PD-1-expressing B cells suppress CD4+ and CD8+ T cells via PD-1/PD-L1-dependent pathway

Xufu Wang, Guoqiang Wang, Zenghua Wang, Bin Liu, Na Han, Jiao Li, Chenghui Lu, Xinfeng Liu, Qin Zhang, Qingbo Yang, Guoming Wang*

Department of Nuclear Medicine, The Affiliated Hospital of Qingdao University, Qingdao, Shandong, 266003, China

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ABSTRACT

B cell-mediated regulatory function is instrumental to the maintenance of tolerance, but may also contribute to immune dysfunction during infectious diseases and malignancies. In this study, we investigated a subset of B cells characterized by PD-1 expression. Data showed that these PD-1+ B cells were rare in peripheral blood, but were significantly upregulated in differentiated thyroid tumors. The PD-1+ B cells also expressed significantly higher level of PD-L1. Continuous, but not short-term, anti-Ig/CD40 L stimulation could upregulate the expression of PD-1 and PD-L1 in B cells. In in vitro experiments, PD-1+ B cells significantly suppressed the proliferation of CD4+ and CD8+ T cells and reduced their viability upon CD3/CD28 stimulation, thus suggesting that these PD-1+ B cells presented regulatory functions. However, unlike other IL-10-secreting Breg cell subsets, the PD-1+ B cells did not express high level of IL-10. Instead, it seemed that PD-L1 was instrumental to the suppressive effects mediated by PD-1+ B cells, since the blockade of PD-L1 significantly increased the proliferation and viability of T cells in the coculture. Interestingly, compared to untreated patients with differentiated thyroid tumor, the thyroidectomy and 131I-treated patients presented significantly lower frequencies of PD-1+ B cells. Together, our investigation demonstrated that the PD-1+ B cells possessed regulatory capacity toward T cell responses, and although rare in peripheral blood, they were significantly enriched in thyroid tumors.

1. Introduction

Based on the etiological and clinical features, thyroid cancers can be distinguished into differentiated thyroid cancer (DTC) and anaplastic thyroid cancer (ATC) (Carling and Udelsman, 2014). DTC is a slow-progressing disease and when diagnosed early, DTC can be effectively managed with surgical resection and radioiodine therapy (131I), with a five-year survival rate near 100%. However, at later stages, DTC has a significantly worse five-year survival rate at approximately 30% to 80% (Carhill et al., 2015; Durante et al., 2006). Distant metastasis is found in a small minority of patients, and a high proportion of these patients eventually succumbs to secondary malignancies (Mazzaferri and Jhiang, 1994). Also, the malignancy can recur in patients who initially responded to treatment (Mazzaferri and Massoll, 2002). The other thyroid cancer subtype ATC, though rare, is one of the most aggressive human malignancies. ATC tumors often invade or encase vital body structures in the patients, making complete resection unachievable (McIver et al., 2001). Response to other treatments is also poor. Patients with ATC have a median survival of less than five months. In addition, it is thought that ATC can arise from well-differentiated tumors (Carling and Udelsman, 2014). Further research into the genetic, environmental, and immunological factors involved in thyroid cancer is needed for the development of better therapeutic strategies.

We previously demonstrated that IL-10-producing B cells were enriched in DTC patients [manuscript under review]. These B cells belong to a broader category of regulatory B (Breg) cells that have the capacity to inhibit pathogenic inflammation and promote peripheral tolerance, mainly through the production of IL-10 (Rosser and Mauri, 2015; Tedder and Leonard, 2014; Xiao et al., 2012). Recently, it is shown that B cells can also regulate immune responses via the PD-1/PD-L1 pathway (Gallego-Valle et al., 2018; Khan et al., 2015; Siewe et al., 2013; Xiao et al., 2016). Activation of B cells via CpG alone or a combination of toll-like receptor agonists, together with CD40 costimulation, could significantly increase the expression of both PD-1 and PD-L1 on those B cells (Gallego-Valle et al., 2018; Siewe et al., 2013). Via PD-L1, Breg cells could reduce the expansion of PD-1+ Tfh cells, thus downregulating humoral immune responses (Khan et al., 2015). Adoptive transfer of PD-L1hi B cells attenuated murine experimental

autoimmune encephalomyelitis. In murine EMT-6 model, tumor-infiltrating B cells demonstrated higher PD-L1 expression than splenic B cells (Zhang et al., 2016). These B cells could suppress the proliferation of $CD4^+$ T cells and $CD8^+$ T cells in a manner reversible by anti-PD-L1 antibody. In advanced hepatocellular carcinoma, PD-1^{hi} B cells comprised approximately 10% of total B cells, and upon encountering PD-L1, these PD-1^{hi} B cells, suppressed the response of tumor-specific T cells, and enhanced tumor growth via IL-10 (Xiao et al., 2016).

