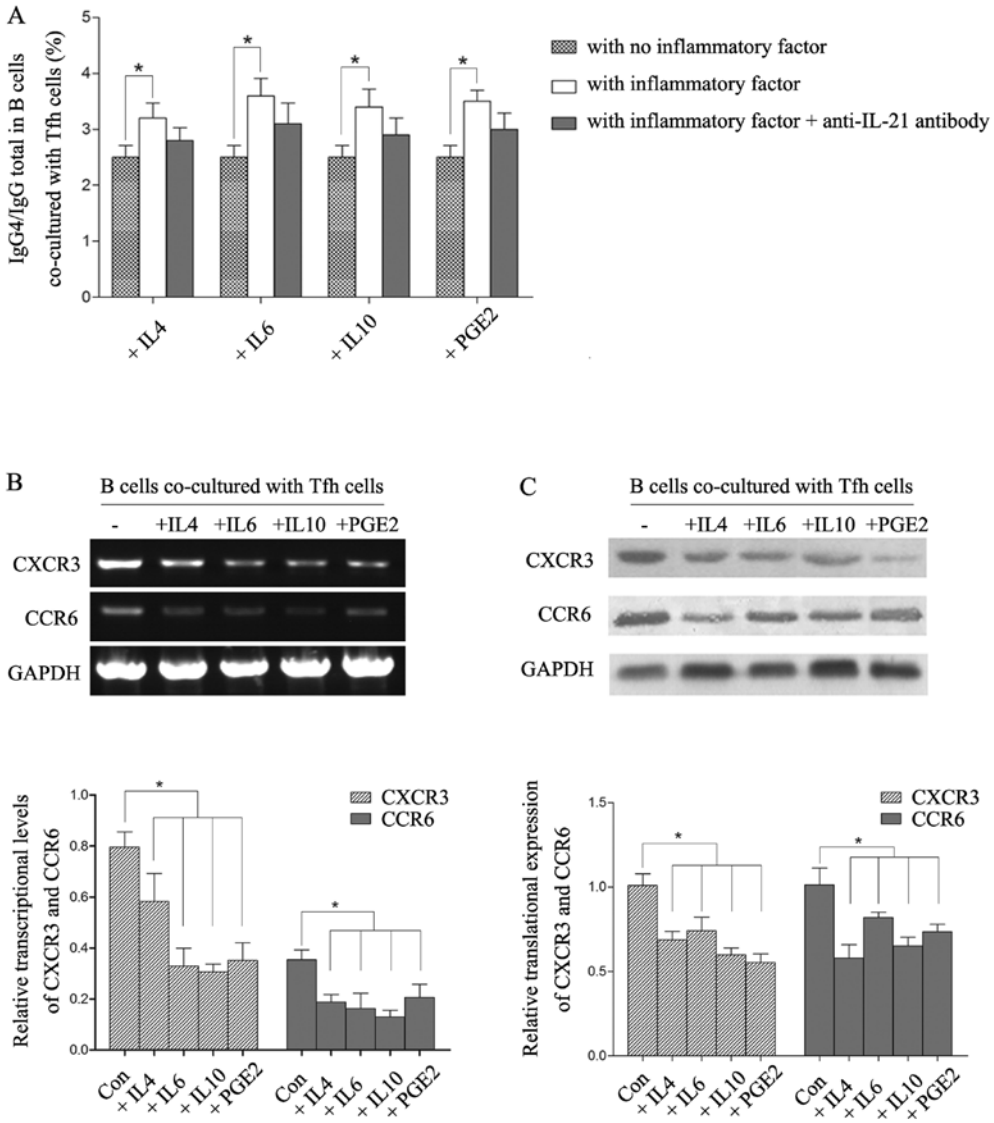

Figure 4. Effect of inflammatory cytokines on increasing B cell polarization. The ratio of IgG4/IgG total was detected in the co-culture system of B cells ($5x10^4$/well) and Tfh cells ($2.5x10^5$/well) before or after IL-4, IL-6, IL-10 and PGE2 treatment. B cells cultured with none of the above mentioned cytokines was set as the negative control. (A) The ratios of IgG4/IgG total detected following IL-4, IL-6, IL-10, PGE2 treatment and in the negative control before or after anti-IL-21 antibody treatment (*P<0.05). (B and C) The transcriptional and translational expression of CXCR3 and CCR6 in Tfh cells treated with the cytokines.

recombinant human IL-4, IL-6, IL-10 and PGE2 respectively into the medium. The ratio of IgG4/IgG total was obviously increased in all IL-4, IL-6, IL-10 and PGE2 treatment groups compared with the ratio in the non-cytokine-treated control (P<0.01). Although the IgG4 ratio was decreased after adding the anti-IL-21 antibody which could inhibit Tfh cell functions, the change was not statistically significant (Fig. 4A). Subsequently, we collected Tfh cells of each group and found that the expressions levels of CXCR3 and CCR6 were both significantly downregulated at the transcriptional level and translational level in the cytokine-treated groups (Fig. 4B and C), which indicated the polarization of the Tfh2 subset. Thus, IL4, IL6, IL-10 and PGE2 facilitated B cells to produce the IgG4 subclass by downregulating CXCR3 and CCR6 to induce Tfh2 cells. Yet, there may exist other regulatory pathways of these inflammatory suppressors to increase the proportion of $IgG4^+$ B cells which is not completely dependent on Tfh cells.

