Immunoregulation of mononuclear phagocytic cells by human chorionic gonadotropin

Immunoregulatie van mononucleaire fagocyten door humaan choriongonadotrofine

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For my parents

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CONTENTS

PREFACE

Pregnancy hormone human chrionic gonadotropin and mononuclear phagocytic cells

The pregnancy hormone hCG is secreted by trophoblasts in the placenta and peaks in the first trimester of pregnancy. hCG is a member of the pituitary glycoprotein family, and is consists of an α-subunit and a β-subunit. The primary function of hCG is to induce the production of progesterone and estrogen by the corpus luteum during early pregnancy. Data are accumulating on a role for hCG in immune regulation. For instance treatment with hCG has been shown to be beneficial in animal models for type I diabetes, Sjögren's syndrome and rheumatoid arthritis. Mononuclear phagocytic cells are derived from progenitor cells in the bone marrow. As initiators of the immune response, these cells are important in both innate and adaptive immunity. Monocytes, macrophages (Mφ), and dendritic cells (DC) are all belong to mononuclear phagocytic cells which reside in the peripheral blood or tissues and exert with different functions. Mononuclear phagocytic cells bear the hCG receptor, and hCG has been shown to influence several properties of these cells. The scope of this thesis is the immunoregulatory effect of hCG on mononuclear phagocytic cells. This aim was approached by investigating the effect of hCG treatment on the function of monocytes, Mφ, and DC in respect to their innate or adaptive function in the immune response. Moreover, the possible mechanisms underlying the regulation by hCG were studied.

Chapter 1

General introduction

GENERAL INTRODUCTION

1.1 MONONUCLEAR PHAGOCYTIC CELLS

The mononuclear phagocyte system (MPS) has been defined as one of the hematopoietic cell lineages derived from progenitor cells in the bone marrow (BM) and involved in both innate and adaptive immunity. In the traditional view of the MPS, committed myeloid progenitor cells differentiate to form blood monocytes, circulate in the blood and then enter tissues to become resident tissue macrophages (Mφ) and dendritic cells (DC) [1]. There is also evidence of a separate embryonic phagocyte lineage, by evidence of local renewal of tissue macrophage populations as opposed to monocyte recruitment [1].

Monocytes are one of the primary effector cells in innate immunity. Circulating monocytes constitute between three and eight percent of the leukocytes in the blood. Monocytes are usually identified in stained smears by their large bilobate mononucleus. They circulate in the bloodstream for about one to three days and then typically move into tissues. While circulating in the blood, monocytes protect the body against blood-borne pathogens. Monocytes move quickly (in approximately 8-12 hours) to sites of infection in the tissues. In the tissues monocytes mature into different types of macrophages and DC at different anatomical locations, depending on the local conditions [2].

Mφ, the Greek word for "big eaters", are also members of MPS, occurring in virtually all organs of the body [3]. Mφ play an essential role in innate immunity by phagocytosis, destruction of microorganisms and the clearance of infected or apoptotic cells. Upon activation, Mφ produce reactive oxygen species (ROS), nitric oxide (NO) synthase and pro-inflammatory cytokines [4, 5]. ROS and NO are also produced by activated monocytes and polymorphonuclear neutrophils (PMN). The production of ROS is initiated by NADPH oxidase, which results in superoxide (O_2^-) from O_2 that can be converted to H_2O_2 and hydroxyl radicals [6]. Moreover, NO produced by inducible NO synthase can combine with O_2^- to generate additional products with enhanced toxicity, such as peroxynitrite ($ONOO^-$) [7] (Figure 1). As part of the antimicrobial response, oxygen radicals generated from ROS and NO are cytotoxic for a variety of microorganisms including viruses, bacteria, protozoa, and fungi [8]. Mφ are potent producers of pro-inflammatory cytokines including IL-6, IL-1β, TNF-α and IL-12, which play an important role in the defense against various infections [9-12]. In addition to this innate function, Mφ express MHC class II and co-stimulatory molecules upon activation and are capable of antigen presentation to T lymphocytes, although generally not as efficient as DC [5]. Mφ can be generated *in vitro* from BM cultures with macrophage-colony stimulating factor (M-CSF) [13].

DC were initially described in the international literature by R.M. Steinman in 1973 [14]. After decades of research, we now know that DC are members of MPS, too. In the immune system DC can influence many different types of lymphocytes (B, NK, NKT), yet their effect on the priming or induction of T cell-mediated immune responses (Th1/Th2 skewing, regulatory T cells induction, peripheral T cell deletion) is the most important one. There are three signals needed for the DC-induced T cell activation. Signal 1: DC capture antigen (Ag) in the periphery and migrate to draining lymph nodes, where they activate T cells. They process Ag and present it to specific naïve T cells in the "MHC I/II-Ag peptide-TCR+CD8/CD4 complex". The peptide binding proteins are of two types, MHC class I

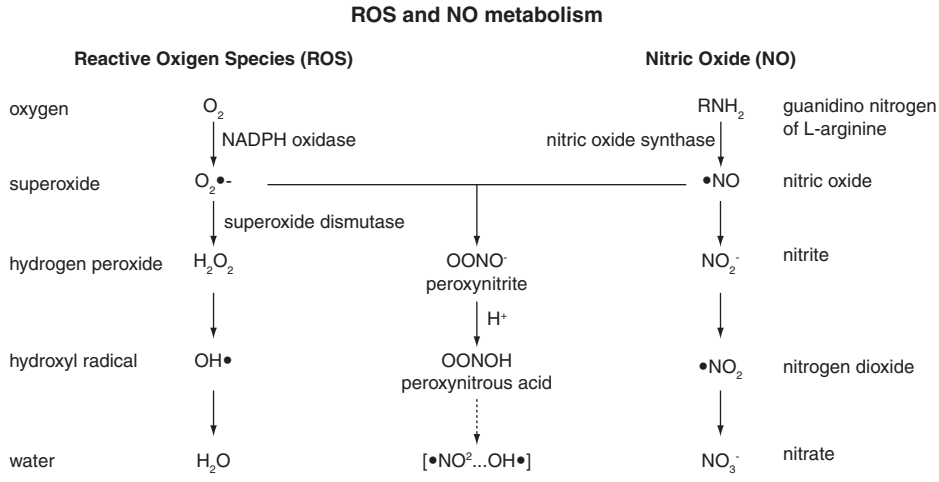

Figure 1. Reactive oxygen species (ROS) and nitric oxide (NO) metabolism.

and II, which can be recognized by the T cell receptor (TCR) on cytotoxic T lymphocytes ($CD8^+$ CTL) and T helper cells (Th $CD4^+$), respectively. Signal 2: DC upregulate surface co-stimulatory molecules such as CD86, CD80, and CD40 followed by interaction with T cell membrane molecules such as CD28, CD152, and CD154, resulting in T cell activation. Signal 3: depending on the cytokine environment, DC can polarize Th0 cells into Th1, Th2 or T regulatory (Treg) cells. This polarization is followed by predominant cell-mediated Th1 immunity, predominant humoral Th2 immunity, or predominant tolerance, respectively [15, 16]. When the Th1/Th2/Treg balance shifts, disease can occur. For example, Type I diabetes is characterized by a predominance of Th1 response, whereas allergic airway diseases are characterized by a Th2-cell response. Knowing that DC play an important role in steering T cell responses, several studies have been done to prevent disease by modifying DC function. *In vivo,* DC undergo continuous differentiation and maturation, from precursors to DC and then from an immature to a mature stage. For *in vitro* studies, cultured DC have been extensively used during the past 30 years all over the world and are still being used to obtain even more information about these unique cells.

In mice, by using different cytokines (e.g. granulocyte/macrophage colony-stimulating factor (GM-CSF)) and growth factors, DC can be generated from BM precursors, named BM-derived DC (BMDC) [17]. Immature DC residing in peripheral tissues as well as secondary lymphoid organs induce T cell anergy by their low levels of MHC, and absent or low levels of costimulatory molecules and proinflammatory cytokines [18]. *In vitro* generated immature BMDC that have been injected intravenously induced $CD4^+$ T cell anergy and enabled the prolonged acceptance of allogeneic heart transplants in mice [19, 20]. *In vitro* IL-10-treated DC display reduced surface expression of MHC and co-stimulatory molecules as well as markedly reduced pro-inflammatory cytokine levels [21]. These DC are rather immature and induce Treg cells that suppress the activation and function

of peripheral T cells in an alloantigen-specific manner [22]. Semi-mature DC, first described as *in vitro* TNF-α-treated DC, are MHC class II^{high} costimulation^{high} cytokines^{low}, and acted in a tolerogenic fashion by preventing experimental autoimmune encephalomyelitis (EAE) by inducing CD4^{+} IL-10^{+} Treg cells [18]. LPS or CD40L stimulation can induce fully mature DC that are MHC class II^{high} costimulation^{high} cytokines^{high}, and induce an inflammatory response that requires the above three signals (Figure 2).

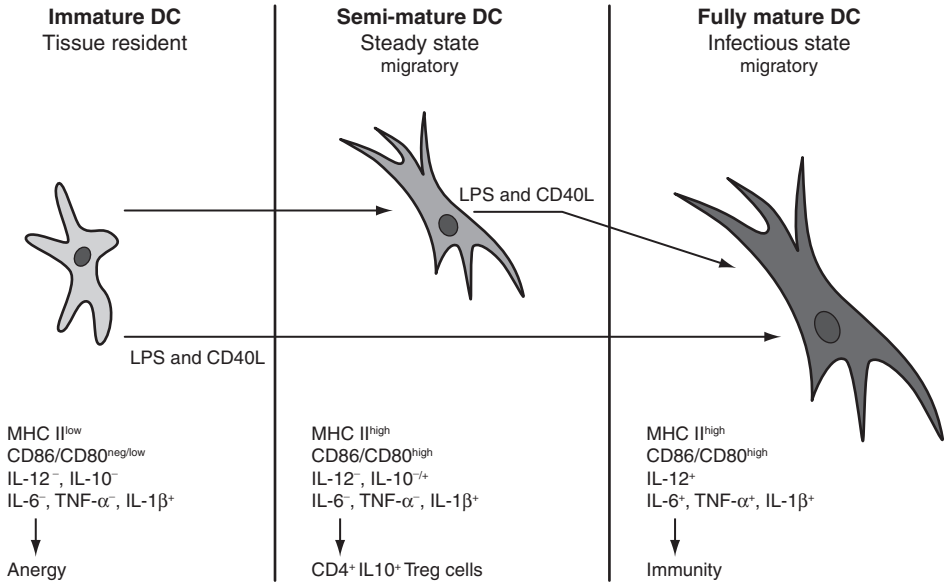

Figure 2. Immature, semi-mature and fully mature dendritic cells (DC) in T cell tolerance and immunity (adapted from *Lutz and Schuler* [18]).

1.1.1 Thioglycollate-induced peritonitis: a study model

Intraperitoneal (i.p.) injection of sterile irritants such as thioglycollate (TG) broth is an established method for inducing acute inflammation in the peritoneal cavity of mice [23, 24]. Prominent features of this inflammatory model are the clearly defined sequential steps of disease development, including intense infiltration of PMN (within 1-4 hours) and monocytes followed by Mφ infiltration (after 40-96 hours) into the peritoneal cavity. This mild disease is accompanied by increased levels of inflammatory cytokines in the peritoneal fluid [25-28]. The mechanisms responsible for eliciting the initial influx of leukocytes into the inflammatory site are not fully elucidated, but resident Mφ have been suggested to be the initiators of PMN recruitment and cytokine production in the peritoneal cavity. Lsp1$^{-/-}$ mice, which have significantly higher levels of resident Mφ in the peritoneum, exhibited accelerated kinetics of neutrophil numbers and an increased influx of these cells upon TG injection [29].

Inhibition of Mφ from releasing PMN chemotactic factor(s) blocked the migration of PMN into the inflamed peritoneal cavity [30]. Release of IL-12 and chemokines (CCL3, CCL4, and CCL5) also need the priming from resident Mφ [31].

The above data suggest that in TG-peritonitis, resident Mφ participate in the control of acute inflammation by acting as 'alarm cells' thus triggering several defense mechanisms. But there is still debate on this subject. Some papers suggest that mast cells also play an important role in the initiation of inflammation, although these cells seem not to be the only responsible cell type [32].

1.2 PREGNANCY AND THE IMMUNE SYSTEM

1.2.1 Hormones in pregnancy

Pregnancy is an incredible orchestration of numerous events, many of which are primarily the result of the hormones in pregnancy. Proper timing of hormone production within and between the placental, fetal and maternal compartments is critical in directing fetal growth and development as well as the coordination of the timing of parturition [33]. Lots of hormones, all with their individual functions, are elevated during pregnancy. Here, three major hormones are discussed to elucidate their importance in pregnancy (Figure 3 and Table 1).

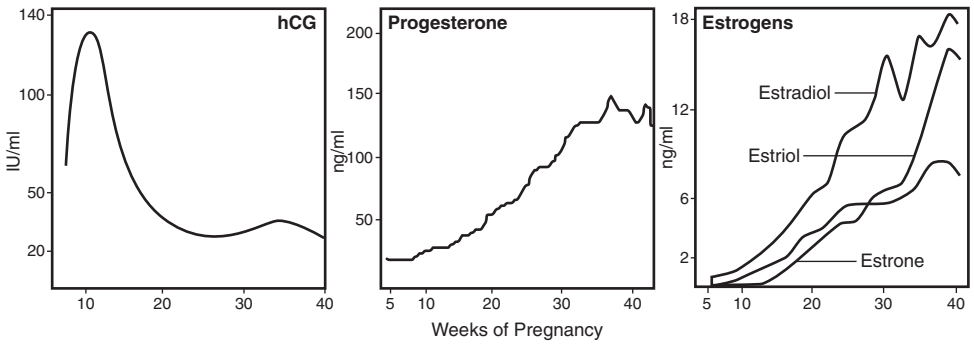

Figure 3. Maternal hormone levels during pregnancy (adapted from *N. Freinkel* [34]).

The first trimester of human pregnancy begins with conception. As egg and sperm unite, and the conceptus implants into the endometrium or uterine lining, human chorionic gonadotropin (hCG) production starts. Except in the case of rare ovarian tumors, hCG is produced mainly during pregnancy by the placenta. hCG values double approximately every two days in the early weeks of pregnancy and peak around 70 days (Figure 3). Elevated levels of hCG initially correlate with gestational age, and are commonly used for pregnancy diagnosis [35]. The most apparent biological effect of hCG is to stimulate the

production of estrogen and progesterone until the placenta is well formed around the tenth week [36]. The first trimester carries the highest risk of miscarriage, which correlates with a low hCG level [37]. hCG has also been suggested to be involved in nausea and vomiting in early pregnancy [38].

Notably, more and more evidence suggests an important role of hCG in immune modulation [36].

Progesterone is a hormone produced by either the ovary or the placenta during pregnancy. Levels of progesterone in the body rise as pregnancy progresses (Figure 3). Progesterone converts the endometrium to its secretory stage to prepare the uterus for implantation. This hormone also functions to inhibit the smooth muscle in the uterus from contracting and decreases prostaglandin formation, both of which allow the fetus to grow in the expanding uterus. Progesterone inhibits lactation during pregnancy. The fall in progesterone levels following delivery is one of the triggers for milk production. In addition, progesterone has a potent anti-inflammatory effect, which maybe involved in the modulation of the immune system during pregnancy [39, 40].

Estrogens are a group of female steroid hormones produced in the ovaries. Naturally occurring estrogens in women are estradiol, estriol, and estrone. During the female menstrual cycle, estrogen and progesterone levels go up and down, falling to their lowest levels while menstruating. Estrogen and progesterone stimulate the endometrium to support the fertilized egg. During pregnancy, estrogen is mainly produced by the placenta and continues to rise till the end of the pregnancy (Figure 3). Among other functions, estrogen increases uterine blood flow and stimulates prolactin production [41-44, 45]. Immunologically, in contrast to progesterone, estrogen is often thought to be an enhancer of the immune response and to be involved in several autoimmune diseases such as rheumatoid arthritis [45, 46].

Table 1. Overview of major maternal pregnancy hormones.

	Source	Main functions	References
human chorionic gonadotropin (hCG)	Placenta	- maintenance of pregnancy - production of progesterone and estrogen - morning sickness - immune modulation	36
Progesterone	First ovary, then placenta	- facilitation of implantation - maintenance of pregnancy - inhibition of uterine smooth muscle contractility - inhibition of lactation - mediation of sexual responsiveness - immune modulation	39, 40
Estrogens	First ovary, then placenta	- augmentation of uterine blood flow - proliferation of the uterine endometrium - progesterone regulation - increase of breast size - immune modulation	41-44

1.2.2 Immunological aspect of pregnancy

1.2.2.1 Some theories on the immunology of pregnancy

Half of the fetal genes are derived from the father, thus the developing embryo and placenta can be considered as a 'semi-allograft'. Normally, such a mismatched organ transplant would be readily rejected. However, the semi-allogeneic fetus is protected from assault by the maternal immune system over an extended period of time. Over the past sixty years, many studies have been done to understand the tolerance of the mother towards the genetically different fetal tissue [47]. In this section, the maternal immune suppression, the skewed Th2 cytokine environment, and the controlled state of inflammation in pregnancy will be reviewed.

- *Immune suppression in pregnancy*

The idea of immunological tolerance was first formulated by Medawar and his colleagues. They proposed three explanations for maternal immunological tolerance: physical separation of maternal and fetal tissues; the antigenic immaturity of the fetal tissues; and immune suppression of the mother [48, 49]. The third idea developed after several studies. Prolonged skin graft survival in mice that had been through multiple matched heterospecific (allogeneic) pregnancies suggested that during pregnancy the immune response was generally [47, 50]. Later on, more and more evidence directed to a specificity of this suppression: antibodies against HLA were found in multiparous woman [51]. Females going through many miscarriages, often only found themselves pregnant after a change of partners [52]. Another elegant study demonstrated that female mice accept an allogeneic tumor graft while pregnant with a conceptus from a father matching the allograft, but not while pregnant by a 'third-party' allogeneic father. After delivery of the pups, the tumors were rejected [53]. These experiments demonstrated the specificity of the suppression to the paternal alloantigens and its temporal nature during pregnancy. The maternal peripheral immune activation still occurs in pregnant HIV^+ women [54], and the maternal response to influenza vaccines and the delayed-type hypersensitivity response remain intact in pregnancy [55], suggesting there is no general suppression of the immune response in pregnancy. Once acute or chronic infection of the uterus or the fetus happens during pregnancy, the immune system might get activated, followed by rejection of the fetus by the mother in order to protect herself from the infection [47].

- *The Th1/Th2 paradigm of pregnancy*

Wegmann *et al.* were the first to propose that fetal survival depends on a bias of maternal immune responses towards Th2 immunity and the inhibition of Th1 responses [56]. This Th2 bias has been demonstrated in mouse models showing that the normal Th1 response to Leishmania infection is downregulated in pregnancy. Conversely, implantation failure and resorption occurred in Leishmania-infected mice due to an exaggerated Th1 response [57, 58].

In humans, there is evidence that Th1 autoimmune diseases (like rheumatoid arthritis (RA), multiple sclerosis (MS), psoriasis) regress and Th2 like diseases (systemic lupus erythematosus (SLE)) worsen during pregnancy [59-62]. This, however, is certainly not a general phenomenon. Hormones elevated during pregnancy, such as progesterone, can promote the production of Th2 cytokines [40]. Although there is undoubtedly a bias towards Th2 cytokine production, which has been demonstrated consistently in mice and humans,

recent findings have challenged the concept of pregnancy as a Th2 phenomenon. This concept therefore requires re-evaluation [61]. Deficiency of the Th2 type cytokines IL-4 or IL-10 has no effect on pregnancy [63]. The systemic Th2 cytokine environment is probably due to the increased natural killer (NK) cell population in early gestation. Therefore, the systemic Th2 expression is present but seems to be a secondary effect [64]. Furthermore, in humans there is no evidence for T cell-mediated reactivity towards the fetus. Instead, there is abundant evidence for T cell-mediated tolerance [53, 64, 65]. First, the maternal $CD4^+CD25^+$ regulatory T cell (Treg) pool is systemically expanded in pregnancy. This population is essential for the suppression of maternal immune aggression against the fetus [66]. Second, trophoblast cells from fetal origin express paternal antigens and invade maternal uterine tissue at the site of implantation. Trophoblasts are lacking human leukocyte antigen (HLA) class II and HLA-A and -B expression, but express and secrete into the blood high levels of HLA-G [67]. This nonclassical HLA class I molecule inhibits T cell proliferation [68] and NK cell activation [69]. Third, trophoblasts secrete hormones like hCG that induce indolamine 2,3 dioxygenase (IDO) expression [70]. IDO depletes tryptophan, an essential amino acid for rapidly dividing cells, and thereby inhibits T cell proliferation [71, 72]. Complement activation, which promotes inflammatory and immune responses, can also be inhibited by IDO. IDO thereby promotes maternal-fetal tolerance [72]. Last, the expression of Fas ligand (FasL) by trophoblast cells may cause apoptosis of activated maternal lymphocytes, which express Fas [73]. All of these mechanisms act locally at the site of fetal antigen exposure as well as systemically and may operate in parallel to sustain gestation.

- *Controlled inflammatory condition in pregnancy*

Recently, new insights led to the suggestion that pregnancy is a controlled state of inflammation with a prominent role for the innate immune system [64]. There are two distinct immunological interfaces in pregnancy: locally in the decidua between maternal immune cells and fetal trophoblasts; and systemically between circulating maternal immune cells and the syncytiotrophoblasts that form the placental villous surface. This syncytiotrophoblast layer releases hormones, like hCG, and cellular microparticles into the maternal blood and interacts with maternal immune cells. At an early stage the inflammatory condition is predominant at the implantation site. Later in pregnancy this is extended to the maternal circulation [64]. There is now strong evidence that in normal pregnancy, compared with the non-pregnant state, not only Th2 cytokine levels are raised, but also the levels of particular Th1 cytokines. This is in contrast to the expected decreased Th1 levels suggested by a decreased Th1/Th2 ratio [64]. This systemic inflammatory response in normal pregnancy is characterized by leukocytosis, increased NK cell numbers in the decidua, increased monocyte priming, increased phagocytic activity, and the production of pro-inflammatory cytokines, including IL-6, IL-12 and TNF-α. It has been suggested that these inflammatory changes can be as strong as those observed in patients with sepsis, but surprisingly do not seem to harm the mother and fetus in any way [74]. This suggests that pregnancy-specific factors as well as the Treg cells mentioned before help to control this inflammatory response.

1.2.2.2 State of innate immunity in pregnancy

In utero, the fetus is physically protected by several layers of maternal and fetal tissues. Moreover, endothelial cells (including those of the endocervix, uterus and fallopian tubes)

and amnion cells have the ability to produce antimicrobial β-defensins. Amniotic fluid has additional bacteriostatic properties [10].

The primary effector cells of innate immunity are phagocytic cells including PMN, MPS, and NK cells. With the help of trophoblasts [75], PMN, monocytes and Mφ protect the fetus against invading micro-organisms [10]. During pregnancy, the phagocytic cells display many aspects of a pro-inflammatory state (Table 2) as indicated by increased cell numbers in the circulation, activation, enhanced phagocytosis and increased production of ROS [76]. Given that NK cells constitute 50-90% of the leukocytes in the decidua (a tissue in which B and T cells are rare), and that the numbers of uterine NK cells fluctuate with both the menstrual cycle and through the midtrimester of pregnancy, NK cells appear to be innate immune modulators in reproduction [10, 77]. NK cells also contribute to the Th2 cytokine condition by a decreased IFN-γ production in pregnancy [64, 78].

Moreover, several molecules associated with innate immunity were found altered during pregnancy. The serum concentrations of complement factor mannose-binding lectin (MBL), mainly produced in the liver, are increased from early pregnancy onwards, resulting in increased activity of the entire MBL pathway [79]. The role of MBL during pregnancy could reside in their ability to bind to apoptotic cells to enhance their opsonization and subsequent removal [79]. The long pentraxin 3 (PTX3), particularly involved in host defense against microbes, is expressed at the maternal-fetal interface, and the circulating levels of PTX3 steadily rise during normal gestation and peak during labor [80-82]. After binding to apoptotic cells, PTX3 edits the cross-presentation of epitopes expressed by the apoptotic cells to T lymphocytes [83]. Elevated levels of macrophage migration inhibitory factor (MIF) were found both locally and systemically during pregnancy [84]. MIF, an inflammatory cytokine, is a pivotal regulator of both innate and adaptive immunity, but especially of innate immunity. Interestingly, hCG can increase MIF synthesis and secretion by human endometrial cells [85].

1.2.2.3 State of adaptive immunity in pregnancy

In section **1.2.2.1** it has been discussed that there is no general suppression of maternal systemic immune function during pregnancy. Apparently, the maternal tolerance to the fetal allograft enables the fetus to develop well and survive. The primary fetal tissue in contact with the maternal immune system is the placenta with alloantigens expressed on trophoblasts. The cooperation of several mechanisms, including IDO-induced T cell anergy, HLA-G-regulated T cell inhibition, FasL-mediated T cell apoptosis, potent Treg cell function and an emerging role for DC, as well as hormones, modifies the maternal immune response at the maternal-fetal interface [66, 67, 70-73, 86]. The humoral immunity seems to be similar in pregnant and non-pregnant women (Table 2).

Taken together, it can be hypothesized that the mother is protected from infection during pregnancy by an increased innate immunity involving monocytes, Mφ, and maybe PMN. This process may be regulated by NK cells. The adaptive immunity against the fetal tissue is in a tolerogenic mode likely due to increased Treg cells and tolerogenic DC activity. Therefore, a strong systemic innate and a dampened specific adaptive immunity, or to say a shift from adaptive to innate immunity, seems to be characteristic for pregnancy.

Table 2. Overview of maternal immunity during pregnancy and the effect of hCG.

Immune system component	Alteration in (early) pregnancy	References	hCG effect	References
B cell number	↔	[10]	↔	[87]
IgM, IgG, IgA	↔	[10]	↓antibody-formation	[88, 89]
T cell number and subsets	total: ↔ $CD4^+$: ↔ $CD8^+$: ↔ Treg: ↑ γ/δ/ T: ↑	[10] [10] [10] [66] [90]	total: ↔ $CD4^+$: ↔ $CD8^+$: ↔ Treg: ↔	[87]
(Local) T cell activation*	↓	[66-68, 70, 73]	↓ & Th2 skewing	[70, 88, 91-93] [94]
Antibody-dependent cellular cytotoxicity (ADCC)	↔	[10]	↓	[95]
(Local) DC number*	↑	[86]	unknown	-
(Local) DC function*	↓	[86]	↓ ↑IDO	[70] [70]
NK cell number	↑	[64]	↑	[87]
(Local) NK cell activation	↓ & Th2 bias	[10] [64]	unknown	-
PMN number	↑	[96] & unpublished data**	↑	[87, 97-99]
PMN phagocytosis	↓	[100, 101]	unknown	-
	↑	[102] & unpublished data**		
Monocytes number	↑	[96] & unpublished data**	↑	[87, 99]
Monocytes activation	↑	[76]	↓ ↓IL-2, ↑IL-6, ↑IL-1β, ↑TNFα	[103] [104]
(Local) Mφ number*	↑	[86]	↑***	[105]
Mφ phagocytosis	↑	[86, 106]	↑NO ↑ROS ↑phagocytosis	[107, 108] [109****] [109****]
Complement activation	↓	[72]	unknown	-

* Local: in uterus and decidua, % and/or absolute number.
** unpublished data from our group.
*** hCG can also increase Mφ number in male testes [110, 111].
**** Described in chapter 3 of this thesis.

1.2.2.4 Features of mononuclear phagocytic cells in pregnancy

The systemic mononuclear phagocytic cells are mainly monocytes, as well as a small proportion of Mφ and DC. Earlier, Sacks *et al.* found that monocytes from normal pregnant women are primed to produce IL-12 but not TNF-α. They further suggested that placental cellular products act systemically to prime and activate monocytes and other cells of the innate immune system [78]. It has been reported that during pregnancy monocyte numbers increase in the peripheral blood [96] and that these cells progressively upregulate the surface markers CD11α, CD54, and CD64 as well as the production of IL-12 and IL-1β compared with the nonpregnant state [76]. In return, IL-12 enhances monocyte activation and phagocytosis [76]. Such a pattern of monocyte activation parallels the increase in placental mass. These findings demonstrate the activation of maternal monocytes during pregnancy and further support the notion that pregnancy results in an elevation of the innate immune system.

It is now well established that immunological recognition of the fetal alloantigens during pregnancy is important for the maintenance of gestation. As mediators of the first encounter with fetal alloantigens, decidual antigen-presenting cells (APC) are involved in the fetal-maternal immune adjustment. In normal pregnancy, an appropriate balance between pro-inflammatory (Th1) and anti-inflammatory (Th2) cytokines is thought to be an important secondary effect for determining pregnancy outcome [56, 112]. Several lines of evidence point to a pivotal role of decidual APC in shaping the cytokine profile towards the establishment of a tolerogenic microenvironment at the maternal–fetal interface. Maternal APC (Mφ and DC) are scattered throughout the decidualized endometrium during all stages of pregnancy (see also Table 2).

Decidual macrophages (dMφ) are BM-derived cells constituting the majority of myeloid cells in human and mouse decidua. Their number increases in the uterus in early pregnancy [86], which is due to pregnancy hormones and the presence of cytokines and chemokines. Estrogen induces the influx of Mφ, but progesterone inhibits the ability of estrogen to recruit Mφ into the uterus [113]. Increased expression of the chemokines CCL2, CCL3, CCL5 and M-CSF has been found in the uterus of pregnant mice regulating the chemotaxis of Mφ [114]. At the maternal–fetal interface, the MIF level also increases, leading to the accumulation of Mφ at this interface. On the one hand, dMφ express low HLA-DR and CD86 compared with peripheral blood $CD14^+$ cells of pregnant and non-pregnant women, which suggests less efficient antigen presentation within the decidua [115]. dMφ are found to be Th2-polarized, resulting in impaired Th1 response [86]. On the other hand, effective scavenger receptor-mediated phagocytosis and destruction of trophoblast cells in the mouse by uterine Mφ facilitates the resolution of the inflammatory condition [114]. An important aspect in this process is the establishment of an adequate microenvironment that allows protection against infection, while at the same time cell growth is promoted and hazardous inflammatory immune reactions are inhibited.

Mature (CD83+) DC (mDC) and more recently, a population of immature ($CD83^-$) Mφ/DC-like cells have been described in human decidualized endometrium [116]. Some of these $CD83^-$ immature cells express high levels of the C-type lectin DC-SIGN [117]. Because human endometrium appears to harbor $DC\text{-}SIGN^+$ cells only during decidualization, these cells might play a crucial role in the local balance between immunity and tolerance. In line with this is the observation that in early human pregnancy decidual DC show a reduced capacity to stimulate allogeneic $CD3^+$ T cell proliferation [116] and a reduced capacity to produce IL-12 p70 [118] in

comparison with mature peripheral blood monocyte-derived DC. In a comparable study in mice, it has been shown that the relative numbers of $CD11c^+$ cells are markedly increased in the pregnant uterus compared with uterus from mice that were not mated [119]. The majority of murine decidual DC that belong to the myeloid ($CD8\alpha^-$) lineage exhibit mainly an immature phenotype and express higher levels of IL-10 than of IL-12 [119, 120]. This suggests that DC at the maternal–fetal interface appear to express a more tolerogenic phenotype. Furthermore, decidual DC regulate the activity of the NK cell population present in the interface [86].

Interestingly, it has been shown that DC function and phenotype can be influenced by several decidual factors, including cytokines such as IL-10 and hormones. IL-10 has been shown to inhibit DC-mediated stimulation of T cells *in vitro*. Progesterone favors DC maturation in mice and humans and could direct a Th2 skewing [118]. In rodents, exogenous estrogen and hCG inhibit DC to stimulate T cell proliferation and support the maturation of DC with tolerogenic properties by upregulating IDO [70, 121]. Additionally non-classical human leukocyte antigen HLA-G and HLA-E molecules may modulate DC cytokine secretion. HLA-G has been found to promote the development of tolerogenic monocyte-derived DC *in vitro* [122]. Surprisingly, decidual $CD83^+$ mDC have been found in decidual lymphatic vessels on their way to draining lymph nodes [116], and mDC accumulate in deciduas of women with recurrent miscarriages [123], suggesting once triggered, these decidual mDC can carry fetal antigen to the regional lymph nodes and there present it to T cells that, once activated, may induce rejection.

The biological plasticity and unique features of peripheral monocytes, decidual Mφ, and decidual DC make them important regulators of the immune system during pregnancy. This modulation would assure effective immunity to protect the mother from pathogens and suppress specific immune responses to tolerate the semi-allogenic conceptus, further supporting the idea that pregnancy results in enforcement of the innate immune system and suppression of the specific adaptive immune system.

1.3 HUMAN CHORIONIC GONADOTROPIN

1.3.1 Introduction

Human chorionic gonadotropin (hCG) was first described by Hirose, and Ascheim and Zondeck more than 80 years ago [124, 125]. hCG is a member of the pituitary glycoprotein hormone (GPH) family, which also includes luteinizing hormone (LH), follicle stimulating hormone (FSH), and thyroid stimulating hormone (TSH). These hormones possess similar heterodimer structures consisting of an identical α-subunit and a unique β-subunit that confers biological specificity. There are significant amino acid sequence homologies among the β-subunits of TSH, hCG, LH and FSH [126, 127].

hCG is mainly secreted during pregnancy by normal trophoblasts of the placenta, and can be found in urine and blood. The primary function of hCG is to encourage the corpus luteum to produce progesterone and estrogen during the early stage of pregnancy. It also stimulates the development of fetal testes [36, 128]. An important role of hCG in immune regulation has been suggested by the relationship between the production of hCG and autoimmune diseases, abortion, and carcinoma, and the still experimental treatment of tumors using anti-β-hCG [129].

1.3.2 The biological structure and source of hCG

The intact hCG molecule consists of an α- and a β-subunit, and has a molecular weight of 36.5 kD. The two subunits of hCG are held together in a non-covalent manner by ionic and hydrophobic forces. The α-subunit is a glycopeptide of 92 amino acids, with two (N-linked) oligosaccharide side chains attached at asparagines (Asn) 52 and 78, stabilized by five disulfide bonds at cysteine (Cys) residues Cys10-60, Cys28-82, Cys32-84, Cys7-31, and Cys59-87 [130]. The β-subunit of hCG is a glycopeptide of 145 amino acids, stabilized by six disulfide bridges at Cys9-7, Cys34-88, Cys38-90, Cys23-72, Cys26-110 and Cys93-100 [131]. It has N-linked sugar moieties attached at Asn 13 and 30 and four serine-linked (O-linked) oligosaccharide units located at serine (Ser) 121, 127, 132 and 138, which principally distinguishes the β-unit of hCG from other GPH and is called the β-carboxy terminal peptides (β-CTP) [126, 127, 132, 133]. These eight N- and O-linked sugar sites account for as much as 35% of the molecular weight. A simplified structure of hCG and other GPH is illustrated in Figure 4A. In addition to intact hCG, several variants of hCG are present in serum, urine and other body fluids, both during pregnancy and in disease conditions. These include free α-subunit, hyperglycosylated or carbohydrate variant hCG, free β-subunit, nicked hCG, β-core fragment from nicked hCG, β-CTP cleaved off from nicked hCG and the remnants from this cleavage. The free hCG subunits and free subunit-degraded products, and the combination of multiple subunits and multiple N-linked and O-linked oligosaccharide side chains cause significant heterogeneity in the circulating hCG [128, 133] (Table 3). These structural features, together with the observation that the individual subunits are inactive, suggest that the variant fractions derived from the subunits are important for hCG function [128].

hCG gene sequences were identified in the 1980s. A single-gene CGA on chromosome 6 encodes the α-subunit protein of hCG. The β-subunit of hCG is encoded by six highly homologous and structurally similar genes CGB1, CGB2, CGB3, CGB5, CGB7, and CGB8. These are arranged in tandem and inverted pairs on chromosome 19q13.3 and are contiguous with the luteinizing hormone beta (LHB, also known as CGB4) subunit gene. The genes for the second human homologue of the bacterial RuvB and neurotrophin 5 demarcate the CGB/LHB gene cluster at either end, with the genes for neurotrophin 6 interspersed between them. This region spans about 77 kb [134, 135] (Figure 4 B).