In DTC, whether B cells could modulate antitumor immunity via the PD-1/PD-L1 pathway has not been demonstrated. Here, we approached this problem by identifying the existence and level of PD-1/PD-L1 expression in B cells from DTC patients. Later, whether these B cells affected the function of autologous T cells was investigated. Finally, effect of treatment on the frequency of PD-1^+ B cells was examined.

2. Methods

2.1. Study subjects

This study was approved by the Ethics Committee of The Affiliated Hospital of Qingdao University. Prior to the collection of samples, all volunteers gave written informed consent for participation in this study. Diagnosis and recruitment were performed at The Affiliated Hospital of Qingdao University. To qualify for this study, the patients must be between 18 to 65 years of age, and present new onset untreated DTC. Controls were recruited from age- and sex-matched healthy individuals. In addition, potential participants with autoimmunity, ongoing infection, renal diseases, hepatic diseases, other thyroid diseases, recurrent thyroid cancer, or other malignancies were excluded. No participant was taking glucocorticoids or other immunosuppressants at the time of sample collection. All cancer patients later received thyroidectomy and radioiodine treatment. Peripheral blood of the patients was collected at diagnosis prior to any treatment and after thyroidectomy and radioiodine treatment. Peripheral blood of healthy controls was also obtained.

2.2. Sample collection

To process peripheral blood, a Ficoll gradient was used to separate peripheral blood mononuclear cells (PBMCs) from erythrocytes and plasma. Additionally, freshly resected tumor samples were obtained from 12 patients. The tumors were minced into 1 to 2 mm^2 pieces and incubated in a triple enzyme digestion mix (collagenase type IV at 0.05%, hyaluronidase type V at 1000 U/mL, and DNase I at 5 U/mL; Sigma) for 6 h at 37 °C. The resulting dissociation product was then filtered using a 70-μm strainer (Falcon) to remove undigested clumps.

2.3. Flow cytometry

PBMCs or tumor-infiltrating lymphocytes (TILs) were incubated with Fixable Violet Live/Dead Cell Stain (Invitrogen) and anti-human CD19, PD-1, and PD-L1 (BioLegend) for 30 min in dark on ice. Excess stains were removed with rigorous washing. The samples were then acquired in a FACSCanto cytometer. Live B cells were identified using lymphocyte-specific FSC vs. SSC gating, followed by Violet-negative and CD19-positive gating. For B cell stimulation, the cells were incubated with 1 μg/mL anti-Ig (anti-IgM/IgG/IgA; Jackson ImmunoResearch) and 1 μg/mL recombinant human CD40 L (Enzo Life Sciences) for a total of 14 days. A portion of the cells was removed every 2 days for flow cytometry analysis.

2.4. mRNA analysis

Total B cells were isolated using Human B Cell Enrichment Kit (Stemcell Technologies). PD-1^+ and PD-1^- B cells were sorted using flow cytometry in a FACSAria cytometer, and were lysed using RNeasy

Mini Kit (Qiagen) to collect total RNA. cDNA was converted from mRNA transcripts via High-Capacity cDNA Reverse Transcription kit (Applied Biosystems). Quantification was then performed using gene expression assay Hs00961622_m1 for human IL10 (TaqMan) with SYBR Green Master Mix (Applied Biosystems) in an ABI PRISM 7000 instrument.

2.5. B cell/T cell coincubation

$CD4^+$ and $CD8^+$ T cells were isolated from PBMCs using Human $CD4^+$ T Cell Enrichment Kit and Human $CD8^+$ T Cell Enrichment Kit (Stemcell Technologies), respectively. PD-1^+ and PD-1^- B cells were coincubated with autologous $CD4^+$ and $CD8^+$ T cells at 1: 2 ratio. T cells were stimulated with 2 μg/mL anti-human CD3 and 4 μg/mL anti-human CD28 (BioLegend). In select experiments, 10 μg/mL anti-PD-L1 antibody MIH3 or irrelevant mouse IgG1, κ isotype control (BioLegend) was added. After 72 h, a portion of the cells was pulsed with ^{3}H-thymidine (PerkinElmer) for 6 h, and the level of ^{3}H-thymidine incorporation was measured in a beta-counter. The rest of the cells were stained with anti-human CD3 and CD4 antibodies (BioLegend) and Fixable Violet Live/Dead Cell Stain for 30 min. Viable $CD4^+$ T cells were identified using lymphocyte-specific FSC vs. SSC gating, followed by CD3-positive, CD4-positive, and Violet-negative gating.

2.6. Statistical analysis

Data were analyzed using Prism 7.0 (GraphPad software). All tests were two-tailed, and $p < 0.05$ was considered significant. The specific tests applied to each experiment were specified in the figure legends.

