Functional characterization of B lymphocytes in autoimmune thyroiditis and associated infertility

PhD Thesis

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1. INTRODUCTION

B lymphocytes are a type of white blood cell of the lymphocyte subtype. They function in the humoral immunity component of the adaptive immune system. B cells have a cell surface receptor (BCR) capable of specific antigen recognition. A single B cell clone is only capable of recognizing one specific antigen, against which it produces specific antibodies when it matures into a plasma cell. Furthermore, B cells play an important role in the antigen presentation of T cells. During the primary immune response, a part of them turns into memory B cells, thereby contributing to the maintenance of immunological memory. The characteristics of the BCR signal convey different information depending on which developmental stage a B cell is in. Naive B cells express IgD and IgM and are negative for CD27. The CD19+ IgD+ CD27- subpopulation also contains the newly formed, transitional B cells which just left the bone marrow to form a naive mature B cell repertoire. As a result of antigen binding, naive IgD+ cells proliferate in the extrafollicular loci of the peripheral lymphatic organs, where they transform into short-lived, antigen-specific, low-affinity IgM-producing plasmablasts (PB). Another group of antigen-activated B cells migrates to the germinal center, where they undergo division and T cell-dependent class switch, which leads to the differentiation of B cells into memory or long-lived plasma cells (PC). Long-lived, memory-type PB and PC populations (circulating antibody-producing cells, ASC) are characterized by the IgD-CD27highCD38high phenotype.

After antigen-specific activation, some B cells differentiate into memory B cells, express CD27, and re-enter the circulation. CD27+ B cells can be further classified based on their IgD expression. Peripheral memory B cells which still express IgD and IgM are termed ‘non-switched memory cells’, whereas those that undergo Ig-class switching and begin to express IgG and further Ig subtypes are known as ‘switched memory’ cells. The latter increase their surface Ig expression and lose the ability to secrete antibodies. Eventually certain memory cells lose their CD27 expression and become IgD- CD27- ‘double-negative’ (DN) memory B cells, which are non-dividing, inflammatory cytokine-producing cells that could play a role in autoimmunity.

Free intracellular (IC) calcium is a multifunctional secondary messenger molecule whose dynamic concentration changes regulate most of the immunological fate and function of B lymphocytes. Migration, cell adhesion, apoptosis, proliferation, cell cycle, protein kinase signaling, mitochondrial and endoplasmic reticulum physiology, translocation and activation of proteins, transcription factors, and gene expression are all processes regulated by IC Ca^{2+}. Factors that dynamically regulate calcium levels in B cells include IC receptors and channels, which are responsible for the release of Ca^{2+} from internal stores; the plasma membrane channels, which are responsible for the introduction of Ca^{2+}; the resting membrane potential, set by voltage-gated and calcium-gated K^+ channels, which provides the electrical driving force for Ca^{2+} entry; voltage-dependent transporters and non-selective cation channels; the sarco/endoplasmic reticulum (SERCA) and plasma membrane (PMCA) Ca^{2+} ATPase pumps, which return Ca^{2+} to IC stores and the buffering of mitochondria. Together, they shape the calcium signals that determine the different functional responses of B cells.

B cell receptor signaling is a major regulator of B cell immunological fate, including positive and negative selection, central and peripheral tolerance, and activation. Ca^{2+} plays a central role in the regulation of the BCR signaling pathway, in which even a small change can have pathological consequences. More and more evidence point to the key role of altered BCR signaling in autoimmunity, which modifies the naive BCR repertoire and promotes the activation of autoreactive B cell clones T cell-dependent and independent manner, promoting the formation of spontaneous autoreactive GCs and the loss of T cell tolerance by recruiting T_{FH} cells. In addition, B cells play a decisive role in the antigen presentation of T cells by the Major Histocompatibility Complex (MHC) II. type molecules, together with costimulatory molecules and cytokines. Although the observations were mainly made in systemic autoimmune models, altered BCR signaling may also play an important role in the development of Hashimoto's thyroiditis, especially since the risk of further autoimmune diseases is also significantly higher in Hashimoto's thyroiditis.

