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CXCL12-driven thymocyte migration is increased by thymic epithelial cells treated with prolactin *in vitro*

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The prolactin hormone (PRL), in addition to its known effects on breast development and lactation, exerts effects on the immune system, including pleiotropic effects on the thymus. The aim of this study was to evaluate the influence of PRL on the epithelial compartment of the thymus. Thymic epithelial cells (TECs) (2BH4 cells) and fresh thymocytes were used. Immunofluorescence assay revealed that PRL treatment (10 ng/mL) increases the deposition of laminin and expression of the chemokine CXCL12 in 2BH4 cells. However, no change was observed in the deposition of fibronectin. Moreover, PRL altered F-actin polymerisation, allowing the formation of focal adhesion complexes in treated cells. When 2BH4 cells were pre-treated with PRL, thymocyte adhesion was not altered. However, in the cell migration assay, pre-treatment with PRL potentiated the chemotactic effect of CXCL12 on the migration of total, double-positive, CD4-positive, and CD8-positive thymocytes. Together, the results of this study demonstrate the effect of PRL on thymic epithelial cells, particularly on CXCL12-driven thymocyte migration, confirming that this hormone is a regulator of thymic physiology.

Keywords. 2BH4 cell line; PRL; TEC; laminin; fibronectin; F-actin

1. Introduction

The thymus is a primary lymphoid organ that provides a specialised microenvironment for the development of T lymphocytes. In the thymus, bone-marrow-derived precursors undergo a complex differentiation process, which involves sequential expression of various membrane proteins and rearrangement of the genes encoding the T-cell receptor (TCR). The final product interacts with the proteins of the major histocompatibility complex (MHC), together with peptides expressed by the cells of the thymic microenvironment (Miller 1961; Ciofani and Zúñiga-Pflücker, 2007; Zdrojewicz *et al*. 2016). The thymic microenvironment consists of a three-dimensional network of stromal cells comprised of thymic epithelial cells (TECs), macrophages, dendritic cells, endothelial cells, and fibroblasts, as well as soluble components, such as cytokines, chemokines, hormones, and extracellular matrix molecules (ECM). This microenvironment provides the signals required for thymocyte migration, proliferation, survival, and apoptosis during the entire process of T lymphocyte differentiation (Savino *et al*. 2004; Petrie and Zúñiga-Pflücker 2007; Nitta and Suzuki 2016).

Among the different types of cells in the thymic microenvironment, TECs are the main component, responsible for the formation of heterogeneous tissues in terms of their morphology, phenotype, and function (Anderson and Takahama 2012). These are found throughout the thymic lobe, both in the cortex (cTECs)

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and in the medulla regions (mTECs), in which they form specific sub-regions. The cTECs and mTECs also express epithelial cell adhesion molecule (EpCAM) in the postnatal thymus, in addition to different cytokeratins. Most mTECs express cytokeratin 5, whereas cTECs express cytokeratin 8 (Alexandropoulos and Danzl 2012). TECs are involved in the development and maturation of thymocytes, secreting factors that are necessary for the dynamic development of the lympho-epithelial compartment. cTECs and mTECs have distinct roles in positive and negative selection processes in the thymus, exhibiting exclusive sets of autoantigens on their surfaces via MHC molecules. Thus, cTECs and mTECs contribute to the generation of diverse and immunocompetent T cells (Alexandropoulos and Danzl 2012).

Several studies have shown that the physiology of the thymus and the differentiation of T lymphocytes are under a complex neuroendocrine mechanism of control (Lannes-Vieira *et al*. 1991; Villa-Verde *et al*. 1995; Smaniotto *et al*. 2005). This includes both endocrine and paracrine/autocrine pathways, which act on stromal cells and thymocytes through specific receptors. Prolactin (PRL) is a lactogenic hormone synthesised and secreted by cells of the anterior pituitary gland, which function as an important mediator of the immunoneuroendocrine response (Kelley *et al*. 2007). Moreover, PRL is also produced in extra-pituitary sites, including neurons and cells of the immune system, mainly lymphocytes (Ben-Jonathan *et al*. 1996; Bole-Feysot *et al*. 1998; Montgomery 2001). Therefore, it can act both as a hormone, through the classic endocrine pathway, as well as a growth factor, neurotransmitter, or immunoregulator in an autocrine-paracrine manner (Méndez *et al*. 2005).

In the thymus, PRL and PRL receptors are expressed by stromal cells, such as TECs, and by developing thymocytes (Savino *et al*. 2016). Once PRL signalling is activated, it induces TEC proliferation, maturation of thymic dendritic cells, and survival and proliferation of early T cell precursors, and inhibits thymocyte apoptosis after exposure to glucocorticoids (Dardenne *et al*. 1989; Carreño *et al*. 2004, 2005; Krishnan *et al*. 2013). However, our understanding of the action of PRL on thymic physiology remains limited. To contribute towards the study of neuroendocrine control over the thymus, we investigated the influence of prolactin on thymic epithelial cells and its participation in the TEC-thymocyte interactions.

2. Materials and methods

2.1 *Culture of thymic epithelial cells (2BH4 TECs)*

The cell line of TECs, namely 2BH4, derived from the thymus of C57BL/6 mice, was used. These cells have a mixed phenotype, expressing cortical and medullary TEC markers (Werneck *et al*. 2000). 2BH4 cells were cultivated in 25 or 75 cm^2 culture flasks with RPMI 1640 medium, supplemented with 10% foetal bovine serum (FBS), 1% L-glutamine, and 40 mg/mL gentamicin (all from Invitrogen, Carlsbad, CA, USA) (designated as complete RPMI medium). The cells were maintained in sterile conditions in an incubator at 37°C in a humidified atmosphere containing 5% CO_2. These cells were passaged when they were in a state of semi-confluence (80%) by treating with 0.25% trypsin solution and 0.02% EDTA in a calcium- and magnesium-free solution (pH 7.2; Gibco, Waltham, MA, USA).