3. Results

3.1. PD-1/PD-L1 expression B cells from DTC patients and controls

To examine the expression of PD-1/PD-L1 on B cells and the role of PD-1/PD-L1-expressing B cells in DTC pathogenesis, we collected the peripheral blood mononuclear cells (PBMCs) from healthy controls (n = 20) and the PBMCs from DTC patients (n = 20). Characteristics of study participants are presented in Table 1. In addition, tumor-infiltrating lymphocytes (TILs) from the stage II and stage III DTC patients (n = 12) were obtained from resected tumors obtained after thyroidectomy. The patients who donated TIL samples were matched with the healthy controls and the other patients in age and sex. The surface expression of PD-1 and PD-L1 on $CD19^+$ B cells was evaluated *ex vivo* using surface staining and flow cytometry (Fig. 1A). Distinct PD-1^+ and PD-1^- populations could be observed in B cells. The frequency of PD-1^+ B cells in blood was generally below 3% in both healthy subjects and DTC subjects (Fig. 1B), with no significant differences between the two groups, though several DTC subjects presented higher PD-1^+ B cell frequency than the whole healthy group. The PD-1^+ B cell frequency in DTC tumor varied between 4.2% and 36% (Fig. 1B), and was significantly higher than the PD-1^+ B cell frequency in either healthy

Table 1
Demographic and clinical characteristics of study participants.

|	Healthy	DTC	*p*
n	20	20	
Sex (F/M)	12/8	12/8	> 0.05
Age (years, mean ± SD)	44.1 ± 6.4	43.3 ± 5.8	> 0.05
Tumor Stage (n)			
I		8	
II		8	
III		4	

p values were examined using Fisher's exact-test for sex ratio and Student's *t* test for age.
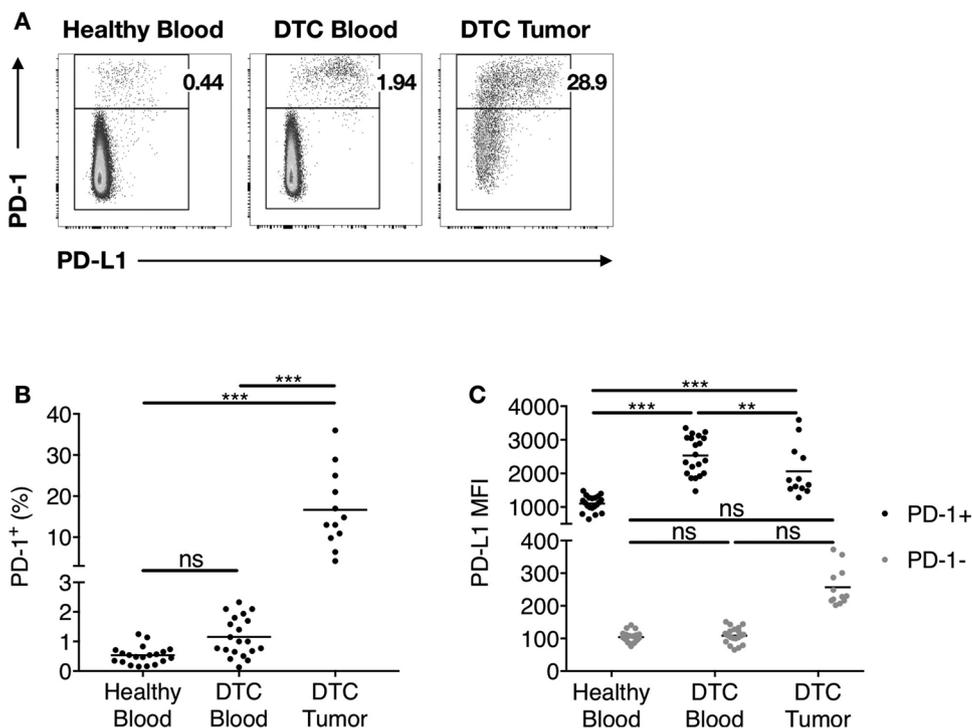
Fig. 1. PD-1/PD-L1 expression in B cells from DTC patients and controls.

