Analysis of B regulatory cells with phenotype CD19⁺CD24^{hi}CD27⁺IL-10⁺ and CD19+IL-10⁺ in the peripheral blood of children with Graves' disease and Hashimoto's thyroiditis

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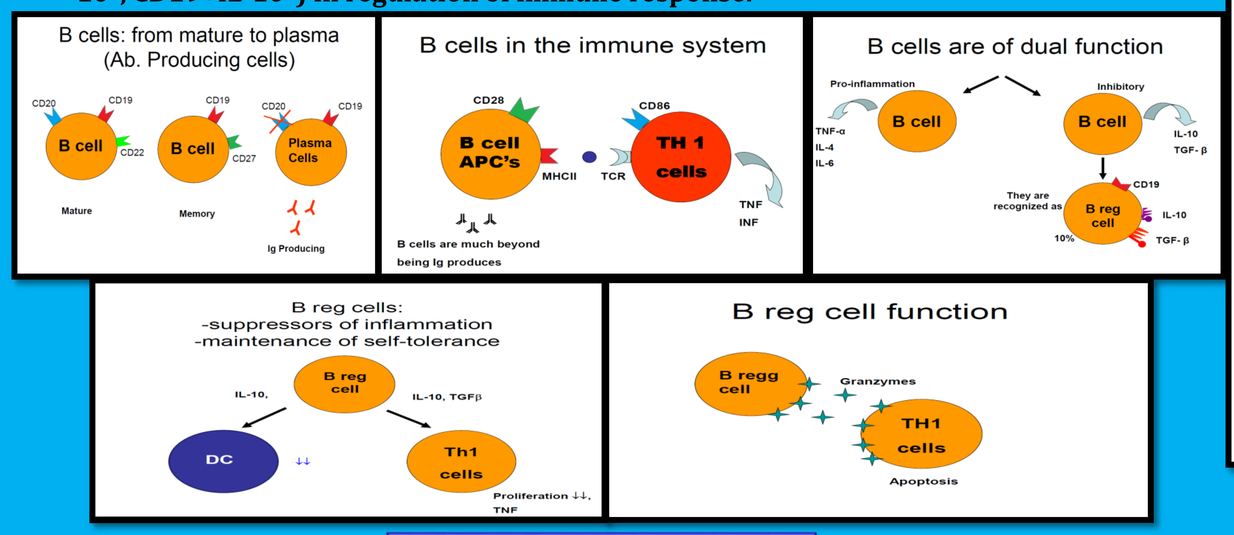
Introduction

Autoimmune thyroid disease (AITD) is the most common organ-specific autoimmune disorder. Genetic background, environmental and endogenous factors are play important roles in determining the activation of immune cells or the efficacy of the immunoregulatory pathways. Recently emphasizes the immunosuppressive role of B regulatory cells (phenotype CD19⁺CD24^{hi}CD27⁺IL-10⁺, CD19+IL-10⁺) in regulation of immune response.



Structure and function of Bregs

Materials

	Group 1- untreated GD (n=24)	Group 2-after 3-6 months of methimazole therapy (n=24)	Group 5- untreated HT (n=22)	Group 6 – HT after 6-12 mc of therapy (n=12)	Group O- Healthy controls (n=20)	
Age (years)	14.9±4	15.6±2	15.2±3	15.8±3	15.4±2	NS* NS** NS*** NS****
BMI (kg/m²)	15.36±1.5	17.93±3	20.69±2.5	21.15±3.5	21.65±3.8	p<0.001* NS**, NS*** NS****
TSH(μIU/ml)	0.02±0.03	2.13±0.2	16.95±1.25	1.77±0.7	1.47±0.87	p<0.001* NS** p<0.0001*** NS****
fT4 (ng/dl)	2.48±1.19	1.2±0.5	1.06±0.05	1.34±0.1	1.17±0.06	p<0.002* NS** NS*** NS****
fT3 (pg/mL)	5.75±2.24	3.3±0.45	2.66±0.41	3.14±0.29	3.5±0.34	p<0.008* NS** NS*** NS****
TRAb (U/l)	10.67±3.68	6.7±3.9	0.54±0.22	0.39±0.12	0.71±0.48	p<0.0001* p<0.001** NS*** NS****
anti-TG (IU/ml)	686.41±85	145.17±42	673±43	183.5±136	21.07±5.7	p<0.0001* p<0.05** p<0.0001*** p<0.013****
anti-TPO (IU/ml)	352.57±65	192.73±28	265±65	151.7±55	19.42±3	p<0.001* p<0.02** p<0.001*** p<0.03****