2.2 *Obtaining fresh thymocytes*

Fresh thymocytes were obtained from C57BL/6 mice aged 4–8 weeks (male or female) provided by Central Biotherium of UFAL. These animals were housed under controlled temperature (22 ± 2°C) with a 12 h light/dark cycle and free access to water and food. After euthanasia, the thymuses were removed, mechanically disrupted, and added to a 24-well plate containing 1 mL of 4% FBS in phosphate buffer solution (PBS) (Sigma-Aldrich, St. Louis, MO, USA) to release thymocytes (all procedures were approved by the Institutional Ethical Committee on Animal Experimentation under protocol number 14/2015 CEUA/UFAL). The supernatant was collected, and the cells were counted in a Neubauer chamber using the exclusion method with 0.02% Trypan blue solution (Sigma-Aldrich).

2.3 *Prolactin treatment*

2BH4 cells were plated for 16 h for cell adhesion and spreading in complete RPMI medium. Next, the medium was replaced, and the cells were treated with PRL 10 ng/mL (Recombinant Mouse Prolactin Protein; RandD systems, Minneapolis, MN, USA) for 24 h in RPMI medium supplemented with 2% FBS (Carreño *et al*. 2005).

2.4 *Flow cytometry*

Cell suspensions (1×10^6) of 2BH4 cells or thymocytes were evaluated for the expression of surface molecules using the following fluorochrome-conjugated antibodies: anti-pan-cytokeratin/Alexa Fluor 488, anti-CD49e/PE, anti-CD49f/FITC (all from eBioscience, San Diego, CA, USA), anti-CD304/APC (Invitrogen), anti-CD184/PE, anti-MHC I/FITC, anti-MHC II/PE, anti-CD4/APC, and anti-CD8/PerCP (all from BD Biosciences, San Diego, CA, USA). The control isotypes IgG1, IgG2a, and IgG2b (conjugated to their respective fluorochromes) were used as negative controls. After 20 min incubation for cell staining (at 4°C in the dark), the cells were washed with PBS containing 4% FBS, centrifuged, and fixed with 2% formaldehyde (VETEC, Duque de Caxias, RJ, Brazil). Analysis was performed using a flow cytometer (FACSCanto II, BD Biosciences), and the data were analysed using WinMDI software version 2.8.

2.5 *Scanning electron microscopy (SEM)*

In a 24-well plate, circular slides were plated and coated with 0.1% bovine serum albumin (BSA; Sigma-Aldrich) for 60 min. After removing this solution, 2BH4 cells (2×10^4) were plated with 500 µL of complete RPMI. After 16 h of incubation, the cultures were washed with PBS and fixed with 0.5% glutaraldehyde (VETEC) in PBS (Lins *et al*. 2020). Images were collected using a large-field secondary electron detector in low-vacuum mode. Prior to analysis, the samples were sputtered with gold for 10 s. Image acquisition was performed at an accelerating voltage of 15 kV, according to the method described by Souza *et al.* (2014). SEM was carried out using a Superscan SSX-550 electron microscope (Shimadzu, Japan).

2.6 *Immunofluorescence assay*

2BH4 cells (2×10^4 cells) were plated in an 8-well Lab-Tek Chamber Slide™ System (Nunc, NY, USA) for 16 h, treated with PRL for 24 h, washed in PBS, fixed with methanol (Synth, Diadema, SP, Brazil) for 10 min, and subjected to an indirect immunofluorescence assay. TECs were pre-incubated for 30 min with PBS containing 1% BSA, followed by the addition of primary antibodies such as anti-fibronectin, anti-laminin (both from Sigma-Aldrich), and anti-CXCL12 (eBioscience) for 1 h in a humid chamber at room temperature (25°C). Next, the cells were washed three times with PBS and incubated for 45 min with FITC-conjugated goat anti-rabbit secondary antibody (Sigma-Aldrich). Subsequently, the cells were washed again in PBS, and the slides were mounted in glycerol/PBS (1:3). As a negative control, the cells were not incubated with the primary antibody, but only with the secondary antibody, so as not to produce any signal. After covering with a glass coverslip, the slides were examined using fluorescence microscopy (Nikon Eclipse 50i; Nikon Instruments Inc., Chicago, IL, USA). The fluorescence intensity was determined in pixels and quantified using Image J version 1.44 (NIH, MD, USA).

2.7 *Direct F-actin cytoskeleton staining*

2BH4 cells (2×10^4), treated with PRL as described above, were washed in PBS, fixed in paraformaldehyde (4%) in PBS, and permeabilised with 0.5% Triton X-100 for 5 min. The cells were stained for F-actin by direct staining with FITC-conjugated phalloidin (Sigma-Aldrich) for 1 h. Subsequently, the cells were washed in PBS, and mounted in glycerol and PBS (1:3). Modifications in the cytoskeleton were evaluated using fluorescence microscopy, and photomicrographs were obtained using a Nikon camera model DS-Ri1 (Nikon Eclipse 50i; Nikon). The fluorescence intensity and cellular area were determined in pixels and quantified using ImageJ version 1.44.

