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BREG cells in Hashimoto's thyroiditis isolated or associated to further organ-specific autoimmune diseases

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ABSTRACT

Hashimoto thyroiditis (HT) may occur isolated or associated with other non-endocrine autoimmune disorders (NEAD). No data are available about Breg cells in these disorders and this represented the aim of the study. Th17 and Breg cells subset were characterized on peripheral blood mononuclear cells isolated from 18 healthy donors (HD), 19 patients with isolated HT and 26 patients with HT + NEAD. Th17 were higher in patients with isolated HT than in HD but no further changes were seen in patients with HT + NEAD. $CD24^{hi}CD38^{hi}$ unstimulated Breg cells were similar in HT patients and in HD, but significantly higher in patients with HT + NEAD than in both HT and in HD. $CD19^{+}CD24^{hi}CD27^{+}$ Breg memory phenotype was similar in HD and in HT patients, but decreased in patients with HT + NEAD (23.4%vs38.5%). Upon CpG-stimulation, $CD24^{hi}CD38^{hi}$ $IL\text{-}10^{+}$ Breg cells were higher in HT patients than in HD (3.9%vs1.8%) but similar in patients with HT + NEAD (2.4%).

1. Introduction

Hashimoto thyroiditis (HT) is the most common autoimmune disease worldwide [1] and it sometime occurs associated with other endocrine and non-endocrine autoimmune disorders [2,3]. The pathogenesis of HT has been updated in the last years, giving more attention to the role of cellular immune response [4]. At first, a prevailing $CD4^{+}$ Th1 polarization has been described in HT [4,5], but recently it has been also described an increased circulating Th17 lymphocytes in hypothyroid HT patients, which was not modified in T_4 treated patients [6,7], suggesting an involvement of Th17 cells in its pathogenesis. Th17 polarization is characteristic of inflammatory phase of autoaggressive disorders and may precede Th1 phase of HT [8]. Only very few studies examined the behavior of these pathways when HT occurs along with other autoimmune disorders [9], while the immunogenetic issues have been more extensively studied [10,11]. In the last years, beside these effector pathways, an emerging role for two lymphocytes populations in autoimmune processes gained attention: regulatory T cells (Treg) and regulatory B lymphocytes (Breg) [12–14]. Treg cells have the role of peripheral clearance of autoreactive clones, whereas Breg seem to suppress the pro-inflammatory response, mostly by production of IL-10 cytokine, also enhancing the activity of Treg cells [13,14]. Focusing on autoimmune thyroid disorders, results on Treg were limited and controversial: both their number and function seem to be impaired in HT [15,16]. As far as concern Breg cells in thyroid disorders, only one recent study characterized them in HT patients, showing similar frequencies of IL-10 producing Breg cells to healthy donors [17]. This phenotype is regarded as a potent inhibitor of proinflammatory processes due to the role of this cytokine in downregulating Th17 pathways [13,14]. No information is available about the behavior of these antagonist pathways when HT is associated with other organ-specific non endocrine autoimmune disorders (NEAD). Therefore, this study was aimed at analyzing Th17 and Breg subpopulations in a cohort of patients with HT isolated or associated with NEAD such as chronic atrophic gastritis, celiac disease and non-segmental vitiligo.

2. Patients and methods

2.1. Patients

A total of 45 patients (40 women and 5 men, median age 47 years) with a definite diagnosis of Hashimoto thyroiditis (HT) were enrolled

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in the study. Nineteen patients (17 women and 2 men, median age 48 years) were affected by isolated HT and 26 (23 women and 3 men, median age 43 years) by HT + NEAD. Among these latter, in particular, 8 patients (8 women, median age 53 years) had HT and chronic atrophic gastritis (CAG), 7 (5 women and 2 men, median age 35 years) HT and celiac disease (CD) and 11 (10 women and 1 man, median age 47 years) HT and non-segmental vitiligo (NSV).

Furthermore, 18 healthy donors, age- and sex-matched with the study group (16 women and 2 men, median age 38 years) were enrolled. Subject's characteristics are shown in Table 1.

