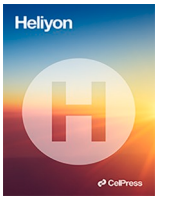
Heliyon 10 (2024) e30556

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon



Research article

Biological changes in human B-cell line Ramos (RA.1) related to increasing doses of human parathyroid hormone

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ARTICLE INFO

Keywords:
Autoimmunity
Autoimmune diseases
B cells
PTH
Hyperparathyroidism

ABSTRACT

Background: The etiopathogenesis of autoimmune diseases is multifactorial, including hormonal factors. Remission of autoimmunity has been observed following treatment for concomitant hyperparathyroidism. Additionally, patients with autoimmune diseases have shown increased expression of parathyroid hormone receptor (PTH1R) and altered distribution of B cells subsets. Hence, this study aims to evaluate potential mechanisms and *in vitro* effects of PTH stimulation on B lymphocytes.

Methods: Using the human B-cell line Ramos (RA.1), various biological effects were evaluated with and without parathyroid hormone (PTH) stimulation at varying concentrations. Flow cytometry was employed to evaluate the phenotype of B lymphocytes based on IgD and CD38 expression, apoptosis induction via Annexin V and proliferation using CFSE. IgM production was quantified through ELISA, and Western blot analysis was performed to assess syk protein phosphorylation as an indicator of cell activation.

Results: Ramos cells (RA.1) evidenced a statistically significant change in the phenotype under human PTH stimulation, demonstrating an increased proportion of germinal centre cells (Bm3-Bm4) when stimulated with high concentrations of PTH.

Conclusions: The *in vitro* effects of PTH in B cells subsets align with previous findings of an altered phenotype in B lymphocytes expressing PTH1R among autoimmune disease patients, suggesting a potential role of this hormone in the pathophysiology of autoimmune diseases. However, further studies are necessary to elucidate the mechanisms by which PTH generates observed effects in B lymphocytes and to determine if PTH plays a role in autoimmunity.

Sources of funding for this manuscript: none.

1. Introduction

Autoimmune diseases (ADs) are clinical syndromes partly caused by aberrant T cells, B cells, or both reactivity to host constituents, provoking tissue injury [1]. Its etiopathogenesis is complex, including diverse biological interactions called "autoimmunity mosaic," which are combinations of genetic, environmental, hormonal, and immunological factors that lead to the loss of antigen self-tolerance

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https://doi.org/10.1016/j.heliyon.2024.e30556
Received 27 January 2024; Received in revised form 26 April 2024; Accepted 29 April 2024
Available online 6 May 2024

and, consequently, the development of inflammation, tissue injury, and subsequent clinical manifestations [2]. Two of the most common systemic ADs are systemic lupus erythematosus (SLE) and primary Sjogren's syndrome (pSS). SLE exhibits a broad spectrum of clinical manifestations, mainly renal, cutaneous, articular, nervous system, and haematological symptoms, among others, with a global incidence ranging between 1 and 5 and 11 per 100 000 person-years and a prevalence ranging from 13 to 7713 per 100 000 individuals [3,4]. pSS is a chronic disease primarily involving exocrine gland dysfunction, with an incidence ranging between 3 and 11 per 100 000 individuals and a prevalence ranging from 0.01 % to 0.72 % [5]. The etiopathogenesis of both diseases is still not fully understood. The exploration of the factors associated with the development of these pathologies must continue, considering the prevalence and significant morbidity of ADs to sustain the search for therapeutic solutions.

Hormonal factors such as the increase in the production of parathyroid hormone (PTH) and/or the overexpression of its receptor (PTH1R) are beginning to be studied as factors that induce the phenotype of B lymphocytes related to the development of the germinal centre.

PTH is a hormone synthesized by the chief cells of the parathyroid glands, acting as a regulator of calcium and phosphate in the kidney, bone, and gastrointestinal tract [6,7]. PTH exerts its function through PTH1R, a G-protein-coupled receptor expressed in bone, kidney, cartilage, pancreas, bone marrow, vasculature, and circulating mononuclear leukocytes [8]. There are many reports describing clinical cases relating to PTH increased synthesis, known as hyperparathyroidism, in haematological and autoimmune pathologies [9–11], and some of them have reported symptom remission of haematological and ADs after parathyroidectomy [10,12,13].

