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ARTICLE

Human chorionic gonadotropin is an immune modulator and can prevent autoimmune diabetes in NOD mice

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Abstract
Aims/hypothesis Expression of T helper (Th)1 cytokine mRNA in pregnant women is known to be inversely correlated with serum human chorionic gonadotropin (hCG). Type 1 diabetes is a Th1-mediated autoimmune disease, in which intervention at an early stage of the autoimmune process can prevent disease progression. We hypothesised that immune modulation by treating young NOD mice with hCG may prevent diabetes.
Methods Female NOD mice were treated with hCG or recombinant hCG from 3 to 15 weeks of age and the incidence of diabetes and development of insulitis was determined. $CD4^+$ and $CD8^+$ T cell populations, T cell proliferation, cytokine production and $CD4^+CD25^+$ regulatory T cells were examined and adoptive transfer experiments were performed.
Results Both purified and recombinant hCG prevented development of diabetes in NOD mice. hCG decreased the proportion and number of $CD4^+$ and $CD8^+$ T cells and inhibited T cell proliferative responses against beta cell antigens. hCG treatment suppressed IFN-γ production, but increased IL-10 and TGF-β production in splenocytes stimulated with anti-CD3 antibody. hCG treatment also suppressed TNF-α production in splenocytes stimulated with lipopolysaccharide. Furthermore, hCG treatment increased the $CD4^+CD25^+/CD4^+$ T cell ratio in spleen and pancreatic lymph nodes. Depletion of $CD4^+CD25^+$ T cells from splenocytes of hCG-treated NOD mice abolished their preventive effect on diabetes transfer.
Conclusions/interpretation We conclude that hCG has an immunomodulatory effect by downregulating effector cells, including Th1 cells, $CD8^+$ T cells and macrophages, and increasing the $CD4^+CD25^+/CD4^+$ T cell ratio, thus preventing autoimmune diabetes in NOD mice.

Keywords Autoimmune disease · Cytokines · Human chorionic gonadotropin · Immune regulation · Insulitis · NOD mice · Regulatory T cells · T cells · Th1 immune response · Type 1 diabetes

Abbreviations

HCG	human chorionic gonadotropin
LPS	lipopolysaccharide
MIN	mouse insulinoma
rhCG	recombinant human chorionic gonadotropin
SCID	severe combined immunodeficient
Th	T helper

L.-Y. Khil, H.-S Jun and H. Kwon contributed equally to this work.

Ji-Won Yoon sadly passed away on 6 April 2006.

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Introduction

Human chorionic gonadotropin (hCG) is a heterodimeric placental glycoprotein required to maintain pregnancy. The level of hCG increases during the first trimester of pregnancy and decreases to 10% of the peak value during the second and third trimesters [1]. The symptoms of autoimmune diseases such as rheumatoid arthritis, Crohn's disease and multiple sclerosis are attenuated during pregnancy [2–4]. In pregnant women, the expression of T helper (Th)1 cytokine mRNA such as *IL2* and *IFN-γ* (also known as *IFNG*) is significantly decreased, while expression of *IL18* mRNA, an inducer of IFN-γ in T lymphocytes and natural killer cells, is inversely correlated with serum levels of hCG [5]. This suggests that hCG may have an immunoregulatory role in addition to its classical reproductive role in the maintenance of pregnancy. There is further evidence to support this view in animal models. Purified hCG and a 400–2,000 Da fraction of clinical grade hCG were shown to prevent diabetes in mice [6]. Another study showed that treatment with hCG prevented the wasting and death of transgenic mice carrying human immunodeficiency virus genes [7]. Recent studies have shown that this wasting syndrome is due in part to high levels of TNF-α, which can be suppressed by treatment with hCG [8]. However, the mechanisms by which hCG prevents autoimmune disease are not clearly understood.

Type 1 diabetes results from the destruction of pancreatic beta cells by T cell-mediated autoimmune responses, which are thought to result from an autoimmune inflammatory process mediated by autoreactive Th1 cells and their secreted cytokines [9–11]. We hypothesised that treatment of prediabetic individuals with hCG might impede the autoimmune processes by modulating immune responses, resulting in the prevention of autoimmune type 1 diabetes. We tested this possibility in the NOD mouse, which is a widely used animal model for the study of human autoimmune type 1 diabetes. We report that hCG treatment downregulated Th1 cytokines and macrophage proinflammatory cytokines and increased Th2 cytokines and TGF-β. In addition, hCG decreased $CD4^+$ and $CD8^+$ T cells, but increased the ratio of $CD4^+CD25^+$ regulatory T cells/$CD4^+$ T cells.

Methods

Animals Female NOD and NOD/severe combined immunodeficient (NOD.SCID) mice were obtained from The Jackson Laboratory (Bar Harbor, ME, USA). The animals were maintained under specific pathogen-free conditions and provided with free access to sterile food and water at the Animal Resources Centre, Faculty of Medicine, University of Calgary. The use and care of the animals were approved by the Animal Care Committee, Faculty of Medicine, University of Calgary.