The diagnosis of each disease was defined on the basis of diagnostic criteria approved by the specific Consensus Conference, and confirmed by serologic and histological tests, where required [1,18–21]. All hypothyroid patients were treated with thyroxine as previously described [22–24]; all CD patients were in gluten free diet from almost 6 months [22].

All study patients and healthy donors were negative for infection and/or inflammatory disorders in the last 6 months; further chronic disorders (cancer, diabetes, COPB, obesity, renal failure), pregnancy and nursing, and treatment with drugs interfering with immune response (NSAIDs, steroids, immunosuppressant drugs, immunomodulatory drugs) were excluded.

All patients were informed about the purpose of the study and a written informed consent was obtained from all patients according to the local ethical rules and to the guidelines in the Declaration of Helsinki.

2.2. Methods

All patients gave morning blood samples, prior to T_4 ingestion, collected in EDTA. Peripheral blood mononuclear cells (PBMCs) were isolated by Lympholyte-H density gradient centrifugation (Cedarlane Laboratories Ltd., CL5020, Canada) and used immediately.

2.3. Analysis of lymphocyte subsets

Effector Th17 lymphocytes were obtained from PMBCs after overnight stimulation with PMA, Ionomycin, Brefeldin A and Monensin (PIB) (Cell stimulation cocktail plus protein transport inhibitor, Affymetrix eBioscience, San Diego, CA, USA). Cells were then surface-stained with anti-CD4 FITC, fixed, permeabilized and stained intracellularly with anti-IL-17A PE (Affymetrix eBioscience, San Diego, CA, USA). The characterized phenotype of Th17 was $CD4^+ IL17A^+$ and the results are expressed as percentages of total $CD4^+$ T lymphocytes.

To detect circulating B and Breg cells, freshly isolated PBMCs were surface stained with anti-CD19 FITC, anti-CD24 APC-eFluor 780, anti-CD38 APC, and anti-CD27 PerCPcy5.5 (Affymetrix eBioscience, San Diego, CA, USA). The following phenotypes were characterized:

Total B cells = $CD19^+$.
Breg cells = $CD19^+ CD24^{hi} CD38^{hi}$.
Breg memory cells = $CD19^+ CD24^{hi} CD27^+$.

Table 1
Characteristics of patients and healthy donors enrolled in the study.

	Healthy donors	HT	HT + NEAD
Subjects (n°)	18	19	26
Sex (women/male)	16W/2M	17W/2M	23W/3M
Age (years) median (IQ1-IQ3)	38 (31–55)	48 (43–56)	43 (34–57)
L-T_4 dose/weight (μg/Kg/die) median (IQ1-IQ3)	/	1.28 (1.15–1.31)	1.19 (1.13–1.30)
TSH (mU/l) median (IQ1-IQ3)	1.48 (0.99–2.13)	1.22 (0.96–1.72)	1.10 (0.95–1.52)

HT = Hashimoto thyroiditis; NEAD = non endocrine autoimmune diseases.

The results for B cells subsets analysis are expressed as percentages of total $CD19^+$ B lymphocytes.

To evaluate IL-10 production in B cells, PBMCs were incubated with 0.1 μM CpG-B ODN2006 (InvivoGen, San Diego, CA, USA) for 72 h at 37 °C. As a negative control, PBMCs were also treated with non-CpG ODN2006 Control (ODN 2137, InvivoGen, San Diego, CA, USA) in the same conditions. In both cases cells were stimulated with PIB in the last 5 h, then collected and stained.

Cells were surface stained with anti-CD19 FITC, anti-CD24 APC-eFluor, anti-CD38 APC, and anti CD27 PerCPcy5.5, fixed, permeabilized and then intracellular stained with anti-IL-10 PE (Affymetrix eBioscience, San Diego, CA, USA). The following phenotypes were characterized:

Total B cells $IL10^+$ = $CD19^+$ $IL\text{-}10^+$.
$IL10^+$ Breg cells = $CD\ 19^+ CD24^{hi} CD38^{hi}$ $IL\text{-}10^+$.
$IL10^+$ Breg memory cells = $CD19^+ CD24^{hi} CD27^+$ $IL\text{-}10^+$.