Considering the clinical context, our research group has hypothesized that high levels of PTH may trigger B-cell-mediated autoimmunity through both direct and indirect mechanisms [14]. Previously, our research group demonstrated that PTH1R expression on B cells could serve as a biomarker in patients with SLE and as a disease activity marker in pSS. Additionally, an altered distribution of B-cell subsets expressing PTH1R was observed in pSS patients, as evidenced by an increase in Bm2 and Bm2' subsets (germinal centre founder cells) [15]. The potential impact of PTH on the immune system has been assessed in some *in vitro* studies. Nevertheless, these studies have yielded inconsistent results due to methodological heterogeneity among them [16].

This study relies on the hypothesis that increased expression of PTH1R on B lymphocytes could influence the activation and selection of these cells, potentially acting as a risk factor. The increased PTH1R expression on B lymphocytes accompanied by some triggering factor, such as a pathological condition which significantly elevates PTH levels, as in primary hyperparathyroidism, may lead to the loss of self-tolerance and the development of autoimmune phenomena. The present research aimed to assess the potential biological effects of PTH interaction with PTH1R on B lymphocytes and its role in autoimmunity development. *In vitro* stimulation analyses with PTH were performed on a human B-cell line expressing this receptor to evaluate the possible mechanisms that generate a cellular biology profile related to the loss of self-tolerance and the induction of autoimmunity. This research attempts to explain the relationship between PTH1R overexpression and high PTH concentrations in patients with ADs.

2. Materials and methods

2.1. Experimental design

This is an *in vitro* experimental study conducted at Universidad Icesi in Colombia and approved by the Human Research Ethics Committee of Universidad Icesi (act. N°158 and N°314).

2.2. Cell lines

The human B-cell line Ramos (RA.1) isolated from a Burkitt lymphoma of a 3-year-old male, was obtained from an ATCC supplier (Manassas, VA, USA; CRL-1596, Lot number: 70016960, CVCL_0597). The cell line was authenticated by the ATCC supplier on October 16, 2017, using a COI assay to determine the species and a STR analysis to determine the DNA profile. Additionally, it underwent testing for mycoplasma contamination on the same date using the indirect Hoechst DNA strand method, direct agar culture method, and PCR-based assay, all of which reported no detection of contamination in the certificate of analysis. Cells were cultured in RPMI-1640 medium (Gibco by Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10 % heat-inactivated foetal bovine serum (FBS, Gibco by Thermo Fisher Scientific, Waltham, MA, USA) and 1 % penicillin-streptomycin (Sigma–Aldrich Co, St. Louis, USA) and incubated at 37 °C and 5 % CO_2. Medium renewal was performed every two days, maintaining a cell density of 1×10^6 cells/mL. In all experiments, the cell line was used up to the tenth passage of thawing. PTH1R expression was characterized by flow cytometry on the Ramos cell line (RA.1) as a model to analyse the *in vitro* effects of PTH stimulation.

2.3. In vitro stimulatory conditions

Ramos cells (RA.1) were stimulated with 0.1 μM CpG (ODN 2006, MACS Miltenyi Biotec, Bergisch Gladbach, Germany) and increasing doses of human PTH (final concentrations: 0.5 ng/mL, 2 ng/mL and 8 ng/mL, Sigma–Aldrich Co, St. Louis, USA). Phosphate-buffered saline (PBS) was used as a negative stimulation control. Cells were seeded in 500 μL of supplemented RPMI-1640 medium in 24-well plates at a cell density of 2×10^5 cells/well and incubated at 37 °C and 5 % CO_2 for different times: 48 h for apoptosis assay, B-cell subset distribution, and immunoglobulin production; 24, 48, 72, and 96 h for evaluation of B-cell proliferation; or 4 h for cell activation analysis. After this incubation period, cells were harvested for each experiment, with a single well used for each experimental condition. Three experimental replicates were performed for subset distribution and five experimental replicates for apoptosis and proliferation assay. The supernatant was obtained by centrifugation at 1200 rpm for 5 min and stored at −80 °C.