Hashimoto’s thyroiditis (HT) is a common organ-specific autoimmune disorder in women of childbearing age. It is characterized by the presence of anti-thyroid antibodies (ATAs), such as anti-thyroid peroxidase (anti-TPO) and anti-thyroglobulin (anti-Tg), and the

infiltration of the thyroid gland by mononuclear cells. The complex interplay of cellular and humoral factors results in a chronic inflammatory process over several years. At a certain level of functional thyroid tissue loss hypothyroidism develops, indicated by the rising of thyroid-stimulating hormone (TSH). This silent disease could be considered clinically irrelevant; however, ATA positivity presents a significant risk for infertility and pregnancy loss, and the odds ratio (OR) of miscarriage is 3.9 even without thyroid dysfunction. The presence of ATAs may also have a negative impact on the success rate of in vitro fertilization (IVF). Additionally, the immune tolerance during pregnancy also appears to be disturbed, indicated by the higher rate of preterm birth (OR 2,07) and pregnancy complications. Levothyroxine, a synthetic thyroid hormone, is prescribed for the general treatment of hypothyroidism.

In contrast to Graves' disease, which is widely recognized as an autoantibody-mediated disorder, Hashimoto's thyroiditis is generally considered to be a T cell-mediated disease. According to the classical hypothesis, auto-reactive B lymphocytes are bystanders in the inflammatory process, activated by the release of thyroid antigens in the appropriate cytokine milieu and in the presence of T helper (T_H) lymphocytes. Recent advances in the field of autoimmunity have revealed that dysregulated B cell signaling via the B cell receptor (BCR) could be the key step and the primary driver of the loss of self-tolerance and the development of autoimmunity, and not just the downstream consequence of autoreactive T_H cell activation. Strikingly, longitudinal studies have shown that disease-associated autoantibodies anticipate disease onset by years in multiple autoimmune disorders.

2. AIMS

1. In the first phase of our work, we standardized a flow-cytometry based method using various stimuli (anti-IgG, anti-IgM and anti-IgG+M activating agents) to investigate the Ca^{2+} flux characteristics of circulating human B lymphocytes from healthy individuals.

With the help of the optimized Fluo-4-AM based flow cytometric method, our goal was to describe the subtle alterations of the activation response of selected B lymphocyte subsets in real-time. This gives us information about the function of B cell subsets and allows the objective comparison of subsets, as each receives the exact same activating stimulus in real-time. This method is suitable for describing the activation characteristics of lymphocytes in various B cell immunological disorders.

2. Next, we aimed to investigate the Ca^{2+} flux response of B lymphocyte subsets to BCR stimulation in Hashimoto's thyroiditis and related infertility using an optimized Fluo-4-AM based flow cytometric method.

- The prevalence, basal calcium signal and calcium kinetics of B lymphocyte subgroups were investigated after BCR stimulation,
    - In hypothyroid, ATA+ patients before and after levothyroxine treatment.
    - In infertile, euthyroid, ATA+ women.
    - In age-matched healthy controls.

We also aimed to assess the effect of levothyroxine on the Ca^{2+} flux kinetics of B cells by sampling hypothyroid patients before and after treatment.