Treatment with hCG and monitoring of diabetes onset Recombinant hCG (rhCG) and hCG from the urine of pregnant women were obtained from Sigma (St Louis, MO, USA). Purity was determined by 12% SDS-PAGE followed by staining using a silver staining kit (Bio-Rad, Hercules, CA, USA). The pattern of protein bands in hCG was the same as in rhCG, indicating that the hCG did not contain other apparent protein contaminants compared with rhCG. NOD female mice (3 or 10 weeks of age) were injected i.p. with 50 IU hCG or rhCG (giving a blood concentration comparable with that in pregnant humans) in 200 μl PBS per mouse or with PBS alone, 5 times per week for 12 weeks. The development of diabetes was monitored by urine glucose measurements using Diastix (Bayer, West Haven, CT, USA) every week and positive glycosuria was confirmed by hyperglycaemia (blood glucose level >16.7 mmol/l). If mice became diabetic during the experiment, they were killed according to guidelines of the animal care protocol.

Histology The pancreata were removed from hCG- or PBS-treated NOD mice at 15 or 30 weeks of age. The pancreata were fixed with 10% buffered formalin, embedded in paraffin and sectioned at 4.5 μm intervals. More than 500 serial sections were prepared from each pancreas and every 20th section was stained with haematoxylin and eosin [12]. The degree of insulitis was scored as one of four categories: intact islet; <25% of the islet infiltrated; 25 to 50% of the islet infiltrated; 50 to 75% of the islet infiltrated; and >75% of the islet infiltrated. The degree of insulitis in 20 to 25 islets per mouse was independently evaluated by two investigators.

T cell proliferation To examine the proliferation of T cells, splenocytes (1×10^5 cells) were cultured for 96 h in the presence or absence of anti-CD3 antibody (0.5 μg/ml) or mouse insulinoma (MIN)6N8a cell extract (25 μg/well) in 200 μl of complete RPMI medium containing 10% fetal bovine serum and antibiotics in a 96-well microplate. To prepare MIN6N8a cell extract, MIN6N8a cells were collected by trypsinisation, suspended in PBS and sonicated for 3 min. The cells were pulsed with [^{3}H]thymidine (37 kBq/well) for 16 h of incubation and then harvested. The incorporation of [^{3}H]thymidine was measured by liquid scintillation counting [12]. A stimulation index was calculated by dividing the cpm from the stimulated group by the cpm from the unstimulated group.

Quantitative ELISA for cytokine production Splenocytes (1×10^6 cells) from 10-week-old female NOD mice treated with PBS or hCG were stimulated with anti-CD3 antibody

(0.5 μg/ml) for IFN-γ, IL-4, IL-10 and TGF-β or lipopolysaccharide (LPS; 10 ng/ml) for TNF-α for indicated times in 1 ml of complete RPMI medium. The supernatant fraction was collected and cytokine release was measured using a specific ELISA kit (Quantikine; R & D Systems, Minneapolis, MN, USA) according to the manufacturer's protocol.

FACS analysis Splenocytes and pancreatic lymph node cells were isolated from hCG or PBS-treated female NOD mice at 10 weeks of age. To examine the $CD4^+$ and $CD8^+$ T cell populations, cells (1×10^6) were incubated with FITC-labelled anti-CD4 and phycoerythrin-labelled anti-CD8 antibodies (Pharmingen, Mississauga, ON, Canada) for 30 min at 4°C in staining buffer (1% fetal bovine serum and 0.1% sodium azide in PBS). To examine the macrophage population, cells were incubated with FITC-labelled anti-CD11b antibody (Pharmingen). To examine the $CD4^+CD25^+$ T cell population, cells were incubated with FITC-labelled anti-CD4 and phycoerythrin-labelled anti-CD25 antibodies. To examine the natural killer T and $CD4^+CD62L^+$ regulatory cell populations, splenocytes and lymph node cells were double-stained with FITC-labelled anti-CD3 and biotin-labelled DX5 antibodies or FITC-labelled anti-CD4 and biotin-labelled anti-CD62L antibodies, respectively, followed by phycoerythrin-labelled streptavidin. The cells were washed with staining buffer and analysed by FACS [13].

