Gonadotropin-releasing hormone receptor pathway affects the function of human EBV-transformed B lymphocytes in an age-independent way

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Immune system function changes during aging, but the molecular mechanisms of this phenomenon are not fully understood. The present study identified pathways that are associated with age-associated changes in human B lymphocytes. Initial in silico analysis of 1355 genes involved in aging revealed the strongest association ($p = 4.36E-21$) with the gonadotropin-releasing hormone receptor (GnRHR) pathway. Extended analysis of 2736 aging-related genes using updated databases confirmed such association ($p = 2.41E-16$). Genes involved in both aging and the GnRHR pathway were significantly involved in lymphocyte B and T activation and aging-related phenotypes, including hyperinsulinemia and diabetes, arthritis, cerebrovascular disease, and cancers. We, therefore, examined non-tumorigenic Epstein-Barr virus (EBV)-transformed B-lymphocyte cell lines that originated from 12 young subjects (20–31 years old) and 10 centenarians (100–102 years old). Gonadotropin-releasing hormone I (GnRH-I) and GnRHR levels did not depend on the age of the cell donors. Inhibition of the GnRHR pathway age-independently decreased cell proliferation ($p < 0.001$) and increased apoptosis ($p < 0.001$). However, the decrease in immunoglobulin G synthesis ($p < 0.01$) was twice as high in centenarian cells than in young cells.

In conclusion, the GnRHR pathway regulated essential properties of B lymphocytes. However, upon EBV transformation, memory class-switched B cells became the dominant cell subpopulation. Therefore, the observed effects of GnRHR inhibition were attributable to this subpopulation.

1. Introduction

Aging is associated with the remodeling of immunity. Age-related alterations are observed in all types of immune cells, including B lymphocytes (Ponnappan and Ponnappan, 2011). Although the total number of B lymphocytes remains stable throughout life, the number of naive cells decreases in favor of antigen-experienced memory cells, including “exhausted” (CD19+IgD+CD27−) memory cells (Ponnappan and Ponnappan, 2011). B-cell lymphopoiesis and migration from bone marrow significantly decrease with age, and B lymphocytes in aged mice are less susceptible to apoptosis (Montes et al., 2006; Souvannavong et al., 1998), which results in a lower turnover of the peripheral fraction of B lymphocytes (Kline et al., 1999). The ability of B lymphocytes to produce antigen-specific antibodies is determined by class-switching recombination (CSR) and somatic hypermutation (SHM), which involve the programmed introduction of point mutations in variable regions of immunoglobulin (Ig) genes. Both of these processes are impaired in age-advanced humans and result in a decrease in B-cell diversity (Gibson et al., 2009). This decline is caused by a failure to properly regulate the expression of activation-induced deaminase (AID), an enzyme that is directly involved in CSR and SHM, and thymus involution that leads to a reduction of T-cell maturation and consequently impairments in T- and B-cell interactions (Blomberg and Frasca, 2013; Palma et al., 2014).

Type I gonadotropin-releasing hormone (GnRH-I) and its receptor (GnRHR) are best known for their role in regulating the hypothalamic-pituitary-gonadal (HPG) axis. Both GnRH-I and GnRHR are also expressed outside the HPG axis, such as in various regions of the central and peripheral nervous systems, ovaries, the endometrium, the prostate, breasts, the placenta, skeletal muscles, and the immune system (Cheung and Wong, 2005). Hypothalamic GnRH-I is rapidly metabolized in the blood and unlikely acts as a ligand in extrapituitary tissues (Tannirverdi
GnRH-I treatment increases the proliferation of Epstein-Barr virus (EBV)-transformed B lymphocytes and human peripheral blood mononuclear cells (PBMCs). GnRH-I treatment in stimulated PBMCs increases the expression of interleukin-2Rγ (IL-2Rγ) mRNA and synthesis of interferon γ (IFN-γ) (Chen et al., 1999; Grasso et al., 1998; Tanriverdi et al., 2005; Tanriverdi et al., 2004). The immunostimulatory effect of GnRH-I has also been suggested by the results of in vivo studies, in which GnRH-I antagonist administration in mouse models of autoimmune diseases reduced Ig levels (Jacobson et al., 1994). Data on the involvement of GnRH-I receptors in immunomodulatory processes are limited. In our preliminary study, we analyzed the expression of GnRH mRNA in PBMCs from 25 young and 27 long-lived (>90 years old) individuals and found that it was significantly lower (p = 0.0394) in cells from older subjects (Supplementary Fig. S1).