3. MATERIAL AND METHODS

3.1. Flow cytometry

Our study was approved by the Regional Research Ethics Committee of the Medical Center, University of Pécs (RIKEB 5913/2015), and written informed consent was obtained from all participants. The study adhered to the tenets of the most recent revision of the Declaration of Helsinki. Peripheral blood samples were collected from each participant. Peripheral blood mononuclear cells (PBMCs) were separated by Ficoll-Paque PLUS gradient centrifugation. During the development of the methodology PBMCs (8 x 10^6 cells) were stained with the appropriate combination of fluorochrome-conjugated anti-human antibodies in 100 µl of media in order to differentiate B lymphocyte subsets: CD3-PerCP, CD19-PE, IgD-APC, CD38-APC-Cy7 and CD27-PE-Cy7. The following fluorochrome-conjugated anti-human antibodies were used to determine the B cell subsets examined in HT: CD5 PerCP, CD19 PE, IgD APC, CD27 PE-Cy7 and CD25 BV421. Cytoplasmic free, ionized calcium level was detected by loading the cells with 5 mM Fluo-4- acetoxymethyl (AM) (supplemented with 20% (w/v) Pluronic F-127 for 15 minutes in the dark at RT. During the methodological measurements Flow cytometry measurements were performed using a BD FACS Canto II. For each sample, 60 s of baseline Ca^{2+} signal was recorded, an activating agent was added, and 720 s were recorded following activation in a new file. The following concentrations were tested: for anti-human IgG+M 0.1, 0.5, 1, 2.5, 5, 7.5, 10, and 20 µg/mL; for anti-human IgG and anti-human IgM 0.5, 1, 10, and 20 µg/mL. Fluorescence data from each sample from HT were measured and recorded for 17 minutes in a kinetic manner. Unstimulated controls were measured for the first 2 minutes for baseline acquisition, then B lymphocytes were activated by crosslinking the BCR with 10 µg/ml anti-human IgG+IgM and samples were measured for another 15 minutes. The data of the flow cytometer measurements were analyzed using the FlowJo v10.7.1 software. The target populations were exported individually, and then curves were fitted to the kinetic measurements using the FacsKin kinetic analysis program.

3.2. Kinetic function, its parameters

Facskin uses an algorithm to fit functions to the kinetic changes of the Ca^{2+} signal, making a precise mathematical description of the Ca^{2+} signal possible. The way Ca^{2+} signals are interpreted as specific cellular responses involves decoding the changes in the concentration, amplitude, steepness, and duration of the response. FacsKin generates parameters to describe each distinct aspect of the activation. The *Max value* reflects the peak of the Ca^{2+} signal. The *Max value* is also the parameter that shows the reactivity of a given subset to an activating agent the most precisely. The *Ending value* in this experimental setup reflects the Ca^{2+} level during the plateau phase of the activation curve, which is a consequence of sustained extracellular Ca^{2+} entry via the CRAC channels. The time and slope parameters indicate how quickly the given cells are able to mobilize Ca^{2+} upon BCR stimulation. The *time to reach the 1st 50% value* is the most sensitive parameter to determine how fast a cell type reacts to a given activating reagent. The *slope parameter at the 1st 50%* closely relates to the distinct activating agent. The *AUC value* shows the total Ca^{2+} mobilizing ability of the given subset in response to a specific activating stimulus.

3.3. Reproducibility

The best laboratory practices for sample preparation, flow cytometry measurements, and intracellular Ca^{2+} mobilization tracking were followed. According to the recommendations of the International Society for Advancement of Cytometry (ISAC), the labeling and measuring steps were all performed at room temperature. Cytometer setup and tracking (CS&T) were performed every day. Compensation matrices were specifically calculated for each measurement day in FlowJo, using compensation beads (BD) for fluorochrome-conjugated antibodies and the test subjects' PBMCs for Fluo-4. The flow rate was kept at a standard low speed. We applied several validation steps and various controls to standardize our measurements and ensure reproducibility. Populations and marker positivity were defined using FMO controls. Not-activated samples were measured and used as a reference to exclude any Fluo-4 signal alterations originating from the technical setup. For each activating agent, a sample without loading the cells with Fluo-4 (Fluo-4 FMO) was measured in a kinetic manner to exclude any alterations in the background fluorescence due to the

activation process, which could resemble a Fluo-4 signal but would be an artifact. FacsKin also allows standardizing all baseline measurements to 1, and all changes can be assessed compared to this. This way, the activation of different cell subsets or individuals with differing basal Fluo-4 signal (Ca^{2+} level) can be objectively compared. The viability of the cells was checked in several steps. In a separate tube, a viability dye (Zombie NIR) was added at the time of cell surface labeling to check the influence of the pre-analytical processes. Viability was verified by analyzing only those intact cells that could be loaded with Fluo-4. After measuring each tube, trypan blue was used to check that the activation process did not lead to rapid cell death.

