Human Chorionic Gonadotropin in the Regulation of T-Helpers Type 17

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Abstract. Chorionic gonadotropin (hCG) is a key pregnancy hormone that regulates steroidogenesis and has immunomodulatory activity. We studied the effects of native and recombinant hCG on the differentiation, proliferation, and production of IL-17 and IFN-ɣ by T-helper cells induced into the phenotype of T-helper type 17 (Th17) *in vitro*. We found that hCG had no significant effects on the level of Th17 cells, as assessed by RORɣτ expression, and the proliferation of these cells (Ki-67^{+}). In addition, no effects of hCG on the production of IL-17 and IFN-ɣ by T-helpers induced in the Th17 phenotype were found. At the same time, recombinant hCG (100 IU/mL) increased the number of non-Th17 T-helpers (RORγt^{-}Ki-67^{+}). Thus, hCG did not modulate Th17 cells in our experimental model.

1 Introduction

Pregnancy from the point of view of immunology is the state of maternal-fetal immune tolerance. There are many factors, thanks to the immunomodulatory properties of which, the normal course of pregnancy is maintained. One of these factors is chorionic gonadotropin (hCG), a glycoprotein associated with pregnancy, which is synthesized by the cells of the developing trophoblast and syncytiotrophoblast after embryo implantation [1]. Since hCG is produced in normal, hyperplastic and malignant cells, it is a potential marker of cellular changes and, as such, is of particular interest as a model for studying the processes of the normal invasive state (pregnancy) in comparison with the processes of tumorigenesis [2].

Due to the presence of unique properties, hCG has a high therapeutic potential. As a pharmacological drug, this hormone is used in the treatment of various diseases associated with the reproductive system in both women and men. HCG is widely used as an analogue of luteinizing hormone (LH) to stimulate ovulation in preparation courses for *in vitro* fertilization (IVF) [3].

It is known that pharmacological preparations of hCG are native and recombinant. Native hCG preparations are obtained from the urine of pregnant women, and are available in lyophilized form at 5,000 or 10,000 IU. Since 2001, recombinant hCG has been

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produced and is available in syringe pens containing 250 μg of pure hCG, equivalent to approximately 6,750 IU of hCG in urine [3].

In terms of the immune system, normal physiological pregnancy is associated with a decrease in the number of pro-inflammatory T-helpers type 17 (Th17) compared with non-pregnant women [4]. At the same time, an increase in the level of Th17 cells is associated with pathological processes, such as preeclampsia or spontaneous abortion [4]. Withal, the role of hCG as a key pregnancy hormone in the regulation of Th17 functions and differentiation has not been studied enough. Despite the fact that we previously studied the effects of the native hCG preparation and established its suppressive effects on this subpopulation [5], we decided to continue this work at a new level. Thus, we considered it necessary to also study the effect of recombinant hCG, which is necessary to understand its action as a drug. In addition, it is not known how hCG regulates Th17 cell proliferation.

The aim of the study was to evaluate the effect of hCG (native and recombinant) on the conversion of naive T-helpers to the Th17 phenotype, taking into account the proliferative status of cells and their production of the main marker cytokines (IL-17 and IFN-ɣ). As part of the study, the following tasks were formulated:

1. To study the role of hCG (native and recombinant) in the regulation of the proliferation of isolated T-helper cells polarized into the Th17 phenotype;
2. Assess the role of hCG (native and recombinant) in the regulation of Th17 differentiation based on the expression level of ROR-γt (the main master regulator gene of Th17) and the proliferative status of the cell (Ki-67 proliferation marker).
3. To evaluate the effect of native and recombinant hCG on the production of the main pro-inflammatory cytokines IL-17A and IFN-γ by T-helper 17 cells.
4. Compare the effect of native and recombinant hCG preparations on the proliferation and differentiation of Th17.

2 Materials and methods

The study was carried out in accordance with the Protocol of the Council of Europe Convention on Human Rights and Biomedicine 1999 and the Declaration of Helsinki of the WMA 2000. The Ethics Committee of the «IEGM of Ural Branch of the Russian Academy of Sciences» (IRB00010009) approved the *in vitro* experimental scheme (Protocol dated 06/12/2016).