Data from these phenotypes are expressed as percentages of IL-10 positive cells referred to the corresponding B lymphocytes subsets.

All intracellular staining was performed with an appropriate isotype control Abs for gate setting (Affimetrix eBioscience, San Diego, CA).

Cells were acquired on a FACS ARIA II flow cytometer (Becton Dickinson). At least 10,000 events were acquired on $CD4^+$ and $CD19^+$ gates, respectively. FACS Diva software was used for analysis.

2.4. Statistical analysis

Data are expressed as median value. The difference among more than two groups was calculated using non parametric Kruskal-Wallis test and Dunn post test to compare all pairs of data.

INSTAT GraphPad Prism 5.0 software for Windows was used for the statistical analysis.

3. Results

3.1. Th17 lymphocytes

In patients with isolated Hashimoto's thyroiditis, an increased percentage of Th17 cells has been observed as compared with healthy donors (2.0% versus 1.4%). When such pathway has been analyzed in patients with HT and further autoimmune disorders, Th17 cells percentage was similar (1.9%) as compared to those with isolated HT and in healthy donors. Kruskal-Wallis test revealed a significant difference among these groups ($p = 0.0368$) (Fig. 1). No difference in the percentage of Th17 was found among the three different associated diseases (not shown).

3.2. Unstimulated B and Breg lymphocytes

No differences in total $CD19^+$ B cells among HT, HT + NEAD patients and healthy donors were observed (Kruskal-Wallis $p = 0.2444$). The percentages of $CD24^{hi} CD38^{hi}$ unstimulated Breg cells were similar in patients with isolated HT and in HD (2.1% versus 2.0%). However, these cells were significantly increased in patients with HT + NEAD (3.8%) as compared with that recorded in both isolated HT and in HD ($p < 0.0001$) (Fig. 2). This increase was evident in all three autoimmune disorders without difference among them (not shown).

As far as concerns with $CD19^+ CD24^{hi} CD27^+$ Breg memory phenotype, again we observed no difference between healthy donors and patients with isolated HT. Noticeably, a significant decrease of this subset in HT + NEAD patients has been observed as compared to isolated HT patients (23.4% versus 38.5%) (Fig. 3). Among the associated disorders, the patients with CD showed the lowest value, significantly different from HT patients ($p = 0.0062$) (not shown).

