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ARTICLE

The impact of sex hormones on BCG-induced trained immunity

L.C.J. de Bree[1,2,3,4] | Robine Janssen[1,2] | Peter Aaby[3] | Reinout van Crevel[1,2] | Leo A.B. Joosten[1,2] | Christine Stabell Benn[3,4] | Mihai G. Netea[1,2,5]

[1]Department of Internal Medicine, Radboud University Medical Center, Nijmegen, The Netherlands

[2]Radboud Center for Infectious Diseases (RCI), Radboud University Medical Center, Nijmegen, The Netherlands

[3]Research Center for Vitamins and Vaccines, Bandim Health Project, Statens Serum Institut, Copenhagen, Denmark

[4]Odense Patient Data Explorative Network, University of Southern Denmark/Odense University Hospital, Odense, Denmark

[5]Department for Genomics & Immunoregulation, Life and Medical Sciences Institute (LIMES), University of Bonn, Bonn, Germany

Correspondence
Dr. Mihai G. Netea, Department of Internal Medicine, Radboud University Medical Center, PO Box 9101, 6500 HB, Nijmegen, The Netherlands.
Email: mihai.netea@radboudumc.nl

Abstract

The anti-tuberculosis vaccine Bacillus Calmette-Guérin (BCG) is a well-known immune modulator that induces nonspecific protective effects against heterologous infections through induction of innate immune memory, also termed "trained immunity." In randomized trials in low weight newborns, BCG vaccination reduced neonatal mortality due to decreased incidence of sepsis and respiratory infections. In many studies, sex-differential nonspecific effects of vaccines have been observed, but the mechanisms behind these differential effects are unknown. We investigated whether the important sex hormones estrogen and dihydrotestosterone (DHT) influence BCG-induced trained immunity in human primary monocytes. Although addition of estradiol and DHT to BCG inhibited the production of proinflammatory cytokines after direct stimulation of human monocytes, they did not influence the induction of trained immunity by BCG. In addition, estradiol or DHT did not induce training or tolerance in monocytes themselves. We conclude that these important sex hormones are unlikely to explain the sex-differential effects after BCG vaccination. Future studies should focus on the investigation of alternative mechanisms as an explanation for sex-differential nonspecific effects of BCG vaccination.

KEYWORDS

Bacillus Calmette-Guérin, dihydrotestosterone, estradiol, heterologous protection, innate immune memory, sex-differential effects

1 | INTRODUCTION

During the last half century, the immune system has been divided into the innate and adaptive immune systems. In contrast to what was thought to be a unique feature of the adaptive immune system, it has recently been discovered the innate immune system is also capable of building an immunologic memory by adapting to previous exposure to vaccines, pathogens, or microbial components, a process that has been termed "trained immunity."[1–4]

Bacillus Calmette-Guérin (BCG), the live attenuated vaccine developed against tuberculosis, induces trained immunity. After BCG vaccination, monocytes respond with an increased cytokine production upon ex vivo stimulation with unrelated pathogens and TLR agonists, effects dependent on epigenetic and metabolic reprogramming of innate immune cells.[5,6] Several epidemiological studies and recent randomized trials have shown that BCG vaccination results in a decreased overall neonatal mortality due to a lower incidence of sepsis and respiratory infections.[7–11]

Strikingly, observations indicate that these nonspecific effects after BCG vaccination are sex specific. A recently published combined analysis of three randomized trials revealed a time dependency in the sex-differential effects of BCG vaccination on neonatal mortality.[12] Whereas beneficial effects in boys were apparent within the first week after vaccination and waned out quickly, the effect in girls started later.[12] During longer-term follow-up, the observed effect of BCG vaccination on overall mortality and reduced incidence of respiratory infections remained more pronounced in girls compared to boys.[13,14]

Other live attenuated vaccines, such as measles and smallpox vaccination, are likewise associated with a more pronounced beneficial effect in girls compared to boys.[15,16] In contrast, detrimental effects after vaccination with non-live vaccines such as the diphtheria-tetanus-pertussis and hepatitis B vaccines were more apparent in girls.[15,17,18] The mechanisms behind these observed sex-differential effects have not been elucidated yet. Hypothetically, sex hormones

Abbreviations: BCG, bacillus Calmette-Guérin; DHT, dihydrotestosterone; E2, estradiol; ER, estradiol receptor; LDH, lactate dehydrogenase

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could play a role in induction of nonspecific immunologic effects of vaccines. The possible influence of sex hormones on BCG-induced trained immunity has been unexplored thus far.