Research objects. The study used heparinized blood of healthy women of reproductive age in the first phase of the menstrual cycle (n=8). The objects of the study were cultures of $CD4^+$ cells isolated from peripheral blood mononuclear cells (PBMCs) of donors. Physiological concentrations (100 and 10 IU/mL) of native (Moscow Endocrine Plant, Russia) and recombinant (Merc Serono, Italy) hCG preparations were used in the work. The concentrations of hCG used corresponded to the level of the hormone in different trimesters of gestation [5].

$CD4^+$ T cells were isolated from the PBMC suspension using the immunomagnetic separation with MACS® technology (Miltenyi Biotec, Germany). After the immunomagnetic separation procedure, we assessed the number of $CD4^+$ T cells and their viability using Trypan blue 0.4% (InVitrogen, USA). It was found that in the obtained cell cultures, the level of $CD3^+CD4^+$ T cells was at least 97.5 ± 1.5%, and the viability was at least 95-98% of the total number of cells.

Culturing $CD4^+$ T-cells, Th17-polarization. Isolated T-helper cells were cultured at a concentration of 10^6 cells/mL in a complete medium (CM) consisting of RPMI-1640 with L-glutamine (Sigma-Aldrich, United States), 10% FBS (Sigma-Aldrich), 10 mM Hepes (Amresco, United States) and 30 μg/mL gentamicin (KRKA, Slovenia) for 60 h at 37°C in

a humid atmosphere containing 5% CO_2. Culturing was carried out in 48-well plates (Nunc, USA).

Recombinant pro-inflammatory cytokines IL-1β and IL-6 were used (final concentration 10 ng/mL, Miltenyi Biotec, Germany) to polarize the $CD4^+$ cells into the Th17 phenotype. The presence of antigen-presenting cells was simulated using the T-Cell Activation/Expansion Kit, human (TCR-activator) (Miltenyi Biotec, Germany). These MACSiBead™ particles are loaded with antibodies against human CD2, CD3, CD28 and are T cell activators and were introduced into cultures at the rate of 1 particle per 2 cells.

After the 60 h-culturing the amount of Th17 was estimated as the percentage of $CD4^+$ lymphocytes (CD4-FITC, Miltenyi Biotec, Germany) expressing the transcription factor ROR-γt (Anti ROR-γt-PE, Miltenyi Biotec, Germany). The expression of ROR-γt was assessed simultaneously with the proliferation marker Ki-67 (anti-Ki-67 PerCP-Vio700, Miltenyi Biotec, Germany) after cell permeabilization according to the instructions for buffers (BioLegend, USA). Measurements were performed on a CytoFLEX S flow cytometer (Beckman Coulter, USA).

In control culture a CM was added instead of hCG. Activation control (cells and CM without TCR activator and cytokines) was used to confirm the work of the experimental scheme.

Assessment of IL-17A and IFN-γ cytokine levels. In culture supernatants, the amount of the main pro-inflammatory cytokines characteristic of the Th17 subpopulation was estimated using enzyme immunoassay: interleukin-17 A (IL-17A) (Human IL-17A ELISA Kit, Thermo Fisher Scientific, USA) and interferon gamma (IFN-γ) (gamma-Interferon-IFA-BEST, Vector BEST, Russia). The results were recorded on a Multiskan Sky Microplate Spectrophotometer (Thermo Fisher Scientific, USA) multichannel spectrophotometer.

Data are analyzed using the Friedman test (the GraphPad Prizm 6 software), and are presented as median, lower and upper quartiles (Me (Q1–Q3)), differences were considered significant at $P<0.05$.

3 Results and discussion

The effect of human hCG on the differentiation of Th17. When studying the effect of hCG, it was found that the hormone at concentrations corresponding to pregnancy did not affect the number of $CD4^+$ ROR-γt^+ cells (Fig. 1). Given the fact that Th17 differentiation was accompanied by proliferative processes, we assessed the level of Ki-67 expression in cells. It was found that hCG did not affect the expression of this protein. Importantly, neither recombinant nor native hCG showed significant effects on Th17 differentiation.