2.4. Cell function by flow cytometry

2.4.1. Subset determination

After stimulus incubation, the cells were collected and analysed for their cell surface markers by flow cytometry. Cells were incubated for 20 min at room temperature in the dark with specific antibodies against CD38 (5 μL, PECy7-conjugated, LS198-4-3; Beckman Coulter Inc., Brea, CA, USA), IgD (10 μL, FITC-conjugated, IgD26; MACS Miltenyi Biotec, Bergisch Gladbach, Germany), and 7AAD as a viability marker (20 μL, Beckman Coulter Company, Marseille, France). Data were collected on a CytoFLEX S flow cytometer (Beckman Coulter, Inc., Brea, CA, USA) and analysed using CytExpert software. Six B-cell subsets were characterized: $IgD^{+}CD38^{-}$ (Bm1), $IgD^{+}CD38^{+}$ (Bm2), $IgD^{+}CD38^{Hi}$ (Bm2'), $IgD^{-}CD38^{Hi}$ (Bm3–4), $IgD^{-}CD38^{+}$ (eBm5) and $IgD^{-}CD38^{-}$ (Bm5).

2.4.2. Apoptosis assay

After stimulus incubation, the cells were collected and stained with propidium iodide (PI) and FITC-conjugated Annexin V using the APOAF kit (Sigma–Aldrich Co, St. Louis, USA). Briefly, cells were suspended in 1× binding buffer, incubated with 5 μL of Annexin V-FITC and 10 μL of PI for 10 min at room temperature, protected from light, and then immediately acquired by flow cytometry on a CytoFLEX S flow cytometer (Beckman Coulter, Inc., Brea, CA, USA) and analysed using CytExpert software.

2.4.3. B-cell proliferation

After stimulation, the Ramos cell line (RA.1) was stained with CellTraceTM CFSE (Invitrogen by Thermo Fisher Scientific, Waltham, MA, USA) to evaluate cell proliferation. Between 1×10^6 cells and 1×10^7 cells were washed twice and resuspended in 500 μL of PBS. Then, 500 μL of 0.125 μM CFSE working solution was added to obtain a final staining concentration of 0.062 μM and incubated for 5 min at room temperature, protected from light. The reaction was stopped by adding 1 mL of FBS for 1 min at 4 °C. Finally, the cells were washed with 10 mL of cold PBS and resuspended in complete RPMI-1640 medium at the desired concentration for stimulation. Cells were collected at 16 h after CFSE stabilization as undivided control. Subsequently, proliferation measurements were performed at 24, 48, 72, and 96 h, incubating with 20 μL of 7AAD as a viability marker (Beckman Coulter Company, Marseille, France) for 20 min. Data were collected on a CytoFLEX S flow cytometer (Beckman Coulter, Inc., Brea, CA, USA) and analysed using FlowJoTM v10.6.1 software (BD Life Sciences).

2.5. Immunoglobulin production

After 48 h of stimulus incubation, the supernatants were collected to measure human IgM using an ELISA-specific kit for human IgM detection (ab214568, Abcam, USA), following the manufacturer's instructions. Seven standard tubes were prepared with concentrations ranging between 390.63 pg/mL and 25 000 pg/mL. Samples, standards, and blanks were processed in duplicate, incubating with the antibody cocktail at room temperature for 1 h. Then, each well was aspirated and washed three times. Finally, the TMB Development Solution was added and incubated for 10 min, followed by the stop solution from the kit to stop the reaction. The concentration of immunoglobulin was calculated by interpolating the optical density (DO) values using the linear regression equation obtained with standard solutions.

2.6. Protein phosphorylation

Phosphorylated protein detection from the B lymphocyte activation signalling pathway was performed using the immunoblot technique with five experimental replicates. Initially, protein lysate was obtained from 1×10^6 cells after 4 h of stimulus incubation. Cells were incubated with Pierce™ RIPA buffer (Thermo Scientific, Rockford, USA), protease inhibitors (HaltTM 78429, Thermo Scientific, Rockford, USA), and phosphatase inhibitors (HaltTM 78426, Thermo Scientific, Rockford, USA) following the manufacturer's recommendations. Lysates were quantified using the bicinchoninic acid (BCA) method with a commercial kit (BCA1 AND B9643, Sigma–Aldrich Co, St. Louis, USA), following the protocol for quantification in 96-well plates. Subsequently, lysates were lyophilized and reconstituted to a final concentration of 1 μg/μL for the subsequent steps.