The first objective of the present study was to characterize the effect of GnRH activation on B-lymphocyte function. The second objective was to establish whether age-associated changes in GnRH signaling are responsible for impairments in B-lymphocyte function in individuals of advanced age.

2. Materials and methods

2.1. In silico analysis

For the initial analysis, we searched the PubMed database, Longevity Map, and Digital Ageing Atlas (Budovsky et al., 2013; Craig et al., 2015). Genes shown as associated with aging (Good et al., 2012; Lunetta et al., 2007; Sebastiani et al., 2013; Shen et al., 2013; Soerensen et al., 2012; Solovieff et al., 2010; Yashin et al., 2010) were annotated using the Webgestalt database (Wang et al., 2017). For the extended, final analysis, we used updated datasets from the following databases: Longevity Map (https://genomics.senescence.info/longevity/), Digital Ageing Atlas (https://ageing-map.org/), GenAge (https://genomics.senescence.info/gene/index.html), Denigma longevity-variant-database (https://www.denigma.de/data/entry/longevity-variant-database, p < 0.05), and genes reported for longevity trait in GWAS catalog (https://www.ebi.ac.uk/gwas/efotraits/EFO_0004300, p < 0.05) (Budovsky et al., 2013; Craig et al., 2015). Aging-related gene expression data (FDR < 0.05) were obtained from the GTEx project for human tissues (adipose, artery, heart, lung, muscle, nerve, skin, thyroid, blood tissues) (Consortium, 2015). We obtained lists of gene symbols from all datasets, which were further processed in R to remove any aggregations, spaces, parentheses, and special characters (Supplementary Table S1). Gene lists associated with the GnRH signaling pathway and immune processes were downloaded from the PANTHER database. Data combining, aggregation, and annotation analyses were performed in R using the wizbionet R package (Wicik et al., 2021). Redundant gene symbols from different studies were deduplicated automatically by Panther database.

2.2. Pathway enrichment analysis

Gene sets obtained during initial analysis were tested using a binomial overrepresentation test to determine non-randomness of the participation of genes in signaling pathways and the Protein Analysis Through Evolutionary Relationships Classification System (PANTHER) database, version 10 (released 2015), to find enriched ontological terms (Mi et al., 2016). Next, these initial results were confirmed during the final analysis performed with the statistical overrepresentation test tool from the PANTHER database, version 16 (released 2020), and Fisher's Exact test with false discovery rate correction. PANTHER 16.0 contains 177 signaling pathways, the components of which are continuously updated based on the latest reports and suggestions from experts in the field. The Homo sapiens reference gene list was used in all steps of the analysis. Interaction networks were constructed using R environment and visualized in Cytoscape 3.7.2 software (Shannon et al., 2003).

2.3. Disease enrichment analysis

Disease enrichment analysis was performed using the EnrichR API plugin and Jensen DISEASES dataset (Chen et al., 2013). In all analyses, FDR corrected p-value cutoff was set at less than 0.05.

2.4. Material

The present study was performed using EBV-transformed B-cell lines from 12 young subjects (20–31 years old) and 10 centenarian subjects (100–102 years old). Cell lines that were derived from centenarians were obtained from the International Institute of Molecular and Cell Biology in Warsaw. Cell lines from young individuals were prepared in the Department of Human Epigenetics using the same standard procedure (Miller and Lipman, 1973). The study was approved by the Bioethics Committee of Warsaw Medical University (KB/284/2013). All of the individuals provided written informed consent to participate in the study.

The cells were stored in liquid nitrogen. After thawing, they were cultured in RPMI-1640 medium that contained 15% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin) and maintained in 5% CO2 at 37 °C in a humidified incubator. Because we sought to examine non-malignant cells, all of the lines were tested using the soft agar colony formation assay, and only cells that did not form colonies were used in the subsequent experiments.

2.5. Viability assessment

For the viability assay, cells were seeded on a 96-well plate (1 × 10^5 cells/well) and treated with the GnRH antagonist Cetrorelix acetate (catalog no. CS249, Sigma, Darmstadt, Germany) at final concentrations of 10 nM to 1 μM. Twenty-four hours later, 10 μl of resazurin from the In Vitro Toxicology Assay Kit (Sigma) was added to each well. After 4 h of incubation, absorbance was read at 600 nm using an Epoch Microplate Spectrophotometer (BioTek, Winooski, VT, USA). The reference wavelength was 690 nm.