Approximately 8-11 days after conception, intact hCG becomes detectable in the blood serum (≥25 IU/l). The level increases until reaching a peak level of 150,000-250,000 IU/l at 8-10 weeks after the last menstrual period. At this stage, there is a large variation in individual hCG concentrations, with extremes of approximately 5,000 to 300,000 IU/l. The concentration then declines to a stable level of about 12,000 IU/l in the remaining second and third trimesters of pregnancy, although, again, wide variations are found among individuals. hCG levels are 30 to 50% higher in twins and multiple gestations [136].

Apart from pregnancy, hCG can also be found under other normal and abnormal circumstances. Prior to evacuation of a hydatidiform mole, intact hCG may be as high as 2,000,000 IU/l [137]; the median serum intact hCG and free β-subunit level in Down syndrome pregnancy (trisomy 21 fetus) is twice the normal level [138]; nicked hCG, hyperglycosylated hCG and free β-subunit in the serum and urine of individuals with post-molar or post-gestational choriocarcinoma are extremely high [139]. Variable levels of hCG,

Table 3. Structure of the hCG-related molecules.

Main hCG-related molecules	Molecular weight (Dalton)	Structure	
		α-subunit	β-subunit
Intact hCG	~36,500	92 amino acids with 2 mono- and biantennary N-linked sugar sites	145 amino acids with 2 mono- and biantennary N-linked and 4 mostly trisaccharide O-linked sugar sites
Free α-subunit	~14,500	92 amino acids with 2 mono- and biantennary N-linked sugar sites	no β-subunit
Free β-subunit	~22,000	no α-subunit	145 amino acids with 2 mono- and biantennary N-linked and 4 mostly trisaccharide O-linked sugar sites
Hyperglycosylated/ carbo-hydrate variant hCG	~40,000	92 amino acids with extra fucose on the 2 N-linked sugar sites	145 amino acids with 2 mostly triantennary N-linked and 4 mostly hexasaccharide O-linked sugar sites
Nicked hCG (unstable)	~36,500	92 amino acids with 2 mono- and biantennary N-linked sugar sites	145 amino acids with 2 mono- and biantennary N-linked and 4 mostly trisaccharide O-linked sugar sites, cut at residues 47-48, 43-44 or 44-45
β-CTP	~7,500	no α-subunit	(degraded β-subunit) β-CTP of residues 93-145 or partly with no N-linked and 4 degraded O-linked sugar sites
The remnant of β-CTP cleaved off from hCG	~29,000	92 amino acids with 2 mono- and biantennary N-linked sugar sites	92-122 amino acids with 2 mono- and biantennary N-linked and 4 mostly trisaccharide O-linked sugar sites, nicked at residues 47-48, 43-44 or 44-45 and β-CTP cleaved off or partly
β-core fragment	~9,500	no α-subunit	(degraded β-subunit) consisting of 2 peptides residues 6-40 and 55-92 with degraded biantennary N-linked and no O-linked sugar sites

mostly intact hCG, may be present in serum and urine samples taken from individuals with persistent trophoblastic disease (dependent on the amount of trophoblast tissue) [140]. Non-trophoblastic cancer cells, germ cell tumors (dysgerminoma), bladder cancer, ovarian cancer and certain other malignancies may generate a small amount of hCG α- and β-subunits [141]. Gonadotroph cells of pituitary normally produce a miniscule amount of hCG (up to 20 IU/l) and hCG β-core fragment (<0.5 IU/l) [142]. Aberrantly low hCG levels can be detected in women with extrauterine or ectopic pregnancy or miscarriage [143].

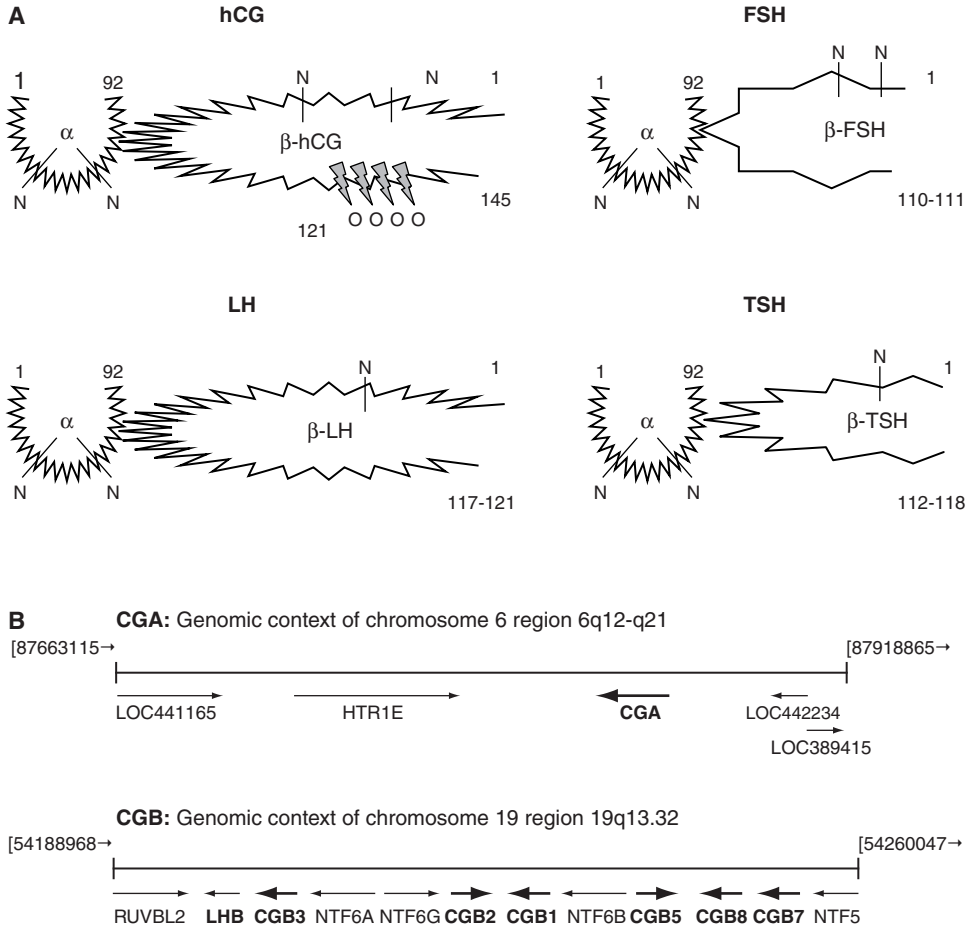

Figure 4.
A. Simplified structure of hCG, LH, TSH and FSH. These hormones have an identical α-subunit which is a glycopeptide of 92 amino acids, with (N-linked) carbohydrates attached at Asn 52 and 78. Their β-subunits are different, consisting of 145, 117-121, 110-111, and 112-118 amino acids, respectively. The β-subunit of hCG has N-linked sugar moieties attached at Asn13 and Asn30 and four serine-linked (O-linked) oligosaccharide units located at residues 121-145, which principally distinguish the β-unit of hCG from other GPH and is called β-carboxy terminal peptides (β-CTP). **B. Gene sequences of α- and β-subunits of hCG.** A single gene CGA on chromosome 6 encodes the α-subunit protein. The β-subunit of hCG is encoded by a region that spans about 77 kb on chromosome 19q13.32.

It has also been reported that intact hCG and free β-hCG are significantly lower than normal in women with a trisomy 18 fetus [138]. Recombinant hCG was synthesized several years ago, and is mainly used therapeutically in reproductive medicine.

1.3.3 Physiology of hCG

The essential role of hCG is supported by the observation that administration of hCG antisera leads to termination of pregnancy [144], and that the risk of miscarriage is correlated with a low hCG level [37]. Although the physiological role of hCG throughout human pregnancy is not fully defined, it is clear that the primary role of hCG in early pregnancy is to extend the functional life of the corpus luteum and maintain pregnancy [128, 145]. A variety of cells bear hCG receptors. For example, through its receptors on the endometrial cells, hCG may promote smooth muscle relaxation and myometrial vasodilation, thus helping the implantation of the embryo; via its receptors on trophoblast cells, hCG can stimulate progesterone production; and hCG stimulates cytotrophoblasts to secrete vascular endothelial growth factor (VEGF) indicating a role in early placental angiogenesis [128].

1.3.4 The immunosuppressive role of hCG in pregnancy, cancer and autoimmune diseases

Apart from its endocrinological role, hCG has been suggested to influence the immune system. Three main fields reveal the immunological role of hCG, and in some cases they are related with each other. These topics are maternal-fetal tolerance, cancer development and autoimmune diseases.

1.3.4.1 hCG and the regulation of maternal-fetal tolerance

An important feature of the immune system is to distinguish between self and non-self antigens. In the majority of the cases, fetal rejection by the maternal immune system is prevented, and the implanted embryo (semi-allograft) is allowed to survive, develop and grow. The embryo is comprised of two groups of cells: fetal cells and trophoblasts. Genetically, trophoblastic cells are of semi-paternal origin and they are the only part of the embryo to directly interact with the maternal immune system [146, 147]. This suggests that the trophoblasts send some kind of "signal" to the maternal side to develop specific immunosuppression against the fetus. Because trophoblasts during early pregnancy secrete hCG, hCG may be crucial in this process.

It has been shown that hCG has an inhibitory effect on cell-mediated immunity *in vivo* and *in vitro* [148]. By breaking down immunological tolerance to hCG via developing anti-hCG autoantibodies in the recipients, vaccination against hCG was successful to prevent pregnancy in clinical trials [149]. Detailed studies in animals showed that vaccination against the hCG β-subunit greatly increased the autoimmune response to hCG [150]. Moreover, hCG induces IDO expression that promotes maternal-fetal tolerance via inhibition of T cell proliferation and complement activation [70, 72]. So, during pregnancy, the adaptive immune reaction is decreased, and the increased levels of hCG contribute to this, whereas on the other hand hCG can increase the function of Mφ implying that the innate immunity is increased to prevent the infection of the mother [107]. Finally, pregnancy is characterized by a Th2 cytokine environment [151, 152]. A negative correlation was found between the Th1:Th2 ratio and serum hCG levels (Figure 5), suggesting that hCG contributes to the secretion of Th2 type cytokines. Taken together, the profound effect of hCG on leukocytes and the hCG receptor gene expression in leukocytes from pregnant women [153-157] suggest that hCG has a key role in the regulation of maternal-fetal tolerance in collaboration with a network of cytokines and leukocytes at the maternal-fetal interface.

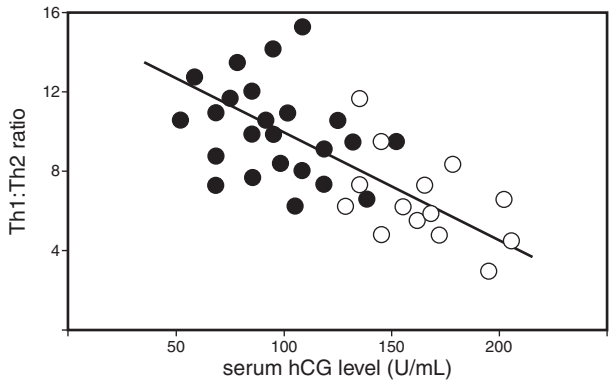

Figure 5. Relation between the Th1:Th2 ratio and serum hCG levels (IU/ml) in singleton (closed circles) and twin pregnant women (open circles). r^2=0.41, p<0.05 (adapted from *S. Suzuki et al.* [94])

1.3.4.2 hCG and its role in cancer development

hCG may facilitate the progression of several tumors in humans. In the early 1970s it was first observed that tumors could secrete hCG [158, 159]. Later on, more and more studies reported that, without pregnancy, elevated serum hCG levels were found in patients suffering from several cancers such as renal, bladder, colorectal, and pancreatic carcinomas [160]. Furthermore, many human cancer cell lines of different types and origins were found to bear cell-surface hCG or its fragments. It was proposed that hCG may act at several different levels to facilitate cancer progression, as a transforming growth factor, an inducer of metastasis, an angiogenic factor, and an immunosuppressive agent [160]. The immunosuppressive function of hCG secreted by tumor cells would be essential to suppress the immune system to be non-reactive to the tumor cells [161]. Because hCG is an established tumor-associated antigen that normal or benign cells do not express without pregnancy, measurement of hCG in serum has become a basic but prognostic indicator for cancer diagnostics and cancer progression. Vaccines based on hCG have been proposed as a means to control cancer progression besides controlling fertility [162-166]. Kalantarov *et al* showed that a vaccine based on a synthetic monoclonal antibody reactive to the 37 amino acid sequence at 109-145 of the carboxy-terminal peptide (CTP) of hCG β-subunit exerted dose-dependent cytotoxic activity in cancer cells [167]. Another study, using a construct containing a β-subunit expressing cDNA, showed that vaccination with this construct elicited CTL responses and protected against tumor formation in mice [168]. More recently, researchers started DC-targeted cancer vaccination that induced cellular immune responses to hCG [169]. Originally, anti-hCG vaccination was created for fertility control by Stevens *et al.*, while it was approved by the World Health Organization (WHO) [170]. It is now utilized more and more in cancer treatment, where anti-hCG vaccines can break down immunological tolerance induced by hCG. The potential of such vaccines against hCG in tumor therapy needs further investigation.

1.3.4.3 hCG and autoimmune diseases

The observation that many autoimmune diseases remit during pregnancy and recur after childbirth due to changes in both the hormonal and cellular immune system led to the idea that pregnancy-related factors such as hCG may have an influence on autoimmune diseases [171, 172]. To study the relationship between autoimmune diseases and hCG, many experiments, both *in vivo* and *in vitro*, have been performed over the last thirty years. The thyroid is a common target of autoimmunity. The high female preponderance of autoimmune thyroid disease (AITD) and the high prevalence of AITD in women after childbearing, but interestingly, improved symptoms of AITD during pregnancy, suggest that pregnancy-associated factors have a strong influence on the maternal immune system and AITD. Meanwhile, the dynamics of hCG and TSH create an inverse image in normal pregnancy. When hCG is at its greatest concentration in the first trimester of pregnancy, serum TSH concentration drops. Afterwards, hCG decreases while TSH increases, forming an opposite kinetics of these two hormones [173]. Under pathological conditions, in which hCG concentrations are markedly higher than in normal pregnancy hCG significantly induces thyroid stimulation [174]. hCG and TSH β-subunits share 85% sequence homology in the first 114 amino acids and contain 12 cysteine residues at highly conserved positions. Therefore, it is likely that their tertiary structures are quite similar and that hCG can mimic the role of TSH [174]. The above-mentioned features of hCG illustrate the two sides of hCG: in a normal pregnancy, hCG levels are accompanied with opposite TSH levels and ameliorated AITD symptoms, but when the hCG levels are too high, thyroid stimulation may occur.

In animal models for type I diabetes, Sjögren's Syndrome, and rheumatoid arthritis (RA), hCG showed direct effects. Non-obese diabetic (NOD) mice are a model for spontaneous type I diabetes and Sjögren's Syndrome. Upon injection with hCG, NOD mice showed postponed onset of diabetes and decreased inflammatory infiltration of pancreatic tissue. Treatment with hCG also resulted in suppressed IFN-γ production, but increased IL-10 and TGF-β production. The transfer of splenocytes from hCG-treated NOD mice into immunocompromised NOD. SCID mice inhibited the development of diabetes [70, 175]. Treatment with hCG prevented Sjögren's syndrome-like exocrinopathy in NOD mice, including decreased lymphocytic infiltration and parenchymal cell damage in the submandibular salivary glands, decreased IFN-γ, TNF-α, IL-1β, IL-10, NO synthase, and MMP9, but caused a marked increase in the salivary flow rate compared with PBS-treated mice [176]. In streptococcal cell wall-induced arthritis in rats, systemic administration of hCG caused a dose-dependent reduction in the clinical arthritis index, and significantly reduced inflammatory cell infiltration, pannus formation, bone and cartilage degradation, and suppressed the production of TNF-α, IL-6, and IL-1β [177]. These findings indicate that hCG exerts a protective effect in these experimental models through the modulation of inflammatory mediators.

Surprisingly, in a guinea pig model of multiple sclerosis (MS), hCG treatment had only marginal effects on the clinical score of disease [178]. Studies of Systemic Lupus Erythematosus (SLE) showed that without pregnancy hCG levels were elevated in sera from SLE patients (both females and males) compared with controls [179]. This hCG was bioactive *in vitro* [180], but the patients response to hCG is low [181]. Therefore, it may be that hCG plays a different role in different types of autoimmune diseases with different underlying mechanisms.

1.3.5 hCG receptors and the downstream pathway

Protein hormones like hCG and LH weigh more than 5,000 Dalton, and therefore cannot act on cells without using membrane receptors. LH and hCG share a common receptor α-chain, transcribed from a single gene on chromosome 2 [182]. Genetic analysis has revealed that exon 10 of the LH receptor could be responsible for discriminating between the LH and the hCG receptor [128]. A variety of tissues in the reproductive system express this receptor, including cytotrophoblasts, umbilical vessels, endometrium, myometrium, fallopian tube, uterine cervix, granulosa cells, and the sperm. Other tissues can also express LH/hCG receptors, such as brain, hippocampus, skin, breast, and urinary bladder. There is no species specificity in the LH/hCG receptor distribution [128, 183, 184]. Recently, Mφ and DC were found to express LH/hCG receptors, suggesting a possible role of hCG in modulating the function of these cells [156, 157]. Overexpression of the LH/hCG receptor has been observed in abnormalities, like choriocarcinoma and trisomy 21 pregnancy [128, 185].

The signaling pathway of the hCG receptor is not completely clear, but the work done by Aggarwal *et al.* on the pathways involved is a breakthrough [186, 187]. Several points were made clear: 1) hCG binding to the hCG receptor can increase cyclic-AMP (cAMP) levels and PKA activity; 2) hCG can inhibit TNF-induced activation of NF-κB, AP-1, JNK, and MEK; 3) dibutyryl cAMP, which can activate PKA and mimic hCG, can prevent TNF-induced NF-κB activation; and 4) dideoxyadenosine, an adenylate cyclase inhibitor, can prevent hCG from inhibiting TNF-induced NF-κB activation (Figures 6 and 7). Fujii S *et al.* further showed that hCG can activate monocytes to produce IL-8 by inducing NF-κB translocation into the nucleus via an alternative pathway instead of the hCG receptor pathway [188]. Another mechanism involved in hCG signaling is based on the Fas and FasL system. Fas, also called CD95, is a TNF-receptor family member. FasL also belongs to the TNF superfamily and is expressed predominantly on activated T cells, Mφ and neutrophils. Ligation of Fas-FasL results in apoptosis. By modulating the Fas-FasL system, hCG can initiate a cascade of the apoptotic pathway that eliminates lymphocytes and suppresses the immune response [93]. Apparently hCG can interact with different intracellular signaling pathways in a variety of immune cell types.

1.3.6 hCG and its cytokine network - endocrinology versus immunology

The Th1 cytokine environment is associated with increased production of IL-2, IL-12, and IFN-γ, and is predominant in cell-mediated immune responses. Th2 type responses are characterized by the secretion of IL-4, IL-5, IL-6, IL-10, and IL-13, and are mainly responsible for humoral immune reactions. The Th1 derived IFN-γ induces Mφ to produce TNF-α and IL-12, which stimulate Th1 cells further and inhibit Th2 cell proliferation. In contrast, IL-10 is able to reduce the synthesis of Th1 cytokines [189].

Pregnancy is characterized by a systemically shifted ratio towards Th2 environment. However, not only the Th2 cytokine levels increase, also raised Th1 cytokine levels were detected in comparison with the non-pregnant state [64]. If the Th1/Th2 balance does not shift towards the Th2 condition when women become pregnant, abortion can occur [61, 151, 152, 190]. Several hormones have been suggested to influence this shift. Steroids promote Th2 responses and inhibited pro-inflammatory cytokines such as TNF-α, IL-1β, IL-6, IL-8, IL-12, and IFN-γ [191]. Estrogens at high levels can stimulate IL-6 production by the placenta itself or by Mφ. Via induction of IL-6, estrogens can facilitate the differentiation of Th0 to Th2

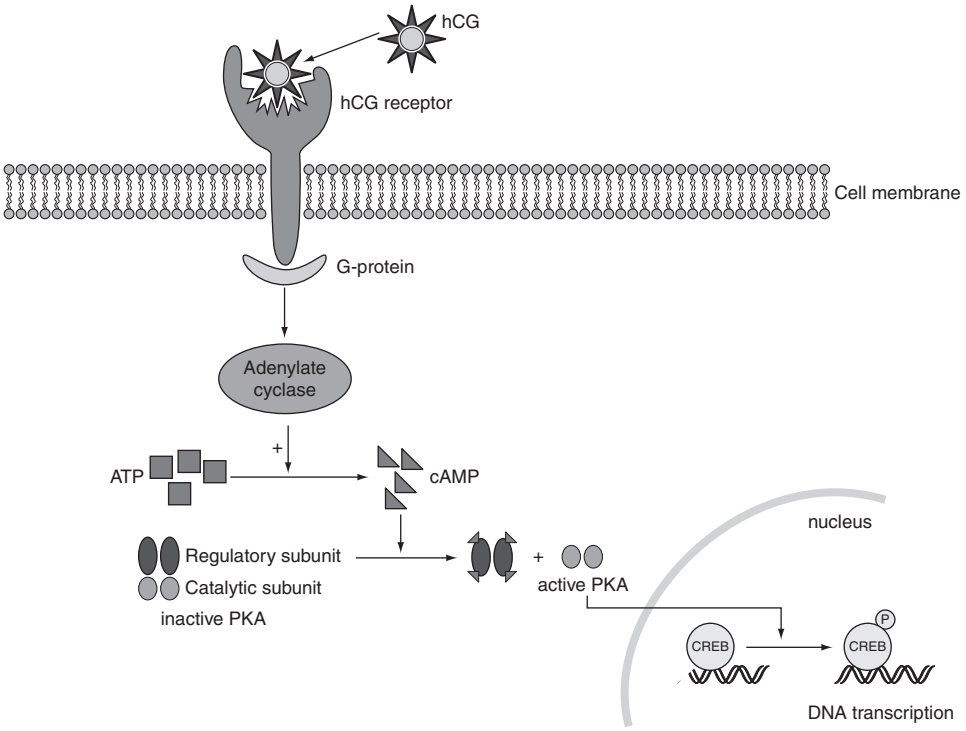

Figure 6. The hCG-receptor induced activation of the cAMP-dependent protein kinase (PKA) pathway.
hCG binds to the G-protein coupled hCG receptor and activates adenylate cyclase which converts (cAMP). PKA consists of two catalytic subunits and two regulatory subunits, and cAMP binds to two sites on each regulatory subunit. Binding of four cAMP molecules causes the release of free and active catalytic subunits, which may phosphorylate serine and threonine residues on target proteins. In this figure, the active subunit phosphorylates a CREB protein, resulting in transcription.

lymphocytes [192]. Progesterone has been shown to induce the production of "progesterone-induced blocking factor" (PIBF), which stimulates the IL-4 and IL-10 secretion by activated T cells, whereas the IFN-γ production is inhibited [193]. Thus, when hCG stimulates progesterone and estrogen secretion by the corpus luteum, it can regulate the maternal immune response. Next to this indirect way, hCG is also able to regulate the immune system directly.

In vitro and *in vivo* hCG treatment can influence the cytokine production, which correlates with the changes in cytokine expression at the maternal-fetal interface during pregnancy (Table 4). The complete and detailed effects of hCG on cytokine production need further investigation. For example, hCG can decrease the IFN-γ production [175], and thereby decrease antigen presentation. hCG also inhibits the release of IL-2 and simultaneously stimulates the secretion of sIL-2R, suggesting that hCG can stop triggering T cell proliferation by interfering with the interaction of IL-2 with its specific receptor [194].

In contrast, generally anti-inflammatory cytokines such as IL-6 and IL-10 increase after administration of hCG. IL-6 plays a crucial role in both local and systemic acute inflammatory

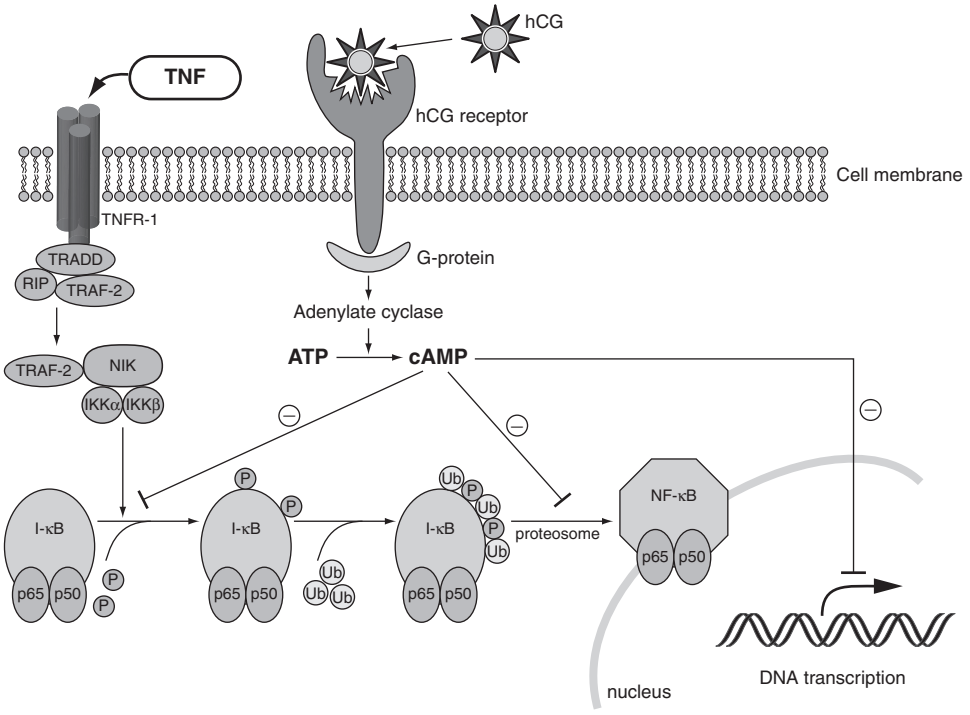

Figure 7. The effect of hCG on the TNF-induced NF-κB pathway.
TNF binding to a p60 receptor induces receptor trimerization. TNF receptor-associated death domain (TRADD) is recruited to the receptor complex, followed by TNF receptor associated factor 2 (TRAF2) recruitment, which associates/activates NF-κB inducing kinase (NIK). Downstream events of NIK involve NIK mediated phosphorylation and activation of a protein kinase complex constituting dimers of IκB kinase (IKK). In the resting cell, NF-κB is found in the cytoplasm as a two subunits (p50 and p65) protein complex binding to the inhibitory κB (IκB) proteins. IKK phosphorylates (P) IκB, which in turn leads to ubiquitination (Ub), then leads to proteasome-dependent degradation of IκB and subsequent liberation of NF-κB in its transcriptionally active form. Liberated NF-κB translocates to the nucleus where it, upon kinase (e.g. ERK and p38) mediated phosphorylation, obtains transcriptional capacity that regulates transcription of the selected genes. hCG, likely mediated through cAMP, can block TNF-induced activation of NF-κB, IκBα degradation, and NF-κB-dependent reporter gene transcription.

responses by controlling the level of pro-inflammatory cytokines, but not of anti-inflammatory cytokines. This promotes the differentiation of Th0 into Th2 cells [11]. IL-10 selectively suppresses Th1-mediated cellular immunity by inhibiting synthesis of Th1 cytokines, such as IFN-γ, TNF-α, and IL-1β. Furthermore, IL-10 suppresses the proliferation of Th1 cells and thereby facilitates a Th2 response [195]. Interestingly, hCG increases the production of the pro-inflammatory cytokine IL-8. This may be due to the hCG-stimulated Th2 activity, as Th2 cytokines like IL-4 and IL-13 can upregulate the production of IL-8 [196].

hCG has also been shown to influence cell growth and VEGF production. The combination of hCG and VEGF protects cells from apoptosis, which is required for fetal development [197, 198].

While hCG can modulate cytokine levels, in return, several cytokines can stimulate the release of hCG from trophoblasts, e.g. IL-1, TNF-α, and epidermal growth factor (EGF) [199-202], but also the Th2-derived cytokines IL-4 and IL-6 [190]. Therefore, hCG can skew the immune system towards a Th2 state by decreasing Th1 cytokines such as IL-2 and IFN-γ and increasing Th2 cytokines such as IL-6, IL-8, and IL-10.

1.3.7 The effect of hCG on mononuclear phagocytic cells

Theoretically, hCG can affect all cells expressing hCG receptors. As stated before, except cells from the reproductive system, hCG also influences cells of the immune system. The effect of hCG on mononuclear phagocytic cells, including DC, Mφ, and monocytes has been extensively studied (Tables 2 and 4).

Peripheral blood mononuclear cells (PBMC) comprise both monocytes and lymphocytes. It was observed that not only hCG but also PBMC obtained from women early in pregnancy can promote progesterone production by luteal cells, suggesting that PBMC may be the target of hCG [188]. In 1973, crude hCG purified from urine of pregnant women was reported to suppress immune reactions [224]. Subsequent studies demonstrated that contaminating factors other than hCG were responsible for these effects, as highly purified hCG had no effect on the function of the lymphocytes [95, 225, 226]. This suggested two possibilities: 1) breakdown products from hCG have an immunoregulatory effect; or 2) hCG does not directly affect lymphocytes, but can influence the immune system indirectly, for example, via modulating the function of monocyte including their cytokine production. The interaction between hCG and monocytes can lead to several effects.

a. hCG was found to be a potent attractor of monocytes *in vitro*. Treatment i.p. of rats with hCG induced migration of mainly monocytes into the interstitial space of the testes [99].
b. Peripheral $CD14^+$ monocytes (as well as NK cells) can secrete hCG. This hCG production can be stimulated by IL-1β, IL-4, IL-6, IL-10, TNF-α, and GM-CSF, and inhibited by IL-2 and IFN-γ [227]. IL-2 also inhibited the hCG-induced cAMP pathway [228].
c. hCG can stimulate monocytes to release IL-6, IL-1β and TNF-α in a dose-dependent manner [104, 229], but hCG inhibits the IL-2 release by monocytes [104, 194, 230].
d. hCG increases the IL-8 production by monocytes. Culture of PBMC from non-pregnant women in the presence of hCG dose-dependently increased the IL-8 production, which was mainly produced by monocytes [188]. The basal IL-8 production by PBMC from women in first trimester of pregnancy was three times elevated compared with nonpregnant women. Interestingly, the stimulation of IL-8 production by hCG was not affected by inhibitors of protein kinases A and C. In contrast, this stimulation was attenuated by D-mannose, which inhibits binding of C-type lectins. This indicates that human monocytes respond to hCG and secrete IL-8 through a pathway different from the hCG receptor-cAMP/PKA signaling [188].
e. hCG modulates monocytes with immunosuppressive properties. hCG clearly inhibits the human peripheral blood monocytes-induced lymphocyte proliferation to phytohaemagglutinin (PHA) and concanavalin A (Con A) [103].
f. hCG has the potential to regulate virus replication. Low doses of hCG have been shown to suppress reverse transcriptase activity in chronically HIV-infected monocytes *in vitro*, thus blocking viral replication and transmission [231]. High doses of hCG that were

Table 4. Cytokine network: pregnancy and hCG effect.

Cytokines	Maternal-fetal interface in 1st trimester	hCG treatment (*in vivo* and *in vitro*)	hCG effect on myeloid cells
IL-1α	↑[10, 190]	unknown	unknown
IL-1β	↔[190]	↑[177, 203-205]	↑[104]
IL-2	↔[190, 206, 207]	↓[208, 209]	↓[104, 194]
(sIL-2R)	unknown	unknown	↑[194]
IL-3	↑[207]	unknown	unknown
IL-4	↑[10, 190, 206, 207]	↓[208, 209]	unknown
IL-5	↑[196, 207]	unknown	unknown
IL-6	↑[10, 190, 210]	↑[177, 211, 212], ↓[213]**	↑[104, 109]
IL-8	↑[10, 190]	↑[205, 214]	↑[188]
IL-10	↑[10, 190, 206, 207, 215]	↑[175, 216]	↑[70, 109, 216]
IL-11	↑[217]	unknown	unknown
IL-12	↑[10, 217]	↔[209, 218]	↔[157]
IL-13	↑[10, 217]	unknown	unknown
IL-15	↑[10, 217, 219]	unknown	unknown
IL-16	↑[217]	unknown	unknown
IL-17	↑[217]	unknown	unknown
IL-18	↑[217]	↑[220]	unknown
IFN-γ	↑[10], ↔[190, 207], ↓[206]*	↓[175, 209, 216]	↓[216]
TNF-α	↑[10, 190]	↑[177, 211, 212], ↓[209, 216]**	↑[104, 157]
TGF-β	↑[10, 190]	↑[177, 216, 221]	↑[216]
GM-CSF	↑[10, 219]	↑[212]	unknown
G-CSF	↑[10, 219]	unknown	unknown
M-CSF	↑[10, 190, 219]	↑[222]	unknown
EGF	↑[190, 219]	↑[223]	unknown
LIF	↑[190, 219]	↑[213, 218, 222]	unknown
SCF	↑[190]	unknown	unknown
VEGF	↑[190, 219]	↑[222]	unknown

*: contradictory data

EGF: epidermal growth factor; M-CSF: macrophage colony stimulating factor; IFN-γ: interferon-gamma; TNF-α: tumor necrosis factor-alpha; TGF-β: transforming growth factor-beta; GM-CSF: granulocyte-macrophage colony stimulating factor; G-CSF: granulocyte colony stimulating factor; M-CSF: macrophage colony stimulating factor; IL: interleukin; LIF: leukaemia inhibitory factor; SCF: stem cell factor; VEGF: vascular endothelial growth factor.

several logs higher than normal levels, however, seemed to increase viral production in monocytes [232]. These data suggest a sensitive hormonal control of the immune response via the modulation of monocyte function.

Mφ are potent participants in innate immunity, by virtue of their properties to phagocytose and to release oxygen species and cytokines [210]. Mφ have also the ability to

secrete hCG, especially villous macrophages [233]. In return, Mφ respond to hCG via their surface hCG receptor [156]. hCG can induce the production of monocyte chemotactic protein-1 (MCP-1), which attracts more Mφ [234]. Administration of hCG also resulted in an increase in Mφ proliferation [235]. hCG alone does not induce NO synthesis, while hCG in combination with IFN-γ increased NO synthesis in cultured peritoneal Mφ and microglia [107, 108]. These data suggest that hCG strengthens the innate function of Mφ. At the start of our study it was not known yet whether hCG influences the phagocytic ability and cytokine production of Mφ that are so crucial for innate immunity. Moreover, the effect of hCG on the role of Mφ in adaptive function needs further study.