Proteins were separated according to their molecular weight by SDS–PAGE electrophoresis on a 7.5 % polyacrylamide gel using 20 μg of protein lysate. Electrophoresis was conducted at 80 V for 120 min. Wet transfer was performed using the Mini Trans-Blot® system (Bio-Rad Laboratories, Inc. California) onto a PVDF membrane at 4 °C and 270 mA for 70 min. Subsequently, the membrane was incubated with the commercial reagent EveryBlot Blocking Buffer (Bio-Rad Laboratories, Inc. Japan) for 5 min with shaking. For detection, the membranes were incubated with primary rabbit antibodies specific to Lyn (dilution 1:500), pLyn (Tyr507, dilution 1:250), and β-actin (dilution 1:2000) (Cell Signaling Technology ®, Massachusetts, USA) for 18 h at 4 °C with shaking, followed by incubation with HRP-conjugated anti-rabbit IgG secondary antibody (1:3000 dilution, polyclonal, Cell Signaling Technology ®, Massachusetts, USA) for 1 h at room temperature with shaking. The revelation was carried out by chemiluminescence using the commercial ClarityTM Western ECL Substrate kit (#170–5060, Bio-Rad Laboratories, Inc. USA), and detection was performed using the Amersham Imager 600 equipment (GE Life Sciences, Buckinghamshire, UK).

2.7. Statistical analysis

The statistical analysis of survival and proliferation assays were performed using two-factor ANOVA, with multiple comparisons conducted using Dunnett test. Immunoglobulin production and cell activation analysis were performed using one-factor ANOVA,

followed by Tukey's multiple comparison test. p values less than 0.05 were considered significant. GraphPad Prism 9 (GraphPad Software, San Diego, CA, USA) was used for data analysis.

3. Results

3.1. Effect of PTH stimulation on the function of B-cell

The expression of PTH1R was characterized in the B cells of the Ramos cell line (RA.1), showing expression in 46.96 % of these cells. Additionally, its phenotype was identified based on the expression of IgD and CD38 surface markers (Bm1-Bm5 phenotype), revealing a predominant distribution of Bm3-4, corresponding to the germinal centre phenotype.

The effects of PTH stimulation were evaluated by flow cytometry after 48 h. Higher concentrations of PTH, co-stimulated with CpG, maintained the Bm3-4 phenotype (93.4 ± 2.3 %) compared to unstimulated cells (77.3 ± 6.4 %), which mainly differentiated into the eBm5 phenotype (6.0 ± 2.2 % with PTH highest stimulation vs. 22.7 ± 6.4 % without), showing a significant result with a p-value of 0.03 according to the ANOVA test (Fig. 1A and B).

3.2. Effect of PTH stimulation on the survival of B lymphocytes

B cells from the Ramos cell line (RA.1) were incubated with increasing doses of PTH for 48 h, and apoptosis was measured by flow cytometry using Annexin V-FITC/PI staining. The evaluation of B-lymphocyte survival did not show a significant difference according to the ANOVA test; conversely, similar percentages of viable cells (81.0 ± 14.7 %) were found in early (5.3 ± 4.6 %) and late (13.4 ± 16.0 %) apoptosis between stimulated and unstimulated cells (Fig. 2A).

3.3. Effect of PTH stimulation on the proliferation of B lymphocytes

After incubating B lymphocytes from the Ramos cell line (RA.1) with increasing doses of PTH, cell proliferation was evaluated by determining CFSE. The proliferation was calculated as a ratio of proliferation every 24 h by the mean fluorescence intensity (MFI). No significant differences were observed in the proliferation ratios among the evaluated conditions according to the ANOVA test (Fig. 2B).

3.4. Effect of PTH stimulation on immunoglobulin production

The supernatant of B cells from Ramos (RA.1), an IgM-producing cell line, was analysed for immunoglobulin quantification by ELISA after stimulation with increasing doses of PTH. The IgM concentration was similar in the supernatant of unstimulated cells (10 365 ± 1792 pg/mL) and PTH-stimulated cells (10 468 ± 2681 pg/mL, on average). Therefore, according to the ANOVA test, no significant differences were found in IgM production when stimulated with PTH (Fig. 2C).