2.8 *Cell adhesion assay*

The effect of PRL on adhesive capacity of thymocytes in TECs was assessed using a cell adhesion assay. Briefly, 2×10^4 2BH4 cells were grown in 25 cm^2 flasks in the presence of complete RPMI medium for 16 h in a CO_2 atmosphere at 37°C for cell adhesion and spreading. Subsequently, the cells were treated or not (control) with PRL 10 ng/mL for 24 h. After treatment, the medium was replaced and 2BH4 cells were co-cultured for 1 h with fresh thymocytes (in a proportion of 50 thymocytes/1 TEC) to allow for heterocellular adhesion interaction. Next, the non-adherent thymocytes were gently removed by washing with PBS at room temperature. The thymocytes that adhered to the TECs were collected by washing with cold PBS and counted. To determine the phenotype of the adhered thymocytes, they were stained with anti-CD4/anti-CD8 antibodies for analysis by flow cytometry.

2.9 *Thymocyte migration towards 2BH4 TECs*

Thymocyte migration assays were performed using a transwell system (Corning Costar, Cambridge, MA, USA), with inserts of 6.5 mm diameter, in polycarbonate membranes, and with 5 μm pores. Initially, 2BH4 cells were plated at 10^4 cells/well, in a 24-well plate in the presence or absence of PRL (10 ng/mL) in complete RPMI medium (500 μL) and maintained at 37°C in a 5% CO_2 atmosphere for 24 h. The inserts were then incubated for 45 min with PBS-0.1% BSA solution, placed into wells of 24-well plates, and freshly obtained thymocytes (2×10^6) were added to the upper chamber. Notably, the TEC medium was not replaced before the co-culture. In some wells, CXCL12 (200 ng/mL, RandD Systems) was added to the lower chamber, on the 2BH4 cultures. After 3 h, the migrating cells, deposited in the lower chamber, were collected and counted. To determine the thymocyte subsets, these cells were subjected to cytofluorimetric analysis.

2.10 *Statistical analysis*

Data are presented as the mean ± standard error of the mean (SEM). Student's *t*-test or one-way ANOVA, followed by Tukey's test, were used for statistical analysis. Values of $p \leq 0.05$ were considered statistically significant. Statistical analysis was performed and graphs were generated using GraphPad Prism 5.0 (GraphPad Software Inc., La Jolla, CA, USA).

3. Results

3.1 *Characterisation of TEC line*

The cell line used in this study was isolated by Amarante-Mendes *et al.* (1995) and characterised as TECs. To confirm this, the characteristics of these cells were evaluated using scanning electron microscopy. As a result, the 2BH4 cells were confirmed to have a typical epithelial appearance, and a polyhedral and irregular morphology, with cytoplasmic extensions, membrane undulations, and centralised nuclei (figure 1A). In addition to this initial characterisation, we evaluated the expression of some molecules with critical epithelial functions. As demonstrated in figure 1B, 97.99% of the cells were positive for cytokeratin (CK), highlighting their epithelial phenotype. Moreover, these cells expressed the receptors VLA-5 (99.95%), VLA-6 (80%), neuropilin-1 (NRP-1) (55.37%), MHC-I (54.29%), and MHC-II (7.99%). Thus, our cellular model was well characterised as TEC.

3.2 *CXCL12 production was enhanced after PRL treatment*

Among the thymic soluble proteins, the chemokine CXCL12 is the most extensively studied protein for its effects on thymocyte migration, and TECs are the main producers of this factor (Lucas *et al*. 2017). Therefore, we investigated whether PRL (10 ng/mL) could modulate CXCL12 production by 2BH4 cells. An immunofluorescence assay showed a significant increase (CTR: 5285 ± 377 vs. PRL: 11910 ± 734, in MFI units, $p<0.001$) in chemokine production by 2BH4 TECs when the cells were treated with PRL (figure 2). This increase was observed through qualitative (2A) and quantitative (2B) analyses of the cells.

The thymic interrelationship between chemokines and ECM molecules has already been established regarding the potentiation of thymocyte migration, since both act synergistically (Savino *et al*. 2015). Thus, after we attested the augmentation of CXCL12 by PRL treatment, we investigated the role of this hormone in the modulation of thymic ECM.

3.3 *Prolactin modulates ECM deposition by 2BH4 TECs*

ECM directly influences the normal functioning of TECs. These molecules facilitate the diffusion of secretory products and enable cell anchorage of thymocytes for further differentiation (Hun *et al*. 2017). To evaluate the influence of PRL on ECM deposition in 2BH4 TECs, an immunofluorescence assay was performed with specific markers for fibronectin (FN) and laminin (LM).

Qualitative (figure 3A) and quantitative (figure 3B) analyses showed that PRL treatment did not alter FN deposition. Regarding LM production, qualitative analysis showed an increase of this glycoprotein by 2BH4 TECs (figure 3C), and quantification of fluorescence intensity confirmed that the treatment enhanced (CTR: 18.97 ± 1.23 vs. PRL: 23.54 ± 1.43, in MFI units, $p<0.05$) the LM deposition when compared to the control group (figure 3D).