After birth, hormonal levels peak shortly due to the activation of the hypothalamo-pituitary-gonadal axis, during the so-called "mini puberty." In this period, sex hormone levels are comparable to the period of early-middle puberty.[19] Estrogens are able to interact with the immune system by binding to estrogen receptors (ERs) on different immune cells, including monocytes.[20] Androgen receptors are present in the monocytes-macrophage cell population as well.[21,22] Because the BCG vaccine is usually administered shortly after birth, possible sex-specific effects after BCG vaccination might be caused by an effect of sex hormones on innate immune memory priming during this temporary peak in hormone levels.

In the present study, we investigated the effect of two of the most important sex hormones during induction of in vitro trained immunity in monocytes. We hypothesized that estrogen might augment the effect of BCG, whereas dihydrotestosterone (DHT) could have an inhibitory effect on BCG-induced trained immunity.

2 | MATERIALS AND METHODS

2.1 | Reagents

5α-DHT dissolved in methanol (D-073 CERILLIANT), water soluble estradiol (E4389) and *Escherichia coli* LPS (serotype O55:B5) were purchased from Sigma-Aldrich (St. Louis, MO, USA). BCG Bulgaria vaccine (InterVax, Toronto, Canada) was obtained via the Dutch National Institute for Public Health and Environment. Human pooled serum was used (containing 0.097 pmol/ml estradiol and 0.97 pmol/ml DHT).

2.2 | Blood donors

Buffy coats from healthy adult donors were obtained after written informed consent (Sanquin blood bank, Nijmegen, The Netherlands). In addition, venipunctures of healthy adult volunteers were performed after written consent. Blood donations were approved by the Arnhem-Nijmegen Medical Ethical Committee.

2.3 | PBMC isolation and monocyte enrichment

PBMCs were isolated by Ficoll-Paque (VWR, Tingalpa, Australia) density gradient isolation. PBMCs were resuspended in RPMI 1640+ (Dutch modified, ThermoFischer, Waltham, MA, USA) culture medium supplemented with 50 mg/mL gentamicin, 2 mM glutamax (ThermoFischer), and 1 mM pyruvate (ThermoFischer). PBMC suspensions were enriched for monocytes using hyper-osmotic Percoll (Sigma-Aldrich) separation as described before.[23] Cells were resuspended in RPMI and counted using a Sysmex (XN-450) analyzer. The monocyte enriched cell suspension was further purified by adherence (100.000 cells/well) in 96-well polystyrene flat-bottom plates (Corning, New York, NY, USA) for 1 h at 37 °C 5% CO2, after which nonadherent cells were removed during washing with warm PBS.

2.4 | In vitro training of human monocytes

Experiments were performed according to the previously described in vitro trained immunity protocol.[24] Monocytes were incubated with 5 mg/L (13,3 uM) phenol red containing RPMI culture medium only, DHT (1, 10, or 100 pmol/mL), estradiol (0.1, 1, 10 pmol/mL), BCG (5 μg/mL), or BCG (5 μg/mL) combined with either DHT (1, 10, or 100 pmol/mL), or estradiol (0.1, 1, 10 pmol/mL) at 37°C 5% CO2. After incubation for 24 h, supernatants were collected and stored at −20°C. Remaining stimuli were removed by washing with warm PBS and fresh medium supplemented with 10% human pooled serum was added. The cells were incubated for an additional 5 days and medium was refreshed once. At day 6 cells were restimulated with either RPMI or LPS (10 ng/mL). After 24 h incubation, supernatants were collected and stored at −20° C for cytokine analysis.