The frequency of blood myeloid DC increases in the late stages of pregnancy, while that of lymphoid DC gradually decreases [118, 157]. Human DC at the maternal-fetal interface have semi-mature DC characteristics and can regulate the Th1/Th2 balance to maintain the Th2 dominant state [118]. Recently, it was described that *in vivo* hCG treatment induces IDO expression by splenic DC, which inhibits the activation of both $CD4^+$ and $CD8^+$ T cells [70]. Our department [175] and others [70, 216] further showed that in NOD mice, i.p. administration of hCG prevented diabetes development by upregulating IDO expression by DC and inducing $CD4^+CD25^+$ regulatory T cells in spleen and pancreatic lymph nodes. DC depletion restores the diabetogenic activity of splenic T cells from hCG-treated mice. This IDO upregulation is transient and declines shortly after hCG withdrawal. The inhibition of IDO activity by 1-methyl-tryptophan abrogates the hCG-induced T cell suppression and resistance to type 1 diabetes. These data suggest that hCG can modulate DC function by upregulating IDO expression, and this may play a major role in the pregnancy-associated remittance of several autoimmune diseases [70].

1.3.8 hCG-derived fractions and their immunoregulatory properties

hCG is a large glycoprotein that can break down into fragments and even small peptides during its metabolism. Over the years, the immunosuppressive activity of hCG preparations has been analyzed by a variety of laboratories using different techniques. Often, the immunosuppressive effects of hCG fractions *in vitro* are thought to be due to uncharacterized fragments rather than to the intact hCG molecule [225, 226, 236, 237]. Yoshimura *et al.* fractionated the hCG molecules according to their isoelectric points, with a linear pH gradient from 3.2-6.1. The relative bioactivity/immunoreactivity, represented as the ratio of cAMP/hCG, was significantly higher in basic components (pI 6.1, 6.2; 5.5, 4.4; pI 5.3, 5.8) [237]. Fractions with low gonadotropic activity (i.e. much less than 500 IU/mg) showed a 100-fold inhibition of lymphocyte proliferation [237]. A study showing that purified hCG did not have immunosuppressive activity in castrated mice suggested that the hCG-derived fractions may induce immunosuppression *in vivo* indirectly through the gonads [225, 226]. On the other hand, some other studies clearly demonstrated the immunosuppressive activity of highly purified hCG and recombinant hCG preparations in a murine delayed-type hypersensitivity model *in vivo* [236, 238].

The metabolism of hCG is still unclear. Even in experiments where highly purified hCG is used, contamination with break down products cannot be excluded. Moreover, intact hCG molecules can also be broken down in the assay system. The above reviewed effects of hCG on pregnancy, tumor growth, and autoimmunity might to a smaller or larger extent be due

to fragments of the hCG molecule. The hCG-derived peptide fragments have been hardly examined, even not the ones derived from the nicking of loop 2 of β-hCG, the sequence MTRVLQGVLPALPQ (residues 41-54). *In vivo* this loop is prone to preferential nicking, which may lead to a variety of oligopeptides, such as MTR, MTRV, LQGV etc [239]. An interesting observation is that some parasites and bacteria bear similar sequences in particular proteins, which might be a way to suppress local inflammation to increase survival [239]. Some hCG fractions have been shown to have higher bioactivity (cAMP activity) than others. Therefore, the biological activity of glycoproteins might be strongly influenced not only by variations in the oligosaccharide side-chain composition but also by the nature and the amount of the break down products in these fractions [175, 240].

1.3.9 Conclusion

hCG might influence the Th1/Th2 balance in both normal pregnancy and in disease such as in autoimmune disease. The level of hCG may be used in diagnostics for disease progression besides pregnancy. hCG exerts its influence on the immune system by influencing the expression of a series of cytokines. This is in agreement with the view of Wilczyński and Licht et al, [241, 242]. The specific adaptive immune system is down-regulated during pregnancy, likely due to an effect on mononuclear cells. On the other hand, hCG can increase the function of Mφ implying that the innate immune system is enforced to prevent sickness from infectious disease of the mother. Although the mechanism by which hCG might mediate immune suppression is not clear yet, two pathways have been indicated: the TNF dependent NF-κB pathway and the hCG receptor-cAMP/PKA pathway, but more pathways may be involved. Furthermore, hCG can induce progesterone and estrogen production in early pregnancy, and these two hormones also have an anti-inflammatory function by altering the Th1/Th2 cytokine profile [243, 244]. In short, hCG can directly influence cells by different pathways, and/or indirectly by cytokines, other hormones and its break down products.

1.4 AIM OF THE STUDY

During pregnancy, the maternal immune system is challenged in two ways. On the one hand, the maternal side has to adapt to tolerate the fetal allograft; on the other hand, the maternal immune system has to maintain an efficient immune response to simultaneously protect mother and child against infection. Extensive studies have shown that pregnancy is a controlled state of inflammation with increased levels of both Th1 and Th2 cytokines, but a ratio shift to Th2 side.

Due to the unique condition of pregnancy, pregnancy related factors have been suggested to account for the adaption of the mother's immune system, especially hormones. hCG, also called the pregnancy hormone, is produced by trophoblast cells during pregnancy and stimulates progesterone and estrogen production. By using mouse models, our department and others have shown that hCG can inhibit Th1 cell mediated autoimmune diseases including type I diabetes, Sjögren's Syndrome, and rheumatoid arthritis. Due to the fact that T cell responses are directed by APC, and hCG receptors are expressed by these cells, we decided to study the effect of hCG on monocytes, Mφ, and DC. Not all these mononuclear phagocytic cell

types are potent APC. For instance, monocytes and Mφ are more effective in innate immunity. We hypothesized that hCG can regulate both innate and adaptive immune responses by modulating the function of the mononuclear phagocytic cells. If so, hCG would not only be a beneficial hormone in the endocrinology of pregnancy, but also in the immunology of pregnancy. To test our hypothesis, we aimed:

a. to clarify the effect of hCG on the innate function of monocytes (**Chapter 2**) and Mφ (**Chapter 3**); moreover, the involved hCG receptor/PKA pathway has been studied to delineate the mechanism underlying the hCG-induced effects;
b. to systemically analyze the effect of hCG on DC differentiation and activation (cell surface markers, cytokine production, and the induction of T cell proliferation) and to unravel the involvement of functional IDO (**Chapter 4**); and
c. to study the beneficial role of hCG in an acute inflammation model and to study the possible target cell types of hCG *in vivo* (**Chapter 5**).

In Chapter 6, the General discussion of this thesis, the data obtained are discussed in the context of the literature data, and suggestions are done for further studies in the field.

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Chapter 2

Chorionic gonadotropin upregulates long pentraxin 3 expression in myeloid cells

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ABSTRACT

Pentraxin 3 (PTX3) is an acute phase response protein that initiates innate immunity against diverse microorganisms. It is produced in response to pro-inflammatory stimuli by many cell types, including myeloid cells. Increased serum levels of PTX3 are found in pregnancy, a condition characterized by increased serum levels of the pregnancy hormone human chorionic gonadotropin (hCG). As myeloid cells bear the receptor for hCG, we hypothesized that hCG can promote innate immunity by affecting the PTX3 production by myeloid cells. In this paper, we demonstrate that hCG increases PTX3 expression by human monocytes, mouse dendritic cells (DC), and mouse macrophages (Mφ) *in vitro*. This increased PTX3 expression by hCG is mediated via the PKA-signaling pathway. hCG injection *in vivo* also increases the PTX3 serum levels. This serum PTX3 is mainly produced by blood monocytes. The hCG-induced hormones progesterone and estrogen also increase the PTX3 production by human monocytes. In conclusion, hCG increases innate immunity via induction of PTX3 in myeloid cells.

KEY WORDS

hCG, PTX3, monocytes, Mφ, DC, pregnancy, innate immunity.

INTRODUCTION

Pentraxins are a superfamily of proteins with cyclic pentameric structure, including the short pentraxins C-reactive protein (CRP) and serum amyloid P component (SAP), and prototypic long pentraxin 3 (PTX3). PTX3 is also called TNF-stimulated gene 14 (TSG14) and is highly conserved in evolution. PTX3 is produced mainly by monocytes, macrophages (Mφ) and dendritic cells (DC) as well as epithelial cells and fibroblasts in response to inflammatory signals such as LPS, TNFα and IL-1β [1-3].

The function of PTX3 has not been fully unraveled, but there are suggestions that PTX3 has a dual role: the protection against pathogens and the control of autoimmunity [4]. First, PTX3 behaves as an acute phase response protein that initiates innate immunity against some specific microorganisms. PTX3 levels increased rapidly and dramatically during endotoxic shock, sepsis and other inflammatory and infectious conditions and correlated with the severity of the disease [4, 5]. PTX3 activates the classical complement pathway by binding to immobilized C1q and facilitates extracellular pathogen recognition by Mφ and DC [4]. Moreover, overexpression of PTX3 results in increased resistance, while PTX3 deficiency causes increased susceptibility to specific microorganisms [4-6]. In PTX3 deficient mice, no generalized impairment of host resistance to microbial pathogens is observed.

Second, PTX3 specifically binds to apoptotic cells and inhibits self-antigen recognition by DC. PTX3, however, does not influence the presentation of exogenous soluble antigens, an event required for immunity against extracellular pathogens [7, 8].

These functions of PTX3 are of great importance during pregnancy where PTX3 is expressed in the placenta in amniotic epithelium, chorionic mesoderm, trophoblast terminal villi, and perivascular stroma. PTX3 serum levels were found to rise steadily during normal gestation and peaked during labor [9]. Implantation and trophoblast invasion are characterized by a progressive and continuous induction of apoptosis in the maternal tissue surrounding the fetus [10, 11]. The removal of apoptotic cells is crucial to the resolution of local inflammation and prevents exposure of self-antigens. The above data suggest that PTX3 is an important enhancer of the innate immune system, which is essential in the host protection against infections and maintenance of a successful pregnancy [12].

During pregnancy there are important changes in the production of hormones like human chorionic gonadotropin hormone (hCG). hCG is a placental glycoprotein, mainly secreted by trophoblasts, that peaks around 300 U/ml in blood with wide variations among pregnant individuals [13]. The most well known function of hCG is to induce the production of progesterone (P4) and estrogen (estradiol, E2). The hCG level peaks in the first trimester of pregnancy and then declines to a stable level in the remaining second and third trimesters, while progesterone and estrogen continue to increase till the end of the pregnancy [14-17]. LH/hCG receptors have been found in several tissues and cell types including ovary, fallopian tube, endometrium, and resident tissue Mφ [12, 18]. The effect of hCG on cells of the immune system has been hardly studied.

Earlier, we showed that hCG enhances innate immunity by stimulating Mφ functions a.o. the phagocytic capacity [19]. Interestingly, an improved phagocytic activity is observed in Mφ from PTX3-overexpressing mice. Furthermore, we found recently an altered PTX3 mRNA expression in hCG exposed dendritic cells (unpublished results). Therefore, we hypothesized

that by induction of PTX3 production by myeloid phagocytic cells, hCG contributes to the increased phagocytosis and the protection against infection during pregnancy. Here, we studied the effect of hCG on PTX3 production by monocytes, Mφ and DC *in vitro* and *in vivo*.

MATERIALS AND METHODS

Human cell culture and stimulation

Human monocytes were isolated from peripheral blood. Briefly, blood was diluted 1.5 times with PBS containing 0.1% BSA (Biowhittaker, Verviers, Belgium), layered on a Ficoll gradient (1.077 g/ml; Pharmacia, Uppsala, Sweden), and centrifuged (2280 rpm, 15 min, room temperature). The cells at the interface were collected, washed, layered on a Percoll gradient (1.063 g/ml; Pharmacia), and centrifuged (1900 rpm, 40 min, room temperature). The monocytes were collected from the interface, washed and a final purity was reached of 85-95%. Monocytes were cultured in 12-well plates (Nunc A/S, Roskilde, Denmark) in a concentration of $2x10^6$ cells/well in 2 ml RPMI1640 (Biowhittaker) supplemented with 10% FCS (Hyclone Perbio, Brevieres, France) overnight. For stimulation, LPS 50 ng/ml (Lipopolysaccharide; Sigma, St. Louis, USA) or hIL-1β 20 ng/ml (R&D system, Minneapolis, USA), were added with or without hCG 300 U/ml (Pregnyl®; Organon, Oss, The Netherlands) for 4 hours. Then the cells were collected, counted and used for RNA isolation.

Human monocytic cell line MonoMac6 (MM6) was a kind gift from Prof. S. Sozzani (University of Brescia, Brescia, Italy). MM6 cells were cultured in RPMI1640 supplemented with 10% FCS, 1% penicillin/streptomycin (Biowhittaker), 1% non-essential amino acids (GIBCO, Eggenstein, Germany), 1% sodium pyruvate (GIBCO) and 1% sodium chloride (solvent for Pregnyl®). The cells were collected into 12-well plates in concentrations of 2×10^6 cells/well in 2 ml culture medium and stimulated with LPS 50 ng/ml, or PBS with or without different concentrations of hCG (10, 50, 150, 300, 600 U/ml). Polymyxin B 50 μg/ml (Sigma, Milan, Italy) was added to study a possible LPS contamination. To inhibit the intracellular PKA signaling pathway, 10 μM H-89 (Biomol, Plymouth Meeting, USA) was applied. Stimulation experiments with progesterone (P4) 100 ng/ml (Sigma) or estrogen (E2) 100 ng/ml (Sigma) were performed in the absence or prescence of hIL-1β 20 ng/ml. After 48h incubation, the culture supernatants were collected and used for further cytokine measurement by ELISA.

Mice

Specific pathogen-free C57BL/6 female mice were purchased from Harlan (Horst, The Netherlands). The mice were 8 weeks of age when sacrificed. All experiments were performed with the approval of Erasmus University Animal Welfare Committee (Rotterdam, The Netherlands).

Murine cell culture and stimulation

Murine bone marrow-derived dendritic cells (BMDC) and bone marrow-derived macrophages (BMDM) were generated by culturing BM cells in petridishes (Becton Dickinson, Le Pont De Claix, France) at a concentration of 2×10^6 live cells per

petridish in 10 ml RPMI-1640 containing 10% FCS, 1% penicillin/streptomycin, and 20 ng/ml recombinant mouse GM-CSF (Biosource International, Camarillo, USA) or 10 ng/ml recombinant mouse M-CSF (PeproTech, Rocky Hill, USA) in a 37ºC, 5% CO_2 incubator. At day 3, another 10 ml of medium with rmGM-CSF or rmM-CSF was added to the cultures.

On day 5 or day 6, BMDM or BMDC were collected by using a cell scraper (Corning Incorporated, Corning, USA), and stimulated with LPS 50 ng/ml in the absence or presence of hCG 300 U/ml. After incubation, the cells were collected, counted and used for RNA isolation.

Similar as described above for human monocytes, murine monocytes and lymphocytes were isolated and cultured with or without hCG 300 U/ml for 5 hours at a concentration of 1 x 10^6 cells/well in 2 ml culture medium in 12-well plates, followed by cell collection for RNA isolation.

Mouse serum collection

C57BL/6 mice were injected i.p. with hCG (500U/20 gram body weight) or PBS in a volume of 200 µl. After 5h the mice were sacrificed and blood was pooled for serum collection.

PTX3 ELISA

ELISA kits for the detection of human and mouse PTX3 were used according to the protocols described earlier [20]. For detection of human PTX3, ELISA plates (96 well; MaxiSorp; Nunc A/S) were coated with 100 µl of monoclonal rat anti-hPTX3 antibody MNB4 in coating buffer (phosphate-buffered saline plus 0.05% Tween 20) followed by overnight incubation at 4ºC. After washing, the plates were incubated for 2h at room temperature with 5% milk powder diluted in coating buffer in order to block the nonspecific binding. After three washes, 100 µl of either recombinant human PTX3 standards (diluted in PBS + 2% BSA) or cell supernatant was added for 2h at 37ºC. Plates were washed and 100 µl of biotin-labeled polyclonal rabbit anti-PTX3 antibody was added for 2h at 37ºC. After three washes streptavidin-HRP was added for 1h at room temperature. Plates were washed and then incubated with 100 µl Chromogen TMB (tetramethylbenzidine) for 30 min, followed by the addition of 2 M H_2SO_4 (50 µl/well). Absorbance values were read at 450 nm by ELISA reader (Thermo Labsystems, Vantaa, Finland).

For detection of murine PTX3, the protocol was similar as for human PTX3, but with the following antibodies and standards: monoclonal rat anti-mPTX3 coating antibody 2C3, recombinant mouse PTX3 standards, and biotin-labeled anti-mPTX3 antibody 6B11.

Quantitative Real-time PCR (qRT-PCR)

Collected cells were lysed in RLT buffer followed by total RNA extraction using an RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the protocol supplied by the manufacturer. Complementary DNA (cDNA) was synthesized using the Superscript first strand synthesis system (Invitrogen, Carlsbad, California, USA) according to the manufacturer's instructions. Mouse quantitative real-time PCR was performed using an ABI 7700 Sequence detection system (Applied Biosystems, Foster City, USA) and Taqman probe-based chemistry. Primers for human PTX3 (Hs00173615_m1) and mouse PTX3 (Mm00477267_g1) and ABL were supplied by Primer ExpressTM (Applied Biosystems). Each PCR sample was run in

duplicate. The mean value of the two reactions was defined as representative for the sample. The resulting PTX3 CT values were corrected relative to an ABL CT. To ease interpretation of results, we have used the following equation for the Figure, 2^(ABL-PTX3) x 100%. Therefore, an increase is proportional to an increase in expression of the particular target gene.

Statistical analyses

Data are expressed as mean values ± SEM in all figures. Statistical analyses were performed using Student's paired *t*-test or Mann-Whitney test. *p*-values <0.05 were considered significant. **p*<0.05, ***p*<0.01, ****p*<0.001.

RESULTS

hCG increases PTX3 expression by human monocytes

Human monocytes were incubated *in vitro* with different stimuli for different time periods to determine the kinetics of the induction of PTX3. PTX3 mRNA expression rose from 2 hrs and peaked at 6 hrs after LPS stimulation and 4 hrs after IL-1β stimulation (Figure 1A). These results are in line with data earlier described for monocytes and DC [1, 21, 22]. The addition of hCG significantly increased PTX3 mRNA expression by non-stimulated human monocytes. No effect on PTX3 expression was observed in LPS or IL-1β-stimulated cells (Figure 1B).

To study the PTX3 protein production, primary human monocytes and the human monocytic cell line MM6 were stimulated with hCG for two days. hCG significantly enhanced PTX3 production by both unstimulated human monocytes and MM6 cells. No effects on

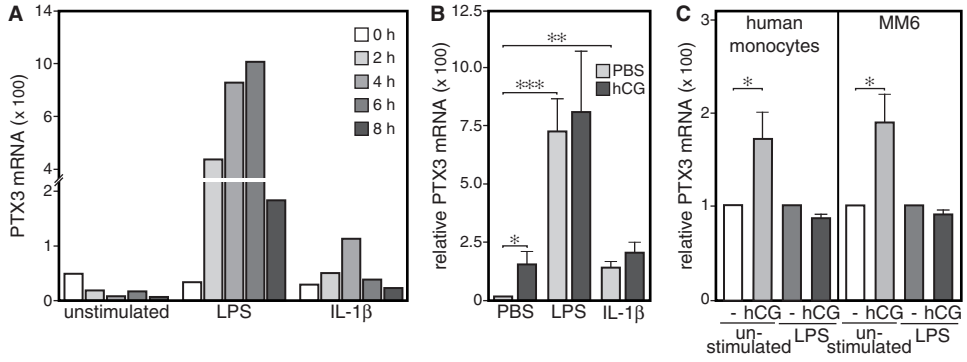

Figure 1. PTX3 expression by human monocytes upon hCG treatment.
A. Human monocytes were isolated from peripheral blood and stimulated with either LPS or IL-1β for 0, 2, 4, 6, 8 hrs. Then the cells were collected for RNA isolation. A representative of three individual experiments is shown. **B.** Human monocytes were stimulated with the above stimulation ± hCG for 4 hrs, then PTX3 qRT-PCR was performed (*n*=4). **C.** Human monocytes and the human monocytic cell line MM6 were stimulated with LPS with or without hCG for 48 hrs. Then the culture supernatants were collected to measure PTX3 protein production by ELISA. hCG increased the PTX3 expression by unstimulated monocytes, but not by LPS-stimulated monocytes. The data shown were relative to non-hCG treatment (human monocytes: *n*=6; MM6: *n*=3).

PTX3 protein production by LPS-stimulated cells incubated with hCG was observed (Figure 1C). As PTX3 was originally described as TNFα inducible gene, the expression of TNFα was studied. The hCG-induced PTX3 expression in human monocytes appeared to be not mediated by TNFα (data not shown).

The increased PTX3 expression by hCG is dose-dependent and PKA pathway-mediated

During normal pregnancy the hCG levels vary from 20 to 300 U/ml [13]. Therefore we tested the effect of different concentrations of hCG (ranging from 0-600 U/ml) on PTX3 expression by MM6 cells. The PTX3 protein expression by MM6 cells upon hCG treatment increased dose-dependently (Figure 2A). In humans, two major monocyte subsets are distinguished, the 'immature' $CD14^{+}CD16^{-}$ monocytes and 'mature' $CD14^{low}CD16^{+}$ monocytes. The latter subset is considered to give rise to different antigen-presenting cells subsets [23]. $CD16^{+}$ and $CD16^{-}$ monocytes were isolated using CD16-beads and separately analyzed for the induction of PTX3 by hCG. Both subsets increased the PTX3 protein expression to a similar level after 48 hrs incubation with hCG (data not shown).

Low concentrations of LPS can induce PTX3 expression by monocytic cells [24]. To exclude that the observed effect was due to LPS contamination within the hCG batch employed, polymyxin B, an antibiotic known to inhibit the biological activity of LPS, was used. Polymyxin B inhibited the PTX3 production induced by LPS but did not change the PTX3 production induced by hCG (Figure 2B).

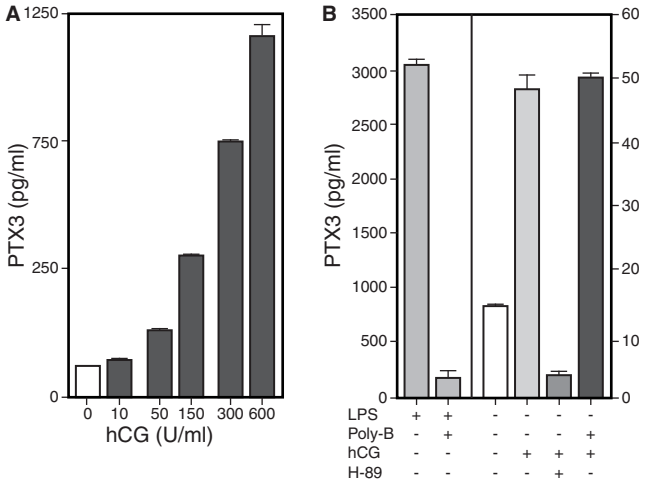

Figure 2. Increased PTX3 expression by hCG is dose-dependent and mediated by the PKA-signaling pathway.

A. MM6 cells were stimulated with different concentrations of hCG (0, 10, 50, 150, 300, 600 U/ml) for 48 hrs. hCG increased their PTX3 production in the culture supernatants in a dose-dependent manner. **B.** MM6 cells were stimulated with LPS with or without hCG (300U/ml) or hCG with or without Poly-B/H-89 for 48 hrs. Then the PTX3 protein production in the culture supernatants was determined by ELISA. LPS increased PTX3 expression, which was inhibited by Poly-B. The increased PTX3 expression by hCG can only be inhibited by H-89, but not by Poly-B (n=3).

hCG binding to its receptor activates the intracellular cAMP/PKA-signaling pathway [25, 26]. We have shown that the increased ROS production induced by hCG in macrophages is mediated via the PKA signaling pathway [27]. To study whether the induction of PTX3 by hCG was also medicated by PKA signaling, the PKA inhibitor H-89 was added together with hCG. H-89 significantly reduced the increased PTX3 production by MM6 cells upon hCG treatment (Figure 2B).

hCG increases PTX3 expression by murine BMDC and BMDM

DC and Mφ are generally believed to be derived from monocytes. DC and Mφ are capable to produce PTX3. The induction of PTX3 expression by murine DC and Mφ upon hCG treatment was investigated. Both BMDC and BMDM responded to LPS by PTX3 mRNA expression (Figure 3). The PTX3 expression level varied among monocytes, DC and Mφ (data not shown), which is in line with the previous findings that DC are better producers of PTX3 than monocytes and Mφ [21]. BMDC stimulated with LPS showed a PTX3 mRNA expression peak at 4 hrs (Figure 3A). hCG treatment for 4 hrs increased PTX3 mRNA expression

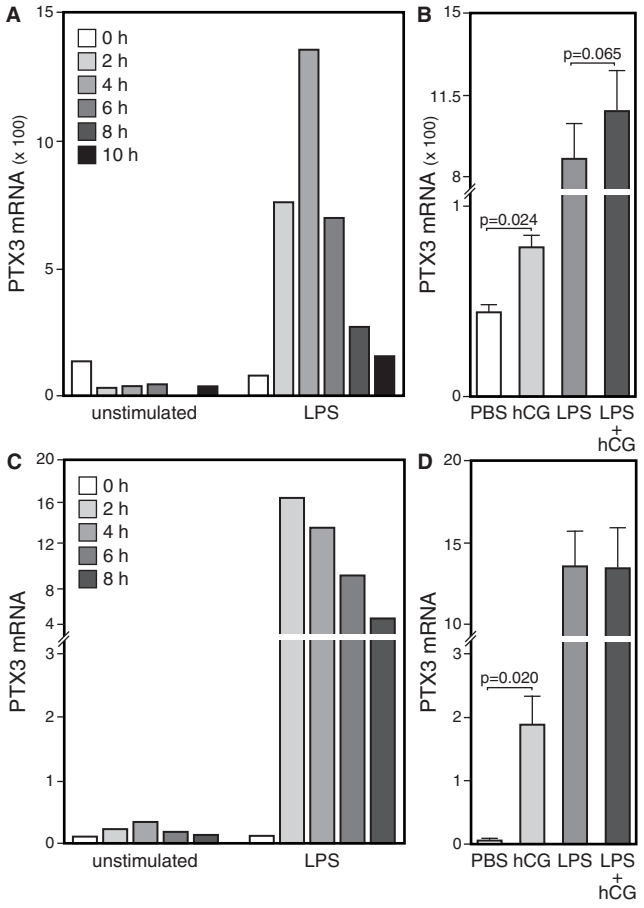

Figure 3. PTX3 expression by murine BMDC and BMDM upon hCG treatment.
BMDC (**A**) and BMDM (**C**) were stimulated with PBS or LPS for 0, 2, 4, 6, 8 hrs. Then the cells were collected for RNA isolation and PTX3 qRT-PCR. LPS significantly increased PTX3 mRNA expression by both BMDC and BMDM, but BMDC expressed 100 times more PTX3 mRNA than BMDM. A representative of three individual experiments is shown. When hCG was added to the cells for 4 hrs, hCG increased PTX3 mRNA expression of unstimulated and LPS-stimulated BMDC (**B**), but only unstimulated BMDM and not the LPS-stimulated BMDM increased PTX3 mRNA expression (**D**) (n=3).

by unstimulated BMDC, but not significantly by LPS-stimulated BMDC (Figure 3B). A maximum induction of PTX3 mRNA by LPS was observed in BMDM at 2 hrs (Figure 3C). hCG treatment increased PTX3 mRNA expression in unstimulated BMDM. PTX3 expression in LPS-stimulated BMDM was not affected by hCG treatment (Figure 3D).

Blood monocytes contribute to the increased serum PTX3 upon hCG injection

It has been shown that pregnancy is accompanied with an increased PTX3 level in the peripheral blood [9]. To investigate the relationship between hCG and PTX3 production by monocytes *in vivo*, mice were injected with hCG followed by collection of blood 5 hrs later and measurement of serum PTX3 protein levels. hCG injection significantly increased the PTX3 protein level in the serum (Figure 4A). To investigate whether blood monocytes contribute to the increased PTX3 production upon hCG administration, mouse monocytes were isolated after hCG injection. Monocytes from hCG-treated mice revealed a higher PTX3 mRNA expression than monocytes from non-treated mice (Figure 4B). To study if other cell types in the blood contribute to the observed increased PTX3 serum level, murine monocytes and lymphocytes were isolated from PBMC and separately stimulated with hCG for 5 hrs *in vitro*. PTX3 mRNA measurement revealed that monocytes expressed PTX3 and increased their PTX3 expression upon hCG treatment *in vitro*. Lymphocytes did not express PTX3 and did not respond to hCG treatment *in vitro* (Figure 4C).

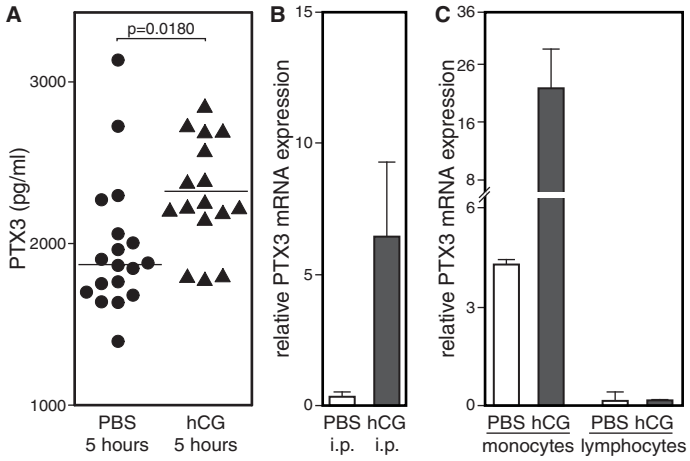

Figure 4. Blood monocytes contribute to the increased serum PTX3 level upon hCG injection.
A. C57BL/6 mice were injected i.p. with hCG or PBS. Five hours later mice were sacrificed and peripheral blood was collected for serum PTX3 protein measurement. hCG injection increased the PTX3 protein level in the serum (n=20); **B.** the blood monocytes from hCG i.p. treated mice were isolated and analyzed for PTX3 mRNA expression by qRT-PCR; **C.** murine blood PBMC, monocytes and lymphocytes were isolated from untreated mice and cultured with hCG *in vitro* for 5 hrs. Then the PTX3 mRNA expression by these cells was measured. Monocytes showed increased PTX3 expression after hCG stimulation *in vivo* and *in vitro*. The data are pooled from 2 separate experiments; each experiment was performed with 20 mice.

Progesterone and estrogen increase PTX3 expression by IL-1β-stimulated human monocytes

In order to study the effect of hCG-inducible hormones on PTX3 expression, human monocytes were stimulated with P4, E2, or P4 or E2 in combination with IL-1β. E2 and P4 significantly increased PTX3 mRNA expression by IL-1β-stimulated cells (data not shown). To further study the PTX3 protein production, MM6 cells were stimulated with LPS, hCG, P4, E2, or P4 or E2 in combination with IL-1β. As shown above, LPS, hCG and IL-1β increased the PTX3 production by MM6 cells. Stimulation with E2 alone did not change PTX3 production. P4 slightly increased PTX3 production but the result was not statistically significant. E2 and P4 addition to IL-1β increased the PTX3 production by MM6 cells compared to cells stimulated with IL-1β only (Figure 5).

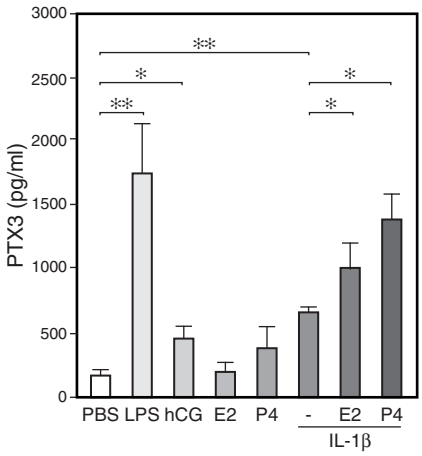

Figure 5. PTX3 expression by human monocytes upon progesterone and estrogen treatment.
MM6 cells were stimulated with PBS, LPS, hCG, progesterone, estrogen, and IL-1β with or without progesterone/estrogen for 48 hrs. Then the culture supernatants were collected for PTX3 protein determination. Progesterone and estrogen increased PTX3 expression by IL-1β-stimulated MM6 cells (*n*=3).

DISCUSSION

Pregnancy has been proposed to be a state of controlled inflammation [28], with increased innate immunity characterized by the activation of macrophages and granulocytes, and the complement system [29-31]. PTX3 is produced by innate immune cells including monocytic cells in response to inflammatory mediators, such as IL-1β, tumor necrosis factor (TNF) and bacterial products [1-3, 32]. Earlier we have shown that hCG influences the innate immune effector functions of Mφ directly [27]. Mφ increased their oxygen radical production and phagocytosis of apoptotic cells upon hCG treatment. This effect of hCG on cells of the innate immune system led us to hypothesize that hCG also modulates PTX3 expression by myeloid cells and as such further contributes to the increased innate immunity during pregnancy. Therefore we studied the effect of hCG on PTX3 production by monocytes, DC and Mφ *in vitro* and *in vivo*.

In the present study, we showed that hCG treatment induced increased PTX3 expression - both at the mRNA and protein level - by unstimulated monocytes, DC and Mφ. This is likely important during pregnancy, where PTX3 was found to be elevated locally as well as systemically [9].

PTX3 is generated in peripheral tissues under the control of inflammatory stimuli and, in particular, plays a non redundant role in the response against a selective set of microbes [32]. Recombinant PTX3 functions as an opsonin after binding to *Paracoccidioides brasiliensis* and zymosan and, thereby increasing the phagocytosis by peritoneal Mφ. Mφ and DC from PTX3-deficient mice did not recognize conidia of Aspergillus fumigatus resulting in increased susceptibility of these mice to invasive pulmonary aspergillosis [5]. In addition, binding of PTX3 to immobilized C1q activates the classical complement pathway and facilitates extracellular pathogen recognition by Mφ and DC [4, 33]. These data together with our findings provide evidence that hCG-induced PTX3 expression in myeloid cells contributes to maintain a robust innate immune system against infections in pregnancy.

Implantation and trophoblast invasion are characterized by a progressive and continuous induction of apoptosis in the maternal tissue surrounding the fetus [10,11,34,35]. Extensive cell death/ tissue necrosis represents a challenge for the immune system because autoantigens are released in a context in which antigen presentation is favored. PTX3 selectively binds to late apoptotic and necrotic cells resulting in decreased phagocytosis by DC [8, 36]. In addition, PTX3 inhibits the cross-presentation of epitopes expressed by apoptotic cells to T lymphocytes [7]. Therefore, in the developing placenta, hCG-induced PTX3 expression may prevent alloimmunization by regulating the clearance of apoptotic cells and quenching cross-presentation.