Adoptive transfer Splenocytes were isolated from hCG-, rhCG- or PBS-treated mice at 15 weeks of age, checked for viability under the microscope and transferred (i.v.) into 6-week-old NOD.SCID mice as described elsewhere [14]. To examine the preventive effect, splenocytes from hCG- or PBS-treated mice were transferred along with same number of diabetic splenocytes. To determine whether $CD4^+$ T cell population contained regulatory T cells, splenocytes were isolated from hCG- or PBS-treated NOD mice and $CD8^+$ T cells were depleted with anti-CD8 antibody-linked micromagnetic beads (Miltenyi Biotec, Auburn, CA, USA). The $CD8^+$ T cell-depleted splenocytes (1×10^7 cells) were co-transferred with splenocytes from diabetic NOD mice (0.5×10^7 cells, 2:1 ratio). To examine the role of $CD4^+CD25^+$ T cells, splenocytes were isolated from hCG-treated NOD mice and $CD25^+$ T cells were depleted using anti-CD25 antibody-linked micromagnetic beads (Miltenyi Biotec). The $CD25^+$ T cell-depleted splenocytes or total splenocytes (1×10^7 cells per recipient) were transferred. In addition, $CD8^+$- and $CD25^+$-depleted splenocytes were co-transferred with splenocytes from diabetic NOD mice. As a positive control, splenocytes from acutely diabetic mice were injected into NOD.SCID mice. The development of diabetes in NOD.SCID recipients was monitored by measuring urine glucose twice weekly and confirmed by measurement of blood glucose levels.

Statistical analyses The statistical significance of the differences between groups was analysed by two-tailed Student's *t* test. Normality of data was verified by the Kolmogorov–Smirnov test. A level of $p<0.05$ was accepted as significant. For comparison of incidence of diabetes, the two-tailed log–rank test was used.

Results

Purified hCG prevents autoimmune diabetes in NOD mice NOD mice were treated with hCG purified from the urine of pregnant women (50 U per mouse, 5 days per week) beginning at 3 weeks of age for 12 weeks and monitored for the development of diabetes. None of the hCG-treated mice developed diabetes (0 of 12), whereas 83% (10 of 12) of the PBS-treated mice developed diabetes by 30 weeks of age (Fig. 1a). An increase in ovarian size and a transient increase in body weight were observed in hCG-treated mice (data not shown). No other physical or behavioural abnormalities were observed. When we examined the islets for insulitis at 15 weeks of age, we found that most of the islets from hCG-treated mice were intact, whereas the majority of the islets from PBS-treated mice showed severe insulitis (Fig. 1b,c). On examining the islets at 30 weeks of age, i.e. 15 weeks after termination of hCG treatment, we found that insulitis was not significantly different from that at 15 weeks of age (Fig. 1c). To determine whether hCG inhibits the development of diabetes after inflammation has already occurred, we injected hCG into NOD mice beginning at 10 weeks of age. The incidence of diabetes was significantly reduced in hCG-treated mice, with 33% of hCG-treated NOD mice (4 of 12) developing diabetes as compared with 83% of PBS-treated NOD mice (10 of 12; Fig. 1d).

To determine whether splenocytes from hCG-treated NOD mice have the ability to transfer diabetes, splenocytes from hCG- or PBS-treated NOD mice at 15 weeks of age were transferred into NOD.SCID mice and the incidence of diabetes was determined. All of the recipients (6 of 6) of splenocytes from PBS-treated or diabetic NOD mice developed diabetes by 8 weeks after transfer, whereas none (0 of 7) of the recipients of splenocytes from hCG-treated mice developed diabetes (Fig. 2a). To determine whether splenocytes from hCG-treated NOD mice have the ability to inhibit the adoptive transfer of diabetes, we co-transferred splenocytes from hCG- or PBS-treated NOD mice along with splenocytes from acutely diabetic NOD mice into NOD.SCID mice. All of the recipients (6 of 6) of a combination of