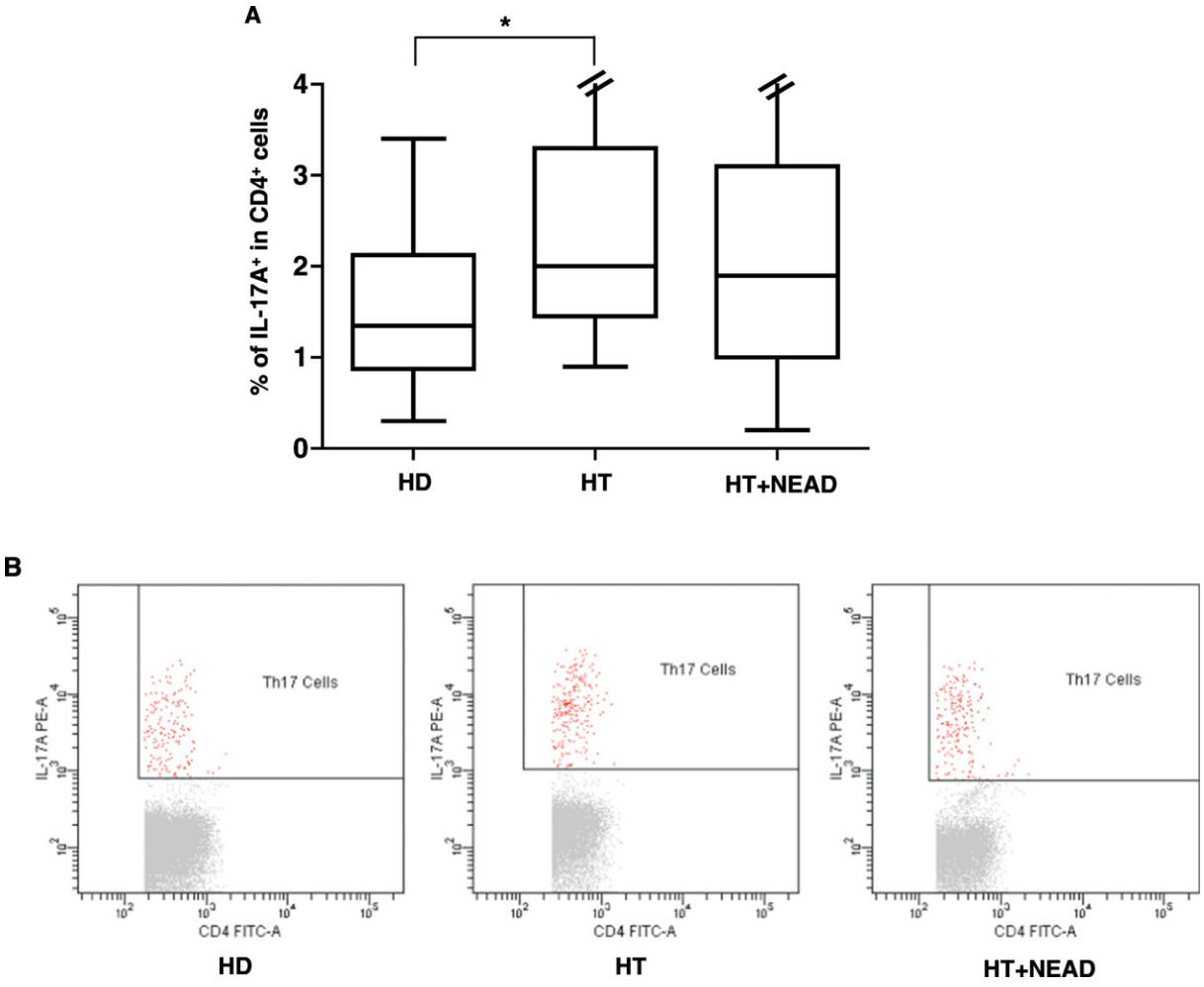

Fig. 1. Th17 lymphocytes. (A) Percentage of Th17 cells in healthy donors (HD), in patients with Hashimoto thyroiditis (HT) and with HT plus non endocrine autoimmune diseases (NEAD). Box plots indicate median, interquartile range (box) and minimum and maximum values (whiskers). Nonparametric Kruskal-Wallis test: $p = 0.0368$; Dunn post test: HD vs HT $^*p < 0.05$ (B) Representative dot plots showing Th17 cells in healthy donors (HD, left panel), in patients with Hashimoto thyroiditis (HT, middle panel) and with HT plus non endocrine autoimmune diseases (NEAD, right panel). Cells were gated on $CD4^+$ population (gray dots), whereas IL-17A positive cells are shown in red.

3.3. Activation response of B and Breg following CpG stimulation

In 35 out of 45 enrolled patients (9 healthy donors, 10 with HT, 16 with HT + NEAD), PBMCs stimulation with CpG oligonucleotide for IL-10 production by B cells was performed. These 35 patients showed superimposable percentages of total B cells, Breg and Breg memory cells when compared to the whole sample of patients.

Total $CD19^+$ B cells, even after CpG-stimulation, was similar in healthy donors, in patients with HT and with HT + NEAD (Kruskal-Wallis $p = 0.4356$). The analysis of total IL-10^+ B lymphocytes ($B10^+$ cells), treated with non-CpG Control and then with PIB, revealed a significantly lower percentage in healthy donors than in patients with isolated HT (0.8% vs 2.0%), but not in those with HT + NEAD (1.6%) (Kruskal-Wallis $p = 0.0390$; Dunn post test: HD vs HT $p < 0.05$). However, following CpG stimulation, total $B10^+$ cells were similar in all three groups of patients ($p = 0.2448$).