The ECM is connected via integrins to the actin cytoskeleton, thereby exerting important functions in

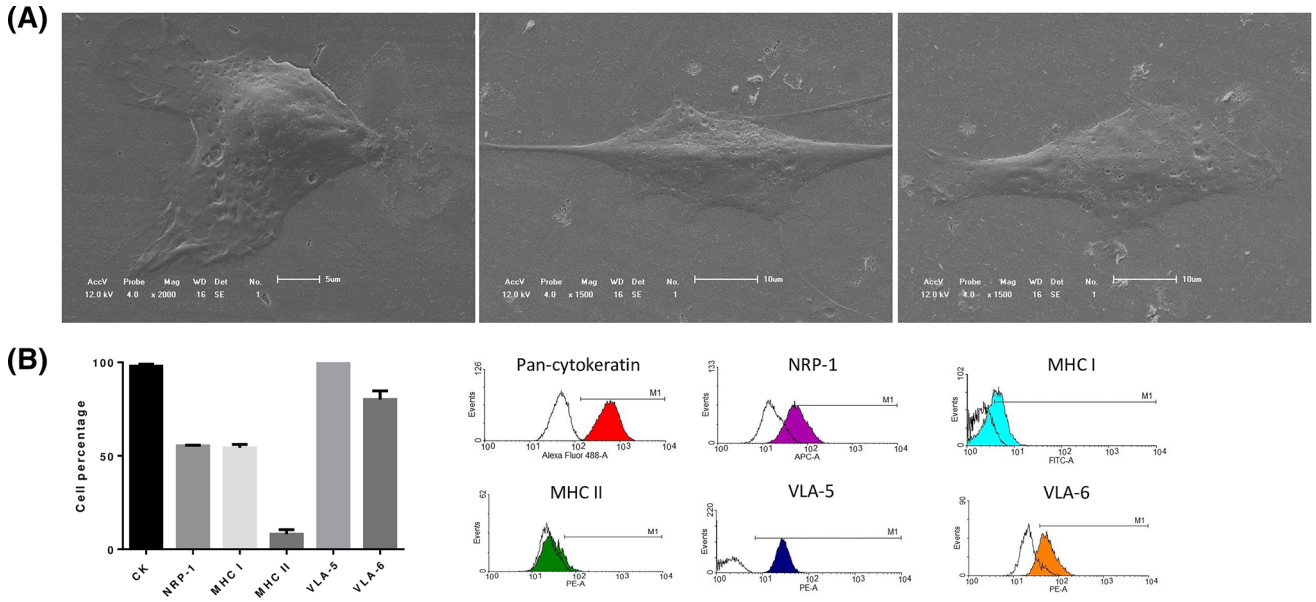

Figure 1. Characterisation of the 2BH4 cells. (**A**) SEM images showing morphological phenotype of 2BH4 TECs plated in 2D arrangement. The cells exhibit typical epithelial appearance, with irregular and polyhedral morphology, discrete membrane extensions, and undulations (magnification: 2000×). (**B**) Functional characterisation by flow cytometry demonstrating the expression of pan-cytokeratin (CK), neuropilin-1 (NRP-1), MHC I and -II molecules, VLA-5 and -6 integrins in 2BH4 cells. The bars graphic (left) indicates the percentage of positive cells for these cell markers (n = 6; two-independent experiments). Representative histograms (right) of the fluorescence intensity show the specific staining (colour curve) and Ig isotype control (white curve).

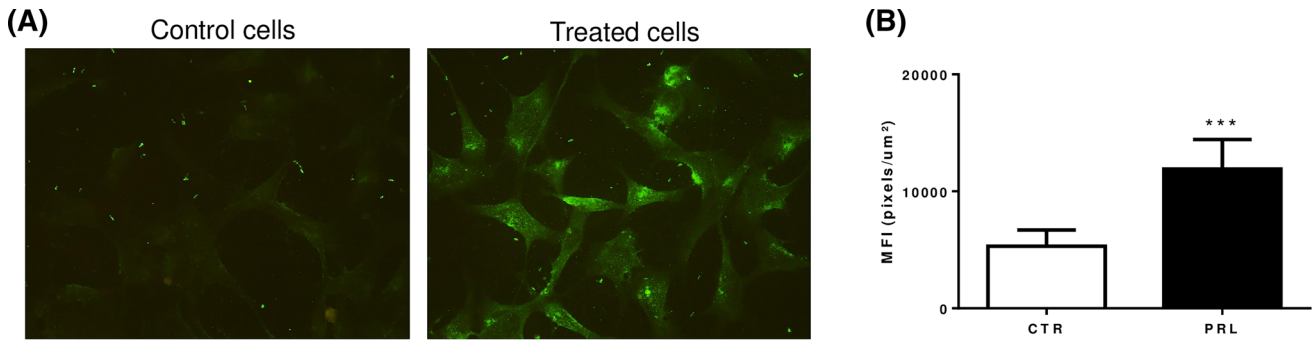

Figure 2. PRL-treated 2BH4 TECs modulate CXCL12 production positively. (**A**) Photomicrographs demonstrating the expression of CXCL12, stained in green, by 2BH4 cells after 24 h of treatment with PRL. As observed, PRL improved CXCL12 production by these cells. Images show higher staining in green, indicating more CXCL12 in TECs. Magnification: 400×. (**B**) Bar graphs showing the quantification of CXCL12 deposition, using ImageJ, in mean fluorescence intensity (MFI). Each column represents the mean ± SEM obtained from two independent experiments, n = 12 in each group, ***p<0.001.

cell behaviour and physiology (Gkretsi and Stylianopoulos 2018). Since we observed changes in the ECM evoked by PRL, it was in our interest to evaluate the F-actin content in treated cells.