2.6. Proliferation assay

The proliferation study was performed using the bromouridine (BrdU) Pharmingen Flow Kit (BD Biosciences, San Jose, CA, USA). Cells were seeded and cultured as above. Bromouridine was added to each well to a final concentration of 10 μM. The cells were then treated with Cetrorelix at a final concentration of 10 μM for 24 h and then fixed and stained according to the manufacturer’s protocol. The cells were analyzed using a FACS Canto II Cytometer (BD Biosciences) and FACS Diva software (BD Biosciences).

2.7. Apoptosis assay

The apoptosis assay was performed using the Annexin V-FITC
Apoptosis Detection Kit (BD Biosciences). Cells were seeded and cultured as described above and treated with 10 μM Cetrorelix for 24 h. The cells were stained according to the manufacturer’s protocol and analyzed using a FACs Canto II Cytometer and FACs Diva Software.

2.8. Immunoglobulin G production

Immunoglobulin G production was assessed using the Human IgG Total ELISA Ready-Set-Go! Kit (eBioscience, Vienna, Austria). Cells were seeded on 24-well plates (1 \times 10^6 cells/well) and treated with 10 μM Cetrorelix for 7 days. In another experiment, cells were treated with another GnRHR inhibitor, WAY-207024 (catalog no. CAS 872002-73-8, Santa Cruz Biotechnology, Dallas, TX, USA), at a final concentration of 10 μM for 24 h with subsequent incubation in WAY-207024-free medium for 6 days. Supernatants were collected and stored at −20 °C. The analysis was performed according to the manufacturer’s protocol. Absorbance was read at 450 nm using an Epoch Microplate Spectrophotometer. The reference wavelength was 570 nm.

2.9. RNA isolation and real-time polymerase chain reaction

Approximately 1 \times 10^6 cells were used for each RNA isolation, which was performed using the GeneMATRIX Universal RNA/miRNA Purification Kit (EURx, Gdańsk, Poland). Next, 200 ng of RNA served as the template for reverse transcription using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, Waltham, MA, USA). The resulting cDNA, corresponding to 1 ng RNA per reaction, was used for real-time polymerase chain reaction (PCR) using a LightCycler 480 Sybr Green I Master Kit (Roche) and Light Cycler 480 Instrument II (Roche). The reaction conditions were the following: pre-incubation for 5 min at 95 °C, 40 cycles of 12 s at 95 °C, 12 s at 60 °C (GnRHR-I) or 62 °C (GnRHR), and 12 s at 72 °C, followed by a melting curve cycle. The primers for GnRH-I amplification were 5′-TTCTACTGACTTGGTGCCTG3′ (forward) and 5′-GGAAATATGGTCAGAATGTGCC-3′ (reverse), and the primers for GnRHR were 5′-GGAGTGGATGGCTGGAACATTA3′ (forward) and 5′-ATGACGATGCACCTGGCTG3′ (reverse). All of the reactions were performed in duplicate. The results were normalized to the expression of TBP. This reference gene was chosen because its expression was not dependent on age.

2.10. Protein isolation and immunoblotting

Protein isolation was performed using RIPA buffer and Pierce Protease Inhibitors EDTA-Free mix (Thermo Scientific) on wet ice with shaking. Approximately 1 \times 10^6 cells and 100 μl of the buffer were used for each isolation. After 10 min of lysis, the samples were centrifuged at 10,000 \times g for 5 min at 4 °C. The total soluble protein concentration was determined using the Pierce BCA Protein Assay Kit (ThermoScientific) in 0.05% Tween with 3% BSA in PBS for 1 h, washed five times with 3% BSA in PBS, and incubated with secondary antibodies that were conjugated to Alexa Fluor 633 (1:10000, catalog no. A21070, Invitrogen). Hoechst 33258 was used for counterstaining.

2.11. Flow cytometry

For each experiment, 1 \times 10^6 cells were washed twice in phosphate-buffered saline (PBS) with 5% FBS and fixed with cold 80% methanol for 15 min at −20 °C. The cells were then permeabilized with 0.5% Triton X-100 for 15 min and blocked with 10% goat serum for 30 min. Next, the cells were then incubated with anti-GnRHR antibodies (1:10, catalog no. ab24095, Abcam, Cambridge, UK) or an appropriate isotype control (1:10, catalog no. ab91353, Abcam) for 30 min. The secondary antibodies were conjugated to Alexa Fluor 488 (1:500, Invitrogen, Carlsbad, CA, USA). The cells were analyzed using FACs Canto II and FACs Diva software. Fluorescence was detected in the FITC channel.