When the frequency of $CD24^{hi}CD38^{hi}$ Breg cells was analyzed after CpG-stimulation, their percentage was significantly higher in patients with HT + NEAD versus HT (4.3 vs 3.5%; Kruskal-Wallis $p = 0.0124$; Dunn post test: $p < 0.05$). Once the intracellular presence of IL-10 was characterized among the Breg phenotype, it appeared that the frequency of $CD24^{hi}CD38^{hi}$ IL-10^+ Breg cells, treated with non-CpG Control and then with PIB, was similar in all group of patients (Kruskal-Wallis $p = 0.5714$). In contrast, upon CpG-stimulation, the frequency of these IL-10^+ Breg cells was higher in patients HT than in healthy donors (3.9% vs 1.8%) and similar in patients with HT + NEAD (Fig. 4).

As far as concerned with IL-10^+ Breg memory ($CD19^+$ $CD24^{hi}CD27^+$), the frequency of this phenotype was similar in all groups of patients both before CpG-stimulation ($p = 0.0772$) and following stimulation with CpG oligonucleotide ($p = 0.7471$).

4. Discussion

Peripheral Th17 cells are increased in patients with HT and their presence has been proven to be positively correlated with activity and severity of the disease [7] and with the titers of anti-thyroid peroxidase autoantibodies, as well [25]. Our data corroborated a Th17 cells polarization in patients affected by isolated HT, but also indicated a concomitant increase of IL-10^+ Breg cells in these patients in keeping with the notion that these latter cells are activated to stem the activation of Th1 and Th17 inflammatory and proapoptotic pathways [13,26]. Candando et al. [14], in fact, emphasized the role of cognate interactions of IL-10^+ Breg cells with $CD4^+$ T cells to produce either an antigen-specific or a generalized cell immune suppression in response to inflammatory stimuli [13,27]. Indeed, in patients with systemic autoimmune diseases (rheumatoid arthritis, lupus erythematosus, Sjogren's syndrome, multiple sclerosis etc) IL-10^+ Breg cells were found elevated being their expansion the result of inflammatory stimuli and/or of