2.5 | Microscopy

Cell morphology was studied by conventional light microscopy at days 0, 1, 3, and 6.

2.6 | Lactate dehydrogenase (LDH) assay

Cytotoxicity was detected by measurement of LDH concentrations in fresh supernatants collected after 24 h stimulation, using the CytoTox96 NonRadioactive cytotoxicity assay (Promega, WI, USA).

2.7 | Cytokine measurements

Cytokine concentrations were determined in supernatants using commercial ELISA kits for TNF-α, IL-6 (R&D systems, Bio-Techne, Minneapolis, Minnesota, USA), IL-8 and IL-10 (Sanquin, Amsterdam, The Netherlands), according to the manufacturer's protocol.

2.8 | BCA protein assay

BCA (bicinchoninic acid) protein assay (Thermo Fischer Scientific, Waltham, MA, USA) was used to measure protein content in lysates of 6 days cultured macrophages. The assay was performed according to the manufacturer's protocol.

2.9 | Statistics

Cytokine, LDH, and BCA protein data were analyzed using Wilcoxon matched-pairs signed rank test. Absolute cytokine concentrations determined in 24 h BCG stimulated conditions were compared with BCG stimulated conditions with additional hormones. As a read-out for trained immunity responses, fold increases of cytokines of BCG (with and without additional hormones) primed (at day 6 LPS restimulated) conditions were calculated over RPMI primed (LPS restimulated) conditions. Obtained fold increases of BCG alone were compared with fold increases of BCG conditions with additional hormones. LDH and BCA protein assay values of all conditions were compared to RPMI control condition. Cytokine data were analyzed separately by sex. All data were analyzed using Graphpad 5.03 (La Jolla, San Diego, CA, USA). A