(A) Expression of PD-1 vs. PD-L1 in pre-gated CD19$^+$ B cells from representative samples. Numbers at the upper right corner indicate the percentage of PD-1$^+$ cells. (B) The frequency of PD-1$^+$ cells in total B cells in healthy blood, DTC blood, and DTC tumor. 1-way ANOVA and Tukey's post-test. (C) The PD-L1 mean fluorescence intensity (MFI) in PD-1$^+$ and PD-1$^-$ B cells from healthy blood, DTC blood, and DTC tumor. The *p* value between PD-1$^+$ and PD-1$^-$ B cells (not labeled) was < 0.001 for every group. 2-way ANOVA and Tukey's post-test. ns, not significant. ***p < 0.001.
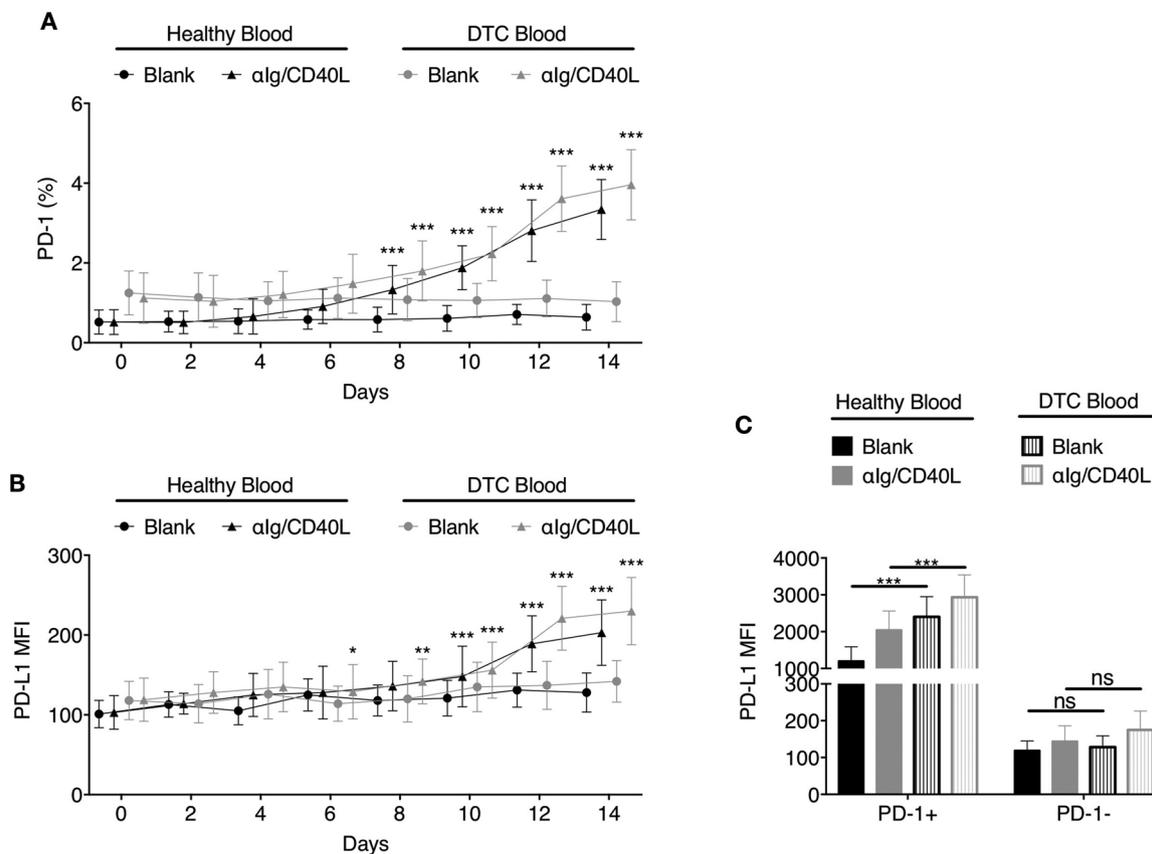
Fig. 2. Expression of PD-1 following B cell stimulation.