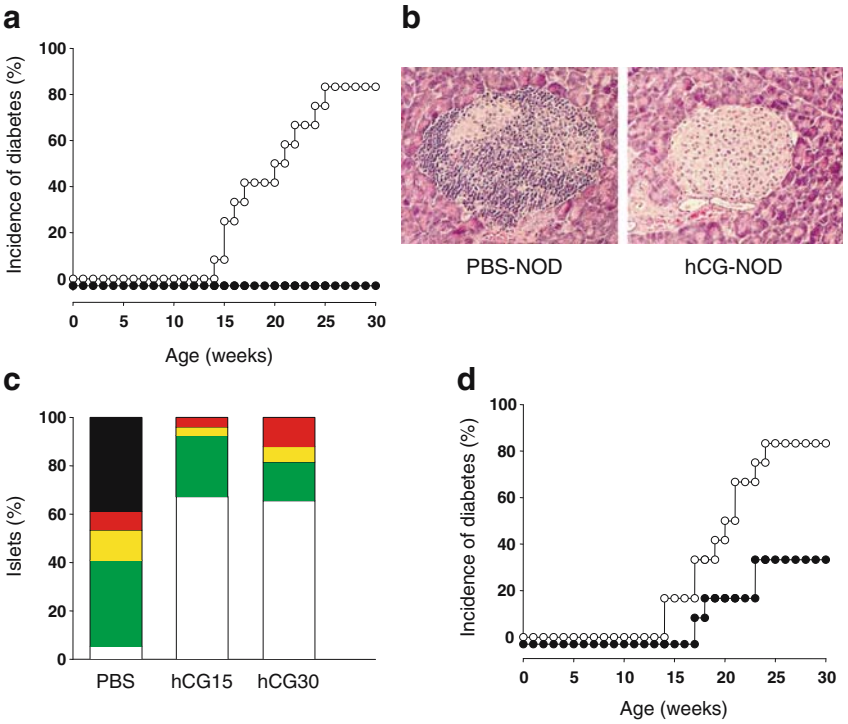

Fig. 1 hCG treatment prevents the development of diabetes in NOD mice. Female NOD mice at 3 (**a**) or 10 (**d**) weeks of age were injected i. p. with PBS (*open circles*, $n=12$) or hCG (*closed circles*, $n=12$) 5 times per week for 12 weeks. The development of diabetes was monitored weekly. $p<0.001$ (**a**) and $p<0.01$ (**d**) by log–rank test compared with PBS-treated mice. **b**, **c** Five non-diabetic mice that had been treated with hCG or PBS for 12 weeks from 3 weeks of age were killed at 15 or 30 weeks of age. Photomicrographs (**b**) were taken of haematoxylin and eosin-stained pancreatic sections from 15-week-old mice. Original magnification ×250. **c** Degree of insulitis was examined in at least 20 islets per mouse ($n=5$ per group). Insulitis grading (% of islet infiltrated): *white column*, intact islet; *green column*, less than 25%; *yellow column*, 25–50%; *red column*, 50–75%; *black column*, >75%

splenocytes from PBS-treated mice and diabetic mice developed diabetes, whereas 44% of recipients (4 of 9) of a combination of splenocytes from hCG-treated mice and diabetic mice developed diabetes (Fig. 2b).

hCG treatment decreases the production of IFN-γ and TNF-α and increases the production of IL-10 and TGF-β To determine whether hCG affects cytokine production, splenocytes from hCG- or PBS-treated NOD mice were stimulated with anti-CD3 antibody. IFN-γ as a Th1 cytokine (destructive cytokine), IL-4 and IL-10 as Th2 cytokines, as well as TGF-β (protective cytokines) were measured by ELISA. hCG treatment significantly inhibited IFN-γ production and increased IL-10 and TGF-β production, but did not affect IL-4 production (Fig. 3a). Examination of TNF-α production in splenocytes after stimulation with LPS showed that TNF-α was significantly decreased in hCG-treated mice (Fig. 3b).

hCG treatment decreases the proportion and number of $CD4^+$ and $CD8^+$ T cells, but increases the ratio of $CD4^+CD25^+/CD4^+$ T cells To determine whether hCG treatment changes the immune cell populations, $CD4^+$ T cells, $CD8^+$ T cells and $CD11b^+$ macrophages were examined in the spleen and pancreatic lymph nodes. The proportion (Fig. 4a) and number (Fig. 4b) of $CD4^+$ and $CD8^+$ T cells were decreased in the spleen and pancreatic lymph nodes of hCG-treated mice. There were no significant changes in the macrophage population (data not shown). We next examined the effect of hCG on the proliferative responses of T cells. Splenocytes from hCG- or PBS-treated mice were stimulated with anti-CD3 antibody or beta cell antigens (extract of MIN6N8a cells) and the proliferative response was examined. Strong T cell proliferative responses were seen with anti-CD3 antibody (Fig. 4c) and medium responses were seen with MIN6N8a cell extract (Fig. 4d). Responses against both of these antigens were significantly inhibited in hCG-treated NOD mice as compared with PBS-treated NOD mice (Fig. 4c,d).

To determine whether hCG can induce regulatory T cell populations, the proportion and number of regulatory T cells were examined by flow cytometry in splenocytes and pancreatic lymph node cells from 10-week-old female NOD mice treated with hCG or PBS. The proportion of $CD4^+CD25^+$ T cells in the $CD4^+$ T population was significantly increased in the spleen and pancreatic lymph node (Fig. 5a), but the