Methods

1. EDTA-anticoagulated peripheral blood has been collected from children with Graves of Hashimoto disease, and from healthy patients. Peripheral blood mononuclear cells (PBMC) were isolated by density-gradient centrifugation using Ficoll (Histopaque-1077, Sigma-Aldrich). Following isolation PBMC were suspended in RPMI medium (RPMI-1640 Medium, Sigma-Aldrich, Missouri) supplemented with 10% Fetal Bovine Serum (Fetal Bovine Serum, Gibco) and 1% Penicillin/Streptomycin (Penicillin-Streptomycin, Sigma-Aldrich, Missouri). Subsequently, cells were incubated for 5 hours at 37° C in 5% CO_2 in presence of TLR9 agonist (CpG ODN, InvivoGen) and PMA/Ionomycin/Brefeldin A (Laukocyte Activation Coctail with GolgiPlug, BD Bioscience). PBMC cultured in enriched RPMI medium alone served as a negative control.

Immunostaining and flow cytometric analysis.

Following incubation PBMC were collected and subjected to extracellular staining with fluorochrome conjugated monoclonal antibodies including: anti-CD1d, anti-CD5, anti-CD19, anti-CD24 and anti-CD27 (BD Biosciences). After incubation for 30 min. at room temperature, in the dark cells were washed and permeabilized prior to intracellular staining (BD FACS Permabilization Solution 2, BD Bioscience). For intracellular staining anti-IL-10 monoclonal antibodies were used. Next incubation for 30 min. (room temperature, in the dark cells) and washing step was followed by acquisition of data on FACSCanto II flow cytometer (BD Biosciences) and analysed with the use of FlowJo software (Tree Star Inc.) Regulatory B10 cells were distinguished within pool of CD19+ B on the basis of stimulation induced production of IL-10. CD1d, CD5, CD24 and CD27 expression markers were used to determine the full phenotype of B10 cells.

2. TSAb levels were measured with a commercial bioassay that uses a chimeric TSH-R and a CRE-dependent luciferase (Thyretain, Quidel Corp., San Diego, CA). TSAb results were expressed as percentage of specimen-to-reference ratio (SRR%). Values TSAb ≥140% & TBAb>40% were considered positive.

Results

Fig 1. Flow cytometric dot plots of peripheral blood Bregs CD19+IL-10+ A) in examined groups, B) gating of lymphocytes CD19-AF, gating of CD19⁺AF/IL10+⁺PE lymphocytes B population after 5h CpG+LA C) gating of CD19⁺AF/IL10+⁺PE lymphocytes B population after 48 h CpG+LA