We then performed a more detailed analysis of the presence of hCG in cell cultures, analyzing the effects of hCG on ROR-γt expression depending on the cell proliferative status. To do this, we assessed the percentage of ROR-γt-positive (ROR-γt^+) T-helpers in gate proliferating (Ki-67^+) and non-proliferating (Ki67^-) cells. It was found that hCG does not affect the number of proliferating and non-proliferating Th17 cells (Fig. 2). However, hCG (rec. 100 IU/mL) has been shown to increase levels of non-Th17 $CD4^+$ lymphocytes (ROR-γt^-Ki-67^+) (Figure 3).

Thus, there is an increase in the percentage of non-pro-inflammatory subpopulation of $CD4^+$ cells. This effect can be explained by the plasticity of Th17, which are able to convert into regulatory T-lymphocytes (Treg) under the influence of an inflammatory environment [6].

These results allow us to aim in further studies to identify the pool of $CD4^+$ cells whose proliferation level increases under the influence of hCG.

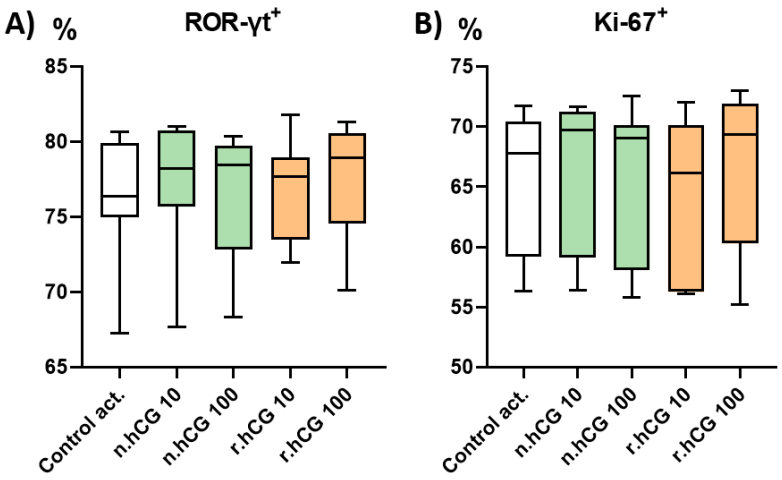

Fig. 1. Effect of hCG on the number of CD4+ lymphocytes expressing ROR-γt and Ki-67 (n=8, Me (Q1-Q3)). Note. (hereinafter): the concentration of hCG in IU/mL; K act. – control with T-activator; n.hCG - native preparation of hCG; r.hCG - recombinant hCG preparation

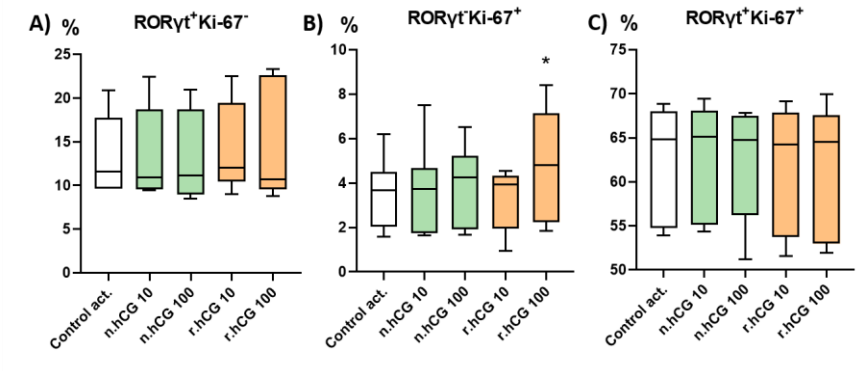

Fig. 2. Effect of hCG on the number of CD4+ lymphocytes expressing ROR-γt and Ki-67 (n=8, Me (Q1-Q3)). Note: * – statistically significant differences from the control with the activator according to the Friedman test at $p<0.05$