B cells were incubated in blank media or with 1 µg/mL anti-Ig and 1 µg/mL CD40 L for a total of 14 days. (A) The frequency of PD-1 and (B) the level of PD-L1 MFI in B cells were examined every 2 days via flow cytometry. Statistically significant differences between the experiment and the day 0 baseline were indicated by asterisks. (C) The PD-L1 MFI in PD-1$^+$ and PD-1$^-$ B cells from healthy blood and DTC blood, after 14-day incubation in blank media or with anti-Ig/CD40 L. 2-way ANOVA and Tukey's post-test. ns, not significant. *p < 0.05. **p < 0.01. ***p < 0.001.
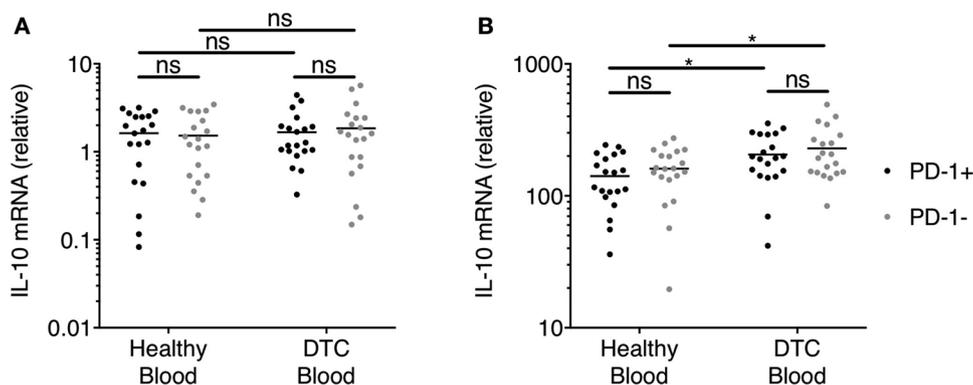
Fig. 3. IL-10 transcription by PD-1$^+$ vs. PD-1$^-$ B cells.
(A) IL-10 mRNA transcript levels in unstimulated PD-1$^+$ vs. PD-1$^-$ B cells. (B) IL-10 mRNA transcript levels in PD-1$^+$ vs. PD-1$^-$ B cells following 1 µg/mL anti-Ig and 1 µg/mL CD40 L stimulation for 14 days. 2-way ANOVA and Tukey's post-test. ns, not significant. $*p < 0.05$.
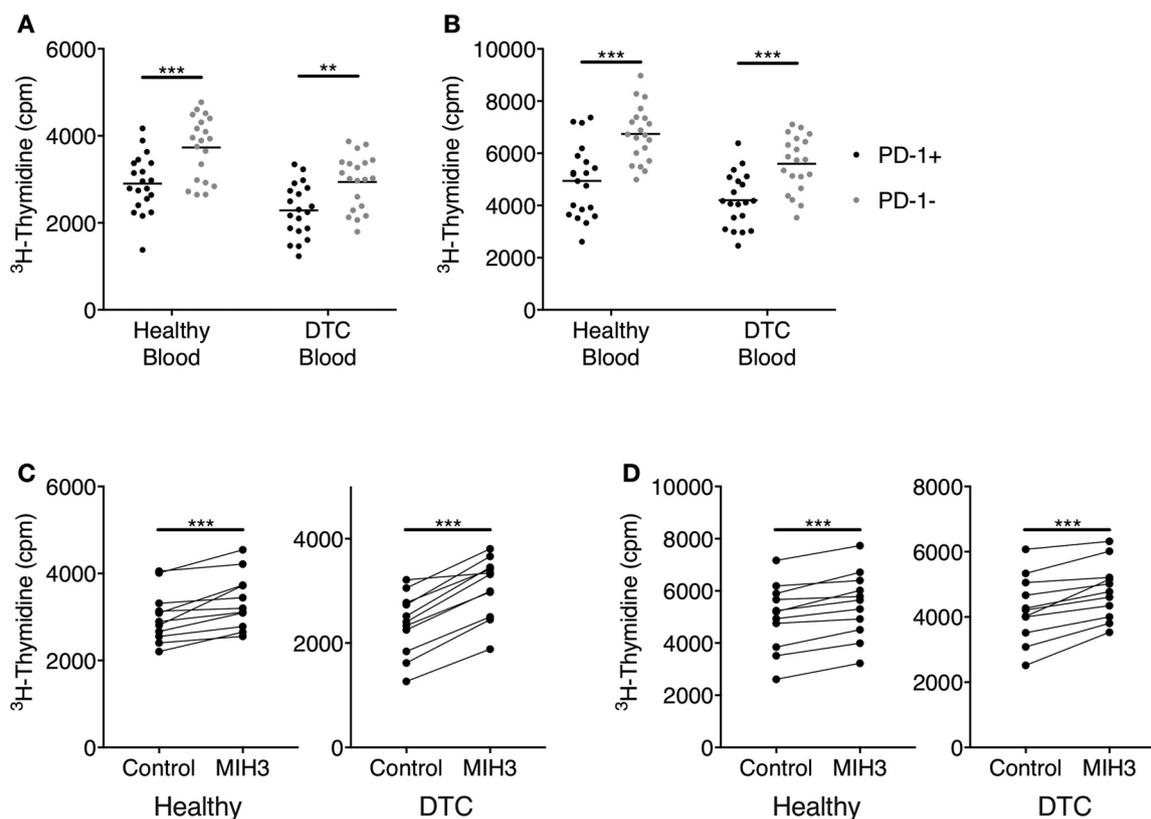
Fig. 4. Proliferation of CD4$^+$ T cells and CD8$^+$ T cells following coincubation with PD-1$^+$ or PD-1$^-$ B cells.
(A) Proliferation of CD4$^+$ T cells after coincubation with PD-1$^+$ or PD-1$^-$ B cells. (B) Proliferation of CD8$^+$ T cells after coincubation with PD-1$^+$ or PD-1$^-$ B cells.
(C) Proliferation of CD4$^+$ T cells with PD-1$^+$ B cells, in the presence of 10 µg/mL PD-L1 blocking antibody MIH3 or isotype control. 2-way ANOVA and Tukey's post-test. (D) Proliferation of CD8$^+$ T cells with PD-1$^+$ B cells, in the presence of 10 µg/mL PD-L1 blocking antibody MIH3 or isotype control. Paired *t*-test $**p < 0.01$.
$***p < 0.001$.

subjects or DTC subjects.