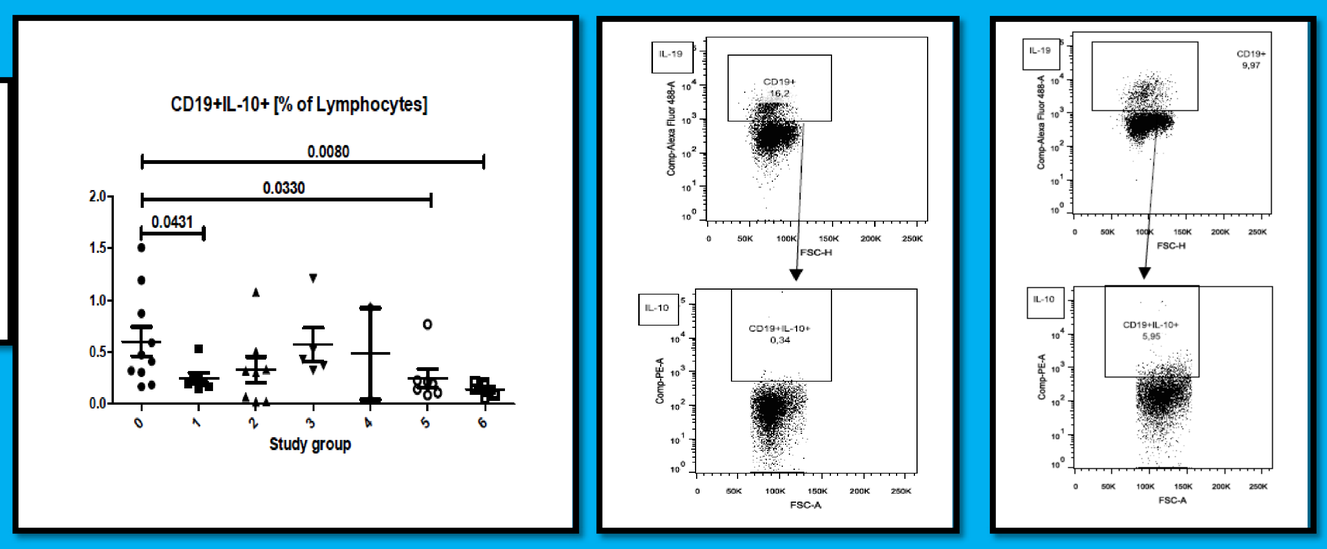
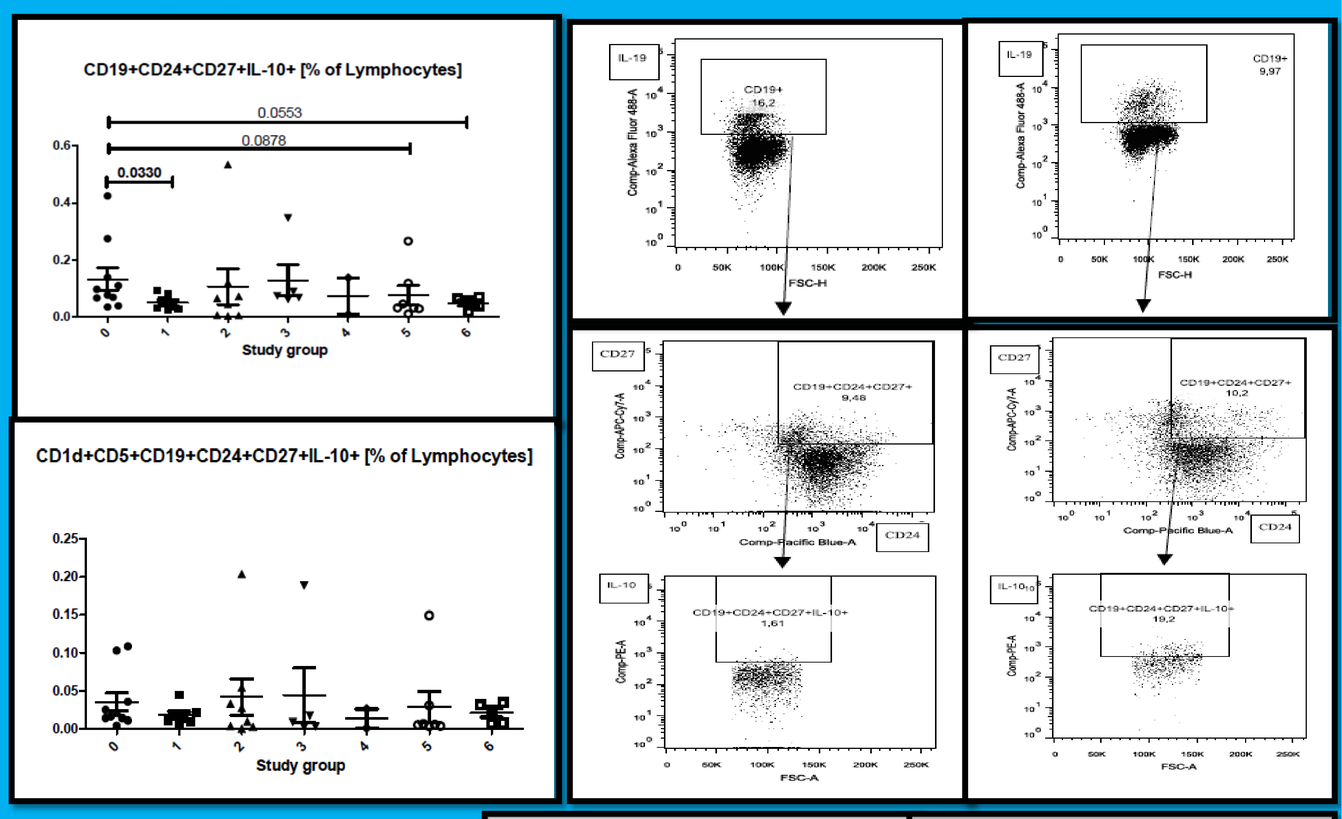


Fig 2. Flow cytometric dot plots of peripheral blood Bregs A) in examined groups, B) gating of lymphocytes CD19-AF, gating of CD19⁺AF/CD27+CD24+/IL-10 lymphocytes B population after 5h CpG+LA C) gating of CD19⁺AF/CD27+CD24+/IL-10 lymphocytes B population after 48 h CpG+LA