2.12. Immunofluorescence

For immunofluorescent staining, 0.5 \times 10^6 cells were washed twice in PBS with 5% FBS and centrifuged at 400 rotations per minute using a Cytospin 3 centrifuge (Thermo Shandon, Astmoor, UK) for 3 min on a glass slide. The cells were then fixed with ice-cold 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, and blocked with 5% goat serum with 0.1% Triton X-100. The slides were then incubated with anti-GnRHR antibodies (1:500, catalog no. sc-13944, Santa Cruz Biotechnology) in 0.05% Tween with 3% BSA in PBS for 1 h, washed five times with 3% BSA in PBS, and incubated with secondary antibodies that were conjugated to Alexa Fluor 633 (1:10000, catalog no. A21070, Invitrogen). Hoechst 33258 was used for counterstaining.

2.13. Statistical analysis

The statistical analysis and plotting were performed using RStudio software with the Tidyverse package. Before the statistical tests, the normality of data distributions and equal variation assumptions were checked using the Shapiro-Wilk normality test and Bartlett’s test, respectively. To analyze the results of the functional studies, we used paired t-tests (two groups) or One-way repeated-measures analysis of variance (ANOVA) (more than two groups). Two-way repeated-measures ANOVA was used for comparisons of more than two groups, with age as an additional factor. For multiple comparisons, Dunnett’s post hoc tests were performed. To analyze the quantitative PCR (qPCR) and immunoblot results, we used one-way ANOVA for independent measures or the Kruskal-Wallis test (when assumptions for the one-way ANOVA were not met). The flow cytometry results were tested using unpaired t-tests. Values of p < 0.05 were considered statistically significant.

3. Results

3.1. Identification of aging-associated pathways by in silico analysis

Our initial bioinformatics analysis identified 1355 aging-associated genes, which were analyzed using version 10 of the PANTHER ontological database. Seventy-six of these genes were also involved (i.e., overrepresented, p = 4.36E-21) in the GnRHR signaling pathway, and this association was the strongest among all enriched aging-related pathways. Next, to validate the obtained results and avoid arbitrary selection of the PubMed articles, we performed a final analysis using the latest updated databases related to aging. The workflow of both analyses is presented in Fig. 1. In the final analysis, we identified 3110 aging-related gene symbols (Supplementary Table S1), from which 2726 were also identified by version 16 of the PANTHER database. This analysis located the GnRHR signaling pathway, involving 97 aging-related genes, on the third position among all aging-associated
Fig. 1. In silico analysis of genes involved in aging and cell-signaling pathways. Workflow of the A. initial and B. final bioinformatics analysis that led to the identification of signaling pathways associated with aging, GnRHR signaling, and immune function. C. Venn diagram showing the overlap between GnRHR signaling genes in the context of aging and immune system function (according to the PANTHER database).

Fig. 2. Interaction network between 15 most significant signaling pathways associated with genes involved in aging and GnRHR signaling. Network shows genes that are associated with at least three of these pathways.
signaling pathways \((p = 2.41E-16)\) (Supplementary Fig. S2).

In order to identify pathways potentially affected by the disrupted GnRHR signaling, we performed another enrichment analysis of 97 genes involved in the GnRHR pathway and aging. It showed that they were significantly involved in the Ras, VEGF, insulin/IGF pathway-mitogen activated MAP kinase cascade, interferon \(\gamma\), and FAS signaling pathways, among others. Forty of these genes were also involved in immune system processes (Fig. 1C); 18 of them were significantly associated with B lymphocyte activation \((p = 5.40E-23;\) Fig. 2) and 17 with T lymphocyte activation \((p = 3.08E-20;\) Fig. 2).

### 3.2. Identification of aging-related diseases associated with the GnRHR signaling pathway

In order to identify pathways potentially affected by the disrupted GnRHR signaling, we performed another enrichment analysis of 97 genes involved in the GnRHR pathway and aging. It showed that they were significantly involved in the Ras, VEGF, insulin/IGF pathway-mitogen activated MAP kinase cascade, interferon \(\gamma\), and FAS signaling pathways, among others. Forty of these genes were also involved in immune system processes (Fig. 1C); 18 of them were significantly associated with B lymphocyte activation \((p = 5.40E-23;\) Fig. 2) and 17 with T lymphocyte activation \((p = 3.08E-20;\) Fig. 2).

#### 3.3. GnRH-I and GnRHR expression in EBV-transformed B lymphocytes

To examine the expression of GnRH-I and GnRHRs in these cells and determine possible age-related differences, we measured mRNA levels in cells from young and centenarian subjects. The mean mRNA levels of both GnRH-I and GnRHR were similar between cells from young and centenarian donors (Supplementary Fig. S3).