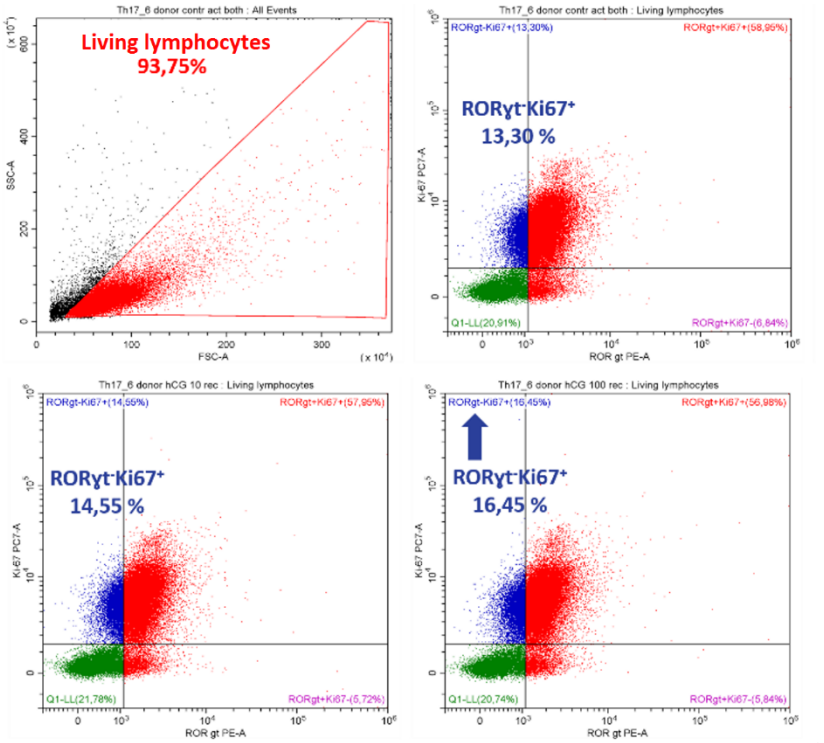

Fig. 3. The effect of rechCG on the number of non-Th17 helper T cells (RORγt-Ki67$^+$) in a culture of Th17-polarized CD4$^+$ cells in one experiment. Note: Representative dot plots of one experiment are shown. The numbers indicate the percentage of the corresponding cell populations from the gate of live lymphocytes.

Evaluation of the level of cytokines IL-17A and IFN-ɣ in supernatants of activated T-helper cultures. In the supernatants of activated T-helpers, the level of marker cytokines that determine whether T-helper belongs to Th17, IL-17A and IFN-γ, was assessed. IL-17 is the main cytokine produced by T h17. It is known that Th17 are able to secrete interferon gamma (IFN-γ), the main pro-inflammatory effects of which include: potentiation of the activity of the type I interferon system and the development of a Th1 type immune response.

It was shown that native and recombinant hCG preparations did not modulate the secretion of these cytokines (Fig. 4).

Thus, it was found that native and recombinant hCG under conditions of T-helper polarization into the Th17 phenotype did not modulate the proliferation and differentiation of Th17, as well as the secretion of cytokines IL-17A and IFN-ɣ by these cells. At the same time, recombinant hCG increased the level of T-helpers that do not belong to the Th17 subpopulation in the experimental model used.