3.4 *F-actin cytoskeleton is a target of the prolactin effects*

The actin cytoskeleton provides mechanical support for organisation of the cellular structure, and its reorganisation generates dynamic changes in cell shape and in cell-cell interactions (Phee *et al.* 2014). Direct labelling of F-actin by phalloidin was used to analyse the morphology and disposition of the actin filaments in the cytoplasm of 2BH4 cells.

In the control group, the cells presented a rounded morphology, scarce cytoplasmic volume, and evident filopodia. In the PRL-treated group, the cells exhibited a longer elongated F-actin cytoskeleton, spindle-shaped morphology, more condensed cytoplasm, and evident focal adhesion complexes (figure 4). Furthermore,

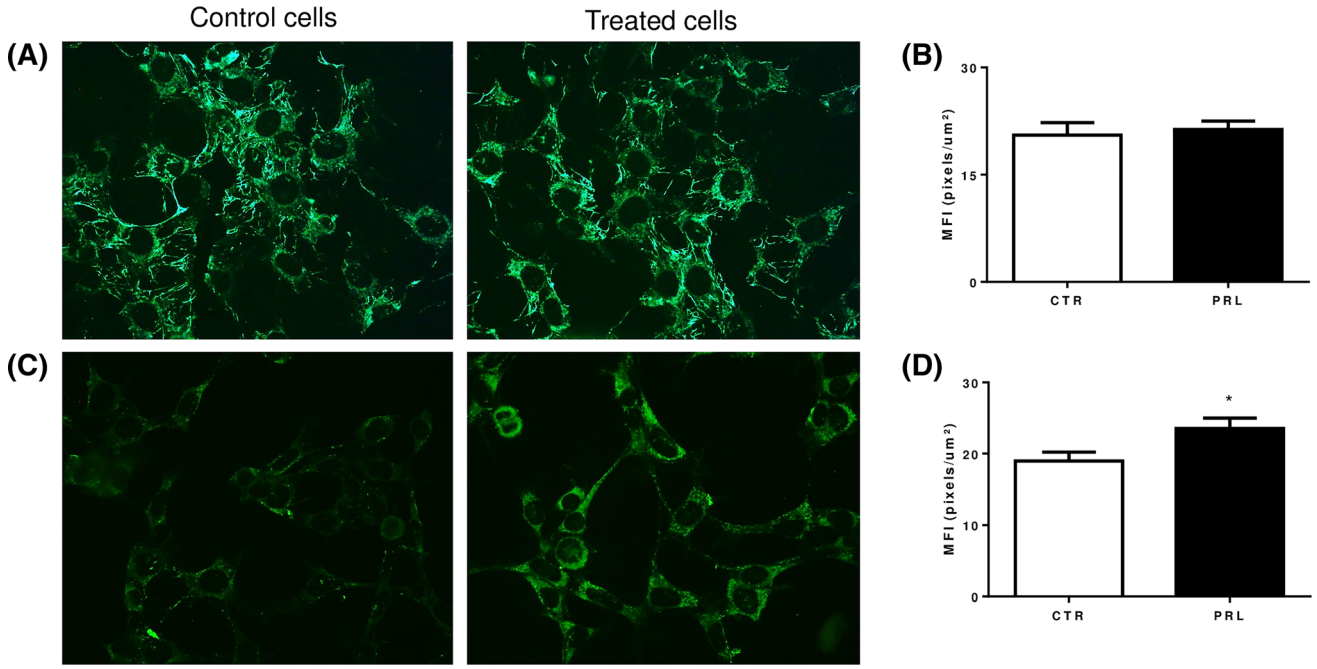

Figure 3. ECM deposition by 2BH4 cells after PRL treatment. TECs were responsive to PRL and modulated the production of LM, but not of FN. Photomicrographs showing the deposition of FN (**A**) and LM (**C**), stained in green. Only LM had a gain in its production by 2BH4 TECs after treatment with PRL. FN production was unchanged. Magnification: 400×. Bars demonstrating the quantitative analysis of FN (**B**) and LM (**D**) deposition, using ImageJ, in mean fluorescence intensity (MFI). Each column represents the mean ± SEM obtained from two independent experiments, $n = 12$ in each group, *$p<0.05$.

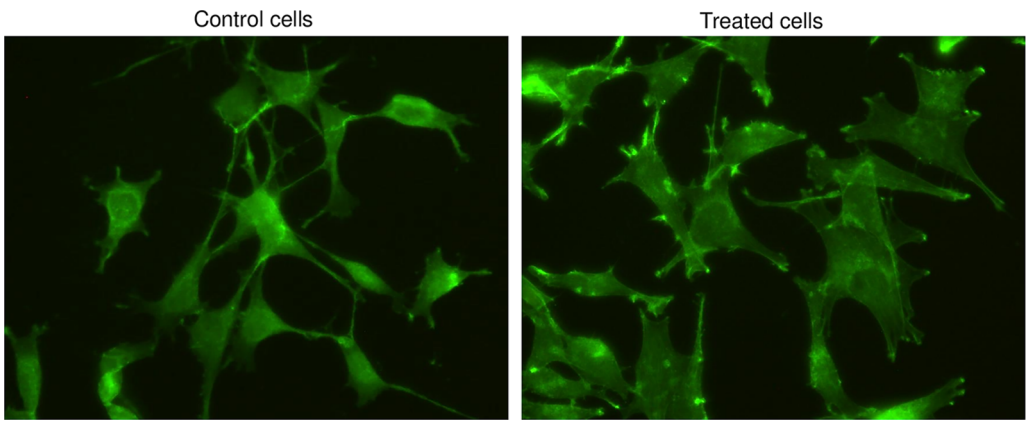

Figure 4. 2BH4 F-actin cytoskeleton is reorganised after PRL treatment. Representative photomicrographs of the control cells and cells treated with PRL. Using phalloidin, it was possible to stain the cytoskeleton (green) and observe the changes in lamellipodia, filopodia and focal adhesions. Furthermore, cellular area was increased by PRL treatment compared with control cells (46% of augmentation). Magnification: 400×.

cellular area was evaluated by ImageJ quantification, and treated cells were more enlarged (4811 ± 426 μm^2 **) than control cells (3293 ± 210 μm^2).