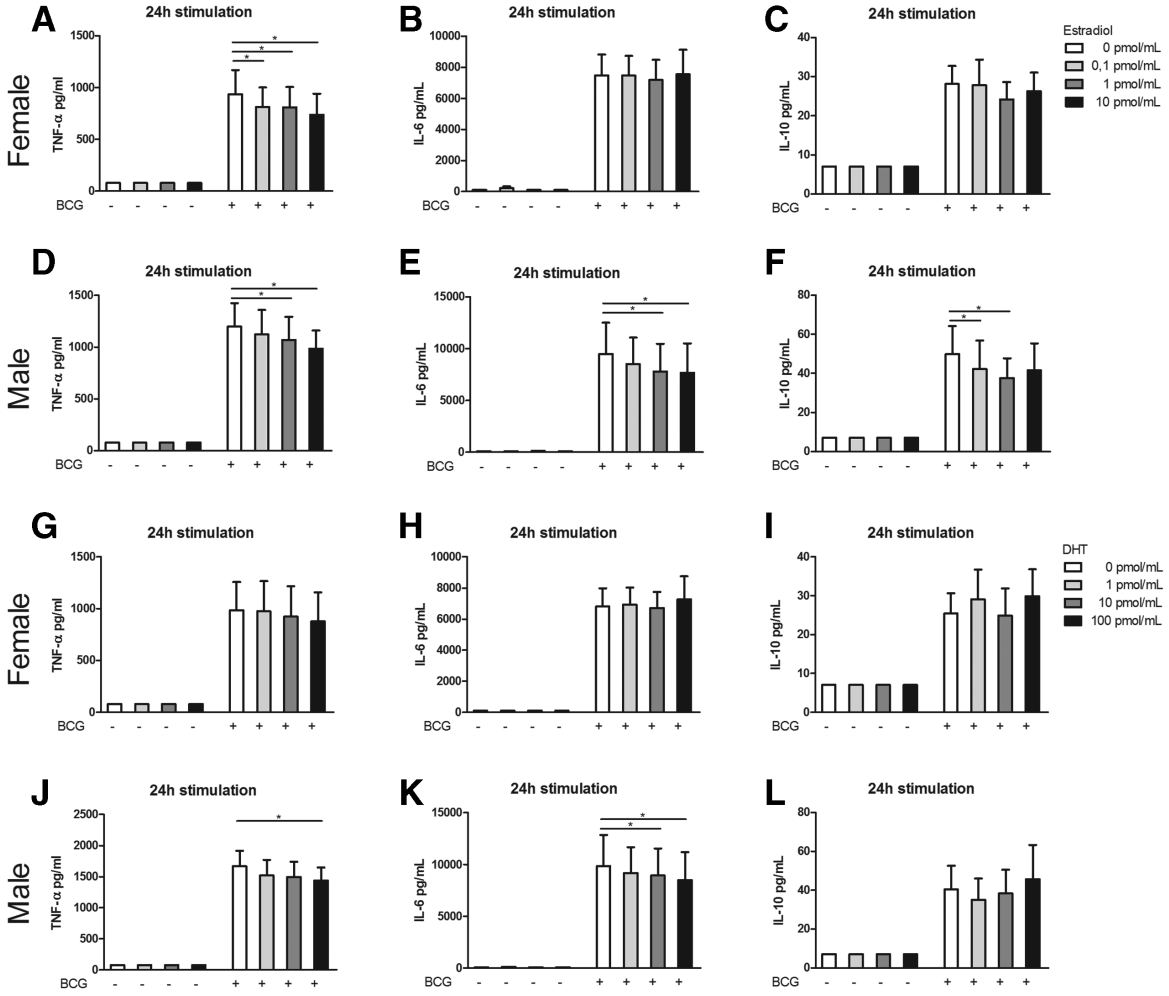

FIGURE 1 **Addition of estradiol or DHT results in diminished cytokine responses after 24 h monocyte stimulation with BCG.** Concentrations of TNF-α, IL-6, and IL-10 after 24 h stimulation with RPMI, or BCG alone, or combined with estradiol (0.1, 1, 10 pmol/mL) (A–F) or DHT (1, 10, 100 pmol/mL) (G–L) are depicted. Stimulation with estradiol or DHT alone did not result in detectable cytokine production (A–L). Compared to stimulation with BCG alone, when added to BCG all concentrations of estradiol resulted in reduced TNF-α responses in females (A). In males, a diminishing dose response on the level of TNF-α, IL-6, and IL-10 was observed when estradiol was added to BCG in comparison with BCG stimulation only (D–F). Addition of DHT to BCG resulted in both diminished TNF-α as well as IL-6 responses in male derived monocytes (J–K). (Wilcoxon matched pairs signed rank test, females $n = 7$, males $n = 7$, $^* = P < 0.05$)

two-sided P value below 0.05 was considered statistically significant. Data are shown as means $\pm$ SEM.

3 | RESULTS AND DISCUSSION

In this study, we aimed to determine the direct effect of estradiol and DHT on primary monocyte derived cytokine production, whether these hormones are able to induce in vitro trained immunity, and to test their possible influence on BCG-induced trained immunity.

3.1 | Addition of estradiol or DHT inhibits the cytokine production by monocyte stimulated with BCG

Freshly isolated human monocytes (females $n = 7$, males $n = 7$) were stimulated for 24 h with estradiol (0.1, 1, 10 pmol/mL) or DHT (1, 10, 100 pmol/mL) alone or in presence of BCG. Stimulation with additional estradiol or DHT did not result in detectable IL-6, TNF-α, or IL-10 production. However, both estradiol and DHT inhibited direct cytokine responses induced by BCG (Fig. 1A–L). In females, a significantly lower TNF-α response was detected when estradiol was added to BCG, compared to BCG alone (Fig. 1A). In males, a diminishing dose response on the production of TNF-α, IL-6, and IL-10 was observed when estradiol was added to BCG in comparison with BCG stimulation only (Fig. 1D–F). Addition of DHT to BCG resulted in both diminished TNF-α as well as IL-6 responses in male-derived monocytes (Fig. 1J–K). In conclusion, when BCG was supplemented with estradiol, a decline in both male as well as female derived monocytic cytokine production was observed, whereas addition of DHT resulted in decreased production in males only. To exclude direct cytotoxicity as a cause for the diminished cytokine responses when estradiol and DHT were added to BCG, LDH concentrations were measured in fresh supernatants collected after 24 h stimulation. LDH