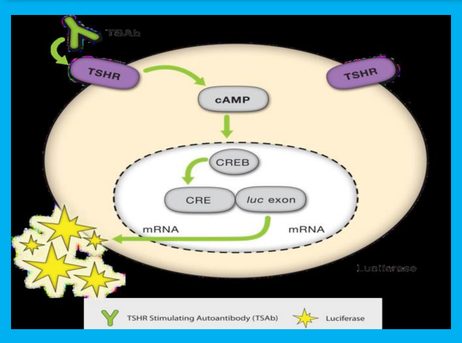
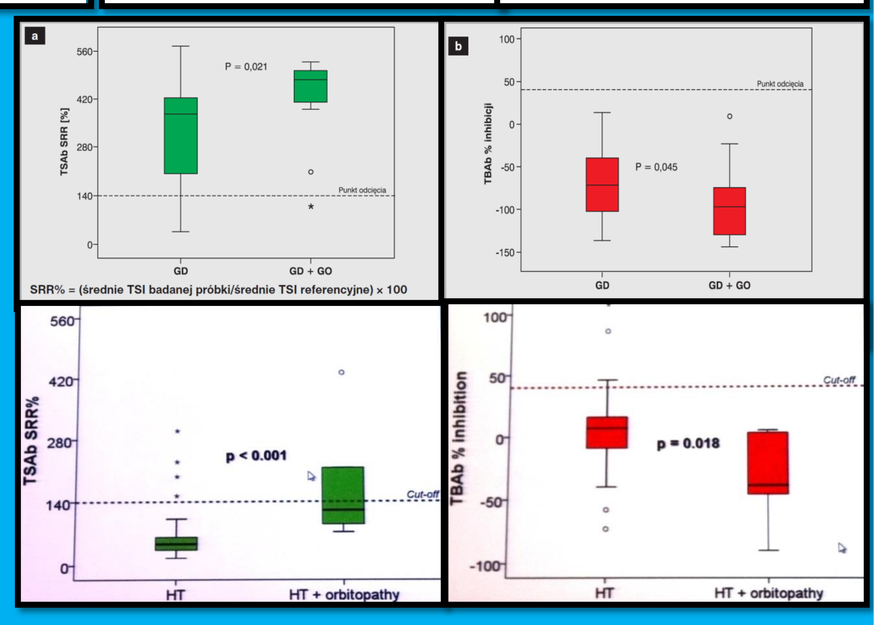


Fig 3 .**Principle of the TSAb bioassay. TSAb binds on the surface of Chinese Hamster Ovary (CHO) cells constitutively expressing a chimeric (Mc4) human TSH receptor and a cyclic adenosine monophosphate (cAMP) luciferase gene. This results in the induction of the cAMP signaling cascade, following in increased production of intracellular cAMP.**



Tab.2 Correlation between TSI & TBI ABs and percentage of Breg cells in children with untreated GD (A) and HT (B).

	CD19+IL-10⁺ GD	CD19⁺CD24^{hi}CD27⁺IL-10⁺ GD
anti-TPO	R=0.35 p=0.21	R=0.36 p=0.33
anti-TG	R=0.37 p=0.45	R=0.44 p=0.32
anti-TRAb	R=-0.45 p=0.025	R=-0.76 p=0.003
TSAb	R=-0.58 p=0.01	R=-0.88, p=0.01
TBI	NS	NS

	CD19+IL-10⁺ HT	CD19⁺CD24^{hi}CD27⁺IL-10⁺ HT
anti-TPO	R=-0.58 p=0.03	R=0.48 p=0.25
anti-TG	R=0.385 p=0.43	R=0.51 p=0.23
anti-TRAb	R=0.52 p=0.12	R=0.58 p=0.76
TSAb	R=0,19 p=0.32	p=NS, R=0,35
TBI	NS	NS

Conclusions

1.The reduction number of Breg cells with expression of CD19⁺CD24^{hi}CD27⁺IL-10⁺ and CD19+IL-10⁺ (B10) could be responsible for loses immune tolerance and development of autoimmune process in thyroid disorders.

2. L-thyroxine therapy in HT patients does not contribute to significant changes in the number of Breg lymphocytes.

3. Methimazole treatment of patients with Graves' disease in the short term leads to normalization of the percentage of lymphocytes Bregs phenotype CD19 + IL-10 + and CD19 + CD24hiCD27 + IL-10 + which indicates immunomodulatory effects used antithyroid drug.

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Thyroid

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