Thus far, the evidence indicated that PRL has significant effects on TEC biology, supporting the argument that this hormone may influence the interaction of TECs with thymocytes. Therefore, functional cell assays were employed in subsequent experiments.

3.5 *TEC-thymocyte adhesion after PRL treatment*

ECM ligands and receptors (as well as F-actin content) have been shown to play a role in TEC-thymocyte interactions, which occur through classical adhesion molecules (Gameiro *et al*. 2010). Thus, whether PRL is able to modulate the degree of thymocyte adhesion to TECs in *in vitro* co-cultures was investigated.

Pre-treatment of 2BH4 TECs with PRL did not affect the number of total thymocytes that adhered to epithelial cells compared with the control group, nor did it change the number of cells in the different subsets of thymocytes (figure 5).

Although the thymocyte adhesion levels were not altered by treatment with prolactin, the possibility that this hormone acts on the migratory capacity of these cells was not ruled out. Therefore, the last set of experiments were used to evaluate this parameter.

3.6 *CXCL12-driven migration of thymocytes is influenced by PRL-treated 2BH4 cells*

Given the importance of thymocyte migration in thymic function, and driven by the lack of studies on the effects of PRL in this process, we evaluated thymocyte migration towards conditioned medium of 2BH4 cells treated with PRL. As shown in figure 6A, PRL pre-treatment did not increase the total number of migrating thymocytes compared to those in the control group. However, as expected, the presence of the CXCL12 chemokine in the conditioned medium increased the total number of migrating cells. Interestingly, a significant augmentation in the migration of total thymocytes was observed in the conditioned medium of PRL-treated 2BH4 TECs in combination with CXCL12. Importantly, the treatment of thymocytes with PRL for up to 3 hours did not alter the expression levels of the CXCL12 receptor, CXCR4, *in vitro*.

Regarding the migrating subsets of thymocytes (figure 6B), an increase in the number of migrating cells (double positive and $CD8^+$ thymocytes) in the groups exposed to CXCL12 alone or in combination with the PRL-conditioned medium was observed compared to the control and PRL group. Moreover, in the $CD4^+$ subset, a significant increase in the cell migration of cells in the PRL + CXCL12 group was observed than in the control group.

4. Discussion

In this study, the participation of PRL in the stromal compartment of the thymus was evaluated. Specifically, we aimed to understand the role of this hormone in the physiology of TECs. Our results showed that PRL affects 2BH4 TECs, as demonstrated by the modulation of the expression of extracellular matrix ligands and CXCL12, reorganisation of the cytoskeleton, and the increase in the number of migrating thymocytes.

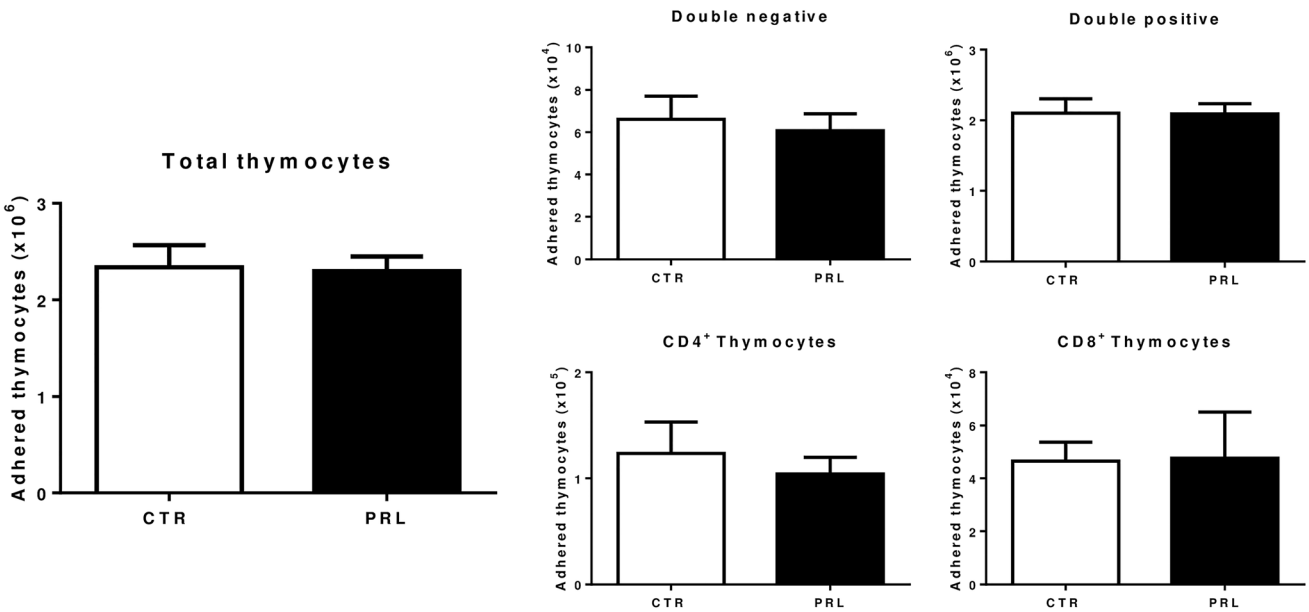

Figure 5. PRL maintains thymocyte adhesion to 2BH4 cells. TECs were pre-treated with PRL (10 ng/mL) for 24 h. After replacement of medium, thymocytes were submitted to adhesion for 1 h on 2BH4 TEC monolayer. Adherent thymocytes were collected, counted, and marked for cytometry. Absolute number of the counting demonstrated no interference of PRL on thymocyte adhesion to TECs. Bars represent the mean ± SEM, $n = 5$. Statistical analysis was performed using Student's *t*-test.