Flow cytometry and immunofluorescence analysis revealed that approximately 90% of the cells expressed GnRHR protein in both the nucleus and cytoplasm (Fig. 4).

However, the expression of total GnRHR evaluated by flow cytometry, and both the 60 and 70 kDa GnRHR isoforms evaluated by immunoblotting, did not change in an age-related manner (Supplementary Fig. S4).

### 3.4. Effect of GnRHR antagonist Cetrorelix on B lymphocyte viability

To investigate whether the GnRHR pathway affects B lymphocyte function, we initially analyzed the effect of GnRHR inhibition on cell viability. Twenty-four hours of treatment with Cetrorelix at final concentrations of 100 nM, 1 \(\mu\)M, and 10 \(\mu\)M significantly decreased cell viability by 17.9% \((p < 0.05)\), 31.9% \((p < 0.001)\), and 24.1% \((p < 0.05)\), respectively, compared with untreated control cells, indicating that B lymphocytes were responsive to GnRHR signaling (Fig. 5).

### 3.5. Effect of GnRHR pathway inhibition on B lymphocyte apoptosis

No age-associated differences in the apoptosis of untreated control B lymphocyte cell lines were found between young and centenarian donors. Upon treatment with 10 \(\mu\)M Cetrorelix, we observed a significant increase in the mean percentage of apoptotic cells by 22.6% \(\pm\) 6.12% for young donors and 16.17% \(\pm\) 13.17% for centenarian donors \((p < 0.001;\) Fig. 6A). B lymphocytes from both age groups were similarly affected \((p = 0.299)\).

### 3.6. Effect of GnRHR pathway inhibition on B lymphocyte proliferation

The percentage of untreated BrdU-positive cells was similar between age groups: 76.09% \(\pm\) 13.95% for young donors, 76.24% \(\pm\) 10.21% for centenarian donors \((p = 0.98)\). The percentage of BrdU-positive cells significantly decreased upon 10 \(\mu\)M Cetrorelix treatment by 14.01% \(\pm\) 4.26% and 14.60% \(\pm\) 5.37% in the young and centenarian groups, respectively, compared with untreated cells \((p < 0.001;\) Fig. 6B). The proliferation-inhibiting effect of Cetrorelix in B lymphocytes was similar between young and centenarian donors \((p = 0.97)\).

The increase in apoptosis could explain such a decrease in the

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**Fig. 3.** Comparison of the top 20 diseases associated with genes involved in the GnRHR signaling pathway and aging (marked in red) with the top 20 diseases associated with the complete set of aging-related genes (marked in blue). Enriched disease terms were ordered based on the FDR \(p\)-value for the GnRHR signaling pathway-related gene set. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
percentage of BrdU-positive cells after Cetrorelix treatment. To verify the interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 4. Immunofluorescent staining of EBV-transformed cells. Cells were centrifuged, fixed, permeabilized, and incubated with A. anti-GnRHR antibodies and secondary antibodies that were conjugated to Alexa Fluor 633 (red) or B. only secondary antibodies. Hoechst 33258 dye was used for nuclear staining. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 5. Effect of GnRHR inhibition on the viability of EBV-transformed B cells. Cell viability was measured in 10 cell lines after 24 h of treatment with 1 nM to 10 μM Cetrorelix using the resazurin-based assay. Untreated cells served as controls. Viability was normalized to untreated cells (set to 100%). The data are expressed as means and standard errors. * p < 0.05, **p < 0.001 (one-way repeated-measures ANOVA and Dunnett’s post hoc test).

3.7. Effect of GnRHR pathway inhibition on IgG production

To examine the effect of 10 μM Cetrorelix on IgG antibody production, we measured total levels of IgG in supernatants originating from B lymphocyte cultures of 6 young and 6 centenarian individuals after 7 days of incubation with this compound. We did not detect IgG in five (2 young and 3 centenarian) of 12 supernatants. In cell lines that produced detectable amounts of IgG, we observed a significant 38.22% decrease in IgG levels after 7 days of Cetrorelix treatment compared with controls (8.32 μg/ml vs. 14.2 μg/ml, p < 0.01; Fig. 7A). Because of an insufficient number of cell lines that produced IgG, we did not analyze age-related antibody production.