We showed that the increased PTX3 expression by hCG is mediated by the PKA-signaling pathway, which suggests hCG receptor involvement. This is consistent with our previous finding that the increased radical production and phagocytosis of Mφ upon hCG treatment was also mediated by the PKA-signaling pathway [27]. In line with our findings are publications from Aggarwal *et al.* [37, 38] showing that hCG binding to the hCG receptor increased the cAMP level and PKA activity. Meanwhile, former studies from Mantovani *et al.* showed that there are several pathways involved in PTX3 induction, including TNF, IL-1β, TLR ligands etc. [32]. Here we addressed a new pathway for the induction of PTX3: the PKA signaling pathway. Taken together, these data suggest that PTX3 induction is mediated via multiple pathways, all of which contribute to an enhanced innate immunity.

To support our hypothesis that hCG affects PTX3 expression by monocytes *in vivo*, we injected mice i.p. with hCG followed by the isolation of monocytes. Indeed, monocytes expressed more PTX3 mRNA upon hCG treatment. We further proved that only monocytes, not lymphocytes, exhibited an increase in PTX3 expression upon hCG treatment. This induction of PTX3 expression in monocytes was accompanied by an increase in PTX3 serum levels. However, we did not observe a direct correlation between PTX3 and hCG serum level from women in the first trimester of pregnancy (data not shown). Several possibilities may account for this: 1) individual differences including inflammatory conditions, blood pressure, body weight, age of pregnancy, and pregnancy number; 2) contribution by other cell types; and 3) unidentified regulatory factors, apart from hCG, in the blood of pregnant women may also influence the PTX3 level, such as progesterone and estrogen.

hCG levels decrease in the second and third trimester of pregnancy, while other hormones

induced by hCG, like progesterone (P4) and estrogen (estradiol, E2) continue to increase and stay high till the end of the pregnancy [14-17]. Upon interaction of hCG with the LH/hCG receptor (or cross-interact with the FSH receptor), the corpus luteum starts to secrete progesterone and estrogen. Interestingly, both P4 and E2 can increase PTX3 production by IL-1β-stimulated monocytes. This observation suggests that the whole period of pregnancy is characterized by increased PTX3 levels, first induced by hCG, later induced by hCG-induced hormones. These increased PTX3 levels contribute to the increased innate immunity in pregnancy.

In this study, we observed that hCG increases PTX3 expression by monocytic cells *in vivo* and *in vitro*. Furthermore, the hCG-induced hormones such as progesterone and estrogen can also increase the PTX3 production by inflammatory monocytes. In conclusion, hCG can enhance innate immunity via induction of PTX3 in myeloid cells, as well as via hCG-induced hormones such as progesterone and estrogen.

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Chapter 3

Chorionic gonadotropin can enhance innate immunity by stimulating macrophage function

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ABSTRACT

Human chorionic gonadotropin (hCG) is a placental glycoprotein mainly secreted by trophoblasts during pregnancy. Its function in endocrine regulation has been well documented, but its immunological role is still largely unclear. For a successful pregnancy an effective innate immunity is needed to protect the mother and fetus against infection, while maintaining tolerance against the paternal antigens of the fetus. The aim of this study was to investigate the effect of hCG on the function of macrophages (Mφ), which are major players in the innate response. hCG treatment of IFN-γ-primed Mφ resulted in increased production of NO, ROS, IL-6 and IL-12p40, and enhanced phagocytosis of apoptotic cells. hCG treatment did not affect the induction of allogeneic T cell proliferation by IFN-γ-primed Mφ. The observed effects were receptor-mediated and involved the PKA signaling pathway, as indicated by blocking studies using specific inhibitors. *In vivo* thioglycollate-elicited Mφ also exhibited increased phagocytic ability upon IFN-γ activation and hCG treatment. In conclusion, hCG enhances Mφ functions involved in innate immunity, while the capacity to stimulate allogeneic T cells remains unchanged.

KEY WORDS

hCG, Mφ, ROS, phagocytosis, innate immunity.

INTRODUCTION

Studies on the role of the maternal immune system during pregnancy have focused mainly on the aspect of immune tolerance to the paternal antigens of the fetus. Pregnancy is associated with a systemic shift towards a Th2 cytokine profile that is characterized by elevated IL-4, IL-5, and IL-10 levels [1-3]. The observation that many Th1-mediated autoimmune diseases remit during pregnancy and recur after childbirth might be related to this changed cytokine profile and the hormonal changes that occur during pregnancy [4]. A prominent role for the innate immune system in the tolerance to the fetus has been suggested [5]. The variety of changes in the immune system led to the concept that the function of the adaptive immune system decreases during pregnancy while a robust innate immunity is needed to maintain an adequate host defense.

During normal pregnancy the decidua is populated by multiple leukocytes [6]. Cells of the innate immune system, NK cells and macrophages (Mφ), are predominant, while the number of lymphocytes is relatively low (1-3%) [7]. NK cells produce 90% of pregnancy-induced uterine IFN-γ [8]. Mφ constitute 20-30% of the decidual cells at the site of implantation [7, 9, 10], and, unlike NK cells, remain present in high numbers throughout pregnancy [7, 11]. Upon activation by IFN-γ Mφ produce reactive oxygen species (ROS), nitric oxide (NO) and pro-inflammatory cytokines [12, 13]. Oxygen radicals generated from ROS and NO are cytotoxic for a variety of microorganisms including viruses, bacteria, protozoa and fungi [14]. It has been suggested that decidual Mφ, employing the above-mentioned mechanisms, play an essential role in pathogen recognition, phagocytosis, destruction of microorganisms and clearance of infected cells during pregnancy in order to protect the fetus against intrauterine infections [15].

Fetal implantation and trophoblast invasion are characterized by a progressive and continuous induction of apoptosis in the maternal tissue surrounding the fetus [16]. Resident Mφ play an essential role in the removal of apoptotic cells in order to prevent exposure of the maternal immune system to paternal antigens in the placenta. Furthermore, clearance of apoptotic cells is crucial to the resolution of local inflammation, which is vital in pregnancy [16]. Together, this indicates that the innate immune system, and the macrophage compartment in particular, is essential in both tolerance towards the fetus and the host protection against infection.

During pregnancy, there are important changes in hormone levels such as the huge production of human chorionic gonadotropin (hCG), which are supposed to influence the immune system. hCG is a placental glycoprotein, also called pregnancy hormone. The best-known function of hCG is to induce the production of progesterone and estrogen during early pregnancy [17, 18]. The biological structure of hCG consists of a unique β-chain and an α-chain shared with other glycoprotein hormones, such as thyroid stimulating hormone. hCG is mainly secreted by trophoblasts during pregnancy and can be detected in urine and blood. We have reported that hCG and an oligopeptide from the beta-chain of hCG inhibit septic shock in mice [19]. The effect of hCG on cells of the immune system is hardly studied. However, this interaction might account for multiple changes in the systemic immune system as well as for the local tolerance induction against the fetus.

Interestingly, Mφ in human reproductive tissues, including the decidua and endometria, express LH/hCG receptors [20] and may thus be influenced by the high local hCG concentration. Binding of hCG to its receptor activates the intracellular cAMP/PKA-signaling pathway

leading to cyclic-AMP response element binding protein (CREB) transcription and contributes to the induction of inducible nitric oxide synthase [21-23]. Indeed, hCG, in combination with IFN-γ has been found to enhance NO production by murine Mφ and microglia [24, 25], but the effect of hCG on other Mφ functions is presently unknown. We hypothesize that hCG can promote the innate function of Mφ, and thereby helps to protect the mother and fetus against infections, while contributing to the maintenance of successful pregnancy. Therefore, we studied the effect of hCG on oxygen radical induction, cytokine production and apoptosis of murine bone marrow-derived Mφ (BMDM) and peritoneal Mφ.

MATERIALS AND METHODS

Mice

Specific pathogen-free C57BL/6 and C3HeB/FeJ female mice were purchased from Harlan (Horst, The Netherlands) and were housed in micro-isolator cages and given mouse chow and water ad libitum in the animal care facility at Erasmus MC. Mice were eight weeks of age when sacrificed for the isolation of bone marrow (BM) cells or subjected to intraperitoneal injection of thioglycollate broth. All experiments were performed with the approval of Erasmus MC animal welfare committee (Rotterdam, The Netherlands).

Bone marrow-derived macrophage cultures and activation

BMDM were generated by culturing BM cells from C57BL/6 mice in petri-dishes (Becton Dickinson, Le Pont De Claix, France) at a concentration of 2 x 10^6 live cells per petri-dish (∅ 10 cm) in 10 mL RPMI-1640 culture medium (Biowhittaker, Verviers, Belgium) containing 10% FCS (Hyclone, Perbio, France), 60 mg/mL penicillin and 100 mg/mL streptomycin (Biowhittaker), and 10 ng/mL recombinant mouse M-CSF (Biosource International, Camarillo, USA) in a 37ºC, 5% CO_2 incubator. At day 3, another 10 mL of medium with rmM-CSF was added to the cultures.

At day 5, cells were collected by using a cell scraper (Corning Incorporated, Corning, USA). For cell activation, BMDM were transferred into 12-well plates (Nunc A/S, Roskilde, Denmark) at a concentration of 1x10^6 cells/well in 1 mL culture medium without rmM-CSF. Cells were stimulated with 50 ng/mL LPS (Lipopolysaccharide; Sigma, St. Louis, USA), 5 ng/mL rmIFN-γ (Biosource) according to previous studies [24, 25], 40 ng/mL rmTNF-α (Biosource), or PBS. After 6 hours, PBS or hCG (Pregnyl, Organon, Oss, The Netherlands) was added at a concentration of 500 U/mL. To inhibit the intracellular PKA signaling pathway, 10 μM H-89 (Biomol, Plymouth Meeting, USA) was added two hours before the addition of hCG. After two days of incubation, supernatants were collected for NO detection and cytokine measurement, and the cells were harvested for phenotyping, ROS detection, and mixed leukocyte reactions (MLR).

Measurement of NO synthesis

NO synthesis in cell cultures was measured using a Griess Reagent Kit (Molecular Probes, Leiden, The Netherlands) according to the protocols supplied by the manufacturer. In short, 150 μL sample, 20 μL Griess reagent (equal volumes of 0.1% N-(1-naphthyl)-ethylenediamine

dihydrochloride and 1% sulfanilic acid), and 130 μL deionized water were mixed and incubated at room temperature for 30 min. Sodium nitrite (1-100 μM) was used as standard, and the absorbance was measured at 540 nm by an ELISA reader (Thermo Labsystems, Helsinki, Finland).

Measurement of reactive oxygen species

ROS production was measured by labeling BMDM with 5-(and 6-) chloromethyl-2',7'-dichlorodihydrofluorescein diacetate acetyl ester (CM-H_2DCFDA; Molecular Probes). Briefly, 1x10^6 cells were resuspended in 1 mL pre-warmed PBS, then 1 μL CM-H_2DCFDA (5 μg/mL) was added and cells were incubated for 10 minutes at 37ºC. Upon reaction of CM-H_2DCFDA with ROS a fluorescent product is generated. Fluorescence was detected and analyzed on a FACSCalibur flow cytometer using CellQuest analysis software (Becton Dickinson).

Flow cytometry

At day 7, BMDM were collected and labeled with the following antibodies: ER-TR3-bio, (MHCII, BMA, Augst, Switzerland), Streptavidin-APC, CD11b-FITC, CD40-PE, IgG control (all from BD PharMingen, Erembodegem-Aalst, Belgium), F4/80-FITC (Caltag, Burlingame, USA), and analyzed using a FACSCalibur (Becton Dickinson). In between incubations, cells were washed twice with PBS + BSA (5%, Celliance Corporation, Atlanta, USA). All steps were performed at room temperature (RT). Data were analyzed by WinMDI 2.8 software and depicted by mean fluorescence intensity (MFI) and percentage (%) of positive cells.

Cytokine detection

ELISA kits for the detection of murine TNF-α, IL-6, IL-10 (Biosource), and IL-12p40 (RnDsystems, Oxen, UK) were used according to the protocols supplied by the manufacturer. In short: plates were coated with capture antibody for 18h at 4ºC and washed with PBS-Tween (0.05%). Diluted culture supernatants and standards were added and incubated for 2h at RT. Thereafter the biotin-labeled detection antibody was added followed by incubation with streptavidin-HRP for 30 min; Chromogen TMB (tetramethylbenzidine) was added for 30 min, followed by the addition of the stop solution. In between incubations the plates were washed with PBS-0.05%Tween. The absorbance was measured at 450 or 450 and 650 nm by ELISA reader (Thermo Labsystems).

T cell proliferation

Allogeneic mixed leukocyte reactions (MLR) were performed in round-bottom 96-microwell plates (Nunc A/S). Naïve T cells were obtained from spleens of C3HeB/FeJ mice, after lysis of the red blood cells by Gey's medium (Millipore, Billerica, USA) and negative depletion by incubation with a combination of antibodies: CD11b (M1/70), CD45R (B220), and MHCII (M5/114) (American Type Culture Collection, Manassas, USA) and anti-rat IgG microbeads (Miltenyi Biotec, Auburn, USA), followed by Automacs separation (Miltenyi Biotec). BMDM were added in various concentrations to T cells (1.5x10^6 cells/well), and co-cultured for three days in RPMI-1640 medium containing 10% FCS, 60 mg/mL penicillin and 100 mg/mL streptomycin. Proliferation of T cells was measured by uptake

of ^{3}H-thymidine (1 μCi/well; DuPont-NEN, Boston, USA) over a period of 16 hours and expressed as counts per minute (cpm).

***In vitro* phagocytosis of microspheres and apoptotic cells**

BMDM were incubated with FluoSpheres® polystyrene microspheres (1.0 μm, yellow-green fluorescent, Molecular Probes) to measure the extent of phagocytosis. In each tube 10^6 cells were mixed with $40x10^6$ beads. After 2h incubation at 37ºC and in 5% CO_2, cells were washed with PBS, then fluorescence was measured using flow cytometry. Another portion of the cells was prepared for immuno-fluorescence microscopy. Cytospins were prepared, air-dried and fixed in acetone for 5 minutes. After blocking with 1% BSA, the slides were incubated with BM8-Biotin, Streptavidin-Texas red (Caltag, San Francisco, USA), and DAPI (detection of DNA content (4,6-diamidino-2-phenylindole), Vector Laboratories Inc.) sequentially, prior to analysis using a fluorescence microscope.

Apoptosis was induced in human Jurkat T cells by exposure to ultraviolet radiation (UVB_{312nm}, equivalent to 800 mJ/cm^2), followed by 24h culture in RPMI-1640 medium containing 10% FCS, 60 mg/mL penicillin and 100 mg/mL streptomycin [26]. This resulted in a population of cells that was ~50% apoptotic as determined by Annexin V and PI staining (Calbiochem, Darmstadt, Germany). To label apoptotic cells, carboxyfluorescein succinimidyl ester (5 μM CFSE; Molecular probes) was added to 10^7 cells/mL and incubated for 10 minutes on melting ice.

For the collection of *in vivo* conditioned peritoneal exudates, C57BL/6 mice were injected i.p. with hCG (300 U/20 gram body weight) or PBS in a volume of 200 μL. One hour later 1 mL of sterile 4% Brewer's thioglycollate broth (Sigma-Aldrich, Poole, UK) was injected i.p. per mouse; PBS was used as a control. At day 3, the mice were sacrificed and the peritoneum was flushed with 4 mL PBS and 1 mL air to harvest cells. A portion of the collected peritoneal cells was incubated with CFSE-labeled apoptotic cells (1:5) for 30 minutes at 37ºC, followed by BM8 staining.

The rest of the cells was cultured *in vitro* in RPMI-1640 medium containing 10% FCS, 60 mg/mL penicillin and 100 mg/mL streptomycin with 5 ng/mL IFN-γ with or without 500 U/mL hCG for two days. The cells were collected and fed with CFSE-labeled apoptotic cells for 30 minutes at 37ºC, followed by BM8 staining. Using flow cytometry, the percentage of double positive cells representing Mφ that have ingested apoptotic cells, so-called phagocytic Mφ, was determined. Phagocytosis of apoptotic cells by Mφ was also analyzed morphologically on cytospins, where cells were stained with Diff-Quick (Dade Diff-Quick, Düdingen, Switzerland).

Statistical analyses

Data are expressed as mean values ± SEM in all figures. All statistical analyses were performed using Student's *t*-test or paired *t*-test. *p*-values <0.05 were considered significant. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

RESULTS

hCG treatment enhances IFN-γ-induced NO production by BMDM

LPS and IFN-γ, but not TNF-α significantly increased NO production by BMDM (Figure 1). hCG alone did not influence NO synthesis by BMDM, but the addition of hCG to IFN-γ-primed Mφ significantly enhanced NO production. hCG did not influence NO production by LPS or TNF-α-activated BMDM (Figure 1).

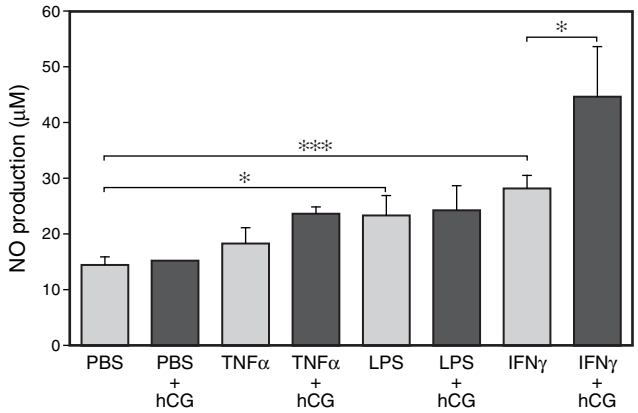

Figure 1. Increased NO production by IFN-γ-primed BMDM upon hCG treatment.
Bone marrow cells were cultured for five days in the presence of M-CSF. Cells were collected and stimulated with the following stimuli: PBS, TNF-α, LPS, or IFN-γ. Six hours later, hCG or PBS was added to each culture. After two days of culture, NO production in the culture supernatants was measured.

hCG increases IFN-γ-induced ROS production by BMDM

BMDM were stimulated with IFN-γ in the presence or absence of hCG to investigate the effect of hCG on the production of ROS. ROS production was determined by fluorescent visualization using CM-H_2DCFDA that upon cleavage and subsequent oxidation by ROS yields a fluorescent product. Flow cytometry revealed two populations: ROS positive cells (gate R1 and R2) and ROS-negative cells (lower part) (Figure 2A). Activation of BMDM with IFN-γ increased the percentage of ROS-positive cells (Figure 2D). hCG treatment of IFN-γ-primed BMDM with hCG significantly increased ROS-production in a dose- and time-dependent manner (Figure 2B and data not shown). ROS production steadily increased in time and reached a maximum after 48 hours of incubation (data not shown). In all cases, the percentage of ROS-positive cells gave similar results as the mean fluorescence intensity (MFI). Differences in pH of the incubation media of stimulated cells were not found at the moment of cell harvest, thus excluding the pH as the cause of the observed differences in ROS production (data not shown).

hCG binding to its receptor activates the intracellular cAMP/PKA-signaling pathway [21, 22]. To study whether the increased ROS production induced by hCG involves this pathway, the

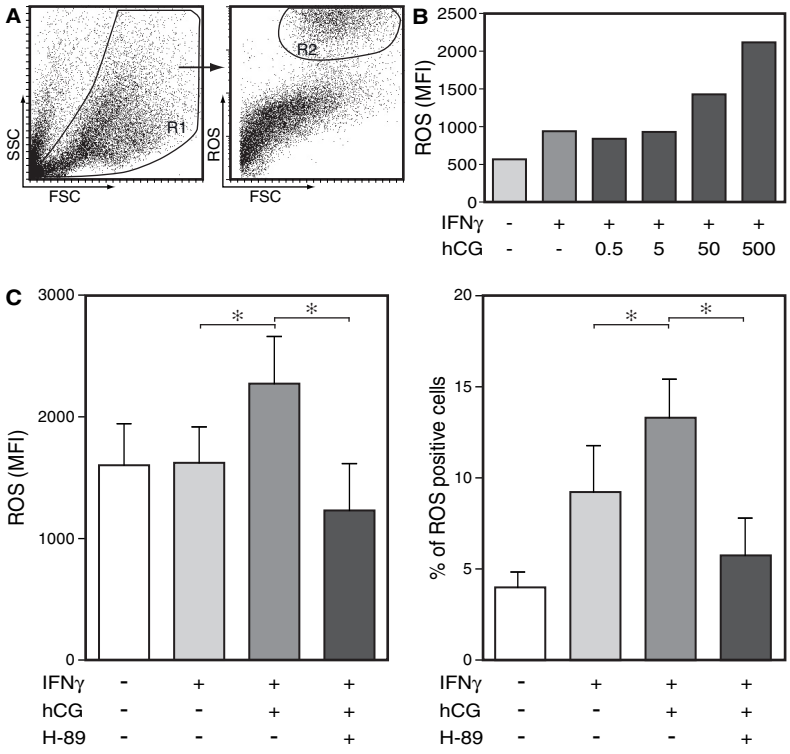

Figure 2. hCG increases receptor-mediated ROS production by IFN-γ-primed BMDM.
Cultured BMDM were stimulated with IFN-γ. Six hours later, hCG (0, 0.5, 5, 50, 500 U/mL) or PBS was added. After two days of culture, ROS production was determined. The CM-H_2DCFDA-positive cells (R2) in the dotplot **A**. were ROS-producing Mφ. **B.** Dose-dependent ROS production by IFN-γ-primed BMDM upon hCG treatment. A representative of three individual experiments is shown. **C.** and **D.** Increased ROS production by IFN-γ-primed BMDM upon hCG treatment was mediated via PKA signaling pathway. BMDM were primed with IFN-γ, after four hours PKA inhibitor (H-89, 10 μM) was added, and two hours later hCG (500 U/mL) or PBS was added. Addition of the PKA inhibitor H-89 blocked the increased ROS production by IFN-γ-primed BMDM upon hCG treatment. The mean fluorescence intensity (MFI) and percentage (%) of ROS positive cells from three to six separate experiments are depicted.

PKA inhibitor H-89 was added two hours prior to the addition of hCG. H-89 significantly reduced the increased ROS production by IFN-γ-primed BMDM upon hCG treatment (Figure 2C and 2D) in a dose-dependent manner (data not shown).

hCG increases cytokine production by IFN-γ-primed BMDM

Unstimulated BMDM secreted small amounts of IL-6, IL-10, and IL-12p40. Activation of BMDM by a low concentration of IFN-γ (5ng/mL) did not increase cytokine production (Figure 3). The addition of an increasing amount of hCG to IFN-γ-primed BMDM caused a dose-dependent increase in IL-6 and IL-12p40 production. IL-10 levels remained

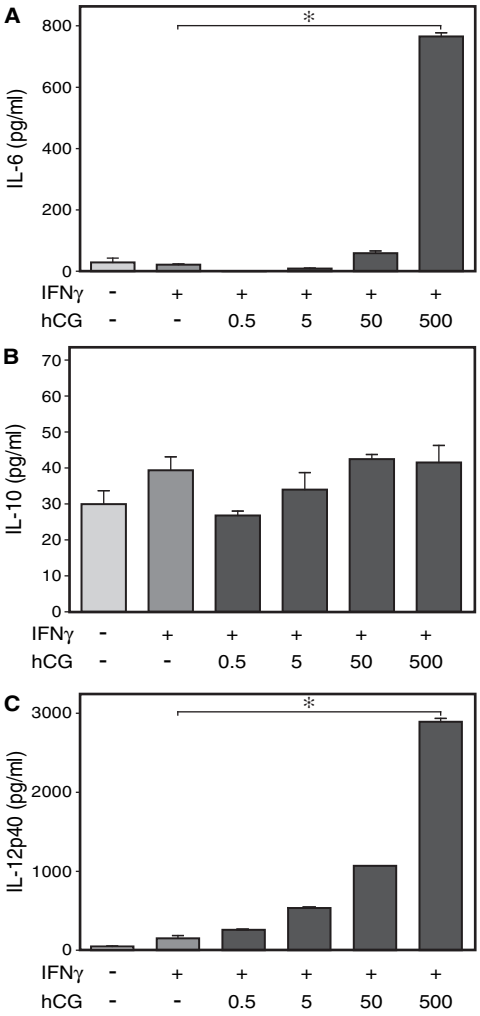

Figure 3. Cytokine production by IFN-γ-primed BMDM upon hCG treatment.
BMDM were primed with IFN-γ, and after six hours hCG (0, 0.5, 5, 50 or 500 U/mL) or PBS was added. After two days of culture, the culture supernatants were analyzed for IL-6, IL-12p40, and IL-10 by ELISA. The results shown are representative from three independent experiments.

unaltered up to 500 U/mL hCG. To characterize further which form of IL-12p40 was induced, IL-12p40 monomer, homodimer (IL-12p80), heterodimer (IL-12p70) and IL-23 concentrations were determined. IL-12p40 monomer and homodimer were significantly increased by IFN-γ-primed BMDM upon hCG treatment, while the levels of IL-12p70 and IL-23 were at a low level and remained unchanged after addition of hCG (data not shown).

We excluded the possible LPS contamination in hCG by showing unchanged TNF-α production by IFN-γ-primed BMDM (data not shown) and insensitivity to polymyxin B, an agent that neutralizes the effects of LPS (data not shown).

hCG treatment does not change the BMDM phenotype and their ability to induce allogeneic T cell proliferation

Treatment of BMDM with IFN-γ increased CD11b, CD40 and MHC class II membrane expression, but the expression of F4/80 remained unchanged. The addition of hCG to IFN-γ-primed BMDM did not change the expression levels of these markers (Figure 4A).

Unstimulated BMDM induced allogeneic T cell proliferation only to a limited extent. Addition of either IFN-γ at low concentration or hCG showed a trend towards an increased

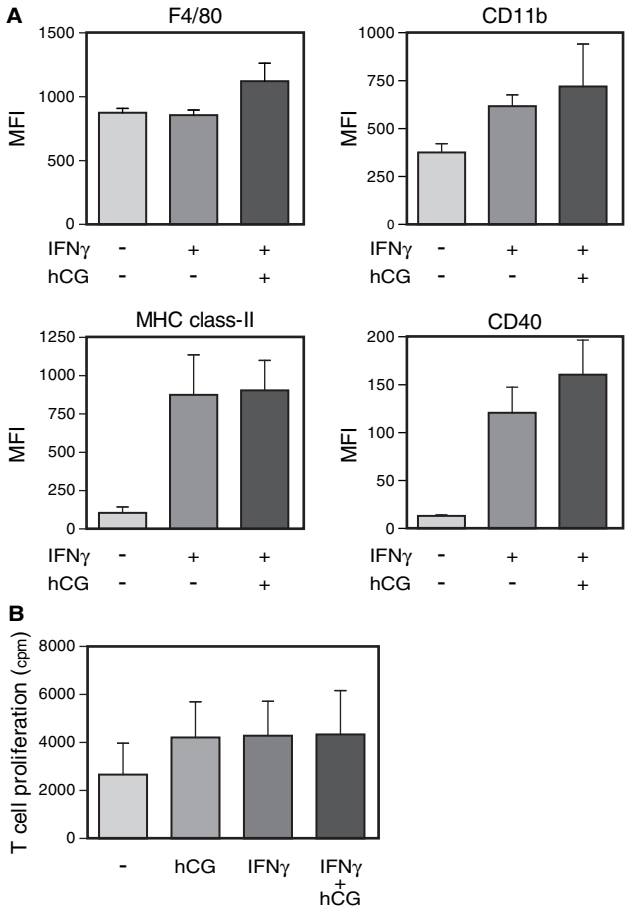

Figure 4. Phenotypic changes and allogeneic T cell proliferation by IFN-γ-primed BMDM upon hCG treatment.

BMDM were stimulated with IFN-γ. After six hours, hCG (500 U/mL) or PBS was added. **A.** After two days of culture, Mφ were collected and labeled for F4/80, CD11b, MHCII and CD40. The expression is depicted as mean fluorescence intensity (MFI). **B.** Mφ were co-cultured with allogeneic spleen T cells. After co-culturing Mφ and T cells (ratio 1:5) for three days, proliferation of T cells was measured by uptake of ^{3}H-thymidine. LPS-activated DC were used as a positive control which gave around 50,000 cpm after co-culture with T cells. The data are pooled from 4 separate experiments.

T cell proliferation. hCG treatment of IFN-γ-primed BMDM also did not change their ability to stimulate T cells proliferation (Figure 4B). LPS-activated DC were used as a positive control.

hCG increases the phagocytic capacity of IFN-γ-primed BMDM

BMDM identified by the expression of the Mφ marker BM8/F4/80, readily phagocytosed FluoSpheres® polystyrene microspheres (Figure 5A). Flow cytometry enabled convenient quantification of fluorescence-positive cells that have phagocytosed variable amounts of labeled microspheres (Figure 5B). IFN-γ-priming of BMDM resulted in 10% increase in cells that phagocytosed beads compared to unstimulated BMDM. Treatment of IFN-γ-primed BMDM with hCG resulted in a further 20% increase of phagocytic cells. The amount of microspheres that were phagocytosed by a single cell was also increased in hCG-treated cells compared to controls. The effect of hCG could be blocked by addition of the PKA signaling pathway inhibitor H-89, suggesting hCG receptor involvement (Figure 5C).

hCG increases phagocytosis of apoptotic cells by thioglycollate-activated peritoneal Mφ

To investigate whether stimulation of phagocytosis by hCG was a general phenomenon, we approached this also in an alternative setting. Mice were pretreated with hCG i.p. and one hour later injected with thioglycollate to induce peritonitis. On day 3, peritoneal cells were collected; the majority of these cells were Mφ (60-80%) as determined by flow cytometry. By using the previously described method of subset analysis [27], hCG did not influence the cellular composition of the infiltrate isolated. Peritoneal cells were either incubated with CFSE-labeled apoptotic T cells and directly analyzed, or were first cultured with IFN-γ with or without hCG for two days prior to analyzing the phagocytic capacity. In Figure 5D, four sequential stages in the phagocytic process of apoptotic cells are depicted (I - IV). From stage IV, Mφ were counted as phagocytic cells. The percentage of $BM8^+CFSE^+$ double positive cells was determined, representing Mφ that have ingested apoptotic cells (Figure 5E). A slightly higher frequency of thioglycollate-recruited Mφ phagocytosed apoptotic cells compared to resident peritoneal cells. Without thioglycollate injection *in vivo,* treatment of hCG did not change the phagocytic ability of peritoneal cells. However, e*x vivo* culture with IFN-γ increased their phagocytic ability significantly. *Ex vivo* culture of peritoneal Mφ, recruited by thioglycollate injection, with IFN-γ resulted in an increased phagocytic capacity; *ex vivo* culture with IFN-γ and hCG further increased the phagocytic capacity compared to IFN-γ culture alone. Moreover, thioglycollate-induced peritoneal cells, after *in vivo* treatment with hCG followed by *ex vivo* stimulation with IFN-γ in the presence of hCG, revealed a higher phagocytic capacity indicating that continuous presence of hCG further increased phagocytic capacity.

DISCUSSION

To test our hypothesis that hCG stimulates innate immunity to protect mother and fetus against infection, we studied the effect of hCG on innate immune functions of BMDM and inflammatory peritoneal Mφ. In general Mφ play an essential role in innate immunity as well

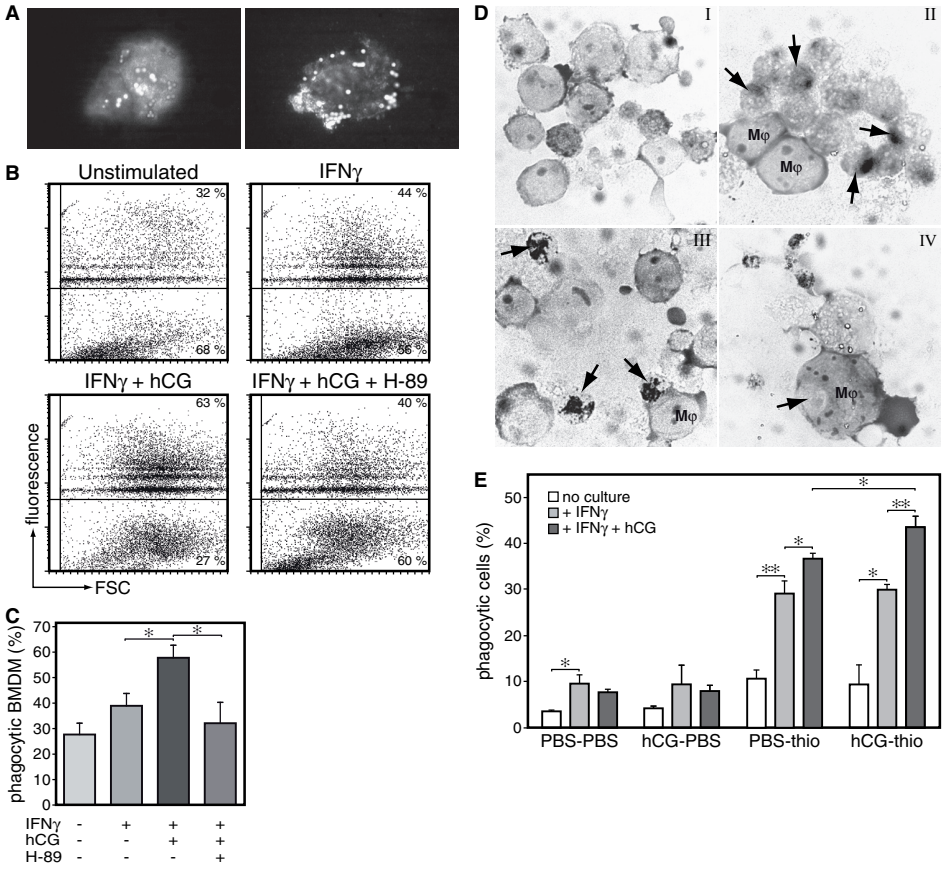

Figure 5. Increased phagocytosis by IFN-γ-primed BMDM and peritoneal Mφ upon hCG treatment.
BMDM were stimulated with IFN-γ. After six hours, hCG (500 U/mL) or PBS was added. After two days of culture, the cells were collected and incubated with fluorescence-labeled polystyrene microspheres for 30 minutes. Subsequently, the cells were prepared for immuno-fluorescence staining on cytospin **A.** red: BM8, green: fluorescence-beads, blue: DAPI; left with DAPI, right without DAPI. **B.** Flow cytometric analysis of phagocytosis of fluorescence-labeled microspheres. The multiple lines in the figure represent cells that phagocytosed different amounts of beads. The result is representative from three separate experiments. **C.** Proportion of phagocytic cells upon IFN-γ priming, hCG stimulation and inhibition of PKA signaling by H-89. Data are pooled from three separate experiments.
C57BL/6 mice were injected i.p. with hCG or PBS. One hour later thioglycollate broth was injected i.p. On day three, mice were sacrificed and peritoneal cells were collected. Part of the cells was incubated with CFSE-labeled apoptotic T cells, followed by BM8 staining; the other cells were cultured *in vitro* with PBS, IFN-γ (5 ng/mL), or IFN-γ plus hCG (5 ng/mL+ 500 U/mL). After two days, cells were collected and incubated with CFSE-labeled apoptotic T cells, followed by BM8 staining. Phagocytosis was recorded on cytospins using Diff-Quick staining (**D**). The peritoneal cells appear large and light blue with diffuse edges; the early apoptotic cells were small and round, appeared dark blue/purple; late apoptotic cells were even smaller and showed shrunken and segmented nuclei; the color was completely pink. The continuous process of phagocytosis of apoptotic cells was recorded as follows: I: non-apoptotic T cells; II: non-phagocytic Mφ and early stage of apoptotic T cells (arrows); III: late stage of apoptotic T cells (arrows) and phagocytic Mφ with protruded arms that encircled the apoptotic T cells (arrow); IV: apoptotic T cell completely phagocytosed by a Mφ (arrow). **E.** In flow cytometry, the percentage of $BM8^{+}CFSE^{+}$ double positive cells was determined, representing the phagocytic Mφ (*n*=5).

as adaptive immunity, by exerting specific functions, such as removal of debris, dead cells and micro-organisms and the production of oxygen radicals and cytokines.