3.4. Testing the Pre-Activating Effect of IgD

We tested the pre-activating effect of the IgD labeling used for the distinction of B-cell subsets by "labeling" five tubes with unconjugated anti-human IgD (same clone as IgD APC) at the same concentration as the APC-conjugated anti-human IgD, five tubes with APC-conjugated anti-human IgD, and five tubes left unlabeled. All tubes were measured in the above-described kinetic manner, activated with anti-IgG, anti-IgM, anti-IgG+M, anti IgD (10 µg/mL was added to all), and ionomycin (1 µg/mL). An additional not-activated sample was measured as a control.

3.5. Statistical analysis

Statistical analysis of prevalence data, median fluorescence intensity, and Fluo-4 baseline data was performed using GraphPad Prism 9. For normality testing, Shapiro–Wilk tests were used. Non-normally distributed datasets were analyzed using Wilcoxon matched-pairs signed-rank tests for single comparisons and Friedman test with multiple comparisons FDR-corrected after Benjamini and Hochberg to compare more than two datasets. Unpaired two-tailed t-tests were used for evaluating two normally distributed datasets, and a two-way ANOVA with Tukey's post hoc test was used to compare more than two normally distributed datasets. To obtain the Max and Ending values from the anti-IgG+M titration series, nonlinear curve fitting was performed as a dose–response stimulation/[agonist] vs. response model in GraphPad Prism 9.

Statistical analysis focusing on the parameters of the Ca^{2+} flux (*maximum value,ending value, AUC, time to reach maximum, time to 1st 50 %, time from 1st 50% to maximum value, time from maximum value to 2nd 50%, Slope at 1st 50%, and Slope at 2nd 50%*) was performed with an R software package. For normality testing, Shapiro–Wilk tests were used with the shapiro.test function from the stats R package. Due to the non-normally distributed datasets, the wilcoxon.test and kruskal.test commands from the stats package, when appropriate, were applied. Dunn's Kruskal–Wallis multiple comparisons were used (dunnTest function from the FSA R package)to perform post hoc tests. *P values* lower than 0.05 were considered significant, and the previously mentioned dunnTest was used to calculate the adjusted *p-values*. From dunnTest, the "bh" adjustment method was used.

In order to detect the differences between the HC-H2, HCH1, HC-HIE, H2-HIE, H1-HIE patient groups we used ordinary two-way ANOVA and Tukey's post hoc test. Differences between the H1 and H2 groups were assessed by a mixed-effects analysis and the Sidak's multiple comparisons test due to repeated sampling and randomly missing values. Levels of comparisons are the follows; (1) between the mean of the patient groups when completely ignoring the grouping of the cells, (2) between the mean of the patient groups within the cell populations and subpopulations, (3) between the mean of cell populations and subpopulations within the patient groups. Statistical analysis was carried out in Prism 8.

AN OPTIMIZED FLOW CYTOMETRIC METHOD TO DEMONSTRATE THE DIFFERENTIATION STAGE-DEPENDENT CA^{2+} FLUX RESPONSES OF PERIPHERAL HUMAN B CELLS

4. RESULTS AND DISCUSSION

During the experiment, We investigated how commonly used B cell-activating agents (anti-IgG+IgM, anti-IgG, anti-IgM) influence the Ca^{2+} flux pattern of circulating B lymphocyte subsets in healthy individuals. In our experiments, anti-IgD-labeled naive B cells gave a much smaller, slower, and more prolonged Ca^{2+} flux response to anti-IgG+M compared to all memory subsets. NSw cells had the highest baseline Ca^{2+} level; it was higher both compared to naive and other memory B subsets. These cells gave a strong and fast Ca^{2+} flux