A continuous gradient-like distribution of PD-L1 was observed (Fig. 1A). While PD-L1 expression was not exclusive to the PD-1$^+$ fraction, it was highly concentrated in the PD-1$^+$ fraction. In DTC blood B cells, the vast majority of PD-1$^+$ fraction presented high PD-L1 expression, while in DTC tumor B cells, both PD-L1^{high} and PD-L1^{low} expression could be observed in the PD-1$^+$ fraction (Fig. 1A). PD-L1 MFI was significantly lower in the PD-1$^-$ B cells than in the corresponding PD-1$^+$ B cells from healthy blood, DTC blood, and DTC tumor (Fig. 1C). Additionally, in PD-1$^+$ B cells, the PD-L1 expression was significantly higher in DTC blood than in DTC tumor, which itself expressed significantly higher PD-L1 than healthy blood (Fig. 1C). In PD-1$^-$ B cells,

the PD-L1 expression was not significantly different among the groups.

3.2. Long-term stimulation upregulated PD-1/PD-L1 in B cells

On T cells, the PD-1/PD-L1 pathway is activated upon antigen-specific stimulation as well as T cell exhaustion (Keir et al., 2008; Wei et al., 2013). Hence, to examine the mechanism of PD-1/PD-L1 upregulation in B cells, we stimulated blood B cells using anti-Ig/CD40 L in a B cell receptor-dependent pathway. Compared to unstimulated B cells, anti-Ig/CD40L-stimulated B cells initially did not upregulate PD-1 expression (Fig. 2A). However, from day 8 to day 14, the frequency of PD-1$^+$ cells gradually increased, to about 3% on average in healthy blood B
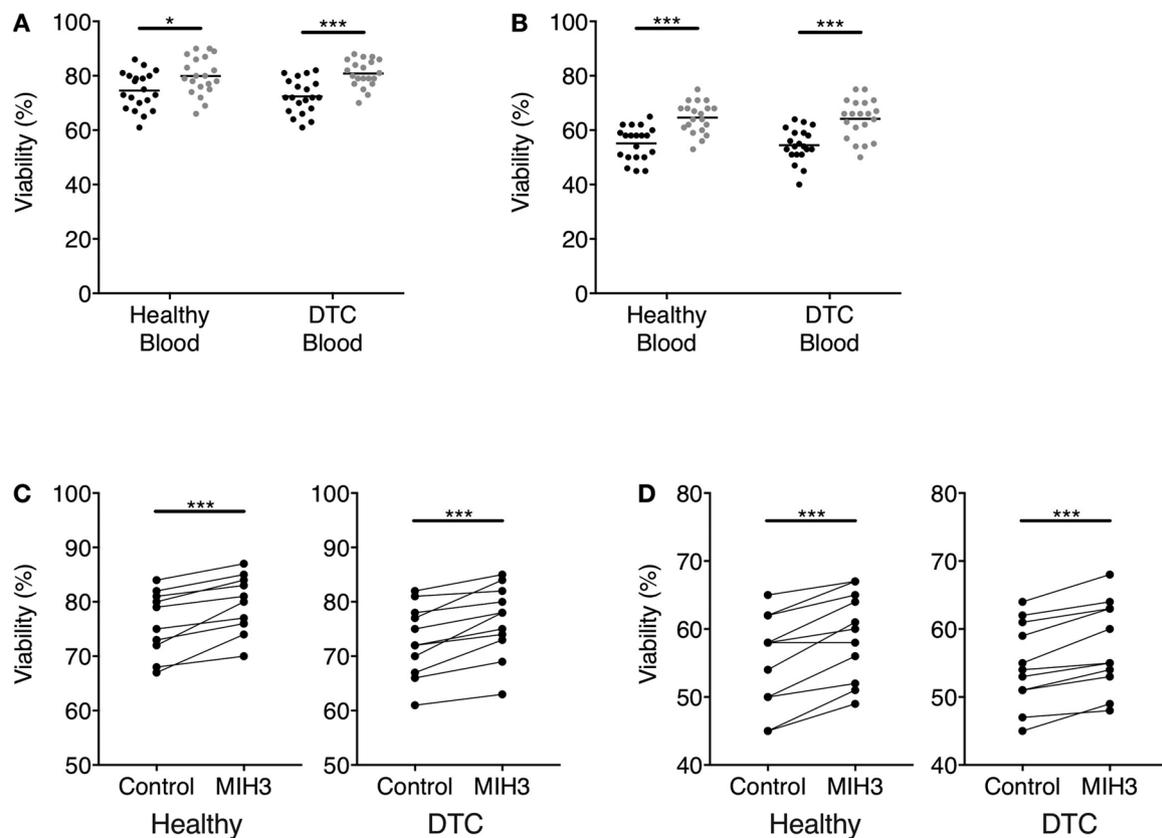
Fig. 5. Viability of CD4$^+$ T cells and CD8$^+$ T cells following coincubation with PD-1$^+$ or PD-1$^-$ B cells.
(A) Viability of CD4$^+$ T cells after coincubation with PD-1$^+$ or PD-1$^-$ B cells. (B) Viability of CD8$^+$ T cells after coincubation with PD-1$^+$ or PD-1$^-$ B cells. (C) Viability of CD4$^+$ T cells with PD-1$^+$ B cells, in the presence of 10 μg/mL PD-L1 blocking antibody MIH3 or isotype control. 2-way ANOVA and Tukey's post-test. (D) Viability of CD8$^+$ T cells with PD-1$^+$ B cells, in the presence of 10 μg/mL PD-L1 blocking antibody MIH3 or isotype control. Paired *t*-test *$p < 0.05$. ***$p < 0.001$.
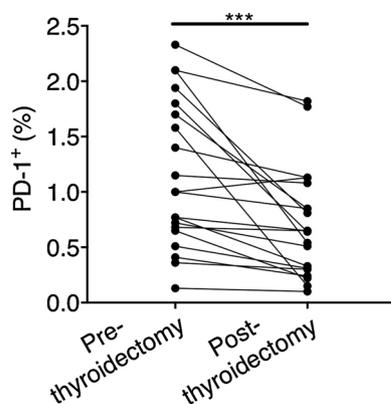
Fig. 6. The frequency of PD-1$^+$ B cells pre- and post-thyroidectomy and ^{131}I treatment. Paired *t*-test ***$p < 0.001$.