In agreement with previous studies on peritoneal Mφ and microglia [24, 25], we found that hCG alone did not influence NO synthesis, while activation by IFN-γ did increase NO production by BMDM. However, hCG further increased NO and ROS production significantly by BMDM treated with IFN-γ Radicals generated from NO and ROS are toxic for a variety of microorganisms, including viruses. Thus, increased NO and ROS production enhance the defensive capacity against invading pathogens [14].

Mφ produce pro-inflammatory cytokines upon activation [13]. hCG significantly increased both IL-6 and IL-12 mRNA (data not shown) and protein production by IFN-γ-primed BMDM, while IL-10 and TNF-α levels did not change. IL-6 is considered to be a pro-inflammatory cytokine, while it also promotes Th2 differentiation from Th0 cells and simultaneously inhibits Th1 polarization [28]. In addition, IL-6 plays a crucial anti-inflammatory role in both local and systemic acute inflammatory responses by down regulating TNF-α and MIP-2, but not the anti-inflammatory cytokine IL-10 [29]. The survival rate of IL-6 knockout mice during endotoxemia was about 50% lower than of IL-6 wild type mice [29], indicating a protective effect of IL-6 under these conditions. Thus, by increasing IL-6 production, hCG can contribute to the Th2 environment during pregnancy as well as enhanced protection against inflammation.

IL-12p40 expression was also increased by hCG. However, no effect was seen on IL-12p70 and IL-23 production. IL-12 exists *in vivo* in three forms: IL-12p70 heterodimer, consisting of IL-12p40 and p35, IL-12p40 homodimer, and IL-12p40 monomer [30]. Mφ are major producers of IL-12, and secrete relatively more p40 than p35 subunits. IL-12p70 plays a key role in the differentiation of Th1 cells and resistance to bacterial infections. Conversely, homodimeric IL-12p40 can compete with the heterodimeric p70 for binding to the high affinity IL-12R thus inhibiting IL-12p70 activity [31-36]. Mice lacking p40 have a higher mortality rate and bacterial burden when infected with Salmonella, compared to mice that were capable of producing p40 but were p35-deficient [33, 37]. Similarly, humans deficient for p40 have a hampered ability to fight infections of Bacille Calmette Guerin and Salmonella and undergo recurrent episodes of pneumonia with sepsis [38, 39]. These data indicate that IL-12p40 is a necessary component of human immunity against bacteria. The observed increase of IL-12p40 by Mφ upon hCG treatment may therefore enhance the innate immune response during pregnancy.

Blocking of the cAMP/PKA-signaling pathway, which is induced upon hCG receptor activation, significantly reduced the increased ROS production by IFN-γ-primed BMDM upon hCG treatment. This indicates that the observed effect of hCG on ROS production by Mφ probably involves receptor mediation. Binding of hCG to hCG/LH receptor activates the intracellular cAMP/PKA-signaling pathway leading to cyclic-AMP response element binding protein (CREB) transcription and inhibition of the TNF signaling pathway [21, 22]. It has also been shown that activation of CREB leads to the induction of NO, IL-6 and TNF-α [23, 40, 41]. In our model the TNF-α production remained unchanged, which may be due to the simultaneous inhibition and induction of TNF-α production by hCG. Inhibition of the PKA signaling pathway did not reduce IL-6 and IL-12p40 protein production by IFN-γ-primed BMDM upon hCG treatment (data not shown), which indicates a separate pathway.

IFN-γ-primed BMDM appeared functional activated upon hCG treatment, indicated by the increased production of NO, ROS, IL-6 and IL-12p40. However, the expression of F4/80, CD11b, MHC class II and CD40 did not alter. Also, the capacity of hCG-treated IFN-γ-primed BMDM to induce allogeneic T cell proliferation was unchanged. This suggests that hCG did not affect the capacity of Mφ to stimulate adaptive immunity via T cell activation.

A major function of Mφ is to phagocytose apoptotic cells, harmful particles, and to take up and destroy microorganisms. Simultaneously, hazardous inflammatory immune reactions should be inhibited. Upon IFN-γ priming and hCG treatment, a higher percentage of Mφ became phagocytic. These phagocytic cells showed an increased phagocytosis of beads. Blocking of the cAMP/PKA-signaling pathway significantly reduced this effect, indicating receptor involvement in the hCG effect. Recruited inflammatory peritoneal Mφ which were isolated after *in vivo* treatment with hCG did not exhibit a change in phagocytic capability. However, further *ex vivo* stimulation with IFN-γ in the continuous presence of hCG did cause an increased phagocytosis, emphasizing the additional stimulating capacity of hCG on the IFN-γ-induced increase of phagocytotic capacity. There are several possible explanations for this observation: 1) *in vivo* thioglycollate stimulation and *in vitro* IFN-γ activation provide different inflammatory signals to hCG treated Mφ, 2) inflammatory recruited Mφ, but not resident peritoneal cells, are more comparable to BMDM; 3) hCG alone can not affect phagocytosis, but an additional inflammatory signal is required for the newly thioglycollate-recruited Mφ to exert the effect. Another difference could be the different hCG concentration between *in vitro* and *in vivo* conditions. For our *in vivo* experiments we chose the effective dose used in other *in vivo* experiments revealing the influence of hCG on the immune system [19, 42, 43]. Our data indicate that the continuous presence of hCG and an activator such as IFN-γ are required for the increased phagocytosis by Mφ in our model. hCG is produced in high amounts by trophoblast cells especially in the first trimester of pregnancy. Simultaneously, apoptosis is prominent in the trophoblast layer of the placenta, suggesting that cell turnover at the site of implantation is necessary for the appropriate growth and function of the placenta [44, 45].

It should be noted that purified hCG preparations contains various breakdown products including peptides and hCG associated fragments, many of which are identified [19, 46]. Although we used clinical grade hCG and our data suggested the whole molecule instead of the breakdown products that exert the observed effect, the function of individual peptides need to be further investigated.

In conclusion, hCG can promote innate functions of Mφ, such as the clearance of apoptotic cells and the resolution of inflammation, which are highly relevant for the maintenance of pregnancy.

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Chapter 4

Chorionic gonadotropin induces dendritic cells to express a tolerogenic phenotype

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ABSTRACT

The pregnancy hormone human chorionic gonadotropin (hCG) has been suggested to play an immunoregulatory role in addition to its endocrine function, thus contributing to the prevention of fetal rejection. We hypothesized that hCG is involved in the maternal-fetal immune tolerance by the regulation of dendritic cells (DC) function. Therefore, we studied the effect of hCG on DC maturation. Upon hCG treatment in combination with LPS, mouse bone marrow-derived DC (BMDC) increased the ratio of IL-10/IL-12p70, downregulated TNF-α, and decreased antigen-specific T cell proliferation. Addition of hCG together with LPS and IFN-γ, blocked MHC class II upregulation, increased IL-10 production and decreased the antigen-specific T cell proliferation by DC. Splenic DC showed similar results. Upon hCG treatment, indoleamine 2,3-dioxygenase (IDO) mRNA expression and its metabolite kynurenine were increased by LPS- and IFN-γ-stimulated DC, suggesting its involvement in the decreased T cell proliferation. To study the effect of hCG on DC differentiation from precursors, bone marrow-derived DC were generated in the continuous presence of hCG. Under this condition, hCG decreased cytokine production and the induction of T cells proliferation. These data are suggestive for a contribution of hCG to the maternal-fetal tolerance during pregnancy by modifying DC towards a tolerogenic phenotype.

KEY WORDS

hCG, DC, IDO, tolerance.

INTRODUCTION

Immune-stimulating DC are potent antigen-presenting cells that process antigens and upregulate co-stimulatory molecules to activate naïve T cells, and induce an adaptive response. In addition, DC regulate the adaptive immune response by Th1/Th2 skewing. The production of IL-12 by DC, especially the increased ratio of IL-12/IL-10, promotes a Th1 response [1-3]. Lipopolysaccharide (LPS) is commonly used to mature DC towards an immune-stimulating phenotype. These immune-stimulating DC are characterized by high MHC class II and co-stimulatory molecule surface expression, increased IL-12 production compared to tolerogenic DC, and high T cell activation capacity [4]. In addition to LPS, IFN-γ is used to activate myeloid cells such as DC and macrophages to increase MHC II expression [5]. Conversely, DC that induce tolerance, the so-called tolerogenic DC, can have either an immature or a mature phenotype. An important difference between immunogenic DC and tolerogenic DC lies in the level of IL-10 production, which is higher in tolerogenic DC than immunogenic DC [6-9].

Human chorionic gonadotropin (hCG) is a placental glycoprotein mainly secreted by trophoblasts during pregnancy. hCG induces the production of progesterone and estrogen during early pregnancy, but in addition it is also proposed to have immunosuppressive effects. During pregnancy the maternal immune system undergoes alterations that help to tolerate the fetus during intrauterine life. High hCG levels were found to coincide with the development of peritrophoblastic immune tolerance [10]. Meanwhile, pregnancy biases towards a Th2 condition supported by increased systemic levels of Th2-type cytokines such as IL-4, IL-5 and IL-10. This Th2 condition shift might be related to the observation that many Th1-type autoimmune diseases remit during pregnancy and recur after childbirth. This has led to the proposal that the change of pregnancy-related factors such as hCG has an immunosuppressive effect and influences the severity of autoimmune diseases [11, 12].

We found that hCG treatment can prevent autoimmune diabetes [13]. The repeated injection of hCG into 14 weeks old NOD mice, a spontaneous model for type I diabetes, prevented the occurrence of diabetes in this model and reversed the inflammatory infiltration of the pancreas by lymphocytes and macrophages. In addition, the transfer of splenocytes, containing DC and lymphocytes, from hCG-treated NOD mice into immunocompromised NOD-*scid* mice inhibited the development of diabetes [13]. Considering the importance of DC orchestrating the immune response [14, 15], the effect of hCG in diabetes development in NOD mice might be due to a direct effect of hCG on DC. Interestingly and in line with this hypothesis, it has been reported that DC bear the receptor for hCG [16]. Earlier it has been shown that hCG itself has activating effect on human peripheral blood-derived DC. A three days culture of these cells in the presence of GM-CSF without other stimulation resulted in increased expression of costimulatory molecules, unchanged HLA-DR, increased induction of allogeneic T cell proliferation and cytokine secretion (IL-12 and IFN-γ) [17]. However, there are no data on the role of hCG on stimulated DC and that is important for both the understanding of the maternal-fetal tolerance during pregnancy. Furthermore, it is relevant for the putative therapeutic effect of hCG in autoimmune diseases.

Ueno *et al.* recently reported that hCG injection into NOD mice inhibited diabetes development and this protective effect was associated with an increased expression of IDO by DC. From this study, it is unclear however, whether the upregulation of IDO is a direct or

indirect effect of hCG on DC [18]. Indoleamine-2,3-dioxygenase (IDO) is an immunoregulatory enzyme that degrades tryptophan to the metabolic products known as kynurenines. IDO has been found to be increased in the placenta in the first weeks of pregnancy [19, 20]. DC are present at the maternal-fetal interface and IDO expression was up-regulated on these DC during pregnancy [21]. Mature DC that express IDO enzyme activity are potent suppressors of T cell responses and promote the development of regulatory T cells [22]. Such regulatory T cells can secrete IL-10, and this cytokine subsequently helps to sustain expression of functional IDO in mature DC [23]. This loop is supposed to result in the development of immune tolerance of the maternal immune system against trophoblasts with paternal antigen expression [21, 24]. Indeed, administration of an IDO inhibitor caused allogeneic fetal rejection [25]. Interestingly, recurrent pregnancy loss has been observed in a patient that developed anti-hCG autoantibodies [26]. The co-localization of IDO and hCG in trophoblasts and placenta, and their similar role in the immunological tolerance between mother and fetus, suggests that hCG could be a direct inducer of IDO.

In this study we investigated the effect of hCG on DC function. Upon activation of DC by LPS and IFN-γ, additional hCG treatment resulted in hampered MHC class II expression, increased IL-10 and increased IDO expression that all resulted in a decreased ability to stimulate T cell proliferation. This is important to understand the role of hCG in successful pregnancy against rejection and its therapeutic effect on autoimmune diseases.

MATERIALS AND METHODS

Mice

Specific pathogen-free C57BL/6 and C3HeB/FeJ female mice were purchased from Harlan (Horst, The Netherlands) and were housed in microisolator cages and given mouse chow and water ad libitum in the animal care facility at Erasmus MC. OT-II (OVA-TcR transgenic) female mice were bred according to standard procedures. Mice were 8 weeks of age when sacrificed for the isolation of bone marrow (BM) cells and naïve T cells from the spleen. All experiments were performed with approval of the Erasmus MC animal welfare committee (Rotterdam, The Netherlands).

Bone marrow cell isolation

Femora were removed from C57BL/6 mice. BM cells were flushed out of the bones with culture medium RPMI-1640 (Biowhittaker, Verviers, Belgium) supplemented with 10% FCS (Perbio, Etten-Leur, Netherlands), and 100 U/ml penicillin, and 100 μg/ml streptomycin (Biowhittaker). The BM cells were filtered through a 100 μm sieve (BD Bioscience, Erembodegen, Belgium) and resuspended in cold culture medium (4ºC). Cells were used after storage in liquid nitrogen with essentially the same results.

Bone marrow-derived DC culture and stimulation

Bone marrow-derived DC (BMDC) were generated by thawing BM cells and culturing in petri dishes (Becton Dickinson, Le Pont De Claix, France) at a concentration of 2 x 10^6 live cells per petri dish (∅ 10 cm) in 10 ml culture medium containing 20 ng/ml recombinant

mouse GM-CSF (rmGM-CSF, Biosource International, Camarillo, USA) in a 37 ºC, 5% CO_2 incubator. At day 3, medium containing rmGM-CSF was refreshed. At day 5, another 10 ml of medium with rmGM-CSF was added to the cultures.

At day 6, non-adherent cells were collected by adding 0.05% EDTA (Fluka, Buchs, Switzerland) for 20 minutes at 37 ºC. The adherent cells were detached and collected. All cells were incubated with an antibody against CD86 (American Type Culture Collection, Manassas, USA) and anti-rat-IgG microbeads, followed by AutoMACS separation (both from Miltenyi Biotec, Bergish Gladbach, Germany). The CD86 negative cells, representing immature DC (iDC), were collected, counted and used in the following experiments.

For cell activation, iDC were transferred into 96-well flat-bottom plates (Nunc A/S, Roskilde, Denmark) $0.5x10^6$ cells/well and stimulated with the following stimuli: 50 ng/ml LPS (Sigma, St. Louis, USA), 100 ng/ml IFN-γ (rmIFN-γ, Biosource, Nivelles, Belgium), or 50 ng/ml LPS + 100 ng/ml IFN-γ, with or without 150 U/ml hCG (Pregnyl, Organon, Oss, The Netherlands). After overnight incubation, the culture supernatants were collected, and the cells were harvested for flow cytometry and T cell proliferation assay.

To study the effect of hCG on DC differentiation, C57BL/6 bone marrow cells were cultured for 6 days in the presence of rmGM-CSF with or without 150 U/ml hCG. Cells were stimulated overnight with LPS in the presence or absence of hCG. At day 7, the culture supernatants were collected and the cells were harvested.

To exclude the possible contamination of hCG by LPS, polymyxin B (Sigma, St. Louis, USA), an antibiotic known to inhibit activities induced by LPS, was applied. The observed effects of hCG were found not to be caused by LPS contamination.

Splenic DC isolation and stimulation

Splenic DC were obtained from C57BL/6 mice. From a spleen cell suspension, red blood cells were lysed by Gey's medium (Millipore, Billerica, USA) cells were incubated with CD11c-labeled microbeads (Miltenyi Biotec), followed by positive selection using the AutoMACS.

$CD11c^+$ DC ($0.5x10^6$ cells/well) were seeded into 12-well flat-bottom plates (Nunc A/S) and stimulated with 50 ng/ml LPS + 100 ng/ml IFN-γ, with or without 150 U/ml hCG. After overnight incubation at 37ºC, the culture supernatants were collected and the cells were harvested for flow cytometry.

Flow cytometry

At day 7, BMDC were collected and labeled with the following antibodies: ER-TR3-bio (MHC II, BMA, Augst, Switzerland), Streptavidin-APC, CD86-FITC, and CD80-PE (all from BD PharMingen, Erembodegen, Belgium). After labeling, the BMDC were resuspended in 7-AAD (7-Aminoactinomycin D, Molecular Probes, Leiden, Netherlands) to exclude dead cells, and analyzed on a FACSCalibur apparatus. Data were analyzed using CellQuest software (Becton Dickinson).

Cytokine detection

ELISA kits for IL-10, IL-6, TNF-α (Biosource, Nivelles, Belgium), and IL-12p40, IL-12p70 (R&D, Oxen, UK) were used according to the protocols supplied by the

manufacturer. Briefly, plates were coated with capture antibody for 18h and washed with PBS-Tween (0.05%). Diluted supernatants and standards were added and incubated for 2h at room temperature. After that the biotin-labeled detection antibody was added followed by incubation with streptavidin-HRP for 30 min; Chromogen substrate TMB (tetramethylbenzidine) was added for 30 min, followed by the addition of the stop solution. In between incubations, the plates were washed with PBS-0.05%Tween. The optical density of the product solution was measured at 450 or 450+650 nm by an ELISA reader (Thermo Labsystems, Finland).

Antigen-specific T cell proliferation

For determination of antigen-specific T cell proliferation, naïve T cells were obtained from the spleens of OT-II mice, after lysis of the red blood cells by Gey's medium (Millipore) and incubation with CD11b + CD45R + MHC II antibodies (hybridoma supernatant M1/70, B220, and M5/114 for CD11b, CD45R, and MHC II, respectively, American Type Culture Collection, Manassas, USA) and anti-rat IgG microbeads (Miltenyi Biotec), followed by AutoMACS separation of the negative cells. DC were collected and pulsed with OVA peptide 323–339 (ISQAVHAAHAEINEAGR, 4 μM) for 2 hours at 37ºC, followed by the addition of OT-II T cells that recognize this peptide. After culturing T cells ($1.5x10^5$ cells/well) and DC ($0.3x10^5$ cells/well) in round-bottom 96-microwell plates (Nunc, Roskilde, Denmark) for a period of three days, proliferation of T cells was measured by uptake of ^{3}H-thymidine (1 μCi/well, DuPont-NEN, Boston, USA) and expressed as counts per minute (cpm). The coculture supernatants were also collected for IL-10 ELISA.

Allogeneic mixed leukocyte reactions

Naïve T cells were obtained from spleens of C3HeB/FeJ mice as described above. BMDC were added to T cells (ratio 1:5). After co-culture in RPMI-1640 culture medium containing 10% FCS, 60 mg/ml penicillin and 100 mg/ml streptomycin for three days, proliferation of T cells was measured by uptake of ^{3}H-thymidine (1 μCi/well; DuPont-NEN, Boston, USA) over a period of 16 hours and expressed as counts per minute (cpm).

Quantitative Real-time PCR (qRT-PCR) of IDO

iDC were stimulated with: 50 ng/ml LPS, 100 ng/ml IFN-γ or 50 ng/ml LPS + 100 ng/ml IFN-γ, with or without 150 U/ml hCG. After 0, 2, 4, 6, 8, 10 hours of incubation at 37ºC, cells were collected and lysed. RNA was extracted using an RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the protocol supplied by the manufacturer. Complementary DNA (cDNA) was synthesized using the Superscript first strand synthesis system for reverse transcription-PCR (Invitrogen, Carlsbad, California, USA) according to the manufacturer's instructions. Quantitative real-time PCR was performed and analyzed using an ABI 7700 Sequence detection system (Applied Biosystems, Foster City, USA) and Taqman probe-based chemistry. Primers for mouse IDO (Mm00492586_m1) and ABL were obtained from Primer ExpressTM (Applied Biosystems). Each PCR sample was run in duplicate. The mean value of the two reactions was defined as representative of the sample. The resulting IDO CT values were corrected relative to ABL CT values. To facilitate interpretation of results, we have used the following equation for all figures: 2^(ABL-IDO) x100%. Therefore, an increase is proportional to an increase in expression of the particular target gene.

Kynurenine detection

Concentrations of kynurenine were determined using an isocratic, reversed-phase HPLC (Agilent) and fluorometric detection (Jasco). The analytical column consisted of a 250 x 2.1 mm i.d. column packed with 5 µm particles of GraceSmart RP-18 (Grace Davison Discovery Sciences), which was protected by a guard cartridge column (4.0 x 2.0 mm i.d.) containing Phenomenex C18 material. An HP ChemStation (Hewlett Packard) was used for data collection and handling.

For kynurenine determination, 100 µl of specimen was mixed with 20 µl of 300 µM 1-methyltryptophan (internal standard) in 14% (w/v) trichloroacetic acid. This mixture was placed on ice for 10 min and centrifuged at 12000g and 4°C for 15 min. Eighty µl of supernatant was transferred to an HPLC vial and mixed with 20µl of 0.6 M LiOH. The sample (15 µl) was injected onto the column and HPLC was carried out at a flow rate of 0.4 ml/min and a column temperature of 40°C. Mobile phase was potassium phosphate buffer (50 mmol/L, pH 3.6) containing 5% (v/v) methanol. Kynurenine (retention time 3.7 min) and 1-methyltryptophan (retention time 9.9 min) were detected via their natural fluorescence at excitation and emission wavelengths of 363 and 500 nm, and 285 nm and 365 nm, respectively. Quantitation was done by measuring peak height relative to a calibration mixture. Recoveries (mean ± SD) of kynurenine and 1-methyltryptophan were 100 ± 13 and 87 ± 4%, respectively. The intra- and interassay coefficients of variation for both compounds were less than 2 and 4%, respectively.

Statistical analyses

Data are expressed as mean values ± SEM in all the figures. All statistical analyses were performed using logarithmic transformation and/or Student's paired *t*-test. *p*-values <0.05 were considered significant. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

RESULTS

hCG treatment hampers upregulation of MHC class II expression by LPS+IFN-γ-stimulated BMDC

The effect of hCG on DC activation was studied by stimulating iDC with LPS or LPS+IFN-γ in the presence or absence of hCG. Unstimulated cells exhibited after overnight culture a small spontaneously matured population (Figure 1A). LPS fully matured DC resulting in high expression of CD86, CD80, and MHC class II. The addition of hCG did not change the expression of these markers; CD40, CD11c, and F4/80 also remained unchanged upon hCG treatment (data not shown). When LPS+IFN-γ were added together, an additional 15% more $CD86^{high}CD80^{high}$ mDC were obtained compared to LPS stimulation alone (Figure 1A). The expression of CD80, CD86 and CD40 was increased twofold in a dose dependent way after stimulation with LPS+IFN-γ compared to LPS stimulation alone. F4/80 and MHC class II expression were unchanged (data not shown and Figure 1C). The addition of hCG hampered the upregulation of MHC class II on LPS+IFN-γ-activated DC, seen both for percentage of cells, and expression level (measured as MFI) (Figure 1A, 1B and 1C). No change in the expression of CD86, CD80, CD40, CD11c, F4/80, chemokine receptor CCR5, and DC marker DEC205 was observed upon hCG addition (data not shown).

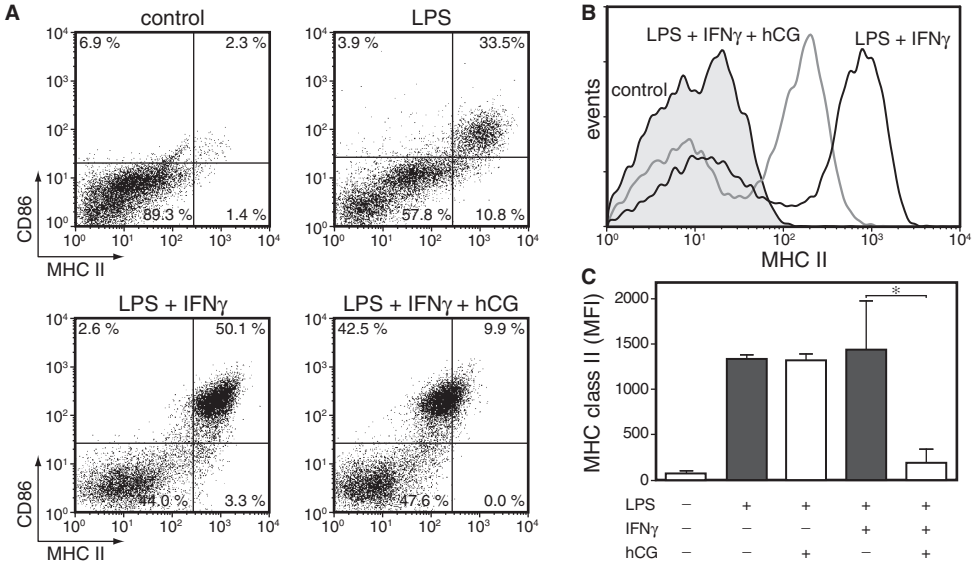

Figure 1. Hampered upregulation of MHC class II expression by BMDC upon hCG treatment.
C57BL/6 bone marrow cells were cultured for six days in the presence of rmGM-CSF. CD86^{-} iDC were isolated and stimulated with PBS, LPS or LPS+IFN-γ with or without hCG. After 18 hours the cells were collected and analyzed by flowcytometry. **A.** and **B.** Dotplot and histogram of a double-staining for MHC class II and CD86 by DC after PBS (control, filled histogram) or LPS+IFN-γ stimulation in the presence (dotted line) or absence (dark line) of hCG. **C.** MHC class II expression (MFI) by gated CD86^{high} CD80^{high} mDC after LPS or LPS+IFN-γ stimulation in the presence or absence of hCG. The data are the mean of 4-6 experiments. * $p<0.05$.

hCG alters cytokine production of BMDC

LPS-activated iDC secreted IL-10, TNF-α, and IL-6. The addition of hCG resulted in an increase in IL-10, and a decrease in TNF-α and IL-6 secretion (Figure 2A).

Activation of iDC with LPS+IFN-γ resulted in a significant upregulation of IL-12p70, IL-10 and TNF-α compared to LPS stimulation alone (Figure 2A and 2B). hCG treatment of LPS+IFN-γ-stimulated BMDC significantly increased TNF-α and IL-10 production by 15% and 30%, respectively, and IL-6 for more than twofold (Figure 2B). Under both stimulation conditions, IL-12p70 production did not change upon hCG treatment. It should be noticed that the absolute production of TNF-α and IL-6 by LPS- and LPS+IFN-γ-stimulated BMDC differs, which suggests that the decreases in TNF-α and IL-6 upon hCG-treatment by LPS-stimulated BMDC, although statistically significant, are biologically irrelevant (Figure 2A and 2B). To further investigate if the decreased MHC class II by LPS+IFN-γ stimulated-DC upon hCG treatment is via the induction of TNF-α or IL-10, BMDC were cultured with neutralizing anti-TNF-α or neutralizing anti-IL-10. Neutralizing TNF-α or IL-10 did not result in restoration of MCH II expression (data not shown). Alternatively, BMDC were cultured with recombinant TNF-α or recombinant IL-10 to approximate the effects of hCG. No effects on MHC class II expression was observed upon the addition of TNF-α or IL-10 (data not shown).

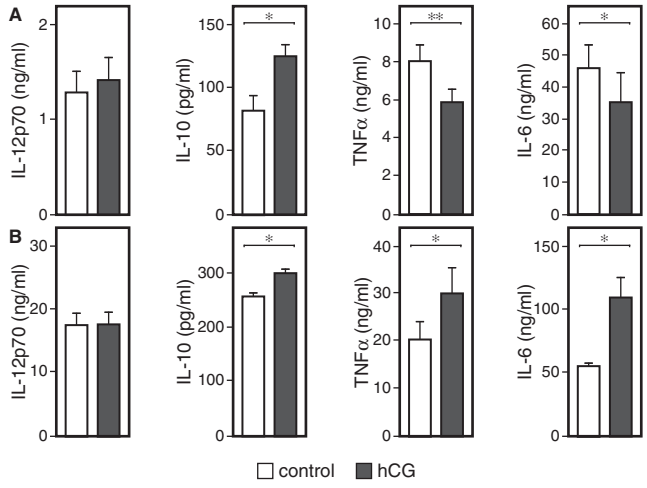

Figure 2. hCG altered cytokine production by LPS or LPS+IFN-γ stimulated BMDC.
iDC were isolated as $CD86^-$ cells from BMDC cultures and stimulated with either LPS or LPS+IFN-γ with or without hCG. After 18 hours the culture supernatants were collected and analyzed for the presence of IL-12p70, IL-10, TNF-α and IL-6 by ELISA. Cytokine production by **(A)** LPS- (*n*=3) or **(B)** LPS+IFN-γ- (*n*=6) stimulated DC with or without hCG treatment were shown. * $p<0.05$, ** $p<0.01$.

Effect of hCG on splenic DC

To study the effects of hCG on a resident DC population, splenic $CD11c^+$ cells were isolated (purity > 85%) and stimulated by LPS+IFN-γ *ex vivo* for 24 hours followed by gating of $CD86^{high}CD80^{high}$ mDC. Addition of hCG to these cultures declined the upregulation of MHC class II (Figure 3A and 3B). In line with the previous result obtained from BMDC, a significant increase of IL-10 production by LPS and LPS+IFN-γ-stimulated splenic DC was observed in the presence of hCG (Figure 3C). Under both conditions studied, IL-12p70 production did not alter upon the addition of hCG (data not shown).

hCG treatment of BMDC hampers their ability to stimulate antigen-specific T cell proliferation

In the next set of experiments we tested the influence of hCG-treatment during LPS, LPS+IFN-γ, and IFN-γ-activation of DC on the induction of OVA-specific $CD4^+$ T cell proliferation. The addition of hCG during DC maturation induced by any of the tested stimuli resulted in a significant decrease in their ability to stimulate antigen-specific T cell proliferation. Treatment of iDC with hCG in the absence of additional maturation stimuli enabled these cells to stimulate showed antigen-specific $CD4^+$ T cell proliferation to a slightly higher extent compared to untreated iDC (Figure 4A).

Th1 and Th2 polarization capacity of DC was determined by the measurement of the IL-4 and IFN-γ production by T cells. Both LPS- and LPS+IFN-γ-stimulated DC induced IFN-γ but no IL-4 production. The addition of hCG did not change this Th1/Th2 balance (data not shown).

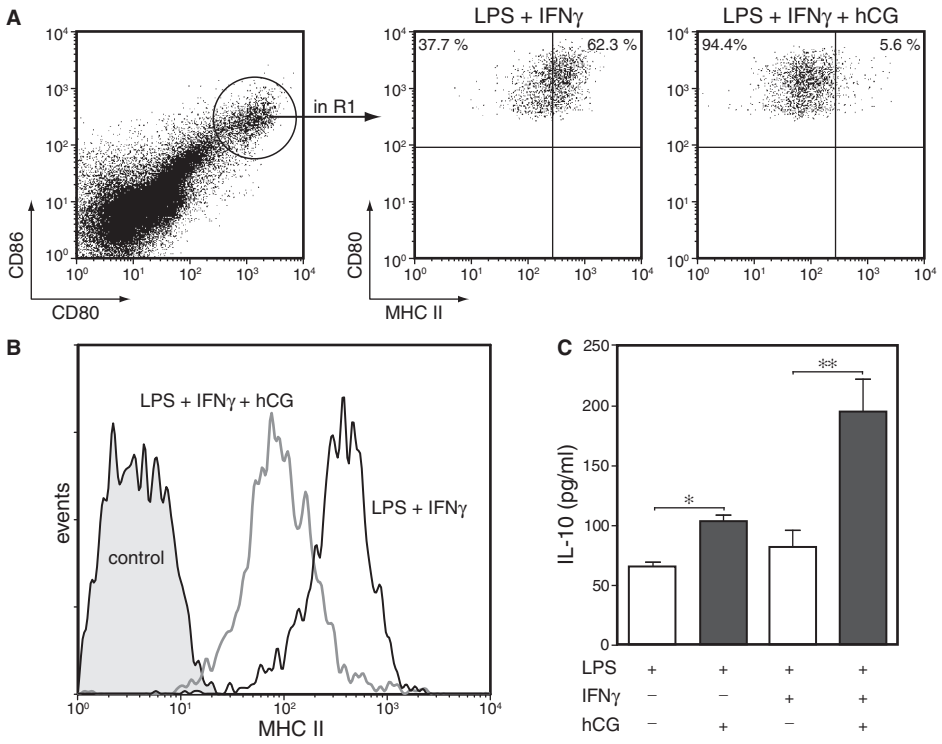

Figure 3. Hampered upregulation of MHC class II expression and increased IL-10 production by LPS+IFN-γ stimulated splenic CD11c$^+$ DC cultured in the presence of hCG.
Splenic CD11c$^+$ cells were isolated from C57BL/6 mice and stimulated with LPS+IFN-γ with or without hCG. 18 hours later, the cells were collected and stained for CD86, CD80, and MHC class II. In dotplot **(A)** and histogram **(B)**, CD86^{high} CD80^{high} cells were gated, followed by analysis of MHC class II expression of LPS+IFN-γ stimulated splenic CD11c$^+$ dendritic cells with (dotted line) or without (dark line) hCG treatment. Filled histogram represents the isotype control. **C.** IL-10 production by splenic CD11c$^+$ cells stimulated with LPS or LPS+IFN-γ with or without hCG. The results shown are a single representative from three independent experiments with similar outcome.

To study whether T cells stimulated by hCG-treated DC display tolerogenic properties, the IL-10 level in the supernatants of cocultures of T cell and DC was measured. Unstimulated and PMA-stimulated T cell did not produce IL-10, neither did iDC activated T cells (Figure 4B). LPS-stimulated DC activated T cells to produce IL-10. hCG treatment to LPS-stimulated DC showed a similar IL-10 level as LPS-stimulated cocultures without hCG. The IL-10 production in LPS+IFN-γ-stimulated cocultures with hCG treatment is significantly higher than LPS+IFN-γ-stimulated cocultures without hCG treatment (Figure 4B).

IDO involvement in the observed effect of hCG on DC

In order to investigate whether the hCG-induced inhibition of T cell proliferation might be mediated directly via the induction of IDO, the mRNA expression of IDO was studied.