response to anti-IgM but not anti-IgG. Despite using IgM as a BCR, NSw cells did not respond like naive cells but showed a Ca^{2+} kinetic pattern like the IgG-type BCR (Sw and DN) memory subsets. However, their Max value remained lower, consistent with the differences between IgM- and IgG-type BCR. Sw and DN memory cells only responded to anti-IgG (and anti-IgG+M) despite containing approximately 10% of IgM-expressing cells. IgG-responding Sw and memory cells had similar basal Ca^{2+} levels, and their Ca^{2+} flux response curve pattern was almost identical. It is important to note that in both Sw and DN compartments, about half of the cells do not express IgM or IgG, likely corresponding to IgA memory cells, which should be considered in an experimental setup aiming to dissect the Ca^{2+} flux responses of B-cell memory subsets.

ASCs are known to downregulate their cell surface Ig expression. Interestingly, we were able to stimulate peripheral ASCs via their IgG receptor. Their basal Ca^{2+} level was similar to Sw and DN memory B cells, which also suggests that they have not yet attained independence from Ca^{2+} signaling. However, circulating ASCs showed a distinct Ca^{2+} flux pattern compared to memory subsets Despite their IgG-expression, their *Max* value was comparable to IgM-expressing NSw memory cells, it remained lower compared to IgG-expressing (Sw and DN) memory subsets. They also reached the *Max* value slower than all memory subsets. This prolonged response to BCR stimulation, and the lower peak is likely the consequence of lower surface IgG expression and possibly inhibitory signaling associated with the BCR. After the peak, the intracellular Ca^{2+} level decreased gradually and almost returned to the baseline by the end of the measurement, indicating further alterations in SOCE and the NFAT signaling.

Defining B-cell subpopulations based on their CD27 and IgD expression is the most common approach; however, in our experiments, anti-IgD-labeled naive B cells gave a much smaller, slower, and more prolonged Ca^{2+} flux response to anti-IgG+M compared to all memory subsets. This curbed response could be the consequence of the robust Ca^{2+} signal generated by the anti-IgD labeling, which not only decreased the responsivity of naive B cells to subsequent stimulation via IgD but also IgM. This anti-IgD pre-treatment seemed to have a minor effect on NSw memory cells. Surprisingly, when leaving the anti-IgD labeling out and only using CD27 to differentiate between naive and memory B cells, we found that naive

cells showed a higher Ca^{2+} flux response to BCR stimulation than memory subsets. This contradicts the common hypothesis that naive B cells are less responsive to stimuli. Both with and without anti-IgD pre-treatment, naive B cells showed a distinct Ca^{2+} flux pattern compared to memory B cells. Furthermore, this naive Ca^{2+} flux pattern was conserved in the IgD-induced response of NSw memory cells, whereas their IgM-evoked response shifted to the memory pattern, resulting in a higher plateau during the SOCE. This further supports the separate role of IgD and IgM engagement in B cells and substantiates that the observed differences between naive and memory subsets are not just the consequence of the Ig-type of the BCR.

After selecting the activating agent, the distinct activation thresholds of functional different B-cell subsets should also be considered, as demonstrated here with the titration of anti-IgG+M. We measured a point above which a clear plateau phase appears, indicative "all or nothing" phenomenon attributed to the SOCE. Above this threshold, the plateau level continued to increase dose-dependently. We observed a lower threshold for activation in IgG-expressing memory B-cell subsets (Sw and DN memory cells) compared to both IgM-expressing NSw memory cells and naive cells. This inherent difference encoded in the BCR type is connected to the function of growth factor receptor-bound protein 2 (Grb2). Grb2 plays a role in the assembly of the inhibitory signalosome in the membrane-bound IgM-type BCR, it also has an activating and signal-amplifying effect by stabilizing the Ca^{2+} signaling scaffold in the membrane-bound IgG-type BCR. This mechanism is presumed to increase the antigen sensitivity of the IgG-type BCR by lowering the activation threshold, and we confirmed this in human peripheral B-cell subsets.