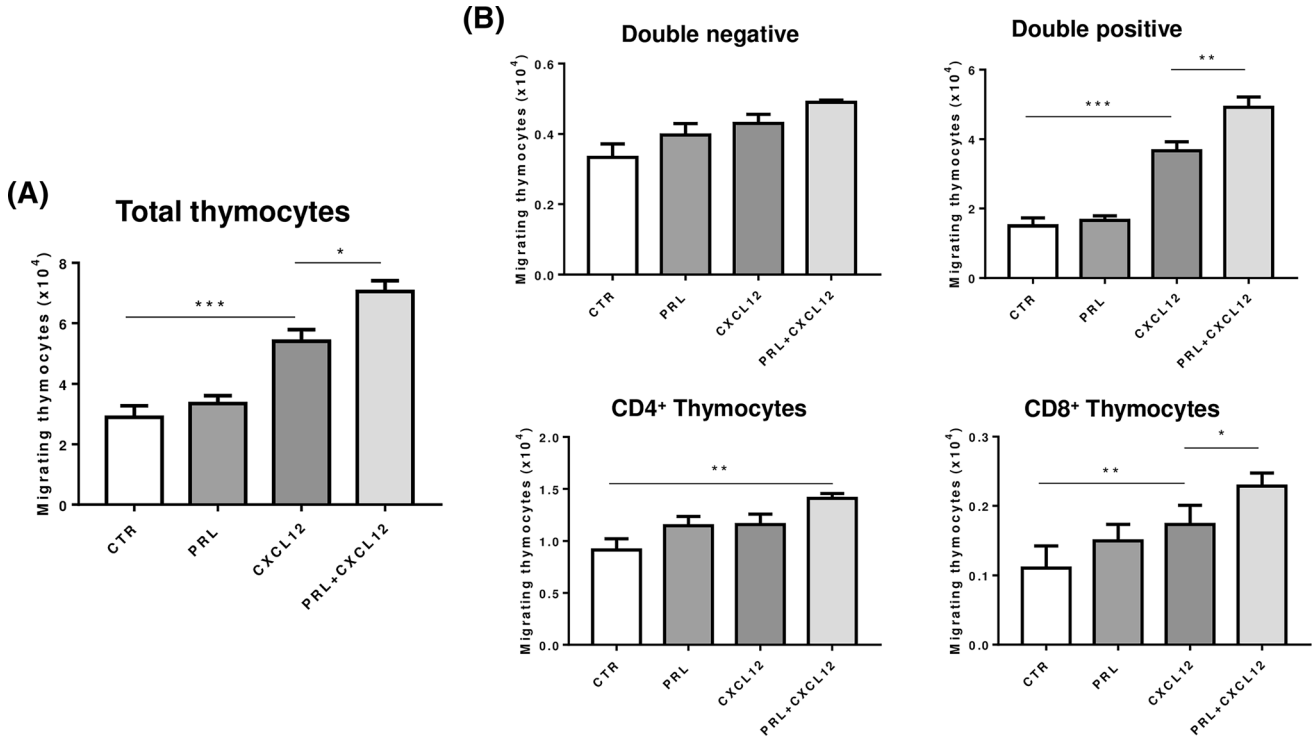

Figure 6. PRL effect on thymocyte migration towards TECs *in vitro*. 2BH4 cells were pre-treated with PRL (10 ng/mL) and thymocyte migration (in the same wells with TECs) was carried out for 3 h. Then, migrating thymocytes were recovered by washing and counted to evaluate PRL influence in this phenomenon. (**A**) Total number of migrating thymocytes. (**B**) Absolute number of thymocyte subsets defined by CD4/CD8 analysed by flow cytometry. Bars represent mean ± SEM. Statistical analysis was performed using the ANOVA test followed by Tukey's test, $n = 6$, * $p<0.05$, ** $p<0.01$ and *** $p<0.001$.

TECs are the main component of the thymic microenvironment, where they produce soluble factors and express ligands and receptors that direct migration and subsequent lymphocyte maturation (Wang *et al.* 2020). In this study, we used a TEC cell line obtained from C57BL/6 mice, namely 2BH4 cells. First, this cell line was characterised through morphological analysis by SEM; subsequently the expression of cytokeratins and surface receptors was evaluated by flow cytometry.

2BH4 cells were found to have morphological characteristics compatible with epithelial cells, in agreement with the analysis carried out by Amarante-Mendes *et al.* (1995), who described 2BH4 to have several shapes – from polygonal to fusiform, with small round nuclei with prominent nucleoli. In addition, TECs can be organised as 'cobblestone' or diffuse cells connected by long cytoplasmic extensions. To confirm the epithelial phenotype of these cells, we evaluated the expression of cytokeratin, which is a constituent molecule of the cytoskeleton of epithelial cells, belonging to the intermediate filaments (Almeida Jr 2004). In our study, a majority of these cells in culture (97.9%) exhibited this marker.