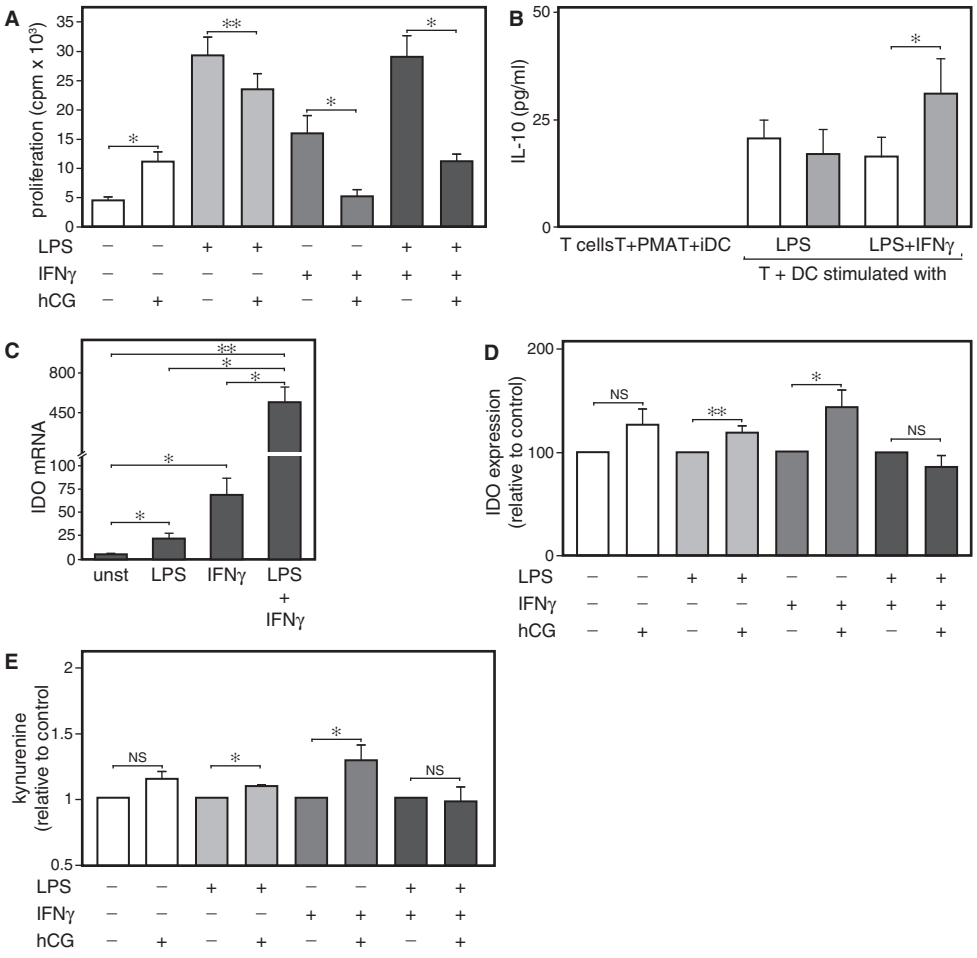

Figure 4. DC treated with hCG revealed a decreased ability to induce antigen-specific CD4⁺ T cell proliferation and an increased IDO mRNA expression.

A. Cultured C57BL/6 BMDC were stimulated with LPS, IFN-γ or LPS+IFN-γ with or without hCG for 18 hours. For OVA antigen-specific CD4⁺ T cell proliferation induction, DC were collected and pulsed with OVA for 2 hours, and then added to splenic T cells from OT-II mice. These DC and T cells (ratio 1:5) were co-cultured for three days. The proliferation of the T cells was determined by ^{3}H-thymidine incorporation. The supernatants from these cocultures were collected and analyzed for IL-10 production **(B)**. Data were obtained from 3 individual experiments. **C.** iDC were stimulated with LPS, IFN-γ or LPS+IFN-γ for four hours, then cells were collected for IDO qRT-PCR analysis (n=3). In the absence or presence of hCG, iDC were stimulated with LPS, IFN-γ or LPS+IFN-γ either for four hours then collected for IDO mRNA measurement by qRT-PCR **(D)**, or supernatants of these cultures were collected at 24 hours for measurement of Kynurenine and tryptophan **(E)**, the picture was depicted as relative to non-hCG treatment in the same stimulation (n=3). * p<0.05, ** p<0.01.

IDO mRNA was found quickly induced from 2 hours after either LPS or IFN-γ stimulation, and peaked at 4 hours (data not shown). After four hours of stimulation, LPS induced IDO mRNA expression about sevenfold and IFN-γ stimulated about twentyfold. The combination of LPS and IFN-γ resulted in a more than hundredfold increased IDO expression compared to unstimulated BMDC (Figure 4C). The addition of hCG significantly increased the IDO mRNA expression by BMDC upon stimulation with LPS or IFN-γ alone, but not by LPS+IFN-γ stimulated and unstimulated BMDC (Figure 4D). In order to analyze whether the increased IDO mRNA expression upon hCG treatment was transcribed into functional protein, the biological activity of induced IDO was investigated by quantification of tryptophan and its catabolite kynurenine in culture medium. hCG treatment significantly increased the kynurenine level by BMDC upon stimulation with LPS or IFN-γ alone, but not by LPS+IFN-γ stimulated and unstimulated BMDC (Figure 4E). The level of tryptophan remained unchanged upon hCG treatment probably due to the high concentration of tryptophan in the culture medium (data not shown).

Effect of hCG on BMDC differentiation from precursor cells

To investigate the effect of hCG on the differentiation of DC from precursor cells hCG was added from the start of the bone marrow cell cultures and refreshed at the same time when medium was changed routinely. By applying double staining for Ly6C and CD31 [27], the differentiation of BMDC was evaluated. The continuous presence of hCG did not change the expression of these differentiation markers as well as MHC class II and CD11c (data not shown). Without LPS stimulation, DC produced low levels of cytokines, and the presence of hCG during their development did not increase cytokine production. LPS-activated DC secreted IL-12p40, IL-12p70, IL-10, TNF-α, and IL-6. The continuous presence of hCG in the BMDC cultures resulted in a decrease in the production of 12p40, IL-12p70, IL-10, and TNF-α (Figure 5A), as well as IL-6 (data not shown). This generalized decrease is not related to increased cell death, since Rhodamine123 staining revealed that similar, low frequencies of dead cells were observed upon hCG addition (data not shown). Different from the hCG effect on the maturation of DC, MHC class II expression by LPS+IFN-γ activated BMDC did not change when hCG was added from the beginning of the bone marrow cell cultures (data not shown).

The allogeneic T cell proliferation after LPS stimulation was significantly decreased when BMDC were cultured in the continuous presence of hCG (Figure 5B).

DISCUSSION

We studied the effect of hCG on DC maturation and function in order to explore its role in the maternal-fetal tolerance and the remission of several autoimmune diseases during pregnancy [12]. BMDC were stimulated to mature by LPS, and IFN-γ in combination with LPS was used for further maturation of DC. hCG addition resulted in hampered MHC class II upregulation by DC stimulated with LPS+IFN-γ but not with LPS alone. Upon stimulation of BMDC with LPS+IFN-γ, hCG significantly increased TNF-α production by DC, whereas upon stimulation with LPS alone, hCG significantly decreased TNF-α production by DC.

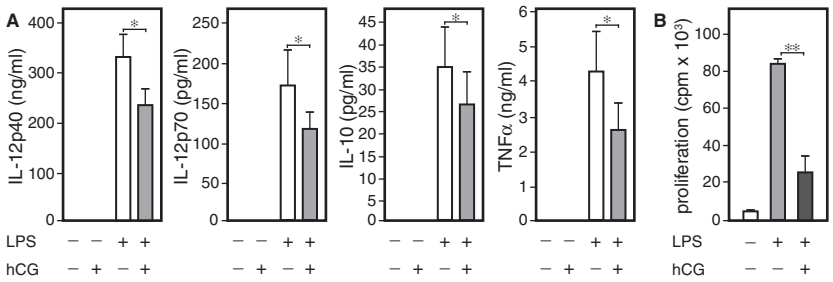

Figure 5. Continuous presence of hCG during development of DC from bone marrow precursors inhibits their ability to produce cytokines and stimulate T cell proliferation.
BMDC were cultured for seven days in the presence or absence of hCG, and stimulated with LPS with or without hCG. After 18 hours of incubation, the supernatants were collected, and evaluated for IL-12p40, IL-12p70, IL-10 and TNF-α content **(A)**, while the cells were tested in the allogeneic T cell proliferation assay **(B)**. Data represent the mean of 3 individual experiments. * $p<0.05$, ** $p<0.01$.

It has been shown that TNF-α can suppress IFN-γ induced MHC class II expression [28]. This suggests that hCG may influence MHC class II via inhibition of IFN-γ-induced upregulation of TNF-α, but our data showed no effects of TNF-α on hCG-induced suppression of MHC class II, neither did IL-10. In general, lower levels of MHC class II expression are correlated with a lower capacity to present antigen and to stimulate T cell proliferation and/or function. Indeed, we observed decreased antigen-specific T cell proliferation by LPS+IFN-γ stimulated DC upon hCG treatment. However, DC stimulated with LPS also exhibited a decreased antigen-specific T cell stimulation while these particular DC did not show impaired MHC class II expression. This indicates that, under these conditions, other hCG-induced factors are involved as well.

A candidate hCG-induced factor that may contribute to decreased APC function is IL-10. This cytokine has important immunosuppressive functions. High production of IL-10 by DC is a characteristic feature for tolerogenic DC. IL-10 is also considered a Th2-type cytokine [3, 8]. In former experiments, we showed that the splenic $CD4^+$ T cells from hCG-treated NOD mice tend to produce more IL-10 [13]. Here, we observed an increased production of IL-10, but unchanged IL-12p70 resulting in an increased IL-10/IL-12p70 ratio, from LPS and LPS+IFN-γ activated BMDC treated with hCG. Increased IL-10 production was also found in splenic $CD11c^+$ DC upon hCG treatment. The change we observed in the IL-10/IL-12p70 ratio implies that hCG might contribute to a Th2 cytokine environment and an immunosuppressive state during pregnancy. However, results from the *in vitro* T cell proliferation assay did not show switching from Th1 to Th2 responses. This is consistent with data in the literature demonstrating that pregnancy is a condition characterized by increased Th2-type cytokine production rather than a change in T cell polarization [29, 30]. Furthermore, hCG treatment increased the IL-10 production by T cells cocultured with LPS+IFN-γ-stimulated DC compared to cultures without hCG treatment. These results suggests that hCG-treated DC can induce T cells with tolerogenic property.

IL-6 is thought to be a pro-inflammatory cytokine involved in chronic inflammatory responses, but IL-6 also plays a crucial anti-inflammatory role in both local and systemic acute inflammatory responses and helps Th0 cells differentiate into Th2 cells [31, 32]. Depending on the stimulation conditions, hCG modified IL-6 production by DC differently. Our data indicate that further studies are needed to elucidate the role of hCG-modulated cytokine production in immunosuppression during pregnancy.

Stimulation of BMDC and splenic DC with LPS+IFN-γ, but not LPS only, revealed a lack of upregulation of MHC class II expression upon hCG treatment, suggesting that IFN-γ is involved in this effect of hCG. IFN-γ was found to trigger IDO production in DC [33].

IDO is an important downregulator of the immune response and is produced in large amounts by regulatory T cells [34, 35]. Induction of IDO expression in trophoblasts suppresses T cell activity against fetus [36]. Retarded intrauterine development is accompanied with a significantly lower IDO activity in placenta [37]. Administration of the IDO inhibitor 1-methyl-tryptophan caused allogenic foetal rejection [25]. Furthermore, in pregnant women kynurenine levels, the metabolite produced upon IDO activation, increase in blood and urine in comparison with non-pregnant women of the same age [38]. The working mechanism of IDO is that it inhibits T cell proliferation *in vitro* by rapidly consuming available tryptophan resulting in anergic T cells and increased levels of pro-apoptotic kynurenines [39, 40]. Therefore, we investigated whether hCG directly influenced the expression of IDO in DC. Indeed, hCG was able to induce substantially more IDO mRNA expression in LPS or IFN-γ stimulated DC compared to stimulation with LPS or IFN-γ alone. This increased IDO mRNA was accompanied by an increase in kynurenine levels. These results are consistent with a role of IDO in the observed hCG-induced inhibition of T cells. By influencing the IDO expression by trophoblasts and DC, hCG might thus directly contribute to the maintenance of tolerance at the maternal-fetal interface, especially under conditions of immunological challenge [22, 41, 42].

hCG levels are systemically elevated in early pregnancy, therefore, it is interesting to investigate what the influence of hCG is on the development of DC from bone marrow precursors. We cultured BM cells in the continuous presence of hCG, followed by maturation of BMDC in the presence of hCG. Under these conditions BMDC revealed a generally hampered cytokine production as well as a decreased T cell proliferation, which was not due to cell apoptosis, or postponed DC differentiation, or downregulation of hCG receptor (unpublished data). Notably, hCG binds to the G-protein coupled receptor that activates Gs/camp pathway which maybe responsible for the hampered IL-12 production by activated DC. The hCG receptor is one of the large GPCR superfamily. Binding of hCG to the hCG receptor triggers a conformational change of the transmembrane region of the receptor facilitating binding and activation of Gs, followed by effector enzyme activation and subsequent intracellular adenylyl cyclase/ cAMP signaling pathway [43, 44]. Activated cAMP pathway inhibits IL-12 expression in human DC [45]. All the above data indicate that hCG may contribute to the maternal-fetal tolerance via modulating DC function.

In conclusion, hCG treatment of activated DC results in hampered upregulation of MHC class II expression, as well as in increased IL-10 and IDO expression that all lead to the decreased ability to stimulate T cell proliferation. This modulating influence of hCG on DC differentiation and function may have an important contribution to maternal-fetal tolerance as well as the remission of several autoimmune diseases during pregnancy.

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Chapter 5

Chorionic gonadotropin alleviates thioglycollate-induced peritonitis by affecting macrophage function

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ABSTRACT

Human chorionic gonadotrophin (hCG) is a hormone produced during pregnancy and present at the implantation site and in the maternal blood. Pregnancy has been proposed to represent a controlled state of inflammation at an early stage at the implantation site and later systemically extended to the maternal circulation. Earlier we reported that hCG can inhibit the development of diabetes in NOD mice and LPS-induced septic shock in a murine model. We hypothesize that hCG can contribute to the reduction of inflammation by modifying Mφ function. Here the thioglycollate(TG)-induced peritonitis model for inflammation was used to investigate the effect of hCG on cytokine production and cell recruitment *in vivo*. hCG pretreatment in thioglycollate-induced peritonitis increased the number of peritoneal cells, especially PMN and monocytes, compared to mice injected with TG only. This increased cell number was partially explained by increased cell survival induced by hCG. Despite the cellular infiltrate, hCG pretreatment decreased intraperitoneal TNF-α, IL-6, PTX3, CCL3 and CCL5 levels. By depleting peritoneal resident Mφ using clodronate liposomes prior to the application of hCG and the TG trigger, we established that Mφ are the main responsive cells to hCG as the suppressed TNF-α and IL-6 production and increased PMN influx are abolished in their absence. Together, these data suggest that hCG contributes to the controlled inflammatory state of pregnancy by regulating Mφ pro-inflammatory function.

KEY WORDS

hCG, thioglycollate-induced peritonitis, resident Mφ, cell recruitment, clodronate liposomes.

INTRODUCTION

Studies on the role of the maternal immune system during pregnancy have focused mainly on the immune response towards the fetus [1]. There are two distinct immunological interfaces in pregnancy: locally in the decidua between maternal immune cells and fetal trophoblasts; and systemically between circulating maternal immune cells and the syncytiotrophoblasts that form the placental villous surface. This syncytiotrophoblast layer releases hCG and cellular microparticles into the maternal blood and interacts with maternal immune cells. Recently, pregnancy is proposed to be a controlled state of inflammation [2]. At an early stage the inflammation is predominantly present at the implantation site and later in the first trimester of pregnancy this is extended to the maternal circulation. This systemic inflammatory response in normal pregnancy is characterized by leukocytosis [3], increased monocyte priming [4], increased phagocytic activity [5], and the production of pro-inflammatory cytokines including IL-6, IL-12 and TNF-α [6,7]. It has been suggested that these inflammatory changes can be as strong as those observed in patients with sepsis, but surprisingly do not seem to harm the mother and fetus in any way [8]. This indicates that pregnancy-specific factors help to control this inflammatory response.

Human chorionic gonadotropin (hCG) is a pregnancy hormone mainly secreted by trophoblasts. It can be detected in urine and blood, indicating that hCG is present at both immunological interfaces between mother and fetus. hCG levels reach the highest in the first trimester of pregnancy, then sharply decline to low levels till the end. The most well-known function of hCG is its role in endocrine regulation, but data are accumulating on its potential immunoregulating properties. Earlier we showed that hCG can inhibit the development of diabetes in NOD mice [9] and LPS-induced systemic inflammatory response syndrome (SIRS) [10]. It has been shown recently that hCG prevents the development of Sjögren's syndrome-like salivary gland exocrinopathy in mice [11].

During normal pregnancy, high numbers of macrophages (Mφ) occur in the decidua, while increased numbers of monocytes are found in the maternal circulation [12-14]. Mφ play an essential role in the maternal defense against micro-organisms and in the removal of apoptotic cells from the placenta to prevent exposure of the maternal immune system to paternal antigens expressed by fetal cells. This clearance of apoptotic cells is crucial to the resolution of local inflammation, and thus vital for the maintenance of pregnancy [15-17]. Recently, we and others have shown that hCG can promote the innate functions of Mφ, such as releasing oxygen radicals and clearing apoptotic cells [18-21]. These *in vitro* studies suggest that hCG can contribute to the maintenance of pregnancy by influencing Mφ function. However, so far no data are available on the effect of hCG on Mφ function *in vivo*.

Intraperitoneal (i.p.) injection of thioglycollate (TG) broth has been broadly used to induce acute inflammation in the peritoneal cavity of mice. This inflammatory response shows clearly defined sequential steps of disease development starting with the influx of polymorphonuclear neutrophils (PMN) and monocytes (1-4 hours) [21] and followed by a Mφ-dominated infiltrate (40-72 hours) recruitment in the peritoneal cavity. The inflammation is accompanied by increased cytokine levels in the peritoneal fluid [22-25]. Resident Mφ have been suggested to be the initiators of the PMN recruitment and cytokine production in the peritoneum. Mice with higher numbers of resident Mφ in the peritoneal cavity exhibited an increased and accelerated

influx of PMN upon TG administration [26]. In addition, inhibition of the release of chemotactic factor(s) by resident Mφ blocked the migration of PMN into the inflamed peritoneal cavity [27]. Resident Mφ also play a role in the induction of IL-12 and the chemokines CCL3 and CCL5 in thioglycollate-induced peritonitis [28]. The above data suggest that in this model, resident Mφ control the acute inflammation by acting as "alarm cells" and triggering several defense mechanisms.

To investigate the hypothesis that hCG reduces inflammation by modifying Mφ function, we used the thioglycollate-induced peritonitis model for inflammation. Therefore we investigated the effect of hCG on cell recruitment and cytokine production *in vivo*. In these experiments, we observed that hCG pretreatment decreased TNF-α, IL-6, PTX3, CCL3 and CCL5 levels in thioglycollate-induced peritonitis, but increased the number of peritoneal cells, especially PMN and monocytes, compared to mice injected with TG only. To study the specific role of Mφ, clodronate liposomes were used to deplete the phagocytic cells from the peritoneal cavity. Mφ phagocytosed the liposomes resulting in accumulation of clodronate liposomes followed by apoptosis [29, 30]. Our study shows that Mφ are the main responsive cells upon hCG pretreatment. This observation is of importance to understand the harmless sterile inflammatory state of pregnancy, as well as the therapeutic effect of hCG in acute inflammation.

MATERIALS AND METHODS

Mice

Specific pathogen-free C57BL/6 female mice were purchased from Harlan (Horst, The Netherlands). The mice and were housed in micro-isolator cages and given mouse chow and water ad libitum in the animal care facility at Erasmus MC. Mice were eight weeks of age when subjected to i.p. injection with TG broth and/or clodronate liposomes. All experiments were performed with the approval of the Erasmus MC Animal Welfare Committee (Rotterdam, The Netherlands).

Thioglycollate-induced peritonitis

Mice were injected i.p. with hCG (300 U/20 gram body weight, Pregnyl®, Organon, Oss, The Netherlands) or PBS in a volume of 200 μL. The hCG concentration was optimized from former experiments [9, 10]. One hour later 1 mL of sterile 4% Brewer's thioglycollate broth (Sigma-Aldrich, Poole, UK) was injected i.p. per mouse; PBS was used as a control. At different time points as indicated, the mice were sacrificed and the peritoneal cavity was flushed with 4 mL cold PBS and 1 mL air to harvest cells. The collected peritoneal cells were either counted and prepared for phenotypic analysis by flow cytometry and immunocytochemistry or incubated with Annexin V and PI (Calbiochem, Darmstadt, Germany) for apoptosis detection. The peritoneal fluids were used for cytokine measurement by ELISA.

LPS contamination in Pregnyl was excluded by experiments using polymyxin B [18].

***In vivo* Mφ depletion by clodronate liposomes**

Clodronate-loaded liposomes (dichloromethylene bisphosphonate, a gift from Roche Diagnostics GmbH, Mannheim, Germany) were prepared as described previously [29].

Mice were injected twice i.p. for two consecutive days with clodronate liposomes in a volume of 200 μL per mouse, containing about 2 mg of liposomes-entrapped clodronate. Consecutive injection was used to obtain a complete depletion of phagocytic cells in the peritoneum. One day after the second liposome application, mice were injected i.p. with hCG (300 U/20 gram body weight) or PBS in a volume of 200 μL. One hour later inflammation was induced by TG as described above. Four hours afterwards, the mice were sacrificed and the peritoneal cells were collected and analyzed by flow cytometry. The peritoneal fluids were used for cytokine measurement by ELISA.

PBS was used as a control for clodronate-loaded liposomes as liposomes without clodronate induced a mild inflammation *in vivo* and *in vitro* (data not shown).

Flow cytometry

The peritoneal cells were collected and labeled with the following antibodies: BM8-bio, (BMA Biomedicals, Augst, Switzerland), Ly-6C-FITC (ER-MP20, Erasmus MC, Rotterdam, the Netherlands), Streptavidin-PercP, CD11b-APC, Ly-6G-PE, IgG control (all from BD PharMingen, Erembodegem-Aalst, Belgium), and analyzed by flow cytometry. In between incubations, cells were washed twice with PBS + 5% BSA (Celliance Corporation, Norcross, GA, USA). All incubations were performed at room temperature (RT). The data were analyzed by WinMDI 2.8 software and depicted by mean fluorescence intensity (MFI) and/or percentage (%) of cells. Determination of different cell types has been described as before [23].

Apoptotic cells were detected by Annexin V and PI staining (Calbiochem, Darmstadt, Germany).

ELISA

CCL3 and CCL5 are also known as "Macrophage Inflammatory Protein-1 alpha (MIP-1α)" and "Regulated upon Activation Normal T cell Expressed and Secreted (RANTES)", respectively. ELISA kits for the detection of murine TNF-α, IL-6, IL-10 (Biosource International, Camarillo, USA), IL-12p40, CCL3, and CCL5 (R&D systems, Oxen, UK) were used according to the protocols supplied by the manufacturer. In short: plates were coated with capture antibody overnight. After blocking by PBS-1% BSA for 2h at RT, peritoneal lavage fluid and standards were then added and incubated for 2h at RT. After washing, the biotin-labeled detection antibody was added followed by incubation with streptavidin-HRP for 30 min; Chromogen TMB (tetramethylbenzidine) was added for 30 min, followed by the addition of the stop solution. In between incubations the plates were washed with PBS-0.05% Tween. The absorbance was measured at 450 or 450 and 650 nm by ELISA reader.

Murine PTX3 mRNA measurement

Total RNA was extracted from lysed peritoneal recruited cells. High-quality RNA was extracted using an RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the protocol supplied by the manufacturer. Complementary DNA (cDNA) was synthesized using the

Superscript first strand synthesis system for reverse transcription-PCR (Invitrogen, Carlsbad, California, USA) according to the manufacturer's instructions. Mouse quantitative real-time PCR was performed and analyzed using an ABI 7700 Sequence detection system (Applied Biosystems, Foster City, CA, USA) and Taqman probe-based chemistry. Primers for mouse PTX3 (Mm00477267_g1) and the household gene Abelson (ABL) were supplied by Primer ExpressTM (Applied Biosystems). Each PCR sample was run in duplicate. The mean value of the two reactions was defined as representative of the sample. The resulting PTX3 CT values were expressed relative to an ABL CT. To ease interpretation of results, the following equation: 2^(ABL-PTX3) x 100% was used for the figure. Therefore, an increase is proportional to an increase in expression of the particular target gene.

Immunofluorescence staining

For immunofluorescence staining, cytospin slides were fixed with methanol for 5 seconds followed by acetone for 15 seconds at -20ºC. Incubation with the first antibody BM8-bio, Ly-6G-PE, or control rat-IgG2a (PH2-4a, own laboratory) was performed for 1h at RT, followed by 30 minutes incubation with Texas Red-coupled goat anti-rat Ab (Caltag, San Francisco, CA, USA) in the presence of 2% normal mouse serum. For double staining, slides were stained for intracellular IL-6-FITC, TNF-α-FITC, or rat IgG2a-FITC (all from BD Pharmingen). In between the steps, slides were washed with PBS-Tween 20. In the end, the slides were mounted in DAPI-containing Vectashield (Vector Laboratories Inc, Burlingame, CA, USA) and examined using a Zeiss Axioplan 2 imaging fluorescence microscope (Zeiss, Göttingen, Germany).

Statistical analyses

Data are expressed as mean values ± SEM in all figures. All statistical analyses were performed using Student's *t*-test. *p*-values <0.05 were considered significant. **p*<0.05, ***p*<0.01, ****p*<0.001.

RESULTS

hCG pretreatment increases PMN and monocyte recruitment in inflammation

To study the cells recruitment in peritoneal cavity, mice were pretreated with hCG i.p. and 1h later i.p. injected with sterile TG to induce peritonitis. Similar to previous experiments [23], 4h after TG injection we found an increase of total inflammatory cells, mainly consisting of PMN (≈70%), identified as Ly-6G^{high}, and monocytes identified as Ly-6G^{-}CD11b^{high}BM8^{med}. At 72h, the infiltrate was characterized by decreased numbers of PMN and increased numbers of monocytes and Mφ (60-80%), identified as Ly-6G^{med}CD11b^{high}BM8^{high} compared to 4h after TG injection (Figure 1A). In the control group without TG injection, 72h after hCG treatment an increased total cell number, mainly consisting of Mφ, was observed (Figure 3A). hCG pretreatment followed by TG injection induced increased total cell numbers. This was due to increased PMN and monocyte recruitment into the peritoneal cavity at 4h compared to PBS-pretreated mice. At 72h after hCG pretreatment no differences in inflammatory cell numbers were observed (Figure 1A). Analysis of the percentages of the different cell types showed similar results as the absolute numbers (data not shown).

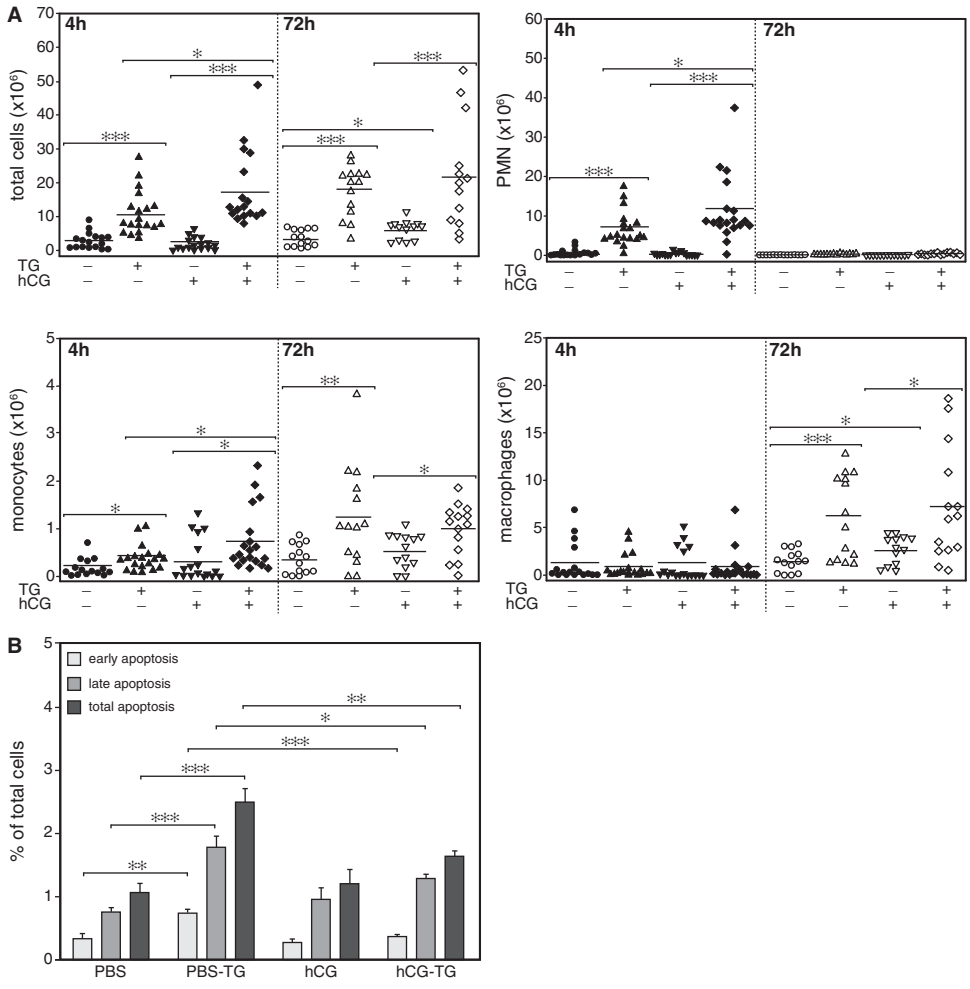

Figure 1. hCG pretreatment induces increased cell recruitment early in peritoneal inflammation.
C57BL/6 mice were injected i.p. with hCG or PBS followed by TG injection one hour later. **A.** Four hours or three days afterwards, mice were sacrificed and peritoneal cells were collected, counted and analyzed for neutrophils (PMN), macrophages and monocytes by flow cytometry (n=15). **B.** Apoptotic cells were determined by incubation of recruited cells 4h after TG injection with Annexin V and PI. Annexin V single positive cells were counted as early apoptotic cells. Annexin V and PI double positive cells were counted as late apoptotic cells. Total apoptotic cells are the sum of early and late apoptotic cells. TG increased cell death in the peritoneal cavity, but hCG pretreatment resulted in a decreased cell death (n=5). *p<0.05, **p<0.01, ***p<0.001

Analysis of T and B cells, DC and NK cells using CD3, B220, CD11c, and NK1.1 labeling revealed increased numbers of all these subsets in the peritoneal cavity upon induction of inflammation, but hCG did not influence their numbers significantly (data not shown).

hCG pretreatment prevents cell death induced by inflammation

To investigate the mechanism of hCG-induced increased cell recruitment, we studied whether hCG influenced the induction of apoptosis. By staining for Annexin V and PI, early and late apoptotic cells were detected as $AnnV^+PI^-$ and $AnnV^+PI^+$, respectively. Without the induction of inflammation by TG, hCG treatment did not affect the number of apoptotic cells in the peritoneal cavity (Figure 1B). Four hours after TG injection, an increased cell death, characterized by both early and late apoptotic cells, was observed. hCG pretreatment decreased the percentage of total apoptotic cells from 2.5% to 1.7% comprising reductions in both early and late apoptotic cells (Figure 1B).

hCG pretreatment decreases IL-6 and TNF-α production

Thioglycollate-induced inflammation is characterized by the increase of several cytokines a.o. IL-6, TNF-α, and IL-12p40. The level of these cytokines increased in peritoneal lavage fluid at 2h and 4h after injection, followed by a decrease towards basal level from 24h onwards (Figure 2A-C and data not shown). Without inflammation, hCG treatment did not affect the proinflammatory cytokine levels (data not shown). hCG pretreatment of thioglycollate-induced inflammation significantly decreased the levels of IL-6 and even more of TNF-α at 2h and 4h without changing the kinetics (Figure 2A and 2B). hCG pretreatment mediated a retained elevation of the IL-10 level at 72h and did not affect the production of IL-12p40 (Figure 2C) compared to PBS pretreatment of thioglycollate-induced inflammation. hCG pretreatment did not affect the expression of IL-12p70 and IL-1β (data not shown).

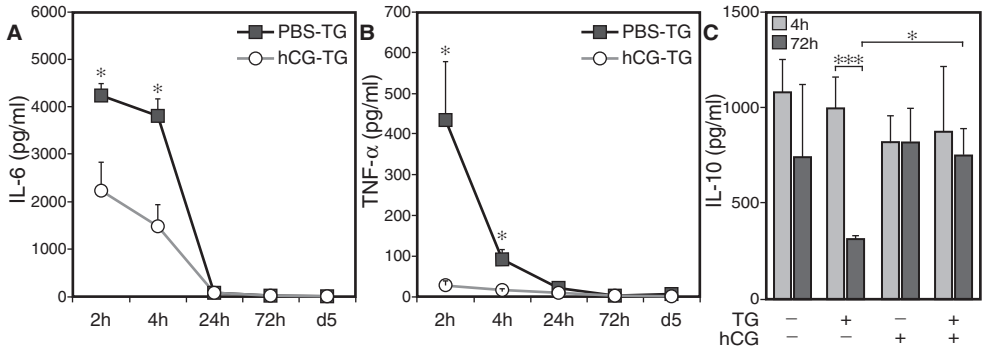

Figure 2. hCG pretreatment decreases the level of IL-6 and TNF-α in peritoneal lavage fluid.
C57BL/6 mice were injected i.p. with hCG or PBS. One hour later TG was injected i.p. After 2h, 4h, 24h, 72h (day 3) and on day 5, mice were sacrificed and peritoneal lavage fluid was collected followed by cytokine measurement. Upon hCG pretreatment of thioglycollate-induced peritonitis, decreased IL-6 (**A**) and TNF-α levels (**B**) at 2h and 4h, and a higher IL-10 level (**C**) at 72h were observed (*n*=15). Kinetic data depicted are from a separate, representative experiment with 5 mice per group (**A** and **B**).

hCG pretreatment decreases the production of TNF-α-inducible factors

Since inflammation-related TNF-α production was most strongly affected by hCG pretreatment, we evaluated the biological significance of this finding. TNF-α-inducible factors,

such as PTX3, CCL3, CCL5 and MIF, which are closely involved in the inflammatory response, were studied in details.

PTX3 is a TNF-α-inducible factor that is quickly produced upon triggering of inflammation. TG application resulted in increased PTX3 mRNA expression by recruited cells at 2h and 4h (Figure 2A). Without the induction of inflammation by TG, hCG treatment did not affect the PTX3 level compared to untreated mice. hCG pretreatment of thioglycollate-induced inflammation significantly decreased the PTX3 mRNA expression at 4h compared to PBS pretreatment (Figure 3A).