5. SUMMARY

In the first phase of our work, we standardized a flow-cytometry based method using various stimuli to investigate the Ca^{2+} flux characteristics of circulating human B lymphocytes from healthy individuals. We found that different activating agents trigger distinct Ca^{2+} flux responses and that B-cell subsets show specific developmental-stage dependent Ca^{2+} flux response patterns. Naive B cells responded with a more substantial Ca^{2+} flux to B cell receptor (BCR) stimulation than memory B cells. Non-switched memory cells responded to

anti-IgD stimulation with a naive-like Ca^{2+} flux pattern, whereas their anti-IgM response was memory-like. Peripheral antibody-secreting cells retained their IgG responsivity but showed reduced Ca^{2+} responses upon activation, indicating their decreased dependence on Ca^{2+} signaling. Ca^{2+} flux is a relevant functional test for B cells, and its alterations could provide insight into pathological B-cell activation development.

B CELLS FROM ANTI-THYROID ANTIBODY POSITIVE, INFERTILE WOMEN SHOW HYPER-REACTIVITY TO BCR STIMULATION

6. RESULTS AND DISCUSSION

In this study we investigated the Ca^{2+} flux of B cell subsets in response to BCR ligation in infertile, euthyroid, ATA+ women; hypothyroid, ATA+ patients before and after levothyroxine treatment and age-matched healthy controls. The functional alterations of B lymphocytes have never been investigated in Hashimoto's thyroiditis and infertility before. The B cells of infertile, ATA+ euthyroid patients showed higher basal level of Ca^{2+} and hyperresponsivity to BCR ligation, indicated by the altered Ca^{2+} flux kinetic parameters compared to healthy controls and hypothyroid patients. To investigate the direct effect of hypothyroidism on B cell function, we evaluated the hypothyroid patients following levothyroxine replacement. After the normalization of TSH levels, the basal Ca^{2+} levels of these patients decreased compared to pre-treatment, although neither the pre- nor post-treatment values differed from the healthy controls. More interestingly, levothyroxine treatment decreased the prevalence of CD25+ B cells, which are presumed to play an important role in peripheral antigen presentation to T cells. One reason for this could be a lower autoantigen release due to decreased metabolic activity of thyroid follicles or it may be a direct effect of levothyroxine. This is a potential mechanism for how the levothyroxine treatment of hypothyroidism could decrease the progression rate of autoimmune thyroid destruction.

In line with previous findings, suggesting that CD25+ B cells represent a functionally different group, we found enhanced responsivity to BCR stimulation in CD25+ B cells compared to CD25- cells within the naive and non-switched subsets in healthy controls as well as all patient populations. It is important to note that increased sensitivity to BCR stimulation between CD25+ and CD25- populations in Sw and DN memory cells was only detected in the H1 and HIE patient groups and was less pronounced than in the naive and NSw subgroups. Understanding the nature of the altered Ca^{2+} flux in CD25+ B cells requires further experiments.

Surprisingly, we only found minimal differences between hypothyroid patients and healthy controls. However, we observed differences between the hypothyroid and the ATA+, infertile, euthyroid patient groups. The overall basal Ca^{2+} level of B cells of infertile patients was higher than that of hypothyroid patients. Basal calcium levels were significantly higher in infertile patients (HIE) in all B cell populations and in all memory subgroups compared to healthy controls.

Interestingly, the elevated basal Ca^{2+} level was not only present in the CD25+ compartment of B cell subsets, but also the CD25- compartment. Furthermore, we were able to demonstrate the increased sensitivity of B cells from euthyroid, infertile patients (HIE) to BCR stimulation. This is also reflected in the subtle differences found in the parameters of calcium kinetics. The naive B cells of infertile patients had enhanced Ca^{2+} flux response compared to the healthy control group, which is especially interesting knowing that immature B cell clones that have enhanced response to BCR stimulation have an advantage during the positive selection. In experimental settings a slight increase in the BCR signaling strength was sufficient to promote autoimmunity in a B cell intrinsic manner. These differences were also present in the memory subgroups. The NSw memory B cells of infertile patients and especially the cells expressing CD25 showed higher calcium levels in the Ending value and AUC values. Sw and DN memory B cells also showed increased sensitivity to BCR stimulation in infertile patients (HIE) compared to healthy controls.