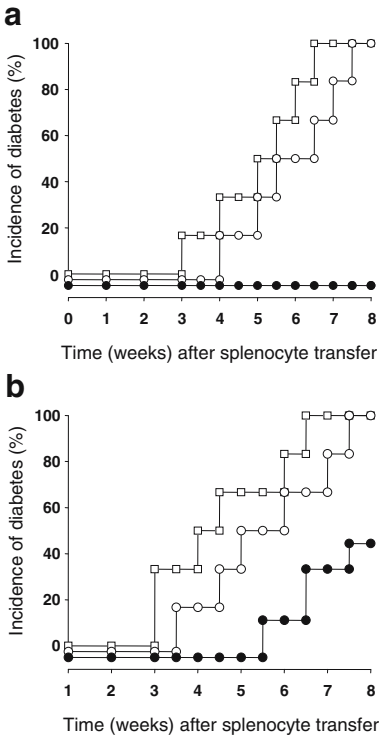

Fig. 2 Splenocytes from hCG-treated NOD mice prevent the transfer of diabetes. Splenocytes were isolated from PBS- or hCG-treated female NOD mice at 15 weeks of age. **a** Total splenocytes from either PBS-treated (*open circles*), hCG-treated (*closed circles*) or diabetic (*open squares*; control) mice were injected intravenously into NOD.SCID mice and the development of diabetes was monitored. **b** Total splenocytes from PBS-treated (*open circles*) or hCG-treated (*closed circles*) mice were mixed with splenocytes from acutely diabetic NOD mice in a 2:1 ratio and injected intravenously into NOD.SICD mice. The development of diabetes was monitored. Splenocytes from diabetic mice were injected as a control (*open squares*). $p<0.001$ (**a**) compared with recipients of PBS splenocytes, $n=6$–7 per group; $p<0.05$ (**b**) compared with recipients of PBS splenocytes+diabetic splenocytes, $n=6$–9 per group

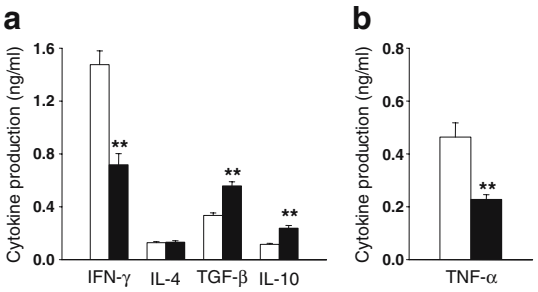

Fig. 3 Human CG treatment inhibits IFN-γ and TNF-α, but increases IL-10 and TGF-β production. **a** Splenocytes from 10-week-old PBS-treated (*open bars*) or hCG-treated (*closed bars*) female NOD mice were stimulated with anti-CD3 antibody for 24 h (IFN-γ), 48 h (IL-4 and IL-10) or 72 h (TGF-β). **b** Splenocytes were stimulated with LPS for 24 h for TNF-α. The supernatants were collected and cytokines were measured by ELISA. $^{**}p<0.01$ compared with PBS-treated group, $n=7$ per group

absolute number of $CD4^+CD25^+$ T cells was not changed in hCG-treated NOD mice (Fig. 5b). Both the proportion and number of natural killer T cells and $CD4^+CD62L^+$ T cells remained unchanged in hCG-treated NOD mice compared with PBS-treated mice (data not shown).

$CD4^+$ and $CD4^+CD25^+$ T cells from hCG-treated mice protect against the development of diabetes To determine whether $CD4^+$ T cells contain regulatory T cells that can prevent the transfer of diabetes, we co-transferred $CD8^+$ T cell-depleted splenocytes from hCG- or PBS-treated NOD mice along with splenocytes from acutely diabetic NOD mice into NOD.SCID mice. All mice (6 of 6) that received $CD8^+$ T cell-depleted splenocytes from PBS-treated NOD mice along with splenocytes from diabetic mice developed diabetes within 8 weeks after transfer. In contrast, only 33% (2 of 6) of mice that received $CD8^+$ T cell-depleted splenocytes from hCG-treated NOD mice along with splenocytes from diabetic mice developed diabetes (Fig. 6a); this reduction is similar to the degree of prevention afforded by transfer of non-depleted splenocytes from hCG-treated mice along with splenocytes from diabetic NOD mice (Fig. 2b).

To determine whether $CD4^+CD25^+$ T cells are responsible for the prevention of diabetes, we depleted the $CD8^+$ and $CD25^+$ cell populations from splenocytes of hCG-treated NOD mice and co-transferred the remaining cells along with splenocytes from diabetic NOD mice into NOD.SCID recipients. All mice (6 of 6) that received $CD8^+$ cell- and $CD25^+$ cell-depleted splenocytes from hCG-treated NOD mice along with diabetogenic splenocytes developed diabetes (Fig. 6b), indicating that $CD4^+CD25^+$ cells play an important role in the prevention of diabetes. We also depleted the $CD25^+$ T cell population from splenocytes of hCG-treated NOD mice and transferred the remaining cells into NOD.SCID mice. All the recipients of $CD25^+$ T cell-depleted splenocytes developed diabetes, whereas none of the recipients of splenocytes that were not depleted of $CD25^+$ T cells developed diabetes (Fig. 6c).

