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Research paper

Dysfunction of CD24 + CD38 + B cells in patients with Hashimoto's thyroiditis is associated with a lack of interleukin 10

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ABSTRACT

Autoimmune thyroid disease (AITD) is characterized by immune attacks on the person's own thyroid. Hashimoto's thyroiditis (HT) is a subtype of AITD and is a common cause of hypothyroidism and related symptoms. Regulatory B (Breg) cells can express interleukin 10 (IL-10) and have recently emerged as a critical participant in suppression pathogenic inflammation and promoting peripheral tolerance. The role of Breg cells in HT is not yet clear. In this study, we first examined the IL-10 production by B cells in healthy controls and HT patients, and found that the healthy control B cells demonstrated significantly higher IL-10 expression than HT B cells after CpG stimulation. In both groups, the IL-10-producing B cells were highly enriched in the $CD24^+CD38^+$ compartment. However, compared to healthy controls, HT patients presented higher levels of circulating $CD24^+CD38^+$ B cells, but lower percentage of $IL\text{-}10^+$ cells in the $CD24^+CD38^+$ B cell compartment. In healthy controls, we performed coculture experiments of T cells with autologous total B cells, $CD24^+CD38^+$ B cells, and non-$CD24^+CD38^+$ B cells, and found significantly lower T cell proliferation as well as tumor necrosis factor (TNF) and interferon gamma (IFN-γ) production in cell cultures containing $CD24^+CD38^+$ B cells. In contrast, the HT $CD24^+CD38^+$ B cells demonstrated reduced capacity in suppressing T cell proliferation and did not suppress TNF and IFN-γ production. This lack of inhibitory activity in HT $CD24^+CD38^+$ B cells was related to a lack of IL-10, since addition of exogenous IL-10 in $CD24^+CD38^+$ B cell-T cell coculture significantly suppressed the proliferation of T cells and reduced proinflammatory cytokine secretion. Together, our study identified an upregulation of $CD24^+CD38^+$ B cells but a downregulation in their regulatory activity in HT patients.

1. Introduction

Autoimmune thyroid disease (AITD) is a relatively common inflammatory disorder affecting 1% to 4% of the total world population (Brown, 2009). Hashimoto's thyroiditis (HT) is a subtype of AITD, characterized by immunity-mediated attacks on the patient's own thyroid, often resulting in hypothyroidism and related symptoms. It is found most commonly in middle-aged women but can occur in every individual (Antonelli et al., 2015). Although HT is a relatively easy disease to treat by using thyroid hormone replacement therapy, patients with HT and generally present significantly elevated risk of developing other autoimmune conditions, such as type 1 diabetes, celiac disease, and rheumatoid arthritis (Boelaert et al., 2010; Jenkins and Weetman, 2002). The consensus now is that HT arises due to immune defects in individuals with genetic predisposition under certain environment triggers, but the precise mechanism of pathogenesis is unclear (Pyzik et al., 2015).

In recent years, a group of B cells with regulatory function has emerged as a critical participant in mediating immune tolerance (Rosser and Mauri, 2015). Through the expression of several regulatory cytokines, including interleukin 10 (IL-10), IL-35, and transforming growth factor beta (TGF-β), these regulatory B (Breg) cells has been shown to suppress proinflammatory responses by inhibiting the expansion and cytokine secretion of pathogenic T cells as well as by promoting the differentiation of regulatory T (Treg) cells (Lee et al., 2014; Liu et al., 2014; Wang et al., 2014). Various cell subsets show the characteristics of Breg cells, such as $CD24^+CD27^+$ B cells and $CD24^+CD38^+$ B cells. In autoimmunity, defect in the Breg compartment was identified in several diseases, such as systemic lupus erythematosus, rheumatoid arthritis, autoimmune thrombocytopenia, and type 1 diabetes (Blair et al., 2010; Li et al., 2012; Ma et al., 2014; Yang et al., 2013). Previous studies have demonstrated that Treg cells in AITD patients were less capable of inhibiting proliferation of pathogenic T cells than Treg cells in healthy controls. Recently, several

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literatures have shown dysregulation of Breg cells in HT (Kristensen et al., 2015; Kristensen 2016; Zha et al., 2012). However, the function of Breg cells in HT is not entirely clear.

Given that the pathogenesis of many autoimmune diseases involves functional impairment of Breg cells, we in this study examined the Breg population in HT patients. First, we observed that IL-10-expressing B cells were concentrated in the $CD4^+CD38^+$ B cells. Compared to $CD24^+CD38^+$ B cells in healthy individuals, the $CD24^+CD38^+$ B cells in HT patients were elevated in frequency but demonstrated deficiency in IL-10 production. HT $CD24^+CD38^+$ B cells were also less potent in suppressing the proliferation and cytokine production of autologous T cells than healthy $CD24^+CD38^+$ B cells. Together, these results demonstrated that a dysregulation of $CD24^+CD38^+$ B cell frequency and function was present in HT patients.

2. Materials and methods

2.1. Study subjects

150 mL to 200 mL of peripheral blood samples were obtained from 8 HT patients and 8 healthy controls upon receiving individual informed consent. All study subjects were females between the ages of 36–55 years. Diagnosis was made in the Second Hospital of Xi'an Jiao Tong University based on the presence of autoreactive immunoglobulins, including the anti-thyroid peroxidase, anti-thyroglobulin, and thyroid stimulating antibodies. All patients were untreated. No HT patient or healthy control demonstrated ongoing infections, cardiovascular diseases, presence of other autoimmune conditions, or malignancies. Patients and controls that had been taking steroids, immunosuppressants, or had experienced pregnancy within 6 months prior to recruitment were also excluded. All procedures were performed according to the Declaration of Helsinki with ethical approval from the institutional review board of the Second Hospital of Xi'an Jiao Tong University.