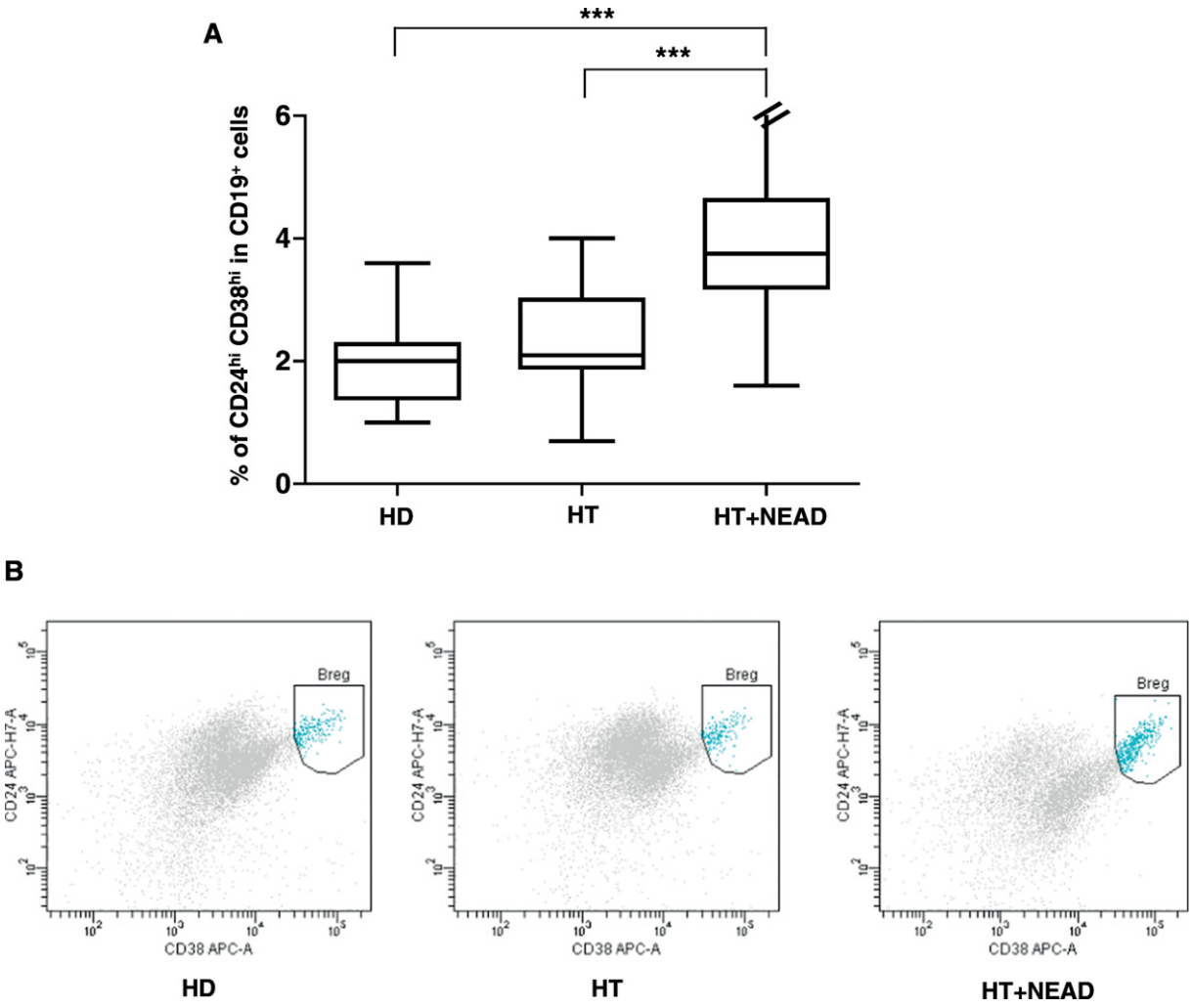

Fig. 2. Unstimulated Breg cells. (A) Percentage of Breg cells ($CD24^{hi}$ $CD38^{hi}$) in healthy donors (HD), in patients with isolated Hashimoto thyroiditis (HT) and with HT plus non endocrine autoimmune diseases (NEAD). Box plots indicate median, interquartile range (box) and minimum and maximum values (whiskers). Nonparametric Kruskal-Wallis test $p < 0.0001$; Dunn post test: HD vs HT + NEAD and HT vs HT + NEAD ***$p < 0.0001$ (B) Representative dot plots showing Breg cells in healthy donors (HD, left panel), in patients with Hashimoto thyroiditis (HT, middle panel) and with HT plus non endocrine autoimmune diseases (NEAD, right panel). Cells were gated on $CD19^+$ population (gray dots), whereas $CD24^{hi}$ $CD38^{hi}$ cells are shown in light blue.

persistent stimulation by autoantigen [14,28]. Up to now just one study analyzed the role of Breg cells in HT [17] describing a similar proportion of IL-10^+ Breg cells in HT patients and in healthy donors. Noticeably, the patients in this previous study [17] were hypothyroid and the analysis of IL-10^+ B cell subsets was performed following different stimuli [17]. In our study, we observed a similar percentage of unstimulated Breg and Breg memory cell in patients with HT and healthy donors. On the contrary, our euthyroid patients with HT showed an increased proportion of functional Breg cells, as measured by their IL10 expression. Hence, in patients with HT, as it was already described for Treg cells, where the variation appears to be qualitative rather than quantitative [15,16], the proportion of functional IL-10^+ Breg rather than the rough number of Breg cells may be the key point. This is in keeping with the lack of a unique phenotype for Breg subsets [13,29,30], which make it necessary to restrict to IL-10^+ Breg the ability to dam inflammatory and proapoptotic pathways [31–33].