Recombinant hCG prevents autoimmune diabetes in NOD mice To determine whether rhCG has a similar effect on the control of autoimmune diabetes as purified hCG, we treated 3-week-old female NOD mice with rhCG and examined the incidence of diabetes, the incidence of diabetes by adoptive transfer and the production of cytokines. We found that 16.7% of mice treated with rhCG became diabetic, which was comparable to the incidence seen in mice treated with purified hCG. Moreover, neither splenocytes from hCG- nor those from rhCG-treated mice caused diabetes when transplanted into NOD.SCID mice. Both hCG and rhCG inhibited the production of IFN-γ and TNF-α, but neither changed the production of IL-4 (Table 1).

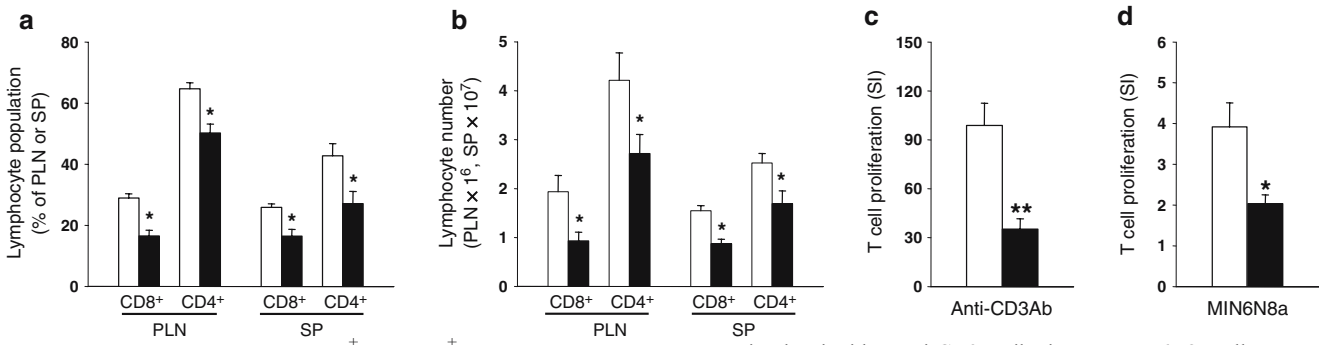

Fig. 4 hCG treatment decreases $CD4^+$ and $CD8^+$ T cells and inhibits T cell proliferation. Pancreatic lymph node cells (PLN) and splenocytes (SP) from 10-week-old PBS-treated (*open bars*) or hCG-treated (*closed bars*) female NOD mice were stained with anti-CD4, anti-CD8 or anti-B220 antibodies and analysed by FACS. The **a** proportion and **b** number of $CD4^+$ and $CD8^+$ T cells were calculated. Splenocytes were stimulated with **c** anti-CD3 antibody or **d** MIN6N8a cell extract. The stimulation index (SI) was determined by dividing the cpm from the stimulation group by that from the unstimulated group. Data are means±SEM. *$p<0.05$; **$p<0.01$ compared with PBS-treated group, $n=10$–12 per group

Discussion

hCG is required for the maintenance of pregnancy [1, 15]. The symptoms of autoimmune disease can be attenuated in pregnant women [2–4, 16, 17]. It was previously found that purified hCG or a fraction of clinical grade hCG can prevent autoimmune diabetes in animal models [6]. However, the mechanisms by which hCG prevents autoimmune disease are not clearly known.

Here, we first examined the effect of hCG on the prevention of diabetes. Treatment of NOD mice with hCG before the development of insulitis resulted in complete prevention of diabetes, while treatment after the development of insulitis was partially effective, suggesting that hCG has a preventive effect before and after the onset of insulitis. In contrast to earlier reports [6], we also found that rhCG could prevent the development of diabetes.

Type 1 diabetes is a T cell-mediated autoimmune disease in both humans and NOD mice, in which activated Th1-type $CD4^+$ and $CD8^+$ T cells are believed to kill pancreatic beta cells by apoptosis [9, 10, 18]. Therefore, we examined the T cell population in hCG-treated NOD mice. Treatment of NOD mice with hCG decreased the number of $CD4^+$ and $CD8^+$ T cells. Consistent with this result, antigen-specific and non-specific T cell proliferation was inhibited by hCG. In addition, we found that hCG treatment decreased IFN-γ production and increased the production of IL-10 and TGF-β, suggesting that hCG may decrease the Th1 immune response. Taken together, these results indicate that treatment of NOD mice with hCG downregulates Th1-type $CD4^+$ and $CD8^+$ T cells. In addition to T cells, macrophages are important contributors to the destruction of beta cells in NOD mice. They achieve this by creating an immune environment that potentiates the Th1 response and by damaging beta cells with macrophage-derived soluble mediators such as IL-1β, TNF-α and IFN-γ [14]. We found that hCG treatment decreased LPS-stimulated TNF-α production, indicating that macrophage function is also downregulated, which may then decrease the Th1 immune response. In addition, the reduction of beta cell-toxic sub-