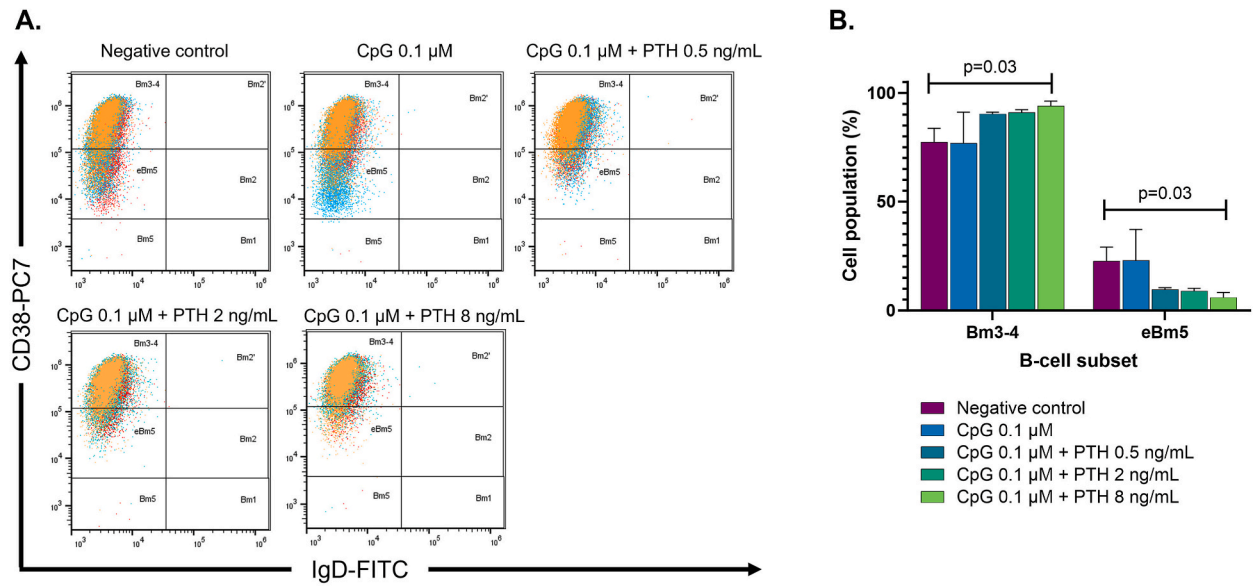

Fig. 1. Phenotype changes of B cells stimulated for 48 h without stimulus, CpG 0.1 μM, CpG 0.1 μM + PTH 0.5 ng/mL, CpG 0.1 μM + PTH 2 ng/mL and CpG 0.1 μM + PTH 8 ng/mL. **A.** Flow cytometry representation **B.** Statistical included 3 experimental replicates and Bm3-4 and eBm5 as the only factors analysed. Two-factor ANOVA and Dunnett's multiple comparison were applied and significant values of $p < 0.05$ were considered.