Further differences were also observed between the hypothyroid (H1) and the autoantibody positive, euthyroid infertile (HIE) patient groups. B cells from infertile patients (HIE) had higher total basal calcium compared to all other groups, including hypothyroid patients (H1).

However, the differences in the Ca^{2+} flux kinetics between the euthyroid infertile and the hypothyroid group were only present in the naive and non-switched memory subsets. The naive subset had higher Ca^{2+} levels at the end of the measurement and both the naive and the non-switched memory cells had higher AUC in the infertile group compared to the hypothyroid group. This indicates a difference in the function of newly formed naive and memory B cells that have not yet undergone isotype switching. The switched and double-negative memory subsets on the other hand showed similar Ca^{2+} flux characteristics in these two patient populations. The double negative memory cells for example had a similarly elevated Max value in both the hypothyroid and the euthyroid, infertile group compared to healthy controls. These findings indicate elevated basal Ca^{2+} levels and enhanced Ca^{2+} flux response in ATA+, euthyroid, infertile patients but not in hypothyroid patients. Two hypotheses could explain these findings, first, that these two patient populations represent different stages of thyroid disease, which could impact the characteristics of the autoimmune response. The ATA+, infertile, euthyroid patients still have maintained thyroid function, thus the observed alterations could be characteristics of an earlier stage of thyroid autoimmunity. In the hypothyroid group, the signs of thyroid tissue damage are evident on the thyroid ultrasound, and they have decreased thyroid function, indicating years of ongoing autoimmune inflammation. This would support the notion that the loss of tolerance for thyroid antigens is initiated in the periphery rather than in the thyroid gland itself. Second, the key difference between the H1 and HIE groups is the presence of infertility. In this context, the enhanced Ca^{2+} flux response of B cells may be a result of systemic immune dysfunction, which could be the common underlying cause of both infertility and HT.

In conclusion, we were able to show systemic BCR hyperresponsivity in ATA+, infertile, euthyroid patients, which was not present in hypothyroid patients. This supports the role of altered Ca^{2+} flux of B cells in the early phase of thyroid autoimmunity and infertility. We therefore suggest a paradigm shift, where B cells could play a role in the development of thyroid autoimmunity and infertility rather than being just bystanders in the inflammatory response. This could also mean that the thyroid gland is just one of the targets of autoreactive B cells and autoimmune thyroiditis might not be the underlying cause of infertility. However, further investigations are necessary to understand the association of thyroiditis and infertility.

7. SUMMARY

In the first phase of Hashimoto's thyroiditis, the central feature - production of autoantibodies against the thyroid gland - raises the role of B lymphocytes and T-B lymphocyte interactions in the pathogenesis. Although Hashimoto's thyroiditis is classically considered a T cell-mediated disorder, many open questions remain regarding the appearance of autoreactive cells. Recent evidence indicates that changes in BCR-induced calcium signaling may be key to the development of autoimmunity.

Therefore, in the second phase of our work, we aimed to examine the calcium current response of B lymphocytes to BCR stimulation in Hashimoto's thyroiditis and associated infertility. Peripheral blood samples were collected from autoantibody positive euthyroid, infertile patients; from hypothyroid, autoantibody-positive patients before and after levothyroxine treatment, and from age-matched healthy controls. We evaluated the calcium flux response following BCR stimulation in naive, NSw, Sw and DN memory B lymphocytes. The kinetic curves of the calcium flux were analyzed using the FacsKin algorithm. All B cell subsets of ATA+, infertile, euthyroid patients showed elevated basal Ca^{2+} level and hyper-responsivity to BCR ligation compared to the other groups, which could reflect altered systemic immune function. The Ca^{2+} flux of hypothyroid patients was similar to healthy controls. The levothyroxine-treated patients had decreased prevalence of CD25+ B cells and lower basal Ca^{2+} level compared to pre-treatment. Our results support the role of altered Ca^{2+} flux of B cells in the early phase of thyroid autoimmunity and infertility.