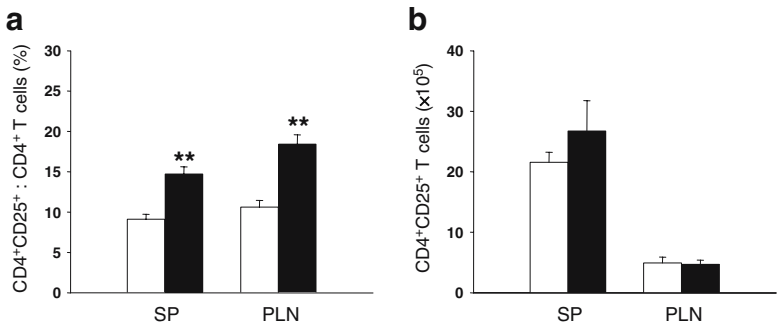

Fig. 5 Human CG treatment increases the ratio of $CD4^+CD25^+/CD4^+$ T cells. Splenocytes (*SP*) or pancreatic lymph node cells (*PLN*) from PBS-treated (*open bars*) or hCG-treated (*closed bars*) 10-week-old female NOD mice were double-stained with anti-CD4 and anti-CD25 antibodies and analysed by FACS. **a** The proportion of $CD4^+CD25^+$ T cells as a percentage of total $CD4^+$ T cells. **b** The number of $CD4^+CD25^+$ T cells. Data are means±SEM. **$p<0.01$ compared with PBS treatment, $n=6$ per group

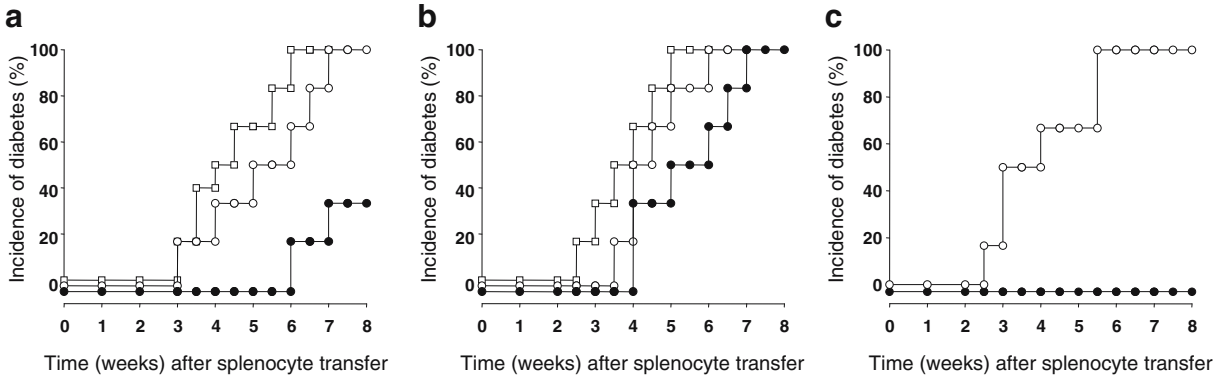

Fig. 6 $CD4^+CD25^+$ regulatory T cells from hCG-treated mice are responsible for the prevention of diabetes. Splenocytes were isolated from PBS- or hCG-treated female NOD mice at 15 weeks of age. **a** $CD8^+$ T cell-depleted splenocytes from PBS-treated (*open circles*) or hCG-treated (*closed circles*) mice were mixed with splenocytes from acutely diabetic NOD mice in a 2:1 ratio and injected intravenously into NOD.SCID mice. Splenocytes from diabetic mice were injected as a control (*open squares*). **b** $CD8^+$ and $CD25^+$ T cell-depleted splenocytes from PBS-treated (*open circles*) or hCG-treated (*closed circles*) mice were mixed with splenocytes and injected as in **a**. Control, as **a** (*open squares*). **c** $CD25^+$ T cell-depleted splenocytes (*open circles*) or total splenocytes (*closed circles*) from hCG-treated mice were injected intravenously into NOD.SCID mice. The development of diabetes was monitored. $p<0.01$ (**a**) compared with recipients of CD8-depleted PBS splenocytes+diabetic splenocytes; $p<0.001$ (**c**) compared with recipients of CD25-depleted hCG splenocytes

stances from macrophages may synergistically act to protect beta cells from destruction. It is known that TNF-α upregulates the transcription factors nuclear factor-κB and activator protein-1, resulting in the expression of a number of inflammatory response genes downstream of TNF-α, and that in vivo treatment of cells with hCG downregulates TNF-α-induced nuclear factor-κB and activator protein-1 [19]. This could explain, in part, the preventive effects of hCG on beta cell destruction.