cells, and about 4% on average in DTC blood B cells (Fig. 2A). The level of PD-L1 expression also remained fairly constant, or with small incremental increases during the initial phase of the anti-Ig/CD40 L stimulation. Significant increases in PD-L1 expression were only observed after day 10 in healthy subjects and day 6 in DTC patients (Fig. 2B). At the end of incubation, the level of PD-L1 expression was examined separately for PD-1$^+$ and PD-1$^-$ B cells (Fig. 2C). The preference of PD-L1 expression by PD-1$^+$ B cells was maintained after incubation, and in both healthy controls and DTC patients, the anti-Ig/CD40L-stimulated PD-1$^+$ B cells presented higher PD-L1 than the unstimulated PD-1$^+$ B cells.

3.3. PD-1$^+$ B cells did not express higher IL-10 than PD-1$^-$ B cells

IL-10 is instrumental to Breg-mediated suppression (Mauri, 2010; Wang et al., 2014). Hence, we examined the IL-10 production in PD-1$^+$ vs. PD-1$^-$ B cells. Unstimulated B cells *ex vivo* expressed only minimal levels of IL-10 transcription, and no differences between healthy controls and DTC patients, or between PD-1$^+$ and PD-1$^-$ B cells were found (Fig. 3A). Anti-Ig/CD40L-stimulated B cells, on the other hand, produced markedly higher levels of IL-10 (Fig. 3B). Both PD-1$^+$ and PD-1$^-$ B cells from DTC patients presented slightly but significantly higher IL-10 than the corresponding B cell subsets from healthy controls. However, no significant difference between PD-1$^+$ and PD-1$^-$ B cells was observed.

3.4. PD-1$^+$ B cells inhibited autologous T cell proliferation via PD-L1-dependent pathway

Whether PD-1$^+$ and PD-1$^-$ B cells possessed regulatory function was then examined. To obtain higher amount of PD-1$^+$ B cells, total B cells were stimulated via anti-Ig/CD40 L for 14 days and were sorted into PD-1$^+$ and PD-1$^-$ subsets. The sorted PD-1$^+$ B cells and PD-1$^-$ B cells were then coincubated with autologous CD4$^+$ and CD8$^+$ T cells at 1: 2 ratio. The T cells were stimulated with anti-CD3/anti-CD28 for 72 h. The proliferation of the T cells was examined. Both healthy blood T cells and DTC blood T cells presented significantly lower proliferation when coincubated with PD-1$^+$ B cells than with PD-1$^-$ B cells (Fig. 4A and B). Given that the PD-L1 expression was largely concentrated in the PD-1$^+$ fraction, we investigated the effect of blocking PD-L1 using anti-PD-L1 antibody MIH3. The addition of MIH3 significantly increased the proliferation of CD4$^+$ and CD8$^+$ T cells, in healthy subjects and in DTC

subjects (Fig. 4C and D). Due to the limitation that PD-1$^+$ B cell frequency was too low, only 11 out of 20 subjects from each group was included in the PD-L1 inhibition assay. These controls and patients were matched with the rest of the participants in terms of age, sex, and disease severity.