The novelty of our study also arises from the characterization of Th17 and Breg cells in presence of other autoimmune diseases associated to HT (NEAD). The results in our patients with isolated HT seem to get along with the hypothesis of a concomitantly increased Th17/IL-10^+ Breg cells [14]. The occurrence of additional autoimmune disorders with HT is again associated to Th17 polarization, but instead, to a clearly increased number of Breg, with reduced suppressive function. In fact, the proportion of these cells was remarkably increased in patients with HT + NEAD, independently from the concurrent disease, but associated to a reduction of IL-10^+ Breg cells. The meaning of this finding has not simplistic explanations. We may speculate that two or more organ specific autoimmune disorders occur when a reduced number of suppressive Breg cells may no longer face an inflammatory stimulus. Also interactions of $CD4^+$ T cells with IL-10^+ Breg cells are usually higher in juvenile forms, whereas it seems to be reduced in the fibrotic stage of thyroiditis [6,7]. However, some cytokines (i.e. IL-12) may favour a transition from proinflammatory to proapoptotic phenotype, as suggested by Rosser et al. [26], and this may be trigger for further diseases. Should be this the case, the inflammatory stimulus might have been dampened down and this may explain the reduced number of IL-10^+ Breg cells in patients with HT + NEAD.

Finally, it must be noted that the Breg $CD19^+$ $CD24^{hi}CD27^+$ memory cells are concomitantly and significantly lower in patients with additional NEAD than in HT patients. The apparent discrepancy with the increased number of $CD24^{hi}CD38^{hi}$ unstimulated Breg cells may be due to a redistribution of different phenotypes. Also, it has been reported that functional Breg may be an inflammation–inducible subset that enters a further differentiation pathway at the end of the inflammatory phase [13].

In conclusion, the presence of NEAD in association with HT affects the percentage, the phenotype distribution and/or cytokine production