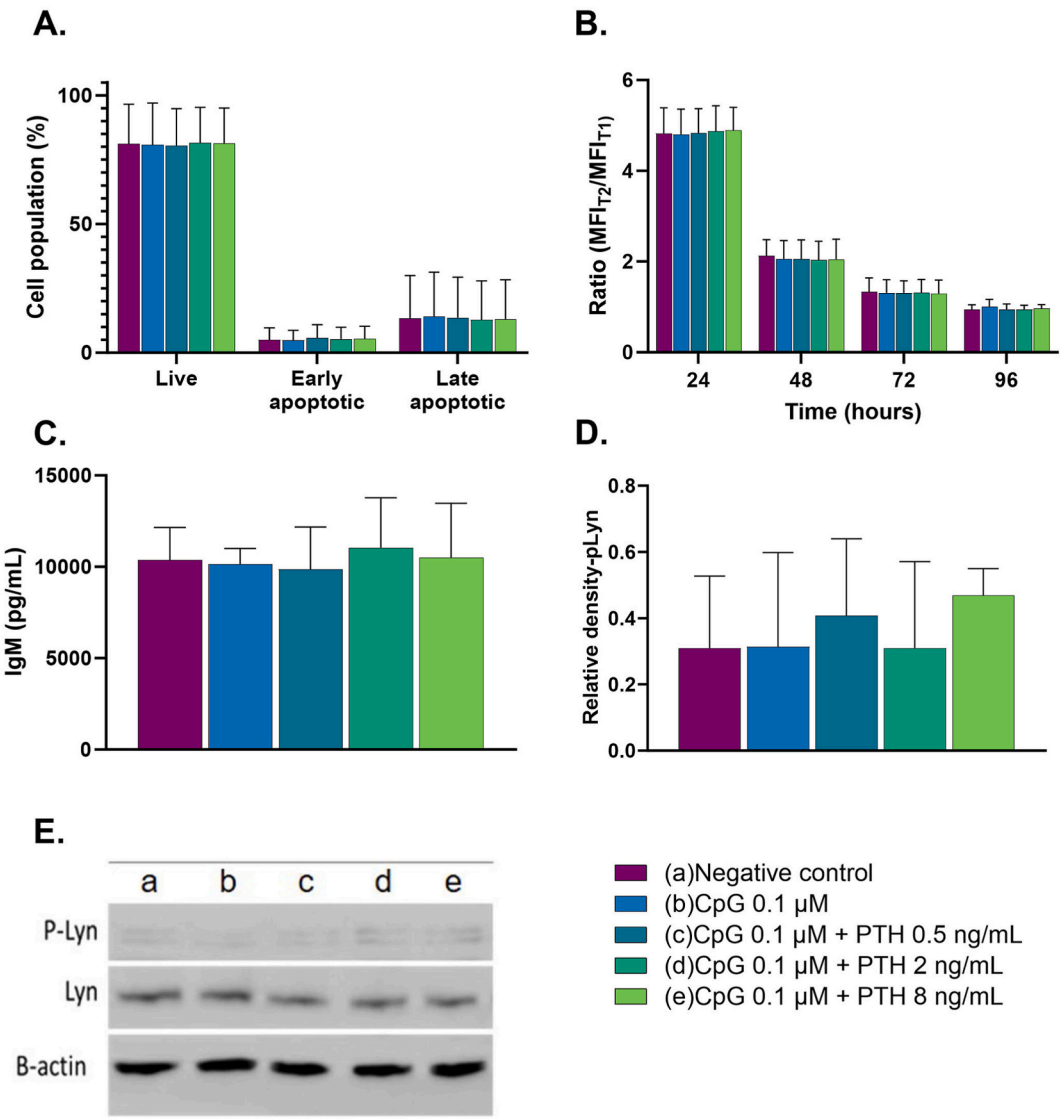

Fig. 2. Effects of PTH on B cells. **A.** Survival analyses of B cells stimulated for 48 h, histograms represent the average obtained from 5 experimental replicates **B.** Cellular proliferation of B cells stimulated at 24, 48, 72 and 96 h, histograms represent the proportion of the CFSE MFI read at time 2 over time 1, where T2 corresponds to the immediately greater time in which the reading was performed, data are representative of 5 experimental replicates **C.** IgM quantification in cell culture supernatant of B cells stimulated for 48 h, data are representative of 5 experimental replicates **D, E.** Effects of PTH on intracellular signaling in B cells stimulated for 4 h, protein levels were measured with Immunoblot and all data represent the mean and standard deviation of 5 experimental replicates. *B cells from the Ramos cell line cultured without stimulus (a), CpG 0.*1 μM *(b), CpG 0.*1 μM+ *PTH 0.*5 ng/mL *(c), CpG 0.*1 μM+ *PTH* 2 ng/mL *(d) and CpG 0.*1 μM+ *PTH* 8 ng/mL *(e). Two-factor ANOVA and Dunnett's multiple comparison were applied to A and B, while Tukey's multiple comparison was applied to C and D. Significant values of* $p < 0.05$ *were considered. Supplementary Material 1 includes the raw data of Lyn and its associated β-actin, and* Supplementary Material 2 *includes the raw data of pLyn and its associated β-actin. The bands 4–8 in both images correspond to conditions a-e respectively.*

3.5. Effect of PTH stimulation on intracellular activation of B lymphocytes

To determine the effect of PTH on B-cell activation, changes in the phosphorylation of Lyn, an essential protein tyrosine kinase in the early B-cell signalling pathway, were analysed by immunoblot. As shown in Fig. 1E, after PTH stimulation, there were no significant changes in the phosphorylation of the Lyn 507 tyrosine residue in B cells of the Ramos cell line (RA.1) according to the ANOVA test (Fig. 2D and E).

4. Discussion

The factors involved in the mosaic of autoimmunity establish scenarios of susceptibility, loss of antigen self-tolerance, and the development of autoimmune phenomena [2]. Based on clinical observations and a previous study, the possibility that PTH might be related to the development of ADs was considered. It was found in this study that there is a marked increase in B lymphocytes expressing PTH1R in LES patients compared to healthy controls. Also, elevated levels of PTH are associated with increased disease activity of pSS [15].