3.5. PD-1$^+$ B cells caused worse viability of autologous T cell via PD-L1-dependent pathway

The PD-1/PD-L1 pathway may also suppress T cell inflammation via the induction of apoptosis (Keir et al., 2008). Hence, we examined the viability of CD4$^+$ T cells and CD8$^+$ T cells using Violet Live/Dead cell staining and flow cytometry. In both healthy controls and DTC patients, the CD4$^+$ and CD8$^+$ T cells coincubated with PD-1$^+$ B cells presented significantly worse viability than the CD4$^+$ and CD8$^+$ T cells coincubated with PD-1$^-$ B cells (Fig. 5A). In addition, blocking PD-L1 using the isotype control antibody MIH3 significantly elevated the viability of CD4$^+$ and CD8$^+$ T cells, in healthy subjects and in DTC subjects (Fig. 5C and D).

3.6. Surgical treatment and radioiodine therapy significantly reduced the frequency of PD-1$^+$ B cells

After collection of PBMCs, all DTC patients in our cohort had undergone thyroidectomy and radioiodine treatment. To investigate whether these processes could affect PD-1$^+$ B cells, PBMCs were collected from DTC patients after treatment. Compared to the frequency of blood PD-1$^+$ B cells pre-thyroidectomy, reduction in the frequency of blood PD-1$^+$ B cells was observed in the majority of patients post-thyroidectomy (Fig. 6).

4. Discussion

In this study, we characterized PD-1-expressing B cells in DTC patients and healthy controls. These PD-1$^+$ B cells expressed high PD-L1, and could be induced upon long-term, but not short-term, anti-Ig/CD40 L stimulation. However, even after long-term stimulation, the PD-1$^+$ B cell frequency was still low at 3%–4%. in vitro incubation with autologous T cells demonstrated that these PD-1$^+$ B cells could suppress the expansion of T cells and reduce their viability, thus suggesting that these PD-1$^+$ B cells presented regulatory functions. However, unlike other IL-10-secreting Breg cell subsets (Carter et al., 2011), these PD-1$^+$ B cells did not express higher IL-10 than the PD-1$^-$ B cells. Instead, it seemed that PD-L1 was instrumental to the suppressive effects mediated by PD-1$^+$ B cells, since the blockade of PD-L1 significantly increased the proliferation and viability of T cells in the coculture.

Xiao et al. in human hepatocellular carcinoma (HCC) patients similarly identified a PD-1^{hi} B cell subset with protumorigenic activity (Xiao et al., 2016). Similar to our observations, the PD-1^{hi} B cell subset was rare in the peripheral blood of healthy controls and HCC patients, but was significantly upregulated in the tumor. Also, these HCC tumor PD-1^{hi} B cells were not proficient at IL-10 expression when stimulated via CD40 L and anti-IgM, similar to our PD-1$^+$ B cells in DTC patients. However, Xiao et al. in addition demonstrated that these PD-1^{hi} B cells from HCC tumors expressed high IL-10 when incubated with anti-PD-1 antibody. This mechanism is unlikely to function in our PD-1$^+$ B cells, since PD-L1 is also expressed by these B cells but no IL-10 upregulation was observed.

Besides IL-10, several studies have demonstrated that the IL-10-expressing Breg cells also suppressed autologous CD4$^+$ T cells a contact-dependent manner that was not specified (Blair et al., 2010; Mauri et al., 2003). Here, we demonstrated that the PD-1/PD-L1 pathway could act as a contact-dependent mechanism of B cell-mediated suppression. However, it remains unclear whether the IL-10-expressing Breg cells identified in previous studies could also utilize this PD-1/PD-L1 pathway. It is also possible that a certain degree of overlap may exist

between PD-1$^+$ B cells and the previous Breg subsets, but the specific details are unknown.

We also found that patients with thyroidectomy and ^{131}I treatment presented lower levels of PD-1$^+$ B cells than untreated patients. The underlying reason for this observation is unknown. It is also possible that the treated patients benefitted from the reduction of PD-1$^+$ B cells in addition to the removal of the tumor mass and radioiodine-mediated tumor ablation. The possibility of eliminating PD-1$^+$ B cells as a form of treatment should be examined in cancer patients.

Conflict of interest

None.

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