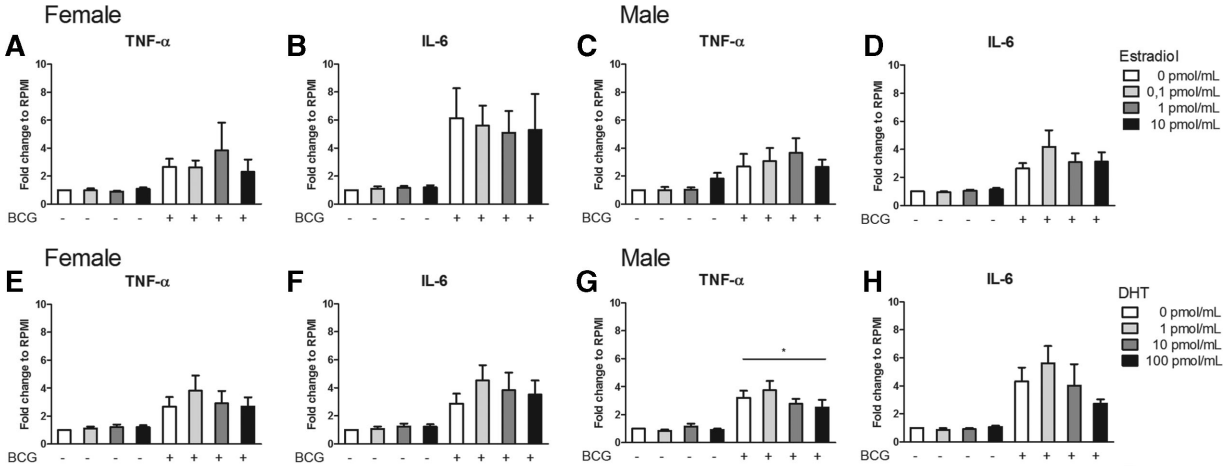

FIGURE 2 Effects of estradiol and DHT during in vitro monocyte training. Adherent monocytes were trained with RPMI, or BCG alone, or combined with estrogen (0.1, 1, 10 pmol/mL) or DHT (1, 10, 100 pmol/mL). After one week, cells were LPS restimulated after which TNF-α and IL-6 responses were determined. Fold increases over RPMI-LPS restimulated conditions are depicted (A–H). Neither estradiol nor DHT did induce training independent of BCG. Addition of either estradiol of DHT did not influence BCG-induced trained immunity, except for high-dose DHT-BCG training, in which a small decrease in training was seen at the level of TNF-α response in male derived monocytes compared to BCG training alone (G). (Wilcoxon matched pairs signed rank test, females $n = 7$, males $n = 7$, * = $P < 0.05$)

concentrations did not differ significantly between conditions (Supplemental Fig. 1). After 24 h stimulation, no microscopically observable differences by means of cell morphology or density were apparent between conditions.