To understand the possible effect of the interaction between PTH and PTH1R on B lymphocytes, *in vitro* stimulation analyses were performed on a cell line expressing this receptor. The effect of PTH stimulation on B-lymphocytes was evaluated, determining its impact on proliferation, survival, subsets, immunoglobulin production, and cell activation. The experiments demonstrate that the B-lymphocyte phenotype is modified with increasing concentrations of PTH, keeping the stimulated cells in the Bm3-4 phenotype. In contrast, the unstimulated cells differentiate toward early memory cells (eBm5). The other biological effects evaluated did not differ with hormone stimulation.

Previously, it was found that in the peripheral blood of patients with autoimmunity, the distribution of the B-cell subsets was altered, showing a higher percentage of Bm2 and Bm2' phenotypes (germinal centre founder cells) expressing PTH1R. The Ramos B-cell line characterized in this study exhibited a predominant Bm3-4 phenotype, corresponding to germinal centre cells. In the germinal centre reaction, activated cells undergo class-switching recombination and somatic hypermutation affinity maturation (SHM) processes [17]. Generally, cells may undergo several SHM processes before being selected and differentiated into memory or long-lived plasma cells [18]. The finding that high concentrations of PTH retain cells at the *in vitro* level in a centroblast/centrocyte phenotype (Bm3 and Bm4) may suggest that these cells undergo more cycles in the germinal centre reaction, indicating increased SHM reactions. These could have at least two types of impacts on autoimmunity development. On the one hand, SHM has been shown to contribute to autoreactive B lymphocyte development, as demonstrated in both a murine SLE model [19] and humans with SLE [20]. Thus, in the hyperparathyroidism model, a potential increase in SHM could be related to the *de novo* generation of autoreactive cells, explaining its relationship with the development of autoimmune pathology.

On the other hand, during germinal centre reaction cycles, a positive selection process occurs, which depends on the affinity of the interaction with dendritic cells or follicular T lymphocytes. If these cells do not receive the necessary survival signals, they undergo cell death [21]. In this case, an increase in affinity in autoreactive cells due to the rise in SHM could induce a rescue of apoptosis of these cells and thus contribute to autoimmunity. The significant result concerning the altered phenotype of B lymphocytes under PTH stimulus supports the hypothesis that high levels of PTH may contribute to B-cell-mediated autoimmunity. However, further studies are required to validate this hypothesis and to elucidate the mechanisms underlying this effect.

5. Conclusions

The effects of PTH on modulating B lymphocyte subsets suggest a potential involvement of the hormone in the pathophysiology of ADs. However, considering the multiple factors and their intricate interactions associated with autoimmunity development, further studies should be conducted to elucidate the mechanism implicated in PTH's potential role in phenotypic changes in B lymphocytes contributing to the development of autoimmunity.

Study limitations: The primary study limitation is the utilization of a single cell line, as it solely represents one phenotype of B lymphocytes. Studies conducted using human primary cells would enable observation of the behaviour and effects of PTH across all subsets, yielding more accurate information. Another limitation is the lack of co-stimulators in the *in vitro* model. Incorporating these factors would allow replication of the conditions seen in the pathophysiology of autoimmunity, acknowledging the multifactorial origins of these diseases.

Ethics Declarations: This study was reviewed and approved by the Human Research Ethics Committee of Universidad Icesi, with the approval numbers: 158 and 314. Informed consent was not required for this study because no human participants were included in the study.

Funding

This work was supported by the Ministry of Science, Technology and Innovation – Minciencias, Colombia [grant number CT. 835-2018].

Data availability statement

Data will be made available on request to the author, Lady J. Rios-Serna.

CRediT authorship contribution statement

Lady J. Rios-Serna: Writing – review & editing, Writing – original draft, Project administration, Methodology, Investigation, Conceptualization. **Angie M. Rosero:** Writing – review & editing, Writing – original draft, Project administration, Methodology, Investigation, Conceptualization. **Gabriel J. Tobón:** Writing – review & editing, Writing – original draft, Project administration, Methodology, Investigation, Conceptualization. **Carlos A. Cañas:** Writing – review & editing, Writing – original draft, Project

administration, Methodology, Investigation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgment

To Tecnoquimicas for supporting CIRAT.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e30556.

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