8. SUMMARY OF THE NEW SCIENTIFIC RESULTS

We standardized a flow-cytometry based method using various stimuli (anti-IgG, anti-IgM and anti-IgG+M) to investigate the Ca^{2+} flux characteristics of circulating human B lymphocytes from healthy individuals. We found that different activating agents trigger distinct Ca^{2+} flux responses and that B-cell subsets show specific developmental-stage dependent Ca^{2+} flux response patterns.

• Naive B cells responded with a more substantial Ca^{2+} flux to BCR stimulation than memory B cells.

• Non-switched memory cells responded to anti-IgD stimulation with a naive-like Ca^{2+} flux pattern, whereas their anti-IgM response was memory-like.

• Peripheral antibody-secreting cells retained their IgG responsivity but showed reduced Ca^{2+} responses upon activation, indicating a reduced dependence on Ca^{2+} signaling.

Functional changes in B lymphocytes have never been studied before in Hashimoto's thyroiditis or related infertility.

• B cell subsets of ATA+, infertile, euthyroid patients showed elevated basal Ca^{2+} level and hyper-responsivity to BCR ligation compared to the other groups, which could reflect altered systemic immune function.

• The levothyroxine-treated patients had decreased prevalence of CD25+ B cells and lower basal Ca^{2+} level compared to pre-treatment.

In conclusion, we were able to show systemic BCR hyperresponsivity in ATA+, infertile, euthyroid patients, which was not present in hypothyroid patients. This supports the role of altered Ca^{2+} flux of B cells in the early phase of thyroid autoimmunity and infertility.

9. PUBLICATION LIST

Publications related to the thesis

Bajnok, A., **Serény-Litvai, T.**, Temesfoi, V., Nörenberg, J., Herczeg, R., Kaposi, A., Berki, T., and Mezosi, E. (2023). An Optimized Flow Cytometric Method to Demonstrate the Differentiation Stage-Dependent Ca(2+) Flux Responses of Peripheral Human B Cells. Int J Mol Sci 24. 10.3390/ijms24109107. *Independent citation: 0 , IF: 5,6*

Serény-Litvai, T., Bajnok, A., Temesfoi, V., Nörenberg, J., Pham-Dobor, G., Kaposi, A., Varnagy, A., Kovacs, K., Pentek, S., Koszegi, T., et al. (2022). B cells from anti-thyroid antibody positive, infertile women show hyper-reactivity to BCR stimulation. Frontiers in Immunology *13*. 10.3389/fimmu.2022.1039166. *Independent citation: 0 , IF: 7,3*

Other publication in this topic

Erdő-Bonyár, Sz., Simon, D., Bajnok, A., Nörenberg, J., **Serény-Litvai, T.**, Várnagy, Á., Kovács, K., Hantosi, E., Mezősi, E., and Berki, T. (2023). Physiological Changes in the Levels of Anti-Cytokine Autoantibodies in Early Pregnancy Are Missing in Pregnant Women with Hashimoto's Thyroiditis. Journal of Immunology Research 2023(2):1-7. 10.1155/2023/5221658. *Independent citation: 0, IF: 4,1*

Other publications not related to the topic

Prenek, L., **Litvai, T.**, Balazs, N., Kugyelka, R., Boldizsar, F., Najbauer, J., Berki, T. (2020). Regulatory T cells are less sensitive to glucocorticoid hormone induced apoptosis than CD4(+) T cells. Apoptosis, 25(9-10), 715-729. *Independent citation: 15, IF: 4,677*

Pap, R., Ugor, E., **Litvai, T.**, Prenek, L., Najbauer, J., Nemeth, P., & Berki, T. (2019). Glucocorticoid hormone differentially modulates the in vitro expansion and cytokine profile of thymic and splenic Treg cells. Immunobiology, 224(2), 285-295. *Independent citation: 8, IF: 2,788*

Summary of independent citation: 23 *Summary of IFs: 24,465*

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