3.2 | Estradiol and dihydrotestosterone do not induce training, nor influence BCG-induced trained immunity

Six days after initial training, macrophages were restimulated with LPS. Previously it has been shown BCG-induced trained immunity results in genome wide epigenetic reprogramming in vivo[25] and up-regulation of several cytokines. Pro-inflammatory TNF-α and IL-6 and anti-inflammatory IL-10 were selected as example cytokines for our primary outcomes based on previous studies.[24] In addition, concentrations of chemokine IL-8 were measured. Priming with different concentrations of estradiol or DHT did not induce training or tolerance in the absence of BCG (Fig. 2A–H). Trained immunity responses on the level of TNF-α and IL-6 responses were observed in all BCG conditions, and they were not influenced by adding various concentrations of estradiol of DHT. Only when BCG was combined with the highest concentration of DHT, a small inhibitory effect on TNF-α production was observed in males (Fig. 2G). No other significant differences were detected when BCG priming was compared to estradiol or DHT supplemented conditions. None of the conditions resulted in an effect on IL-10 or IL-8 production after LPS restimulation (Supplemental Figs. 2 and 3). Intracellular protein concentrations were measured at day 6 (before LPS restimulation) in lysates of cultured macrophages and compared between conditions. Protein concentrations did not differ between conditions, indicating similar number of cells between conditions (Supplemental Fig. 4). In an additional set of experiments RPMI medium was replaced by phenol red-free RPMI medium, in order to exclude an effect of pH indicator phenol red, which possesses low affinity for the ER. BCG training on the level of TNF-α and IL-6 could be induced in the absence of phenol red, regardless of additional estrogen (Fig. 3A–D).

Overall, these data show that although direct diminishing effects of estradiol or DHT on BCG-induced cytokine response after 24 h stimulation were detectable, induction of in vitro BCG training of monocytes is not altered by addition of estradiol or DHT. As reviewed by Bouman et al.,[26] there is mounting evidence regarding sex and gender differences in innate immune composition and function in vivo. Fluctuations during the menstrual cycle, use of contraceptives, pregnancy, and menopause hint toward a role for sex hormones in the mechanism behind these differences. However, with inconclusive results from in vitro studies focusing on effects of sex hormones on monocyte function, it remains a challenge to draw firm conclusions about the interplay between sex hormones and monocyte function and the actual contribution of these hormones to observed sex-differences in immune responses in vivo.[26]

One obvious limitation of the current study is the use of an in vitro model of trained immunity, which may not be a perfect mirror of the induction of trained immunity in vivo. Estradiol and DHT might as well have different effects on neonatal cells compared to the adult cells used in our model. Therefore, we cannot exclude a possible effect of sex hormones on the cytokine production after BCG vaccination and thereby on the induction of trained immunity in vivo.

Due to medium supplementation with 10% human pool serum in our model, medium control conditions contained 1% estrogen and 10% DHT of the lowest concentration of added hormones to other conditions. A significant effect of these low concentrations of hormones in our medium control on training would be very unlikely. However, controls containing low amounts of sex hormones may actually better reflect physiologic conditions with detectable estrogen and DHT levels in both sexes.

At last, it is widely known medium pH indicator phenol red possesses very low ER affinity,[27] but huge variation in sensitivity between