Twenty-four hours of incubation with 10 μM of another GnRHR inhibitor, WAY-207024, followed by 6 days of incubation in WAY-207024-free medium induced a significant 26.9% decrease in IgG levels compared with controls (23.8 μg/ml vs. 32.6 μg/ml, p = 0.038, Fig. 7B). In this experiment, antibodies were detected in supernatants that were collected from six cultures that originated from young donors and four cultures that originated from centenarians. Notably, WAY-207024 decreased IgG levels by 20.9% (from 40.9 μg/ml in treated cultures to 32.4 μg/ml in controls) in the young group and by 45.4% (from 20.1 μg/ml in treated cultures to 10.98 μg/ml in controls) in the centenarian group. In both age groups, however, the level of significance was not reached.

4. Discussion

The GnRHR pathway was the focus of the present study based on an in silico analysis of genes involved in the aging process. The association between the GnRHR pathway and aging identified in our initial analysis (p = 4.36E-21) was stronger than the association with the insulin/insulin-like growth factor-1 or p53 pathway, the well-known regulators of this process (Moskalev et al., 2014). This strong association was confirmed in our most recent, extended analysis (p = 2.41E-16). Comparison of phenotypes associated with GnRHR signaling and aging versus phenotypes associated with a complete list of aging-related genes revealed a substantial overlap of aging-associated abnormalities such as hyperinsulinemia, hyperglycemia and diabetes, arthritis, fatty liver disease, cerebrovascular disease, and cancers (Fig. 3).

We also identified B and T lymphocytes as elements of the immune system significantly associated with GnRHR and aging pathways. During aging, the function of both lymphocyte types is severely affected. A pool of naïve T cells decreases, and their terminal differentiation increases, cells lose the co-stimulatory receptor CD28 critical for their activation, and abnormal function of T-helper cells negatively affects B lymphocyte stimulation. In turn, the production of B cells and B-cell receptor diversity decrease with aging. Their capacity to initiate primary or secondary responses and production of antibodies also decrease (Crooke et al., 2019).

Taking into account the results of our in silico analyses, both lymphocyte types were good candidates for studies of the effect of the GnRHR pathway on their function and aging. However, we were unable to repeatedly obtain large amounts of fresh cells from study subjects because of ethical and medical reasons. Moreover, we were already in possession of several EBV-transformed B lymphocyte cell lines...
constituting an efficient source of non-malignant cells and were able to prepare new ones quickly. Due to these reasons, during the implementation of this project, we focused on B lymphocytes.

To identify the mechanisms that co-regulate activity of B lymphocytes and affect their aging, we analyzed the effects of GnRHR pathway modulation in these cells. Treatment with GnRH-I, a natural GnRHR agonist, produced only slight and nonsignificant effects on B-lymphocyte function compared with untreated cells (data not shown). As all of our tested cell lines produced GnRH-I and GnRHR, their endogenous expression may have been sufficient to exert maximal biological effects that were not further modified by an increase in GnRH-I. Consequently, we inhibited activity of the GnRHR pathway, which suppressed B-cell viability and proliferation and IgG synthesis but increased apoptosis. These results indicated that the GnRHR pathway co-regulated properties of B lymphocytes.

Our findings are consistent with previous studies that explored the effects of GnRH-I on the proliferation of various immune cell types. Treatment with GnRH-I and GnRHR agonists stimulated the proliferation of rat splenocytes and thymocytes, human PBMCs, EBV-transformed B lymphocytes, and Jurkat cells (Azad et al., 1997; Morale et al., 1991; Tanriverdi et al., 2005; Tanriverdi et al., 2004). Blockade of the GnRHR pathway during early postnatal life affected immune system function in rats and monkeys (Mann et al., 2000; Mann et al., 1994; Morale et al., 1991). Newborn monkeys that were treated with a GnRHR antagonist exhibited lower levels of circulating B and T lymphocytes and a decrease in mitogen-induced B and T lymphocyte proliferation (Mann et al., 2000).

The effects of GnRHR inhibition on Ig levels were previously
explored in in vivo studies that utilized mouse models of autoimmune diseases. For example, in a model of systemic lupus erythematosus, GnRHR inhibition decreased autoantibody and total Ig levels (Jacobson et al., 1994). The authors suggested that these effects were at least partially attributable to a reduction of the number of B cells. Treatment with a GnRHR antagonist decreased serum IgG levels in a non-obese mouse model of autoimmune diabetes and decreased the production of IL-6 and IFN-γ by spleen cells in vitro (Ansari et al., 2004). The results of our in vitro study complement these in vivo findings. Notably, not all of the tested cell lines produced detectable amounts of IgG. This was consistent with previous studies that showed that only ~60% of EBV-transformed B-cell lines expressed IgG on their surface (Winiarska et al., 2017) and ~65% of EBV-transformed B-cell lines released IgG (Wroblewski et al., 2002). We found that IgG levels in the cell culture medium decreased after Cetrorelix and WAY-207024 treatment. Experiments with WAY-207024 showed that the mean decrease in IgG production was twice as large in cultures from centenarian subjects compared with young subjects (Fig. 7C), suggesting that B lymphocytes from centenarians responded more unfavorably than young subjects to deficiencies in GnRHR pathway function.