Another characteristic found in 2BH4 cells was the expression of MHC I, MHC II, VLA-5, VLA-6, and NRP-1 surface receptors. 2BH4 expressed both MHC molecules; however, MHC II was expressed at low levels. Studies have shown that MHC I is expressed in large quantities in murine and human TECs, while MHC II displays different levels of expression (Yang *et al.* 2006; Saldaña *et al.* 2016; Benhammadi *et al.* 2020). NRP-1 is a transmembrane glycoprotein that responds to the semaphorin-3A and VEGF family members. This receptor plays a role in embryonic development, angiogenesis, and cell migration. In addition, NRP-1 expression has been detected in thymic stromal cells and TECs, and has been demonstrated to have an effect on thymic physiology (Lins *et al.* 2020).

The VLA-5 and VLA-6 integrins are heterodimers composed of two chains, α and β, which mediate interactions between the extracellular environment and actin cytoskeleton. VLA-5 integrin is a receptor for fibronectin and is composed of an α5 chain associated with a β1 subunit (Linhares-Lacerda *et al.* 2010). The VLA-6 integrin is formed by an α6 chain, which also

binds to a β1 subunit to form the laminin receptor (Golbert *et al*. 2013). Our results showed that more than 80% of 2BH4 cells in culture express these integrins, which are important for migration, adhesion, and differentiation of thymocytes on the substrate of extracellular matrix.

A clear comparison between 2BH4 TECs and primary TECs should be described here. Primary TEC cultures can be readily obtained from mouse or human thymus, applying enzymatic dissociation associated with FACS- or bead-sorted methods to purify TECs. These cells kept the phenotypic, physiologic and functional features, however they had decreased cell viability due prolonged time of culture (Villegas *et al*. 2018). In our study, we extended the detailed characterisation written by Amarante-Mendes *et al.* (1995) and reinforced the cellular model for TEC of 2BH4 cells.

The chemokine CXCL12 has pleiotropic effects, acting in the migration of T cell precursors for specific microenvironments, wherein there are basic factors present for their development. This chemokine is selectively expressed in most thymic stromal cells (Lucas *et al.* 2017). Our results demonstrated the modulation of CXCL12 production by PRL treatment in 2BH4 TECs *in vitro*. Consistent with these results, other hormones are also able to modulate the expression of chemokines. For example, treatment with growth hormone (GH) increases the expression of CXCL12 in thymic nurse cells and in the thymic microenvironment (Smaniotto *et al.* 2005).

PRL treatment promoted an increase in the production of the LM glycoprotein. The deposition of LM in the thymus is under hormonal regulation, such as GH, which increases the deposition of LM in TECs *in vitro*, or under the regulation of triiodothyronine (T3), which enhances intrathymic expression of LM. Also, Sema-3A increases LM production in thymic stromal cells (Lins *et al.* 2020). Similarly, mice that were administered intraperitoneal glucocorticoids (GCs) exhibited a greater amount of LM within the thymic lobes. Interestingly, some pathological conditions such as type 1 diabetes and infections by *Trypanosoma cruzi* and *Plasmodium berguei* also exacerbate the distribution and density of LM in the thymus (Savino *et al*. 2015).

Next, the actin cytoskeleton, the main support of cells and the organisation of which is essential for cell movement, was evaluated. Its interaction with integrins and focal adhesion complexes facilitates cell migration. After PRL treatment, 2BH4 cells were found to form structures including focal adhesion complexes, lamellipodia, and filopodia, which are key to the migration process. These changes in the cytoskeleton, caused by PRL, agree with those in a study by Da Silva *et al.* (2015), in which PRL changed the distribution of F-actin filaments in mammary cells. This type of remodelling promoted a greater migration of cells.

Lymphocyte migration depends on sequential cell adhesion/de-adhesion events, in which ECM proteins are involved. We showed that PRL did not interfere with thymocyte adhesion to 2BH4 cells. Previous studies have shown that the treatment of TECs (IT-76M1 cell line) from BALB/c mice with PRL (10^{-8} M, for 24 h) promoted an improvement in thymocyte adhesion, due to the increased amounts of ECM components and their respective integrins (De Mello-Coelho *et al*. 1997). However, further studies are required to better understand the role of PRL in TEC-thymocyte adhesion. This invariant adhesion may be due to the short contact time (1 h) between cells, or the greater amount of LM was not high enough to retain more thymocytes on the surface of the 2BH4 cells.

The final experiment analysed thymocyte migration towards 2BH4 TECs pre-treated with PRL. Our results showed that PRL acted on TECs to increase the total number of CXCL12-driven migrating thymocytes. Smaniotto *et al.* (2005) showed that thymocytes derived from GH-transgenic mice migrated more efficiently through a transwell chamber in the presence of CXCL12. In addition, the transendothelial migration of thymocytes pre-treated with GH was increased in response to CXCL12 (Smaniotto *et al.* 2011). These results contribute to the advancement of knowledge related to thymic physiology, demonstrating, for the first time, the effect of PRL on CXCL12-driven migration, thus opening a new field of scientific investigation.

Taken together, the data presented in this study provide an insight into the effects of PRL on the thymic microenvironment, particularly on TEC interaction with soluble factors, as the chemokine CXCL12 was found to be important for the migration of developing thymocytes during differentiation.

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