2.2. Cell preparation

Immediately after collection, heparinized blood was centrifuged across a Ficoll (Sigma-Aldrich) density gradient. Peripheral blood mononuclear cells (PBMCs) were collected from the murky layer between plasma and Ficoll. The PBMCs were then washed twice in PBS (Gibco) and incubated in RPMI 1640 supplemented with 15% fetal bovine serum, 1 × GlutaMax, and 1 × Pen Strep (Gibco) under 37° C and 5% CO_2 before further experiments.

2.3. Flow cytometry

For surface marker staining and cell sorting, cells were incubated with anti-human CD3, CD19, CD24, and/or CD38 (all from eBioscience) for 30 min under 4° C. Excess antibody was then removed by washing. If staining was not followed by a sorting step, cells were further labeled with Violet Dead cell stain (Invitrogen) according to instruction manual provided by the manufacturer, and then fixed in 2% formaldehyde.

For CFSE labeling, cells were incubated with CellTrace CFSE staining solution (Invitrogen) for 20 min at 37° C in dark, mixed with complete RPMI medium for an additional 5 min at 37° C, and then washed twice before stimulation and incubation.

For intracellular staining, surface-stained cells were treated with CytoFix/CytoPerm (BD) for 20 min under 4° C, washed with 1 × Perm Wash (BD), and then stained with anti-human IL-10, for 30 min under 4° C. Excess antibody was then washed off.

For analyses of surface marker expression and intracellular IL-10 secretion, the BD FACS LSR II machine was used. For sorting $CD19^+$ total B cells, $CD24^+CD38^+$ B cells, non-$CD24^+CD38^+$ B cells, and $CD3^+$ T cells, the BD FACS Aria machine was used. Data were analyzed in FlowJo.

2.4. B cell-T cell stimulation and coculture

1 μg/mL CpG ODN2006 (Invivogen) was added to isolated B cells or B cell subsets for 3 days. For intracellular IL-10 detection, 6 h before intracellular staining, GolgiPlug (BD) was added. For IL-10 expression ex vivo, B cells were incubated with GolgiPlug only for 6 h before staining.

CpG-stimulated B cells or B cell subsets were then incubated with sorted $CD3^+$ T cells for an additional 72 h, in the presence of plate-bound anti-CD3 and anti-CD28 antibodies. The plate was coated with 2 μg/mL each of anti-CD3 and anti-CD28 antibodies in 100 μL PBS overnight, and rinsed with complete culture medium before the addition of B cells and T cells. B cells and T cells were added at 2.5×10^4 and 5×10^4 per 200 μL per well in a 96-well plate. In some experiments, 10 ng/mL rhIL-10 (R & D Systems) was added in the beginning of the B cell-T cell coculture.

2.5. Proliferation assay

After B cell-T cell coculture, the $CD3^+$ T cells were isolated using a negative selection method with Human T Cell Enrichment kit (Stemcell), and were labeled with 0.5 μCi/well tritiated thymidine (New England Nuclear) in 100 μL/well complete medium for an additional 6 h. 100 μL/well 1 N NaOH/0.1% SDS was then added to each well with mixing. Cell lysate was harvested in a Unifilter-96 Harvester (PerkinElmer) and the level of radioactivity was counted in a MicroBeta scintillation counter (PerkinElmer). Each experiment had three replicates and the average value was presented.

2.6. ELISAs

Supernatants were diluted at 1:4 ratio with PBS and then the ELISAs were performed using commercial kits from eBioscience, following instructions provided by the manufacturer.

2.7. Statistical analyses

The statistical tests were performed using Prism software. For two group analyses, *t*-test with Welch's correction for unequal variances was used. For multiple group analyses, ANOVA was used. The specific tests for each experiment were described in the corresponding figure legend. Two-tailed P values less than 0.05 were considered statistically significant.

3. Results

3.1. HT patients presented downregulated numbers of IL-10-producing B cells but elevated Breg frequency

Previous studies have attributed Breg capacity to the level of IL-10 production (Blair et al., 2010; Matsumoto et al., 2014). Therefore, we first examined the prevalence of $IL\text{-}10^+$ B cells in circulation (Fig. 1A). In both healthy individuals and HT patients, the frequencies of $IL\text{-}10^+$ B cells in circulation were very low (Fig. 1A and 1B). Previous works showed that CpG could potently stimulate IL-10 production in human B cells without inducing cell death (Iwata et al., 2011). After CpG stimulation, both groups presented upregulated frequencies of $IL\text{-}10^+$ B cells (Fig. 1C), and the frequencies in healthy individuals were significantly higher than in HT subjects.

Previously, it was identified that IL-10-producing B cells were enriched in the $CD24^+CD38^+$ compartment (Blair et al., 2010; Matsumoto et al., 2014). Here, we found that the HT patients presented significantly higher frequencies of $CD24^+CD38^+$ B cells than healthy individuals (Fig. 2A and B). Subsequently, we found that in healthy individuals, from 4.4% to 11.9% of $CD24^+CD38^+$ B cells expressed IL-10, while in HT patients, from 1.5% to 5.6% of $CD24^+CD38^+$ B cells