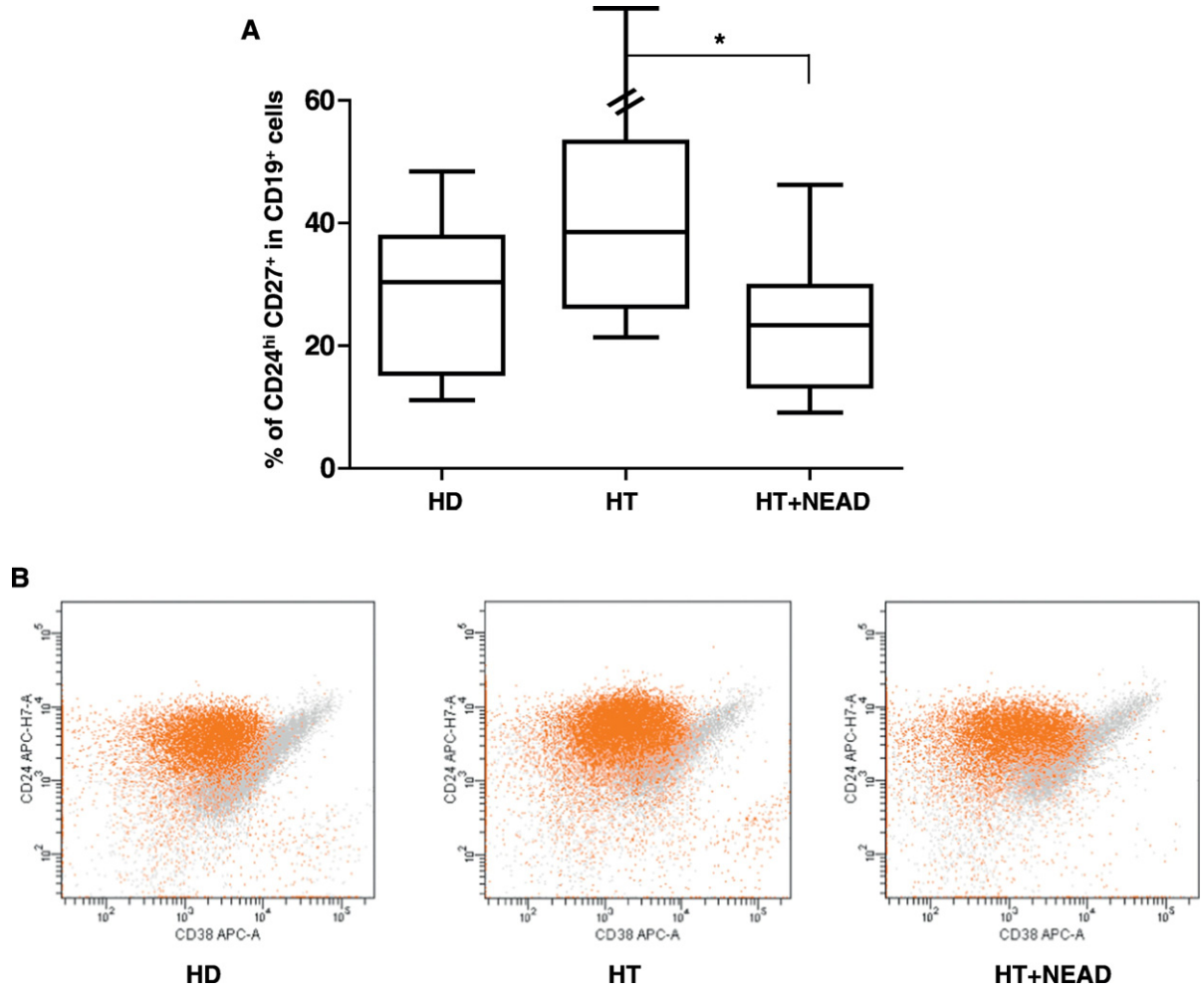

Fig. 3. Breg memory cells. Percentage of Breg memory cells ($CD24^{hi}$ $CD27^{+}$) in healthy donors (HD), in patients with isolated Hashimoto thyroiditis (HT) and with HT plus non endocrine autoimmune diseases (NEAD). Box plots indicate median, interquartile range (box) and minimum and maximum values (whiskers). Nonparametric Kruskal-Wallis test $p = 0.045$; Dunn post test: HT vs HT + NEAD $^{*}p < 0.05$ (B) Representative dot plots showing Breg memory cells in healthy donors (HD, left panel), in patients with Hashimoto thyroiditis (HT, middle panel) and with HT plus non endocrine autoimmune diseases (NEAD, right panel). Cells were gated on $CD19^{+}$ population (gray dots), whereas $CD24^{hi}$ $CD27^{+}$ cells are shown in orange.

of Breg cells, leading to an increased number of Breg with reduced function as compared with patients with isolated HT. Our findings, although preliminary and in need of more extensive studies on different Breg phenotypes, shed light on the behavior of Breg cells in HT, the most frequent autoimmune disorder either as isolated disease or framed in a syndromic association.

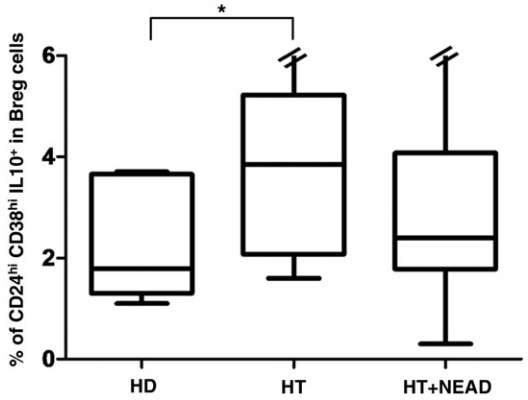

Fig. 4. Stimulated IL-10^{+} Breg cells. Percentage of $IL10^{+}$ Breg cells ($CD24^{hi}$ $CD38^{hi}$ $IL10^{+}$) after CpG stimulation in healthy donors (HD), in patients with isolated Hashimoto thyroiditis (HT) and with HT plus non endocrine autoimmune diseases (NEAD). Box plots indicate median, interquartile range (box) and minimum and maximum values (whiskers). Nonparametric Kruskal-Wallis test $p = 0.0287$; Dunn post test: HD vs HT $^{*}p < 0.05$. Analysis of IL-10 producing cells was performed on the previously shown B lymphocytes subsets ($CD24^{hi}$ $CD38^{hi}$ cells).

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