We did not observe age-related differences in the expression of either GnRH-I or GnRHR or age-related differences in the sensitivity to GnRH-I antagonist treatment. These results contrast with the results of our preliminary study, in which we observed a decrease in the expression of GnRHR mRNA in PBMCs from age-advanced individuals compared with young controls (Supplementary Fig. S1). This inconsistency may have a few explanations. First, it could be attributable to the heterogeneity of PBMCs, in which B cells account for only up to 15% of the total cell pool. Second, PBMCs were directly isolated from blood and were not cell lines. Third, we used EBV-transformed B lymphocytes. Although these cell lines are used in studies of aging and longevity as a surrogate for fresh B cells (Hussain and Mulherkar, 2012), their similarity to parental lymphocytes are used in studies of aging and longevity as a surrogate for fresh B lymphocytes. In young humans, the largest subpopulation of circulating B lymphocytes consists of naïve B cells (Simon et al., 2015). Notably, we found that transforming B lymphocytes of young individuals with EBV results in a significant switch in subpopulation sizes towards this seen in age-advanced humans in whom memory class-switched B cells predominate (Supplementary Table S2). Consequently, all tested EBV-transformed B-cell lines, regardless of the age of donors, consisted mostly of such cells. This could explain why using EBV-transformed cell lines prevented us from observing age-related GnRHR pathway inhibition-dependent differences in B lymphocyte viability, proliferation, apoptosis, and IgG synthesis. We conclude that the observed effects of Cetrorelix are attributable only to this subpopulation of B lymphocytes.

In summary, we found that the GnRHR pathway affects EBV-transformed B lymphocytes proliferation and apoptosis and IgG synthesis. While the results of our in silico analysis indicated strong involvement of the GnRHR pathway in the aging process, we did not observe age-related changes in either GnRH-I or GnRHR expression or the sensitivity to GnRHR antagonist treatment in these cells. This is most likely due to EBV transformation, making memory class-switched B cells dominant in all cultures, including those originating from young donors. Consequently, the current state of knowledge suggests that the EBV-transformed cells should not be used as a model in studies on mechanisms of aging of the immune system. However, they can be used to study the effects of aging on the function of the B lymphocyte pool.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.exger.2021.111471.

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Data availability statement

Datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

CRediT authorship contribution statement

Conceptualization, Z.W.; Methodology, D.W., Z.W. and M.P-K.; Validation, D.W., Z.W. and M.P-K.; Formal Analysis, Z.W., D.W.; Investigation, D.W.; Resources, M.P-K.; Data Curation, Z.W., M.P-K.; Writing – Original Draft Preparation, D.W.; Writing – Review & Editing, Z.W., M.P-K.; Visualization, D.W., Z.W. and M.P-K.; Supervision, M. P-K.; Project Administration, M.P-K.; Funding Acquisition, D.W.
Declaration of competing interest

The authors declare no conflict of interest.

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References

Aguilar-Rojas, A., Huerta-Reyes, M., 2009. Human gonadotropin-releasing hormone receptor-activated cellular functions and signaling pathways in extra-pituitary tissues and cancer cells (review). Oncol. Rep. 22, 981–990.

Amari, M.A., Dhar, M., Spieker, S., Bakht, N., Rahman, A.M., Moore, W.V., Jacobson, J. D., 2004. Modulation of diabetes with gonadotropin-releasing hormone antagonists in the nonobese mouse model of autoimmune diabetes. Endocrinology 145, 337–342.

Azad, N., LaPaglia, N., Kirsteins, L., Uddin, S., Steiner, J., Williams, D.W., Lawrence, A. M., Emanuele, N.V., 1997. Juxta cell proliferative activity is increased by luteinizing hormone-releasing hormone. J. Endocrinol. 153, 241–249.

Blomberg, B.B., Frasca, D., 2013. Age effects on mouse and human B cells. Immunol. Res. 57, 294–300.