Similar to the previous findings, hCG pretreatment did not change the minimal presence of CCL3 and CCL5 in the peritoneal lavage fluid compared to PBS-injected mice in the absence of inflammation (Figures 3B and 3C). Upon TG injection, CCL3 and CCL5 were strongly

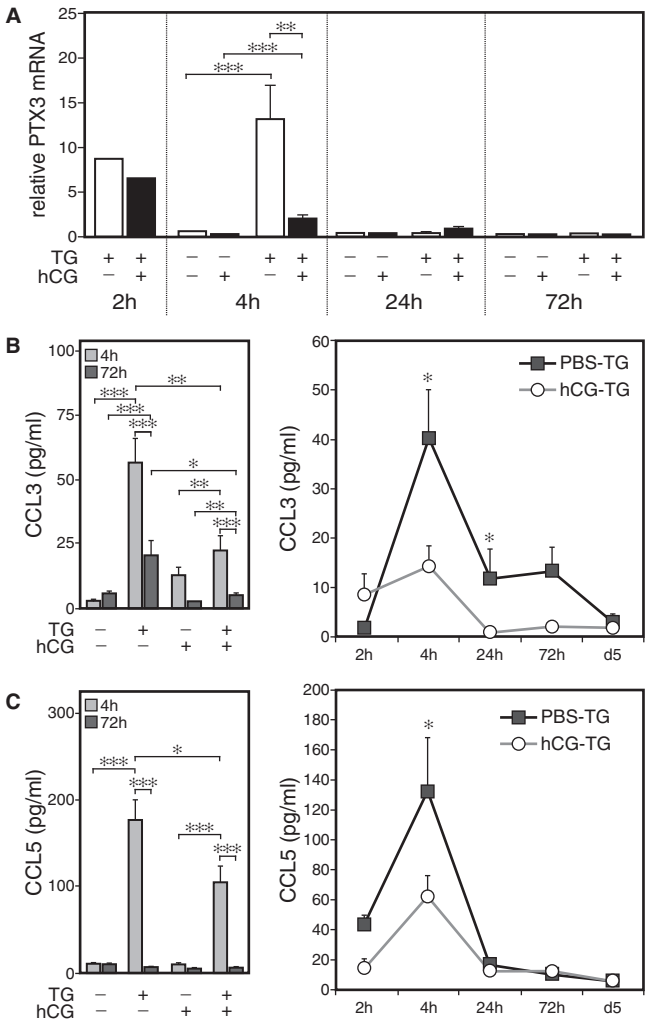

Figure 3. hCG pretreatment decreases PTX3 mRNA expression by peritoneal recruited cells and decreases the CCL3 and CCL5 levels in peritoneal lavage fluid. C57BL/6 mice were injected i.p. with hCG or PBS. One hour later TG was injected i.p. and at 2h, 4h, 24h, 72h (day 3) and on day 5 afterwards, mice were sacrificed and thioglycollate-recruited cells and peritoneal lavage fluid was collected followed by measurement of PTX3 (**A**), CCL3 (**B**) and CCL5 (**C**) (n=15) Kinetic graphs depicted are from a representative experiment with 5 mice per group (**B** and **C**, right panels).

induced, reaching a maximal level at 4h followed by a decrease from 4h onwards to reach a basal level at d5 and 24h, respectively (Figures 2B and 2C). hCG pretreatment significantly decreased the production of CCL3 at 4h and 72h and decreased the production of CCL5 at 2h and 4h, without changing the kinetics of CCL3 and CCL5 production (Figures 3B and 3C). Macrophage migration inhibitory factor (MIF) is also a TNF-α-inducible gene relevant to the observed increased cell recruitment. Therefore MIF was studied in hCG pretreated mice. At 4h after hCG pretreatment of thioglycollate-induced inflammation, also MIF was significant decreased (data not shown) in line with the findings mentioned above.

Both Mφ and PMN produce IL-6 and TNF-α upon peritoneal inflammation

To establish which cell type(s) were responsible for the production of IL-6 and TNF-α upon TG administration, double staining for $BM8^+$ monocytes/Mφ or Ly-$6G^+$ PMN in combination with intracellular IL-6 or TNF-α was performed on cells recruited to the peritoneal cavity 2h after TG injection. At this time point, the majority of the cells in the lavage fluid were Ly-$6G^+$ PMN, while a minority were $BM8^+$ monocytes/Mφ (Figure 4A). TNF-α^+ cells were either $BM8^+$ or Ly-$6G^+$ (Figure 4B and 4C). In contrast, four hours after TG injection, all TNF-α^+ cells were $BM8^+$; no TNF-α^+Ly-$6G^+$ cells were present (data not shown). Two hours after TG injection, IL-6^+ cells were $BM8^+$ monocytes/Mφ or Ly-$6G^+$ PMN (Figure 4D and 4E). Most of the IL-6^{bright} cells were only $BM8^+$; some IL-6^{low} cells were $BM8^-$Ly-$6G^+$ at 4h (data not shown). Similar results were obtained in hCG pretreated mice after the induction of peritoneal inflammation.

Resident Mφ contribute significantly to the observed decrease of inflammation after hCG pretreatment

Considering the role of resident Mφ in the initiation of inflammation (upon TG injection), we depleted resident Mφ by clodronate liposomes to investigate whether the modulating effect of hCG pretreatment in thioglycollate-induced peritonitis was mediated by resident Mφ.

Clodronate liposomes treatment quantitatively depleted resident Mφ, defined as $SSC^{med-high}BM8^{high}$Ly-$6C^{med}CD11b^{high}$ cells (Figure 5A). Conversely, PMN are $BM8^{med}$Ly-$6G^{high}$.

Four hours after TG injection into clodronate liposomes-treated animals, we observed the full depletion of resident Mφ, and the appearance of $SSC^{med}BM8^{med}$Ly-$6C^{high}CD11b^{high}$ cells, which are considered to be the newly recruited monocytes/Mφ (Figure 5A). After Mφ depletion hCG pretreatment did not result in an increase of Mφ numbers in the peritoneal cavity (Figure 5B).

Upon TG injection, hCG pretreatment increased the PMN number as depicted in Figure 1A and 5B. Without hCG treatment, Mφ depletion resulted in decreased PMN recruitment after TG injection in line with a role for resident Mφ in the PMN recruitment. When resident Mφ were depleted, hCG pretreatment did not increase PMN recruitment in the peritoneal cavity of thioglycollate-injected mice. Instead, a significant decrease in PMN number was observed compared to non-depleted, hCG-treated mice, suggestive of a role of resident Mφ in the hCG-induced increased PMN influx. When the data were expressed as percentage of cells instead of absolute numbers, the result was similar (Figure 5B).

To investigate the contribution of resident Mφ to the observed hCG-mediated decrease in inflammatory cytokine production in thioglycollate-injected mice, peritoneal lavage

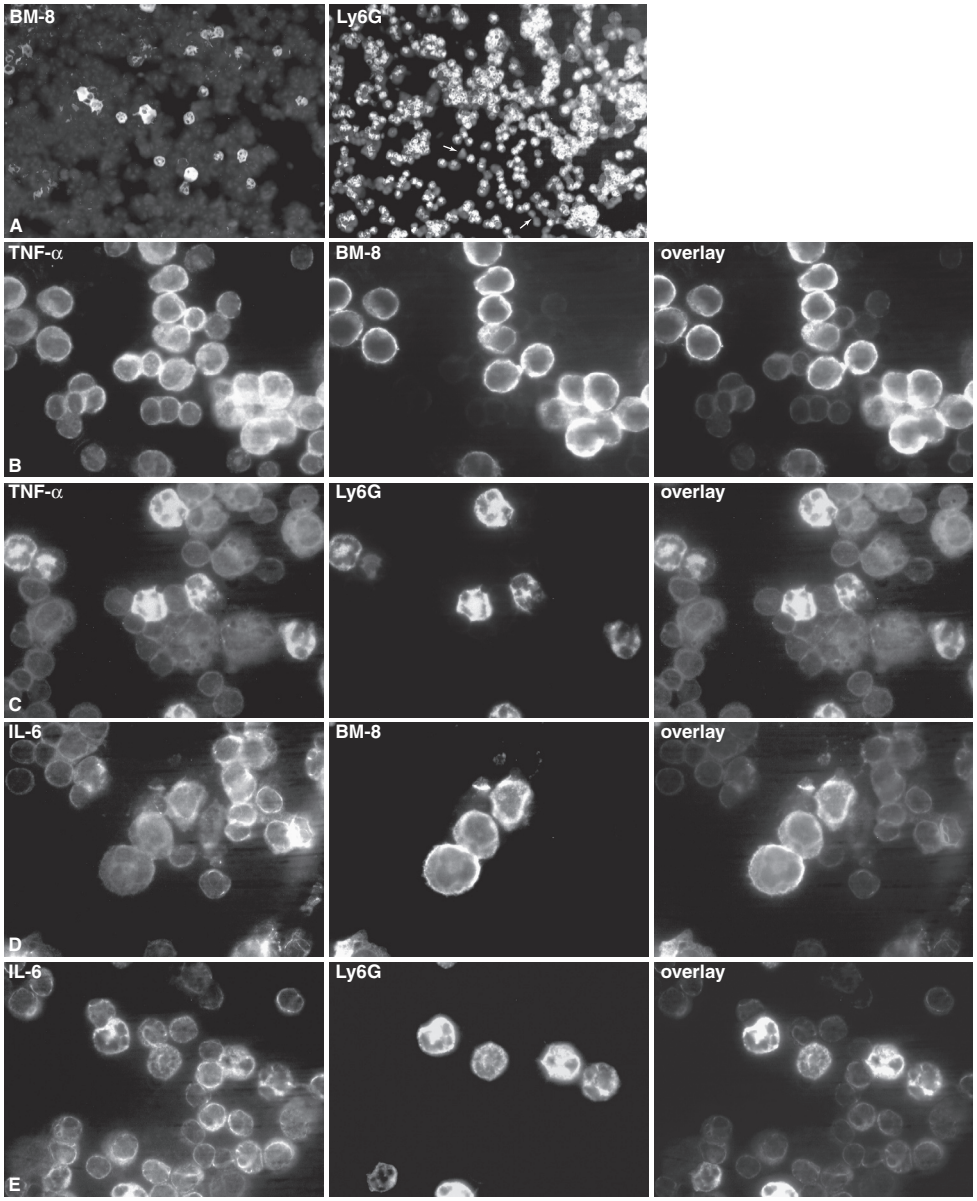

Figure 4. PMN and Mφ are the main producers of IL-6 and TNF-α in thioglycollate-induced peritonitis.
A. 4h after TG injection, peritoneal recruited cells were collected and stained for BM8 and Ly-6G, blue: DAPI; red left: BM8, red right: Ly-6G. The majority of the cells is Ly-6G$^+$ (arrow: Ly-6G$^-$), and a few cells are BM8$^+$; magnification x400. Recruited cells from 2h after TG injection were double stained for BM8 (red, **B** and **D**) or Ly-6G (red, **C** and **E**) and intracellular TNF-α (green, **B** and **C**) or IL-6 (green, **D** and **E**). The pictures show single color staining (left and middle) and overlay (right). Most BM8$^+$ cells are TNF-α$^+$ and IL-6$^+$. All Ly-6G$^+$ cells are TNF-α$^+$ and IL-6$^+$; magnification x630.

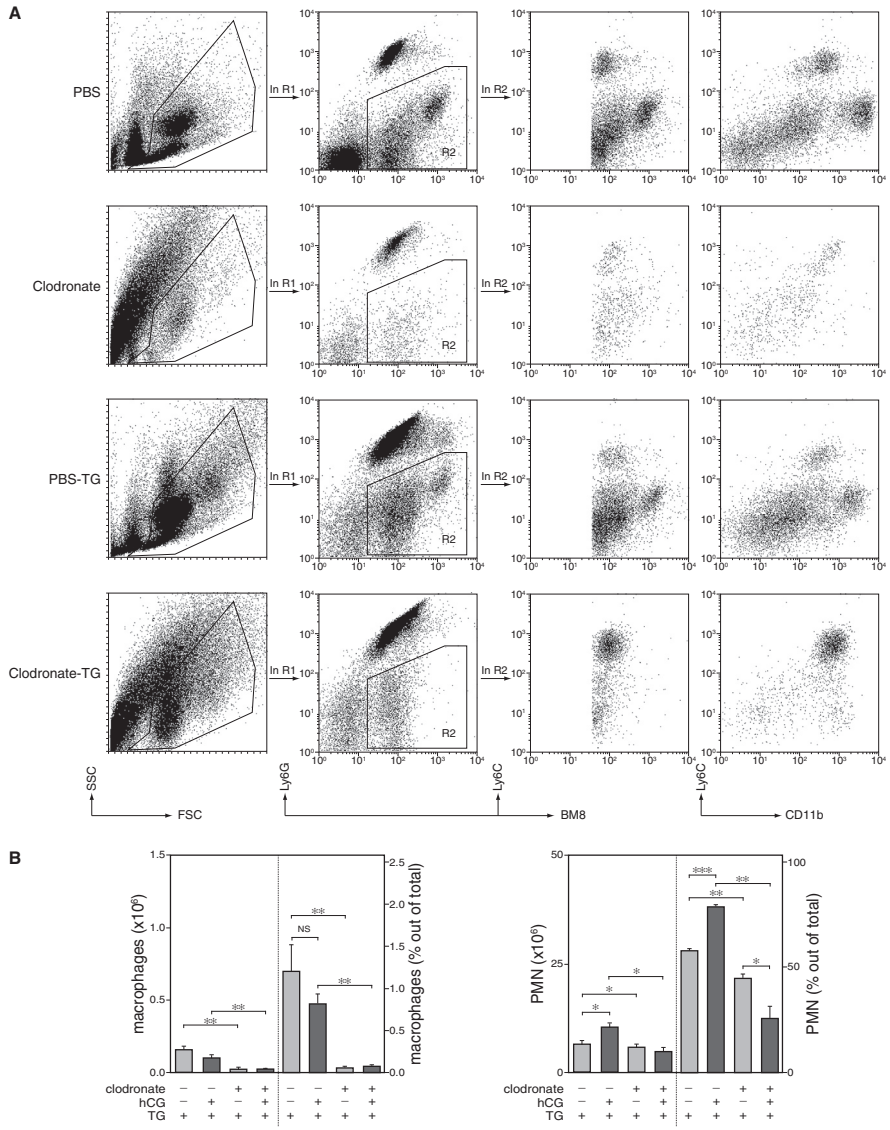

Figure 5. Upon hCG pretreatment, Mφ are the prime responsive cells accounting for the decreased IL-6 and TNF-α levels following an inflammatory trigger.

A. Flow cytometry analysis of Mφ in thioglycollate-induced peritonitis upon resident Mφ depletion by clodronate liposomes. Mice were either or not injected with clodronate liposomes, followed 2 days later by PBS or hCG stimulation and, after 1h, the induction of acute inflammation by TG injection. Four hours later, peritoneal cells were harvested and prepared for analysis. Clodronate liposomes depleted BM8^{high} resident Mφ. With TG injection, newly recruited BM8^{med} monocytes reached the peritoneal cavity at 4h. **B.** BM8^{high}Mφ and Ly-6G^{high}PMN (absolute number and %) in the peritoneal cavity upon different treatments are indicated above. **C.** Mice were depleted of resident Mφ, then pretreated with hCG, and 1h later TG was injected. Another 4h later, the mice were sacrificed for measurement of IL-6 and TNF-α in the peritoneal fluid. After Mφ depletion, hCG treatment did no longer influence the IL-6 and TNF-α levels in thioglycollate-induced peritonitis. (*n*=5).

fluid was collected 2 days after depletion of the resident Mφ and 4 hrs after induction of inflammation. Independent of hCG stimulation, this Mφ depletion resulted in a lower IL-6 level demonstrating that resident Mφ contribute significantly to the IL-6 production in inflammation (Figure 5C). When resident Mφ were depleted, hCG pretreatment did not further decrease the IL-6 production in thioglycollate-induced inflammation, suggestive of a contribution of resident Mφ to the decrease of the IL-6 level by hCG pretreatment (Figure 5C).

Upon TG injection, hCG pretreatment not only decreased the IL-6 level, but also the TNF-α level (Figure 2B and 5C). However, Mφ depletion did not affect the TNF-α level in thioglycollate-induced inflammation, suggesting that cells other than the resident Mφ were the main TNF-α producers (Figure 5C). hCG pretreatment in combination with depletion of resident Mφ did not cause a decrease of the TNF-α production compared to hCG pretreatment without depletion, strongly suggesting a role for resident Mφ in mediating hCG-induced suppression of TNF-α production by other cell types (Figure 5C).

DISCUSSION

Our earlier *in vitro* experiments have shown that hCG influences Mφ directly [18]. In particular, their innate immune effector functions, such as radical production and phagocytosis of apoptotic cells, are increased by hCG. Pregnancy has been proposed to be a state of controlled inflammation [2]. We hypothesized that hCG could play an important role in the control of inflammation via modifying Mφ function and therefore we studied the effect of hCG cytokine production and cell recruitment in thioglycollate-induced peritonitis in a murine *in vivo* model.

In the present study, we showed that hCG pretreatment induced increased cell recruitment, especially of PMN and monocytes, in the peritoneal cavity at 4h after TG injection. In line with literature data indicating that resident Mφ are important triggers of PMN recruitment [26, 27], we found that resident Mφ depletion resulted in diminished PMN recruitment into the peritoneal cavity. In addition, hCG pretreatment of resident Mφ-depleted mice did not increase, and even decreased PMN numbers upon thioglycollate injection, strongly suggesting that the observed increased PMN influx upon hCG pretreatment is via modulating the resident Mφ function. The increased cell recruitment upon hCG pretreatment in thioglycollate-induced peritonitis was partially due to diminished cell apoptosis. However, the observed decrease in the percentage of apoptotic cells was small compared to the increased cell recruitment upon hCG pretreatment. The possible reason for this is that the apoptosis measurement is at a single time point, while the cell recruitment is the accumulated effect until the moment of sampling. The decreased apoptosis we observed is in line with the former findings on hCG and apoptosis as hCG has been found to induce vascular endothelial growth factor (VEGF) production and the combination of hCG and VEGF protected cells from apoptosis [31, 32]. Changes in cell adhesion may also contribute to the observed increased cell influx upon hCG pretreatment. We found that hCG increased the adhesive properties of human monocytes *in vitro* (data not shown). These data suggest that, in addition to the decreased apoptosis, other mechanisms may also contribute to the observed accumulation of cells in the peritoneal cavity upon hCG pretreatment. It remains to be established why hCG induces an influx of PMN and monocytes

when their pro-inflammatory functions are blocked afterwards in acute inflammation. This feature to reduce further damage from infection while prevent a massive cell death can be beneficial to pregnancy.

Earlier we and others showed that hCG inhibits SIRS, autoimmune type I diabetes development and Sjogren's syndrome-like exocrinopathy in mice [9-11]. The present study sheds some light at the factors and cell types affected by hCG. Upon TG injection, hCG pretreatment resulted in decreased levels of TNF-α, IL-6, MIF, CCL3 and CCL5 at 4h and an increased IL-10 level at 72h compared to control thioglycollate-injected mice. TNF-α, IL-6, MIF, CCL3, and CCL5 are all pro-inflammatory factors that significantly increase upon the induction of inflammation and can amplify inflammatory responses leading to immunopathology, whereas IL-10 is generally believed to be an anti-inflammatory cytokine. Furthermore, CCL3 and CCL5 mainly attract mononuclear phagocytes, including monocytes and Mφ [33]. The observed decrease in CCL3 and CCL5, as well as CCL2 (MCP-1) (data not shown), upon hCG pretreatment excludes the possibility that these chemokines account for the increased numbers of PMN and monocytes in the peritoneal cavity after hCG pretreatment. Thus, our data support a beneficial role for hCG in reducing inflammation in thioglycollate-induced peritonitis in mice by influencing cytokine and chemokine expression. The increased cell number in the peritoneal cavity is partially due to prolonged cell survival rather than increased cellular influx. However it should be noticed that we studied a selection of the chemokines considered relevant. Whether the same mechanism also plays a role in the inhibition of SIRS and diabetes development in mice remains to be established.

PTX3 is a recently defined acute phase protein that is quickly produced in inflammatory conditions such as endotoxic shock and infection. Furthermore, its expression level correlates with the severity of such conditions [34]. High levels of PTX3 are observed in serum from pregnant females [35] in line with the hypothesis that pregnancy is a controlled inflammatory condition. These increased PTX3 levels are suggestive of induction by factors elevated in pregnancy. Interestingly, after hCG pretreatment of thioglycollate-induced peritonitis we found a decreased PTX3 mRNA expression in the cells recruited to the peritoneal cavity. This emphasizes that hCG can influence the inflammation, but this does not explain the increased PTX3 levels in pregnancy. The decreased PTX3 expression after hCG pretreatment may be explained by the decreased TNF-α level because PTX3 was first described as a TNF-stimulated gene (TSG-14) [36]. Interestingly, CCL3, CCL5 and MIF are also TNF-α-inducible genes [37-39]. Therefore, the decreased production of these TNF-α-inducible factors points towards a dominant role of hCG-induced decrease of TNF-α production in the control of inflammation. The kinetic data are indicative of such a mechanism since hCG pretreatment decreased the TNF-α level in thioglycollate-induced peritonitis, followed by a decrease in PTX3, CCL3, CCL5 and MIF expression.

Furthermore, it has been shown that hCG inhibits NFκB and AP-1 activation in cancer cells and ovarian cells [40, 41]. The inhibitory effect of hCG on NFκB was also observed in human Jurkat T cells (unpublished data). Once activated, NFκB regulates the expression of almost 400 different genes, including cytokines such as TNF-α, IL-6, and several chemokines. Further studies are needed to elucidate the role of NFκB in the immunomodulatory effect of hCG.

Shortly after inflammation induction, IL-6 can be produced by both Ly-6G$^+$ PMN and

$BM8^+$ monocytes/Mφ. However, peritoneal mesothelial cells can also secrete IL-6 after inflammatory signals such as IL-1β and TNF-α [42, 43]. Mesothelial cells are potential producers of TNF-α in peritonitis, too [44, 45]. Resident Mφ depletion and hCG pretreatment resulted in similar decreased levels of IL-6 compared to control thioglycollate-injected mice, suggesting a contribution of recruited Mφ to the hCG effect on IL-6 production.

Similar to a former study describing that PMN can produce TNF-α upon inflammation [46], we found that TNF-α is detectable in $Ly\text{-}6G^+$ PMN as well as in $BM8^+$ monocytes/Mφ shortly after induction of inflammation. Upon resident Mφ depletion the level of TNF-α remained unchanged suggesting that these cells are not the main producers of TNF-α in our TG model, although immunochemistry showed that two hours after TG injection most of $BM8^+$ cells are $TNF\text{-}\alpha^+$. By FACS analysis we observed two subpopulations of $BM8^+$ cells: $BM8^{high}$ resident Mφ, which are depleted by clodronate liposomes application, and $BM8^{med}$ myelomonocytic cells, including newly recruited inflammatory Mφ, which are unaffected by the prior clodronate liposome-mediated depletion. It has been described that thioglycollate-elicited peritoneal macrophages are more proliferative compared to resident Mφ [47, 48] and produce about 10 times more TNF-α [49]. Therefore, we suggest that the $TNF\text{-}\alpha^+BM8^+$ cells are newly recruited Mφ. Upon resident Mφ depletion, hCG pretreatment did not decrease TNF-α production, suggesting that resident Mφ are prime responders to hCG. Therefore, these Mφ likely mediate the suppression of TNF-α production by other cells such as PMN and resident/recruited Mφ.

There are seeming contradictions between our previous *in vitro* data on enhanced Mφ function by hCG [18] and the present *in vivo* results on hCG alleviating inflammation by dampening Mφ function. However, several points need to be highlighted in this regard: 1) the sources of Mφ differed, as in our previous study we used M-CSF-stimulated bone marrow-derived Mφ cultured *in vitro*, and here we found the suppressive effect to be related to resident peritoneal Mφ, which have a distinct derivation, phenotype and responsiveness, 2) the triggers are different, as formerly we used IFN-γ to stimulate Mφ, and in this study TG (chemical) was applied to create a sterile inflammatory condition targeting not only Mφ but many more cell types present in the peritoneal cavity. Despite of these disparities, hCG seems to have beneficial immunological effects in pregnancy, as, on one hand, it enhances innate immunity by increasing the cellular function of Mφ, such as killing bacteria through release of NO and ROS, and, on the other hand, hCG downregulates systemic pro-inflammatory factor production and thus contributes to the control of the inflammatory condition during pregnancy.

Taken together, in this study we observed that hCG contributes to the modulation of thioglycollate-induced peritoneal inflammation by affecting resident Mφ function, which results in decreased production of pro-inflammatory factors like IL-6, TNF-α, PTX3, CCL3 and CCL5. This may explain why hCG can resolve inflammatory conditions such as SIRS, and may contribute to the control of the sterile inflammatory condition during pregnancy.

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Chapter 6

Conclusions and discussion

6.1 CONCLUSIONS

The results described in this thesis support the notion that hCG, via modulating mononuclear phagocytic cells, contributes to an increased innate immunity and a decreased adaptive immunity during pregnancy (Figure 1).

*1. hCG increased the PTX3 production by monocytes (**Chapter 2**).*

PTX3 is a cytokine that rises in response to inflammatory signals. hCG dose-dependently increased the PTX3 production by unstimulated monocytes as well as Mφ and DC. This effect was mediated by the PKA signaling pathway, which is known to be activated by hCG and hCG receptor association. Experiments in mice further proved that the *in vivo* target cells for hCG-induced PTX3 production are monocytes and not lymphocytes. Monocytes are the predominant mononuclear phagocytic cells in the blood. Via modifying monocyte function, hCG can contribute to the increased PTX3 serum level in pregnancy.

*2. hCG stimulated the innate function of Mφ (**Chapter 3**)*

Mφ are also important players in innate immunity, due to their ability to phagocytose pathogens in the tissues. Here, we describe that hCG increased the production of NO, ROS, IL-6 and IL-12p40 by IFN-γ-primed BM-derived Mφ. These observations suggested an enhancement of the innate function of Mφ by hCG. This was further studied by analyzing the phagocytosis capacity of the hCG treated cells. Mφ phagocytosed more labeled-beads and apoptotic cells upon hCG treatment. Although i.p. injection of hCG did not result in increased phagocytosis of peritoneal Mφ, culture of Mφ from hCG injected mice with hCG did result in an increased phagocytosis. This suggested the requirement of the continuous presence of hCG for increasing phagocytosis by Mφ. Again, the PKA signaling pathway appeared to be involved in the observed effect of hCG on Mφ. The T cell proliferation was not affected by hCG treatment, indicating that the function of Mφ in adaptive immunity remained unchanged.

*3. hCG induces DC to express a tolerogenic phenotype (**Chapter 4**).*

DC are potent APC, which can activate naive T cells and thereby induce an adaptive immune response. In addition, DC regulate the adaptive immune response by Th1/Th2 skewing. We studied the effect of hCG on DC in adaptive immunity. Upon hCG treatment of LPS or LPS+IFN-γ stimulated DC, the cytokine production ratio of IL-10:IL-12p70 shifted towards a Th2 response. This is indicative for a contribution of hCG to the Th2 cytokine environment in pregnancy. hCG blocked the LPS and IFN-γ induced MHC class II up-regulation of BMDC. Upon hCG treatment, IDO expression was increased by LPS- and IFN-γ-stimulated DC. The decreased MHC class II expression and the increase of IDO are both associated with a decreased T cell proliferation. Interestingly, hCG also affected DC differentiation from their precursors, resulting in decreased cytokine production and decreased induction of T cell proliferation. These data suggest a contribution of hCG to the maternal-fetal tolerance during pregnancy by modifying DC towards a tolerogenic phenotype.

*4. hCG helped to alleviate thioglycollate-induced peritonitis by dampening Mφ function (**Chapter 5**).*

Recent data suggest that early stage pregnancy can be considered as a controlled state of inflammation at the implantation site and that later on this inflammation extends to the maternal circulation. We used an *in vivo* model of acute inflammation that has clearly defined sequential steps of disease development: thioglycollate-induced peritonitis. In this model peritoneal resident Mφ have been suggested to initiate the inflammation. hCG pretreatment decreased the intraperitoneal TNF-α, IL-6, PTX3, CCL3 and CCL5 levels despite the increased cellular infiltration. The increased peritoneal cell number was partially due to the increased cell survival by hCG. We demonstrated that upon hCG treatment Mφ are the main responsive cells accounting for the increased PMN influx and suppressed TNF-α and IL-6 production. Together, these data suggest that hCG contributes to the controlled inflammatory state of pregnancy by down regulating the pro-inflammatory function of Mφ.

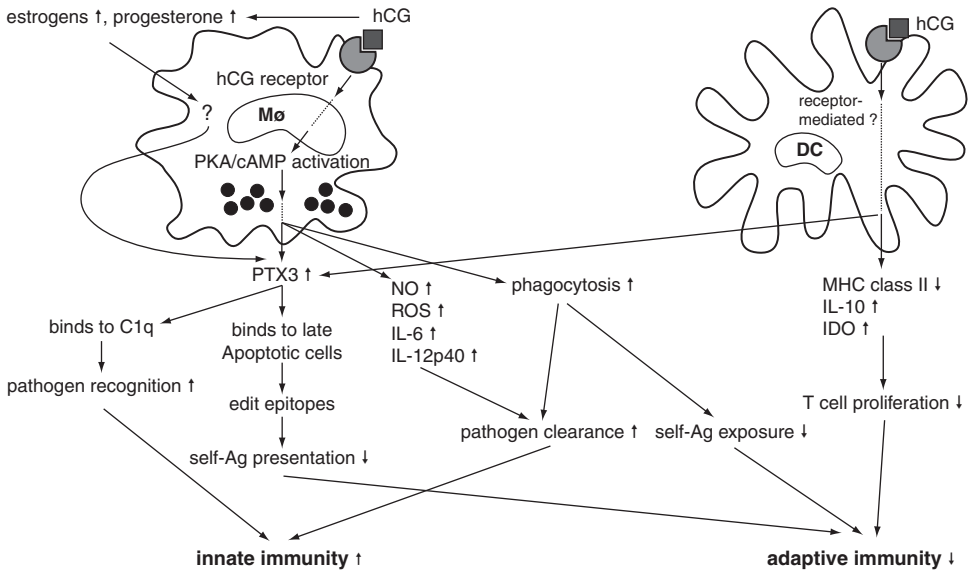

Figure 1. Schematic representation of the conclusion from the experimental work of this thesis.
Innate Immunity: hCG treatment induces increased PTX3 expression by mononuclear phagocytic cells. hCG-induced hormones such as progesterone and estrogen can increase the PTX3 expression, too. PTX3 activates the classical complement pathway by binding to immobilized C1q and facilitates extracellular pathogen recognition by Mφ, which further leads to increased innate immunity against pathogens. hCG also increases the phagocytosis of Mφ. Meanwhile, by increasing NO, ROS, IL-12p40, and IL-6 production by these Mφ, hCG treatment enhances the defensive capacity against invading pathogens. Adaptive immunity: Upon hCG treatment, DC increase their IL-10 production and, increase their IDO activity, but reduce their MHC class II expression, resulting in decreased T cell proliferation. In addition, the capacity of hCG-treated Mφ to induce allogeneic T cell proliferation is unchanged. hCG stimulates the local Mφ to phagocytose apoptotic cells, avoiding the exposure of self-Ag, and decreasing the adaptive autoimmunity. Furthermore, next to the protective function of PTX3 against pathogens, PTX3 selectively binds to late apoptotic cells and edits the cross-presentation of epitopes expressed by apoptotic cells to T lymphocytes, thus also contributes to prevent alloimmunization.

6.2 CONCERTED ACTION OF hCG AND OTHER IMMUNOREGULATORY FACTORS IN PREGNANCY

Although we and others showed that hCG plays an immunoregulatory role in pregnancy, hCG is not the sole factor influencing the maternal immunity. Various studies have been conducted to unravel the mechanisms and to identify other factors relevant to the decreased adaptive immunity in pregnancy (Figure 2).

1. Galectin-1 is a tolerance signaling molecule in pregnancy.

An immunoregulatory factor identified in this context is glycan-binding protein galectin-1. This protein has been shown to inhibit the proliferation and survival of effector T cells, to block the secretion of proinflammatory cytokines, and to suppress Th1-dependent inflammation *in vivo* by skewing towards a Th2-dominant cytokine profile [1-4]. In humans, galectin-1 expression is increased in the endometrium and decidual tissue, and is proposed to play a crucial role in maternal-fetal tolerance [5]. Galectin-1-deficient mice show higher rates of fetal loss. Treatment with recombinant galectin-1 prevented fetal loss and restored tolerance through the induction of tolerogenic DC and Treg cells, as well as increased apoptosis of effector T cells [6, 7]. It has been shown that galectin-1 inhibited the production of hCG and progesterone by trophoblasts, suggesting a role in hormone regulation as well as in immune regulation [8]. It would be interesting to further study the relationship between hCG and galectin-1 in immune tolerance and pregnancy.

2. Apoptosis induction contributes to the maternal-fetal tolerance.

Recently the inhibitory T cell costimulatory molecule programmed death ligand 1 (PDL1), has been found to play an important role in maternal-fetal tolerance. PDL1 blockade or deficiency resulted in increased fetal rejection rates [9-11]. This effect was found to be mediated by PDL1 expressing regulatory T cells. We are not aware of studies on a relationship between hCG and PDL1. Previously hCG was suggested to regulate FasL expression on endometrial cells, thus contributing to immune tolerance by activating the apoptotic signaling pathway in T cells [12]. It would be interesting to investigate the effect of hCG on other apoptotic pathways, such as PDL1.

3. TGF-β induces T cell tolerance in pregnancy.

TGF-β is a critical cytokine that promotes the development of tolerogenic APC and Treg cells [13]. The uterus and placenta produce TGF-β. In other cell types in the ovary, e.g. granulosa cells, theca interna cells, and oocytes, hCG could induce the expression of TGF-β [14]. No data are available on the effect of hCG on the induction of TGF-β expression in APC and Treg cells. Taking in consideration the important role of TGF-β in tolerance induction, the influence of hCG on TGF-β production should be studied in APC and Treg cells.

4. hCG influences cell trafficking via modulating MIF production.

Macrophage migration inhibitory factor (MIF) is another factor that has been found to contribute to the maintenance of the immune privilege at the maternal-fetal interface. MIF plays an important role in the trafficking of monocytes and Mφ [15]. A number of reports established

the induction of MIF by hCG in ovarian cells [16]. hCG pretreatment in thioglycollate-induced inflammation resulted in a decrease in MIF production (**Chapter 5**). Earlier we observed a decreased MIF expression by spleen cells upon treatment with an oligopeptide from the β-chain of hCG [17]. These studies suggest that the effect of hCG on MIF production by cells from the immune system is different from that in ovarian cells.