$CD8^+$ T cell-depleted splenocytes from hCG-treated mice were able to substantially prevent the transfer of diabetes by splenocytes from acutely diabetic NOD mice (Fig. 6a), achieving a similar degree of prevention to that seen in recipients of total splenocytes from hCG-treated NOD mice (Fig. 2b). This suggests that hCG treatment may induce a regulatory T cell population that can prevent diabetes transfer and that the $CD4^+$ T cell population may contain these regulatory T cells. Regulatory T cell numbers and function have been shown to be impaired in NOD mice [20, 21] and in human patients with type 1 diabetes or other autoimmune diseases [11]. Thus, we examined whether there were any changes in regulatory T cell populations, such as natural killer T cells, $CD4^+CD62L^+$ T cells and $CD4^+CD25^+$ T cells. We found that only the $CD4^+CD25^+$ T cell population was significantly increased in hCG-treated NOD mice. An increase in the $CD4^+CD25^+$ T cell population was also found in male NOD mice treated with hCG (data not shown). Although hCG treatment did not increase the absolute number of $CD4^+CD25^+$ regulatory T cells, the increase in the ratio of $CD4^+CD25^+$ T cells relative to $CD4^+$

Table 1 Comparison of recombinant hCG with purified hCG with regard to control of autoimmune diabetes in NOD mice

	PBS	hCG	rhCG
Diabetes incidence[a]	10/12 (83.3%)	0/12 (0%)****	1/6 (16.7%)*
Diabetes incidence by adoptive transfer[b]	6/6 (100%)	0/6 (0%)****	0/6 (0%)****
Production of cytokines (ng/ml)[c]			
IFN-γ (n=9)	1.400±0.115	0.764±0.077***	0.790±0.0921**
IL-4 (n=7)	0.129±0.011	0.131±0.012	0.133±0.013
TNF-α (n=5)	0.479±0.076	0.236±0.033*	0.287±0.030*

Data are n/n (%) or means±SEM
*$p\leq0.05$
**$p<0.01$
***$p\leq0.005$
****$p\leq0.001$ compared with the PBS control
[a] Three-week-old female NOD mice were treated with hCG, rhCG or PBS and cumulative incidence of diabetes at 30 weeks of age determined, as described in Methods
[b] The cumulative incidence of diabetes in NOD.SCID recipients of splenocytes isolated as described in Methods was determined at 8 weeks after transfer
[c] The production of IFN-γ, IL-4 and TNF-α in splenocytes isolated as described in Methods was measured by ELISA

T cells was able to inhibit the proliferation of effector T cells, as $CD4^+CD25^+$ regulatory T cells are potent suppressors of activation of both $CD4^+$ and $CD8^+$ T cells [22, 23]. Adoptive transfer of $CD4^+CD25^+$ T cells can prevent the development of autoimmune thyroiditis, gastritis, inflammatory bowel disease and diabetes [24–26]. Moreover, $CD4^+CD25^+$ T cells have been shown to play an important role in controlling the progression of type 1 diabetes in NOD mice [27–29]. Therefore, hCG may increase the ratio of $CD4^+CD25^+/CD4^+$ T cells, which could restore the immune balance between regulatory and effector T cells, resulting in the prevention of autoimmune disease. Depletion of $CD4^+CD25^+$ T cells resulted in complete loss of the preventive effect of splenocytes from hCG-treated NOD mice, indicating that $CD4^+CD25^+$ T cells play a role in the prevention of autoimmune diabetes by hCG treatment. It is possible that the increase in IL-10 and TGF-β in hCG-treated mice may contribute to the increased ratio of $CD4^+CD25^+/CD4^+$ T cells, as it is known that these cytokines play an important role in the generation and function of $CD4^+CD25^+$ regulatory T cells [30, 31]. There is growing evidence that hCG can have a direct immunomodulatory effect on immune cells [6, 32]. In this regard, we found from in vitro experiments that treatment of immune cells with hCG inhibited IFN-γ and TNF-α production (L.-Y. Khil, H.-S. Jun and H. Kwon, unpublished results). However, we cannot exclude the possibility that hCG, which is a pleiotropic hormone, might also exert its immunosuppressive effects by modulating the level of other reproductive hormones such as progesterone and testosterone [1, 33, 34] and thereby prevent the development of type 1 diabetes in NOD mice.

In this report, we have shown that hCG treatment: (1) downregulates Th1 cells, $CD8^+$ T cells and macrophages; (2) upregulates Th2 cells; and (3) increases the ratio of $CD4^+CD25^+/CD4^+$ T cells in the spleen and pancreatic lymph nodes, contributing to the prevention of autoimmune diabetes in NOD mice. These results raise the possibility that hCG, a subunit of hCG (e.g. beta hCG) or a molecule associated with hCG [6, 35–39] might be useful in the treatment of diseases characterised by immune deviation or abnormal cytokine release. Although NOD mice are widely used as an experimental model of human type 1 diabetes, caution must be exercised when extrapolating results from mice to humans.

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Duality of interest The authors declare that there is no duality of interest associated with this manuscript.

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