Discussion

Studies have focused on B cell development, and the multiple functions of B cells were explored including modulation of humoral immunity, activation of T lymphocytes by antigen presentation and regulation of the tissue immune microenvironment by crosstalk with other soluble cytokines. The main mediators of B cells are different immune globulin subtypes (IgA, IgG, IgM and IgE) and IgG was proven to be essential in the regulation of tumorigenesis, invasion and metastasis (30,31). The proportion of the IgG subclass (IgG1, IgG2, IgG3 and IgG4) is 65, 25, 6 and 4% of the total IgG, respectively, in healthy adult serum, but these proportions were found to be altered in certain diseases such as various types of cancer (14,32).

In the present study, we observed an abnormal distribution of 4 IgG subclasses existing in patients with prostate cancer as well. The ratio of IgG4/IgG total was positively increased

in the patient groups (6.7±1.4% in clinical stage ≥T3 group, 3.6±1.5% in stage T2 group, 1.9±0.5% in stage ≤T2 group and 1.4±0.3% in healthy controls, respectively). In contrast, the IgG1/IgG total ratio was gradually decreased with the progression of cancer. The result indicated a quite abnormal polarization toward IgG4 in patients with aggressive prostate cancer, implying a potential interaction between $IgG4^+$ B cells and features involved in tumor progression.

Considerating the development of B cells, we further detected Tfh cells, which were considerate as the main helpers to promote B cell proliferation and maturation. Since the percentage of Tfh2 cells increased positively in the progression of prostate cancer, we concluded there existed a polarization of Tfh cells for producing the Tfh2 subset. To illustrate the different effects of Tfh subsets, we co-cultured B cells with Tfh1, Tfh2 and Tfh17 cells respectively, and tested the IgG distribution. As we predicted, the IgG4/IgG total ratio was obvious higher in the Tfh2 co-cultured group than that in the Tfh1 or Tfh17 co-cultured group. Thus in Tfh cells, Tfh2 may act as a key regulator to improve B cell polarization, resulting in antibody class switching to IgG4.

The immune tolerant environment in the interior of malignant tumors has been recognized as a key characteristic which promotes tumor proliferation or invasion and what is more important facilitates the escape from immune surveillance (33,34). Various immune cell types have been shown to be related to immune tolerance in different diseases or experiments, including regulatory T cells (Tregs), myeloid-derived suppressor cells (MDSCs), tumor-associated macrophages (TAMs), nature killer T (NKT) cells and regulatory dendritic cells (DCs). These immune response inhibitors act mainly by suppressing Th1 and Th2 responses through IFN-γ dependent NO or PGE2 production, secretion of IL-4/6/10, inducing Treg cells and mutually interacting with each other (11,35-38). Taking into account the upregulated $IgG4^+$ B cells and Tfh2 cells in patients with aggressive prostate cancer, we hypothesized that these inflammatory suppressors could improve the alteration of the subtypes in B or Tfh cells during cancer development. By co-culturing B cells with Tfh cells *in vitro*, IL-4, IL-6, IL-10 and PGE2 were added into the medium, respectively. We observed an obviously increase in the level of the ratio IgG4/IgG total in each IL-4/IL-6/IL-10 and PGE2 treated group, which confirmed the ability of these anti-inflammatory cytokines to induce the concentration of IgG4 antibodies. However, inhibition of Tfh cell functions using the anti-IL-21 antibody was not effective in preventing the upregulation of IgG4 stimulated by IL-4, IL-6, IL-10 and PGE2. Meanwhile, we found that the expression levels of CXCR3 and CCR6 were decreased after IL-4/6/10/PGE2 treatment, which implied the polarization of the Tfh2 subset. Thus, the above inflammatory suppressors may contribute to the polarization of B cells for producing IgG4 antibodies by downregulating CXCR3 and CCR6 to promote the Tfh2 cell proportion. Yet, there still may exist other regulatory pathways of these cytokines causing an increase in the proportion of $IgG4^+$ B cells, which is not completely dependent on Tfh cells.

In conclusion, these data provide additional evidence of the B cell response to the immunesuppressiven environment by evaluating IgG4 antibodies and established the relationship between $IgG4^+$ B cells and Tfh2 cells. Although alterations in the specific T/B subtypes were confirmed to be strongly associated with tumor proliferation and malignancy, the definite mechanisms such as immunoregulatory signaling pathways remain obscure. Clarification of the lymphocyte functions in the inflammatory microenvironment of tumors may be of potential therapeutic value.

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