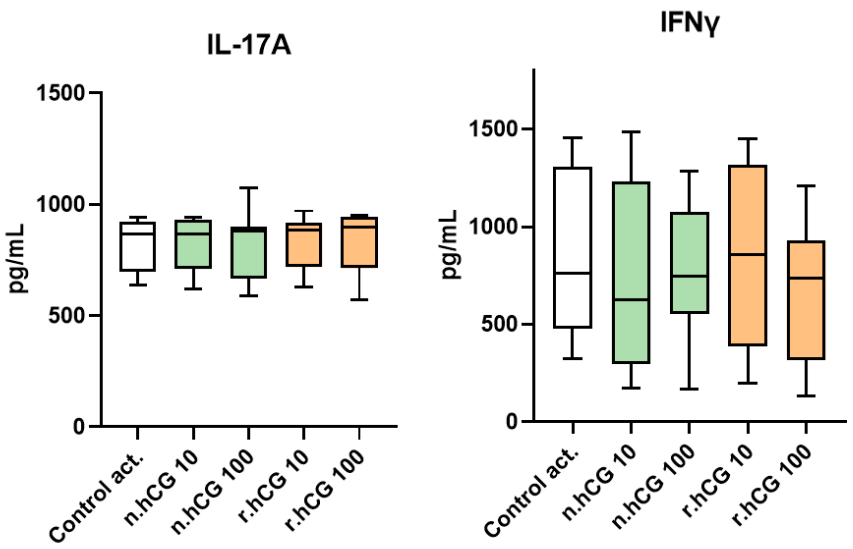

Fig. 4. The effect of hCG on the production of IL-17A and IFN-γ cytokines (n=8, Me (Q1-Q3)).

Therefore, as a result of our experiments, it was shown that hCG did not modulate the processes of proliferation and differentiation of Th17 under conditions of T-helper polarization into the Th17 phenotype, which was carried out with the help of pro-inflammatory cytokines and a TCR activator.

Quite surprisingly, that hCG showed such effects, as the hormone had previously been shown to inhibit the differentiation of these cells. Apparently, in our experimental system, we were unable to detect these effects. Perhaps this is due to the period of cell incubation with the hormone (60 hours), during which the effects of hCG do not appear phenotypically. In previous studies, this period was 72 hours [5], and hCG suppressed the expression of ROR-γt at this incubation period. In addition, in 2022, it became known that hCG suppresses the activity of Th17 cells in an experimental mouse model [7]. However, in our model, when using human cells, the effects of hCG were not detected.

Given that the processes of Th17 differentiation are associated with the proliferative processes of these cells, we consider it important to evaluate the level of intracellular expression of the Ki-67 marker [8]. This protein is an adequate marker for assessing the growth phase of the Th17 population, since Ki-67 is present in all active phases of the cell cycle (G (1), S, G (2) and mitosis), but is absent in non-dividing cells (G (0)). However, we did not find any effect of hCG on proliferation processes in our experimental system. The most interesting result we got is that hCG (recombinant 100 IU/mL) increased the number of proliferating T helpers, but not the Th17 phenotype. This effect can be explained by the plasticity of Th17, which are able to convert into regulatory T-lymphocytes (Treg) even under the influence of pro-inflammatory cytokines [6]. Thus, the goal of further research may be to identify the pool of T-helper cells whose proliferation level increases under the influence of hCG.

4 Conclusions

1. It was found that native and recombinant hCG preparations in our study did not affect the proliferative status of activated $CD4^+$ lymphocytes polarized into the Th17 phenotype.

2. It was shown that the introduction of native and recombinant hCG preparations into cultures at concentrations of 100 and 10 IU/mL did not affect the level of $CD4^+$ lymphocytes expressing ROR-γt (Th17). However, hCG (rec. 100 IU/mL) has been shown to increase levels of non-Th17 $CD4^+$ lymphocytes (ROR-γt^-Ki-67+). Thus, hCG under conditions of Th17- polarization does not regulate the proliferation and differentiation of Th17.

3. When assessing the level of IL-17A and IFN-γ in cell culture supernatants, it was found that hCG (nat., rec.) had no effect on the secretion of these cytokines.

4. In the course of the work, the following differences in the action of hCG preparations were revealed: the native preparation (10, 100 IU/mL) and recombinant (10 IU/mL) hCG did not affect the differentiation and proliferation of Th17 cells. However, the recombinant hCG preparation (100 IU/mL) increased the level of non-Th17 $CD4^+$ lymphocytes. There were no differences in the effects of native and recombinant hCG preparations on the production of cytokines (IL-17A and IFN-γ) by these cells.

5. It has been established that the introduction of native and recombinant hCG preparations into cultures of activated lymphocytes at concentrations of 100 and 10 IU/mL does not affect the proliferative status of activated $CD4^+$ lymphocytes polarized into the Th17 phenotype.

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