Budovsky, A., Craig, T., Wang, J., Tacutu, R., Coard, A.,Lourencio, J., Fraifeld, V.E., de Magalhaes, J.P., 2013. LongevityMap: a database of human genetic variants associated with longevity. Trends Genet. 29, 559–560.

Chen, H.F., Jacobson, J.D., Nisula, B.C., Steinberg, A.D., 1994. Modulation of the expression of GnRH receptors in neonatal rats with a potent LHRH-antagonist inhibits the morphogenetic development of the thymus and maturation of the cell-mediated and humoral immune responses. Endocrinology 128, 1073–1085.

M. A., Alikaper, A.M., Smit-McBride, Z., Buzdin, A., Zhavoronkov, A., 2014. Genetics and epigenetics of aging and longevity. Cell Cycle 13, 1063–1077.

Crooke, S.N., Ovsyannikova, I.G., Poland, G.A., Kennedy, R.B., 2019. Immunosenescence signaling and interleukin-2 receptor gamma-chain messenger ribonucleic acids that are modulated by GnRH in vitro. J. Clin. Endocrinol. Metab. 84, 743–750.

Chen, E.Y., Tan, C.M., Koo, Y., Duann, Q., Wang, Z., Meirelles, G.V., Clark, N.R., McIvor, A. R., 2015. Enrichr: interactive and collaborative HTML5 gene list enrichment analysis tool. BMC Bioinf. 14, 128.

Cheung, L.W., Wong, A.S., 2008. Gonadotropin-releasing hormone signaling in extrapituitary tissues. FEBs J. 275, 5479–5495.

Consortium, O.T., Yang, J., Huang, T., Petralia, F., Long, Q., Zhang, B., Armgren, C., Zhao, Y., Mobbs, C.V., Schad, E.E., Zhu, J., Tu, Z., 2015. Synchronized age-related gene expression changes across multiple tissues in human and the link to complex diseases. Sci. Rep. 5, 15145.

Craig, T., Smelick, C., Tacanu, R., Wuttke, D., Wood, S.H., Stanley, H., Janssens, G., Savitskaya, E., Moskalev, A., Arikng, R., de Magalhaes, J.P., 2015. The digital ageing atlas: integrating the diversity of age-related changes into a unified resource. Nucleic Acids Res. 43, D878–D887.

Crooke, S.N., Osvyannikova, I.G., Poland, G.A., Kennedy, R.B., 2019. Immunosenescence and human vaccine immune responses. Immun. 16, 25.

Ersing, I., Bernhardt, K., Gewurz, B.E., 2013. NF-kappaB and IRF7 pathway activation by Epstein-Barr virus latent membrane protein 1. Viruses. 5, 1587–1606.

Feng, Y., Wang, Y., Gu, M., Zhang, H., Zhang, F., Ge, M., Xiong, Y., Zhao, Y., Li, H., Zhang, H., Hu, J., 2014. Comparison of the viral genome with the human genome in EBV-transformed B-lymphocyte cell lines (LCLs). J. Immunol. Methods 264, 19–28.

Fernick, interactive and collaborative HTML5 gene list enrichment analysis tool. BMC Bioinf. 14, 128.

Feng, Y., Wang, Y., Gu, M., Zhang, H., Zhang, F., Ge, M., Xiong, Y., Zhao, Y., Li, H., Zhang, H., Hu, J., 2014. Comparison of the viral genome with the human genome in EBV-transformed B-lymphocyte cell lines (LCLs). J. Immunol. Methods 264, 19–28.

Feng, Y., Wang, Y., Gu, M., Zhang, H., Zhang, F., Ge, M., Xiong, Y., Zhao, Y., Li, H., Zhang, H., Hu, J., 2014. Comparison of the viral genome with the human genome in EBV-transformed B-lymphocyte cell lines (LCLs). J. Immunol. Methods 264, 19–28.

Feng, Y., Wang, Y., Gu, M., Zhang, H., Zhang, F., Ge, M., Xiong, Y., Zhao, Y., Li, H., Zhang, H., Hu, J., 2014. Comparison of the viral genome with the human genome in EBV-transformed B-lymphocyte cell lines (LCLs). J. Immunol. Methods 264, 19–28.

Feng, Y., Wang, Y., Gu, M., Zhang, H., Zhang, F., Ge, M., Xiong, Y., Zhao, Y., Li, H., Zhang, H., Hu, J., 2014. Comparison of the viral genome with the human genome in EBV-transformed B-lymphocyte cell lines (LCLs). J. Immunol. Methods 264, 19–28.