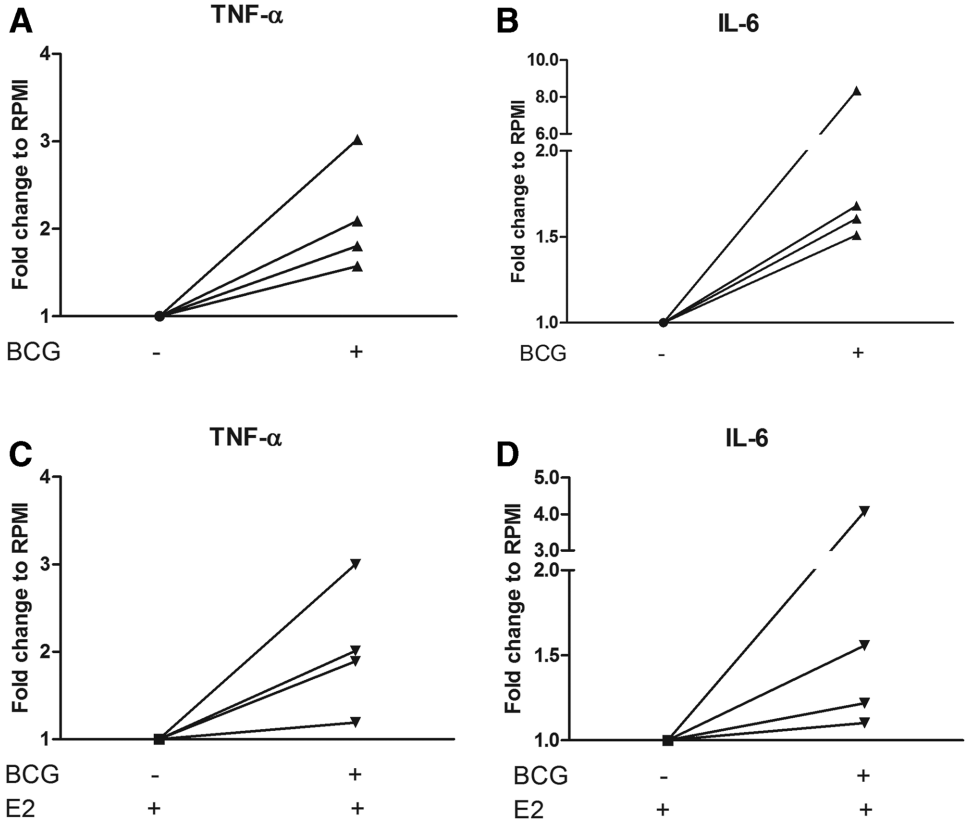

FIGURE 3 Training in absence of medium pH indicator phenol red. Fold increases of TNF-α and IL-6 over RPMI-LPS restimulated adherent monocyte conditions are depicted. Training was induced in phenol red free RPMI medium (A–B), and supplementation of estradiol did not have an additional effect (D–C) (Wilcoxon matched pairs signed rank test, $n = 4$)

cell types has been described.[28] To our knowledge no studies have been conducted to determine the effect of phenol red—ER binding and cytokine production in primary human monocytes. In this study, we have shown BCG training can be induced in the absence of phenol red, regardless of estradiol supplementation.

Although our findings do not directly support that the epidemiologically observed sex-differential effects after BCG vaccination can be explained by effects of estradiol or DHT on monocyte training capacity, the influence of sex hormones on other immune cells in the context of BCG-induced trained immunity cannot be excluded. It has been shown BCG vaccination enhances NK cell, as well as heterologous Th1 and Th17 cytokine responses, after ex vivo restimulation with related and unrelated pathogens,[29,30] and these processes may be under control of sex hormones.

Alternatively, genetic or epigenetic differences could be responsible for observed sex-differential effects. For example, several transcriptional and translational factors are located on the X chromosome.[31] Moreover, it has been shown that miRNAs play important roles in regulating development and function of immune cells.[32] Noncoding miRNAs are able to control gene expression, and 10% of all miRNAs in the genome are found within the X chromosome. In contrast, the Y chromosome only contains 2 miRNA genes.[32] The X-linked gene composition together with the influence of sex hormones on gene expression,[31,33] might play important roles in inducing the more pronounced beneficial long-term effects in girls compared to boys after BCG vaccination.[13,14]

In conclusion, the sex hormones estradiol and DHT do not influence BCG-induced in vitro trained immunity of either male or female derived adult monocytes. Future immunologic studies in large cohorts of vaccinated individuals should assess which are the other potential mechanisms that may control the epidemiologically observed sex-differential effects of BCG vaccination.

AUTHORSHIP

L.C.J.d.B. and M.G.N. conceived and designed the experiments. L.C.J.d.B. and R.J. performed the experiments. C.L.C.J.d.B. and R.J. analyzed the results. C.L.C.J.d.B. and R.J. wrote the manuscript. L.A.B.J., P.A., R.v.C., and C.S.B. critically read the manuscript.

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DISCLOSURE

The authors declare no conflicts of interest.

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SUPPORTING INFORMATION

Additional information may be found online in the Supporting Information section at the end of the article.

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