5. hCG-induced hormones also contribute to the immunology of pregnancy.

hCG-induced hormones like progesterone and estrogen can also regulate the immune system during pregnancy. For example, progesterone suppresses lymphocyte proliferation and thereby helps to prevent rejection of the developing fetus and placenta [18]. Progesterone has also been shown to skew T cells towards a Th2 response. This effect of progesterone was due to induction of apoptosis in Th1 cells and inhibition of the differentiation of Th1 cells, while Th2 cells could escape from this regulation [19]. Estrogens, a group of hormones rising during pregnancy, seem to have an opposite effect [18, 20]. However, their roles in immune regulation are still controversial [21]. Estrogens were found to support trauma/sepsis and some chronic autoimmune diseases such as RA [20]. Interestingly, estrogen can activate DC by increasing their proinflammatory cytokine production and their induction of T cell proliferation. In contrast, progesterone inhibits DC functions [22]. There are also reports on the anti-inflammatory properties of estrogens in postmenopausal females [21]

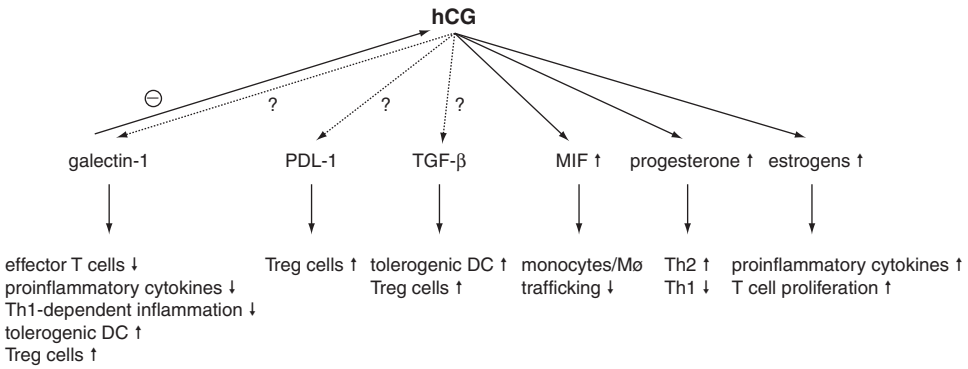

Figure 2. Concerted action of hCG and other immunoregulatory factors in pregnancy.
hCG increases the production of MIF that decreases the trafficking of monocytes and Mφ. hCG also stimulates the production of progesterone and estrogen. Progesterone shifts T cell responses towards Th2 type, whereas estrogen increases the inflammatory response. Galectin-1, PDL-1 and TGF-β induce Treg cells and tolerogenic DC that contribute to the maternal-fetal tolerance during pregnancy. The ability of hCG to regulate these three factors remains to be established.

6.3 TWO DIFFERENT SIGNALING PATHWAYS ARE INVOLVED IN THE hCG EFFECT ON MONONUCLEAR PHAGOCYTIC CELLS

The glycoprotein hormones weigh more than 5.000 Dalton. Due to their relatively big sizes, membrane receptors are required for their regulatory effects. LH and hCG are thought to share a common receptor. By studying this receptor, two pathways of hCG function have been suggested by Aggarwal *et al*. [23, 24] (Figure 3).

- *hCG receptor-cAMP/PKA signaling pathway:*

hCG binds to the hCG receptor, which is a G-protein coupled receptor, and activates adenylate cyclase that converts ATP to cAMP, followed by binding to the regulatory subunits of PKA. This binding causes the release of the active subunits of PKA, which may phosphorylate serine and threonine residues on target proteins. Blocking of this pathway significantly reduced the hCG-induced increased PTX3 expression by monocytes (**Chapter 2**) and reduced the increased ROS production and the increased phagocytosis by IFN-γ-primed Mφ (**Chapter 3**). This indicates that binding of hCG to the hCG/LH receptor activates the intracellular cAMP/PKA-signaling pathway leading to cyclic-AMP response element binding protein (CREB) induced transcription, which may lead to the induction of inflammatory mediators such as ROS and IL-12 [25]. Interestingly, in our study inhibition of the PKA signaling pathway only partially reduced the increased IL-6 and IL-12p40 protein production by IFN-γ-primed Mφ upon hCG treatment. This indicates that other pathways are also influenced by hCG receptor activation.

- *inhibition of the TNF-induced NF-κB pathway:*

It has been accepted broadly that TNF binding to the TNF receptor activates the NF-κB pathway. hCG, likely mediated through cAMP, can block this TNF-induced activation of NF-κB, IκBα degradation, and NF-κB-dependent reporter gene transcription [26]. It is unknown whether this pathway is involved in our studies. In **Chapter 5**, hCG pretreatment decreased the levels of TNF-α-induced PTX3, CCL3, CCL5 and MIF expression in thioglycollate-induced peritonitis, indicating the necessity of studying this pathway in our model.

Another study carried out by others suggested an opposite mechanism: hCG induced the production of IL-8 by monocytes, which is dependent on NF-κB activation (NF-κB translocation into the nucleus) without the involvement of the hCG receptor-cAMP/PKA signaling pathway [27]. It was further shown that cell surface lectins bind to hCG and thereby influenced the IL-8 production by monocytes. This effect can be interfered by the competitive binding of mannose to C-type lectins [27].

In addition to these pathways, we did some interesting observations with regard to hCG stimulation, although the mechanisms are still unclear. First, hCG treatment hampers the upregulation of MHC class II expression by LPS+IFN-γ-stimulated DC, but not by LPS-stimulated DC (**Chapter 4**). Another observed difference between LPS+IFN-γ and LPS-stimulated DC upon hCG treatment is the increased TNF-α, IL-6 and IL-10 production by LPS+IFN-γ stimulated cells. To further investigate whether the decreased MHC class II expression by LPS+IFN-γ stimulated-DC upon hCG treatment is due to the induction of TNF-α or IL-10, DC were cultured with neutralizing anti-TNF-α or neutralizing

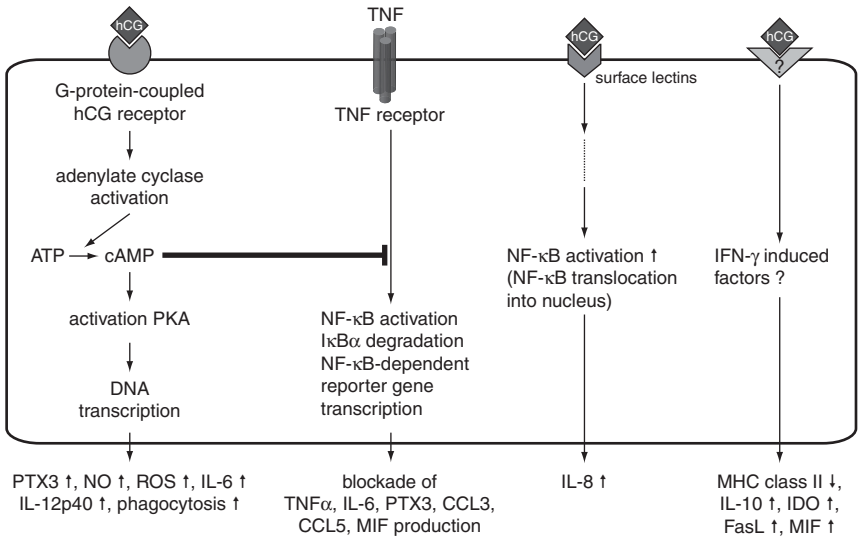

Figure 3. hCG-induced signaling pathways in mononuclear phagocytic cells.
The most common pathway for hCG effect is the hCG receptor/PKA signaling pathway, which is responsible for the increased production of proinflammatory factors and increased phagocytosis. hCG was also shown to inhibit TNF-induced NF-κB activation, which may be responsible for the decreased TNF-inducible factors in thioglycollate-induced peritonitis. On the other hand, The binding of hCG sugar sites to cell surface C-type lectin results in NF-κB activation that leads to IL-8 production by monocytes. The pathways induced by HCG treatment resulting in increased IL-10, IDO, FasL and MIF, as well as the decreased MHC class II remain to be established.

anti-IL-10. Neutralizing TNF-α or IL-10 did not result in restoration of the MHC II expression. Alternatively, DC were cultured with recombinant TNF-α or recombinant IL-10 to approximate the effects of hCG. No effect on MHC class II expression was observed upon the addition of TNF-α or IL-10 (**Chapter 4**). This suggests that neither TNF- α nor IL-10 is responsible for the hCG-induced suppression of MHC class II by LPS+IFN-γ stimulated-DC. Due to the involvement of IFN-γ in the observed effect of hCG, IFN-γ-induced factors may be the candidates to mediate the hampered MHC class II expression.

Second, hCG induces IDO expression by DC (**Chapter 4**). Ueno *et al.* recently confirmed our previous study that hCG treatment of NOD mice inhibited diabetes development. This protective effect was associated with an increased expression of IDO by DC [28]. From this study, it is unclear whether the upregulation of IDO is a direct or indirect effect of hCG on DC. We showed that upon *in vitro* hCG treatment, IDO expression and its metabolite kynurenine were increased by LPS- and IFN-γ-stimulated DC, which is related to the decreased T cell proliferation. The function of IDO in tryptophan metabolism and its role in tolerance induction has been studied broadly, but the induction of IDO needs further investigation. It has been suggested that the IDO induction in trophoblasts by hCG requires the hCG receptor and is cAMP-dependent but PKA-independent, and involves the mitogen-activated protein kinase 3/1 (MAPK3/1) signaling pathway [29]. This indicates that other pathways may regulate this

effect of hCG in addition to the two pathways mentioned above. By molecular techniques, specific target molecules in the hCG-IDO interaction in DC can be studied to unravel the underlying mechanism.

Third, hCG induces PTX3 expression by monocytes (**Chapter 2**). We proved that this effect of hCG involves the cAMP/PKA signaling pathway. This pathway is completely new for PTX3 induction. Former studies from Mantovani *et al*. showed that there are several pathways involved in PTX3 induction, including those involving TNF, IL-1β and TLR ligands [30]. Together these data suggest that PTX3 induction is mediated via multiple pathways by multiple cells, which all contribute to an enhanced innate immunity. It would be interesting to know how hCG induces the PTX3 expression. Can hCG breakdown products such as small peptides directly bind to the PTX3 promoter and stimulate the PTX3 expression? Or does hCG regulate other factors and indirectly influence PTX3 production? We have studied this in different cell types, including monocytes, Mφ, DC, endothelial cells, fibroblasts, and human embryonic kidney cells (unpublished data). Only mononuclear phagocytic cells showed increased PTX3 production upon hCG treatment. So far this effect of hCG is specific for myeloid cells. Trophoblasts also bear the hCG receptor [31] and respond to hCG, a.o. by producing IDO [29].

It would be interesting to investigate the capacity of trophoblasts to produce PTX3 after hCG stimulation.

6.4 hCG CAN PLAY A BENEFICIAL ROLE IN BOTH AUTO-IMMUNITY AND ACUTE INFLAMMATION

hCG has been shown to be effective in several chronic inflammatory disease models (summarized in Table 1). In NOD mice, it has been shown by three groups that hCG down regulates autoimmune diabetes [28, 32, 33]. First, our group reported that i.p. injection of purified hCG before the onset of clinical symptoms of diabetes lowered the increased blood glucose levels, inhibited the inflammatory infiltrate of pancreatic tissue, and induced profound inhibition of the functional activity of Th1 cells [32]. Ueno A. *et al.,* showed that recombinant hCG did not affect the proliferation of splenic purified T cells and their production of IFN-γ and IL-4. Moreover, the expression levels of MHC class I, MHC class II, CD40, CD80, or CD86 by splenic $CD11c^+$ DC were similar. Administration of hCG inhibited both the activation of diabetogenic $CD4^+$ and $CD8^+$ T cells. This suppression was mediated by IDO-expressing DC upon hCG treatment [28]. The third group showed that both purified and recombinant hCG prevented the development of diabetes in NOD mice [33]. In this study, hCG decreased the proportion and number of $CD4^+$ and $CD8^+$ T cells in the spleen and pancreatic lymph nodes and inhibited T cell proliferative responses against pancreatic β cell antigens.

hCG treatment suppressed the IFN-γ production, but increased the IL-10 and TGF-β production in splenocytes stimulated with anti-CD3 antibody. hCG treatment also suppressed the TNF-α production in splenocytes stimulated with LPS. Furthermore, hCG treatment increased the $CD4^+CD25^+/CD4^+$ T cell ratio in spleen and pancreatic lymph nodes [33]. The development of diabetes in NOD mice seems to be initiated by myeloid cells. Before the massive infiltration of lymphocytes around and into the islets of Langerhans at 7-12 weeks of age, DC and Mφ already accumulated at the islet periphery at 4-7 weeks of age. This aberrant accumulation

Table 1. Beneficial role of hCG in autoimmune diseases and acute inflammation.

Disease	Model	Effect	Mechanism
Diabetes	NOD mice	Prevented/postponed	IDO ↑ IFN-γ, IL-10 ↑ and TGF-β↑ Treg cells ↑
Sjögren's syndrome	NOD mice	Saliva production ↑	proinflammatory cytokines ↓ NO ↓
RA	Streptococcal cell wall-induced arthritis in rats	Reduced	proinflammatory cytokines ↓ NO ↓ TGF-β↑
Septic shock	LPS-induced septic shock in mice	Reduced/postponed	MIF ↓ plasma alanine aminotransferase ↓
Peritonitis	TG-induced peritonitis in mice	Reduced	proinflammatory cytokines ↓

of DC/Mφ resulted in Th1-mediated β cell destruction followed by diabetes symptoms [34]. Apparently, hCG treatment prevents autoimmune diabetes via modulating both the DC and T cell function.

hCG has also been shown to prevent Sjögren's syndrome-like exocrinopathy in NOD mice [35]. hCG treatment decreased the lymphocytic infiltration and parenchymal cell damage in the submandibular salivary glands, and decreased the mRNA levels of IFN-γ, TNF-α, IL-1β, IL-10, and NO. Functional analysis revealed a marked increase in the salivary flow rate in hCG-treated mice compared to control mice.

In streptococcal cell wall-induced arthritis in rats, systemic administration of hCG caused a dose-dependent reduction in the clinical arthritis index [36]. Consistent with the amelioration of clinical symptoms, hCG significantly reduced the inflammatory cell infiltration, pannus formation, and bone and cartilage degradation. Suppression of the production of TNF-α, IL-6, and IL-1β, associated with less synovial pathology, was found in the hCG pretreated animals. Furthermore, reduced circulating NO and elevated circulating TGF-β levels after hCG administration suggested the monocytes/macrophages as a potential target.

Consistent with the systemic immunosuppressive effects of hCG observed in patients [37], hCG seems to contribute to the pregnancy-associated improvement of autoimmune diseases. Especially in Th1-mediated autoimmune diseases, such as insulin dependent diabetes mellitus, Sjögren's syndrome, multiple sclerosis and rheumatoid arthritis, mononuclear phagocytic cells seem to be crucial players initiating disease development and directing the involved T cell responses. The therapeutic effect of hCG in animal models of these autoimmune diseases suggests that mononuclear phagocytic cells must be considered as a potential *in vivo* target of hCG.

Interestingly, hCG has also been shown to be effective in acute inflammatory disease models. Earlier experiments from our group showed that a single treatment with purified hCG

24h after a high dose LPS injection inhibited septic shock in mice [17]. In acute inflammation, neutrophils and macrophages are the first mobilized cells. These cells play a prominent role in the inflammatory process. To investigate the mechanism by which hCG mediates its effect in acute inflammation, we set up a murine model for local acute inflammation: thioglycollate-induced peritonitis. This model allows a clear dissection of the different phases in acute inflammation. hCG pretreatment in thioglycollate-induced peritonitis resulted in an increased number of peritoneal cells, especially neutrophils and monocytes, compared to mice injected with thioglycollate only. This increased cell number was partly explained by increased cell survival induced by hCG rather than increased inflammatory chemotaxis. Despite the increased cellular infiltration, hCG pretreatment decreased the intraperitoneal TNF-α, IL-6, PTX3, MIF, CCL2, CCL3 and CCL5 levels. The decreased PTX3, MIF, CCL2, CCL3 and CCL5 expression after hCG pretreatment may be due to the decreased TNF-α level, because the involved genes were all described as TNF-α-inducible genes. This points towards a dominant role of the hCG-induced decrease of TNF-α production in the control of inflammation. The kinetic data support such a mechanism since hCG pretreatment decreased the TNF-α level in thioglycollate-induced peritonitis, followed by a decrease in PTX3, MIF, CCL2, CCL3 and CCL5 expression. By depleting peritoneal resident Mφ using clodronate liposomes prior to the application of hCG and the thioglycollate trigger, we showed that Mφ are the main responsive cells to hCG. The increased neutrophil influx and the suppressed TNF-α and IL-6 production upon hCG treatment did no longer occur in the absence of Mφ (**Chapter 5**).

6.5 REMAINING QUESTIONS AND FUTURE DIRECTIONS

6.5.1 Mechanisms underlying the observed regulation of hCG

Many different *in vitro* and *in vivo* effects of hCG have been found in the past. However, the mechanisms underlying these effects remain largely unclear. For example, the pathway for hCG-induced IDO expression is not known, although several possibilities have been excluded such as the Stat6, IL-10, and IFN-γ signaling pathways [28]. Therefore, the next steps are to further unravel the pathways involved. Blocking of the known pathways step by step should give insight into the underlying mechanisms. Meanwhile, studies of messenger RNA (mRNA) from highly purified cells or cell lines should be performed to elucidate the genes whose expression levels change upon hCG treatment. This can yield a fingerprint of the cellular activation by hCG.

6.5.2 Research on hCG-derived peptides

hCG is a relatively large glycoprotein (36.5 kD) and is broken down into smaller fractions and even small peptides during its metabolism. Even in experiments where highly purified hCG was used, the presence of a small percentage of such contaminants can not be excluded.

In the past years, our department showed that hCG-derived oligopeptides can also have immunoregulatory activity [17, 32]. The loop 2 of β-hCG contains a nicking sequence MTRVLQGVLPALPQ (residues 41-54). Based on the known preferential cleavage sites, oligopeptides have been synthesized, and tested in a variety of inflammation models.

For example, the synthetic hexapeptide VLPALP showed a protective effect in LPS-induced septic shock syndrome. This treatment was accompanied by reduced pro-inflammatory cytokine production. This study has also been extended to Rhesus monkeys showing in a similar effect (Khan *et al*., to be published). In addition, in a model for ischemic kidney reperfusion damage, mice were treated with hCG-related peptides i.v. prior to occlusion of the renal arterial vein of the remaining kidney, followed by releasing the clamp. Reperfusion of the kidney caused damage characterized by increased serum levels of blood urea nitrogen (BUN). Treatment with AQGV or LQGV reduced the serum BUN values significantly. Determination of relative gene expression levels by mRNA study revealed that treatment with AQGV or LQGV caused a decreased expression of genes encoding pro-inflammatory cytokines (Khan *et al.,* to be published). The mechanisms underlying these regulatory effects of oligopeptides derived from hCG remain unknown, but these results suggests that these oligopeptides may become a new treatment modality in inflammatory diseases.

More studies on hCG-derived oligopeptides need to be done, especially on their working mechanisms *in vivo* and *in vitro*. This may also help to optimize treatment regimens for therapeutic applications of these oligopeptides in various diseases based on a disturbed immune homeostasis.

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SUMMARY

During pregnancy the mother accepts a semi-allogeneic fetus. The lack of rejection suggests that the immunological response from the mother to the fetus is suppressed. However, there is no generalized suppression of the maternal immune response.

Fetal cells, especially villous trophoblast cells, are in direct contact with maternal leukocytes. The absence of rejection resulting in the acceptance of the fetus by the mother is due to a number of factors and mechanisms. HLA-G and HLA-E expression by trophoblasts, a unique population of uterine NK cells, indoleamine2,3-dioxygenase (IDO) induction, biased Th1/Th2 cytokine environment, apoptosis of maternal T cells, and increased regulatory T (Treg) cells have been suggested to contribute to the acceptance of the fetus by the mother. Moreover, accumulating evidence suggests that the maternal innate immunity increases whereas the adaptive immunity decreases during pregnancy.

During pregnancy, hormone levels, such as the human chorionic gonadotropin (hCG) level, change dramatically. hCG is a hormone produced by trophoblasts. Early pregnancy testing is based on the detection of hCG in urine. hCG induces the corpus luteum of the ovary to produce progesterone and estrogen. These hormones are critical to maintain pregnancy. In addition to this endocrinological role of hCG, more and more evidence points towards an immunological role for hCG. For instance, treatment with hCG has been shown to be effective in animal models for several autoimmune diseases including type I diabetes, Sjögren's syndrome and rheumatoid arthritis.

hCG binds to the hCG receptor followed by activation of the cAMP-PKA signalling pathway, resulting in CREB transcription. Cells of the reproductive system bear hCG receptors, and thus can be affected by hCG. Interestingly, mononuclear phagocytic cells, including monocytes, Mφ, and DC, also bear the hCG receptor.

Mononuclear phagocytic cells are derived from progenitor cells in the bone marrow and are involved in both innate and adaptive immunity. Committed myeloid progenitor cells differentiate into monocytes, circulate in the blood and then enter the tissues to become resident Mφ and DC depending on the local conditions. Monocytes are primary effector cells in innate immunity and protect the body against blood-borne pathogens. Mφ play an essential role in innate immunity in the tissues by their ability to phagocytose and destruct microorganisms, to clear infected or apoptotic cells, and to produce oxygen radicals and pro-inflammatory cytokines. Mononuclear phagocytic cells are capable of presenting antigens to T cells and thereby initiate adaptive immune responses. DC are the most potent antigen-presenting cells and thereby can activate naive T cells. In addition, DC regulate the adaptive immune response by Th1/Th2 skewing and the induction of Treg cells.

Due to the fact that, first, mononuclear phagocytic cells play an important role in the initiation of autoimmune diseases; second, hCG treatment has been shown to improve symptoms of several autoimmune diseases in animal models; and last, mononuclear phagocytic cells bear hCG receptors, we hypothesized that hCG can influence the immune system via modulating the function of mononuclear phagocytic cells. This hypothesis was approached by studying the effect of hCG on monocytes, Mφ, and DC.

In **Chapter 2** we investigated the effect of hCG on the PTX3 production by monocytes. PTX3 is an acute phase response protein that initiates innate immunity against various

microorganisms. It is produced in response to pro-inflammatory stimuli by many cell types, including myeloid cells. Increased serum levels of PTX3 are found in pregnancy. hCG dose-dependently increased the PTX3 expression by human monocytes, mouse DC and mouse Mφ. This increased PTX3 expression was mediated via the PKA-signalling pathway. hCG injection *in vivo* also increased the PTX3 serum levels. This serum PTX3 was mainly produced by blood monocytes. The hCG-induced hormones progesterone and estrogen also increased the PTX3 production by human monocytes. From this study we conclude that hCG contributes to the increased innate immunity during pregnancy via induction of PTX3 in mononuclear phagocytic cells.

In **Chapter 3**, we described that hCG treatment of IFN-γ-primed Mφ increased the production of NO, ROS, IL-6 and IL-12p40, and enhanced the phagocytosis of labelled-beads and apoptotic cells. These observations suggested an enhancement of the innate function of Mφ by hCG. hCG treatment did not affect the induction of allogeneic T cell proliferation by IFN-γ-primed Mφ. The observed effects were receptor-mediated and involved the PKA signalling pathway. Although i.p. injection of hCG did not result in increased phagocytosis of peritoneal Mφ, cultures of thioglycollate(TG)-elicited Mφ from hCG injected mice exhibited an increased phagocytic ability upon IFN-γ activation and hCG treatment. This suggests the requirement of the continuous presence of hCG for increasing phagocytosis by Mφ. The induction of T cell proliferation was not affected by hCG treatment, indicating that the function of Mφ in adaptive immunity remained unchanged.

In **Chapter 4**, the effect of hCG on DC and their function in adaptive immunity was studied. Upon hCG treatment in combination with LPS, mouse bone marrow-derived DC increased their ratio of IL-10/IL-12p70, downregulated TNF-α production, and decreased antigen-specific T cell proliferation. Addition of hCG together with LPS and IFN-γ to DC blocked their MHC class II upregulation, increased their IL-10 and TNF-α production and decreased the induction of antigen-specific T cell proliferation. The hampered MHC class II expression was not due to increased IL-10 and TNF-α production, because neutralizing antibodies and recombinant IL-10 and TNF-α did not have an effect on MHC class II expression. Splenic DC showed similar results. Upon hCG treatment, LPS- and IFN-γ-stimulated DC increased the expression of IDO mRNA and its metabolite kynurenine. This suggests their involvement in the observed decreased T cell proliferation upon hCG stimulation. To study the effect of hCG on DC differentiation from their precursors, bone marrow-derived DC were generated in the continuous presence of hCG. Under this condition, hCG decreased cytokine production and the induction of T cell proliferation. These data suggest that hCG contributes to the maternal-fetal tolerance during pregnancy by modifying DC towards a tolerogenic phenotype.

Recently, pregnancy at an early stage has been proposed to represent a controlled state of inflammation. Later in pregnancy this state of inflammation is extended to the systemic maternal circulation. Earlier our group reported that hCG can inhibit the development of diabetes in NOD mice and LPS-induced septic shock in a murine model. We hypothesized that hCG can contribute to the reduction of inflammation by modifying the Mφ function.

In **Chapter 5**, we used an *in vivo* model of acute inflammation that has clearly defined sequential steps of disease development: TG-induced peritonitis. In this model, peritoneal resident Mφ play an important role in the initiation of the inflammation. hCG pretreatment in TG-induced peritonitis resulted in an increased number of peritoneal

cells, especially PMN and monocytes, compared to mice injected with TG only. This increased cell number was partially caused by increased cell survival induced by hCG. Simultaneous with the increased cellular infiltration, hCG pretreatment decreased the intraperitoneal TNF-α, IL-6, PTX3, CCL3 and CCL5 levels. By depleting peritoneal resident Mφ using clodronate liposomes prior to the application of hCG and the TG injection, we established that Mφ are the main responsive cells to hCG in this model. In the absence of Mφ, the hCG induced increased PMN influx and suppressed TNF-α and IL-6 production did not occur. Together, these data suggest that hCG *in vivo* can downregulate the pro-inflammatory activity of Mφ.

In conclusion, the results described in this thesis suggest that hCG, via modulating the function of mononuclear phagocytic cells, contributes to an increased innate immunity and a decreased adaptive immunity. This modulation likely helps to protect the mother against pathogens and to suppress specific immune responses against the fetus.

SAMENVATTING

Gedurende de zwangerschap accepteert het immuunsysteem van de moeder de foetus, hoewel deze voor de helft van vaderlijke oorsprong is. De afwezigheid van afstoting suggereert dat de immunologische afweerreactie van de moeder tegen de foetus is onderdrukt. Er is echter geen sprake van een totale onderdrukking van het afweersysteem van de moeder.

Foetale cellen zoals trofoblasten komen in nauw contact met de witte bloedcellen van de moeder. De afwezigheid van afstoting, leidend tot de acceptatie van de foetus door de moeder, wordt veroorzaakt door een scala aan factoren en mechanismen, zoals HLA-G en HLA-E expressie door trophoblasten, een unieke populatie NK cellen in de uterus, inductie van indolamine-2,3 dioxygenase (IDO), een veranderd Th1/Th2 cytokine micromilieu, apoptose van maternale T cellen en een toename van regulatoire T cellen. Verder zijn er aanwijzingen dat er tijdens de zwangerschap bij de moeder een verhoogde activiteit is van de niet-specifieke afweer terwijl de specifieke afweer, wordt onderdrukt.

Tijdens de zwangerschap zijn er grote veranderingen in de serum concentraties van hormonen, zoals humaan choriongonadotrofine (hCG). hCG is een hormoon dat wordt geproduceerd door trofoblasten. Zwangerschapstesten zijn gebaseerd op de detectie van dit hormoon in de urine. hCG stimuleert het corpus luteum in het ovarium om progesteron en oestrogeen te produceren. Deze hormonen zijn van belang voor het behoud van de zwangerschap. Naast deze endocrinologische rol van hCG zijn er steeds meer aanwijzingen voor een immunologische rol van hCG. Zo bleek behandeling met hCG effectief te zijn in proefdiermodellen voor een aantal autoimmuunziekten, zoals type I diabetes, het syndroom van Sjögren en reumatoïde artritis.

hCG bindt aan de hCG receptor en activeert in de cel vervolgens de cAMP-PKA signaleringsroute, die vervolgens resulteert in transcriptie van CREB. Cellen van het reproductieve systeem hebben hCG receptoren en kunnen dus door hCG worden beïnvloed. Mononucleaire fagocyten, zoals monocyten, macrofagen en dendritische cellen, hebben ook hCG receptoren.

Mononucleaire fagocyten ontstaan uit voorlopercellen in het beenmerg en spelen een rol bij zowel de specifieke als niet-specifieke afweer. Myeloïde voorlopercellen differentiëren tot monocyten, circuleren in het bloed en gaan de weefsels in om, afhankelijk van de plaatselijke condities, verder te differentiëren tot macrofagen of dendritische cellen. Monocyten zijn effector-cellen van het niet-specifieke afweersysteem. Zij beschermen het lichaam tegen ziekteverwekkers in de bloedbaan. Macrofagen spelen een belangrijke rol in de niet-specifieke afweer in de weefsels, door fagocytose, vernietiging van micro-organismen, het opruimen van apoptotische cellen en de productie van zuurstofradicalen en pro-inflammatoire cytokinen. Mononucleaire fagocyten zijn in staat antigeen te presenteren aan T cellen en zo een specifieke afweerreactie te initiëren. DC zijn de meest krachtige antigeen-presenterende cellen van het afweersysteem. DC kunnen naïeve T cellen stimuleren en zo een specifieke afweerreactie induceren. Bovendien kunnen DC het specifieke immuunsysteem aansturen door de Th1/Th2 balans te veranderen en door regulatoire T cellen te induceren.

Omdat mononucleaire fagocyten een belangrijke rol spelen bij de initiatie van autoimmuunziekten, hCG behandeling de klinische verschijnselen van verschillende autoimmuunziekten in diermodellen vermindert, en mononucleaire fagocyten hCG receptoren hebben, hypothetiseerden we dat hCG het afweersysteem kan beïnvloeden door modulatie

van de functie van mononucleaire fagocyten. Deze hypothese hebben we getoetst door het effect van hCG op monocyten, macrofagen en DC te bestuderen.

In het kader van ons onderzoek hebben wij het effect van hCG op de productie van PTX3 door monocyten bestudeerd (**Hoofdstuk 2**). PTX3 is een zogenaamd "acute fase" eiwit dat het specifieke afweersysteem stimuleert tot een afweerreactie tegen bepaalde micro-organismen. PTX3 wordt geproduceerd door verschillende celtypen, waaronder myeloide cellen, na stimulatie met ontstekingsmediatoren. Tijdens de zwangerschap zijn er verhoogde PTX3 serum spiegels. hCG verhoogt op dosisafhankelijke wijze de PTX3 expressie door humane monocyten, muizen macrofagen en DC. Deze door hCG geïnduceerde PTX3 expressie verliep via de PKA-signaleringsroute. Monocyten in het perifeer bloed bleken de voornaamste producenten van PTX3 in het serum te zijn. De door hCG geïnduceerde hormonen progesteron en oestrogeen verhoogden ook de PTX3 productie door humane monocyten. Uit deze studie concluderen we dat hCG tijdens de zwangerschap bijdraagt aan een versterking van het niet-specifieke afweersysteem via de inductie van PTX3 in mononuclaire fagocyten.

In **Hoofdstuk 3** hebben we beschreven dat hCG behandeling van IFN-γ gestimuleerde macrofagen resulteert in een verhoging van de productie van NO, ROS, IL-6 en IL-12p40, en een verhoogde fagocytose van gelabelde bolletjes en apoptotische cellen. Ook deze waarnemingen suggereren dat hCG een versterking van het niet-specifieke immuunsysteem veroorzaakt. hCG behandeling had geen effect op de inductie van T cel proliferatie door IFN-γ gestimuleerde macrofagen. De waargenomen effecten waren receptor-gemedieerd en maakten gebruik van de PKA signaleringsroute. Hoewel intraperitoneale injectie van hCG niet resulteerde in een verbeterde fagocytose door peritoneaal macrofagen, vertoonden gekweekte, door thioglycollaat (TG) opgewekte macrofagen van hCG geïnjecteerde muizen een verhoging van fagocytose activiteit na IFN-γ stimulatie en hCG behandeling. Dit suggereert dat continue aanwezigheid van hCG noodzakelijk is voor de verbeterde fagocytose activiteit door macrofagen. Het induceren van T cel proliferatie werd niet beïnvloed door hCG behandeling. Dit geeft aan dat de functie van macrofagen in het aansturen van het specifieke afweersysteem onder deze omstandigheden door hCG niet beïnvloed wordt.

In **Hoofdstuk 4** hebben wij het effect van hCG op DC en hun aansturende functie in het specifieke afweersysteem bestudeerd. Door behandeling van DC met hCG in combinatie met LPS werd de verhouding IL-10/IL-12p70 verhoogd en de TNF-α productie en de antigeen-specifieke T cel proliferatie verlaagd. Toevoeging van hCG samen met LPS en IFN-γ, blokkeerde de verhoging van MHC klasse II, verhoogde de productie van IL-10 en TNF-α en verlaagde de antigeen-specifieke T cel proliferatie door DC. De verminderde expressie van klasse II werd niet veroorzaakt door een toename in IL-10 en TNF-α productie, omdat neutraliserende antistoffen en recombinant IL-10 en TNF-α geen effect hadden op de MHC klasse II expressie. hCG behandeling verhoogde de expressie van IDO mRNA en de metaboliet kynurenine. Dit is suggestief voor de betrokkenheid van deze mediatoren bij de verminderde T cel proliferatie, die werd waargenomen na stimulatie met hCG. Om het effect op de differentiatie van DC uit precursors te bestuderen, werden uit beenmerg DC gekweekt in de continue aanwezigheid van hCG. Onder deze omstandigheden verminderde hCG de cytokine productie en de inductie van T cel proliferatie. Deze data suggereren een bijdrage van hCG aan de maternale-foetale tolerantie tijdens de zwangerschap door de modificatie van de DC naar een tolerogeen fenotype.

Recent is verondersteld dat zwangerschap in de vroege fase te vergelijken is met een locale gecontroleerde ontsteking. Later in de zwangerschap breidt deze ontsteking zich uit tot in de maternale circulatie. Eerder rapporteerde onze onderzoeksgroep dat hCG een remmend effect heeft op de ontwikkeling van diabetes in NOD muizen en LPS-geïnduceerde septische shock in een muismodel. Wij veronderstellen dat hCG bijdraagt aan de afname van de ontsteking door modificatie van de functie van macrofagen. In **Hoofdstuk 5** maken wij gebruik van een *in vivo* model voor acute ontsteking dat zich ontwikkelt volgens duidelijk gedefinieerde sequentiële stappen: TG-geïnduceerde peritonitis. In dit model spelen macrofagen een belangrijke rol bij de initiatie van de ontsteking. Voorbehandeling met hCG in TG-geïnduceerde peritonitis resulteerde in een toename van met name granulocyten en monocyten in de buikholte ten opzichte van TG injectie alleen. Deze toename in cel- aantallen werd gedeeltelijk veroorzaakt door een verbeterde overleving van de cellen na hCG behandeling. Gelijktijdig met de toegenomen ophoping van cellen namen de intraperitoneale spiegels van TNF-α, IL-6, PTX3, CCL3 en CCL5 toe. Door verwijdering van de residente peritoneale macrofagen door clodronaat liposomen vóór de behandeling met hCG en de TG injectie hebben wij vastgesteld dat macrofagen de belangrijkste cellen zijn die op hCG reageren in dit model. In afwezigheid van macrofagen werd de hCG geïnduceerde ophoping van granulocyten en de onderdrukking van de TNF-α en IL-6 productie niet waargenomen. Deze gegevens suggereren dat hCG *in vivo* een verlaging van de pro-inflammatoire activiteit van macrofagen veroorzaakt.

Concluderend kan worden gesteld dat de resultaten van onze studies suggereren dat hCG door modulatie van de functie van mononucleaire fagocyten zorgt voor een versterking van het niet-specifieke immuunsysteem en een verminderd functioneren van het specifieke immuunsysteem. Deze modulatie draagt waarschijnlijk bij aan de bescherming van de moeder tegen ziekteverwekkers en onderdruking van de afweerreacties tegen de foetus.

ABBREVIATIONS

7-AAD	7-amino-actinomycin D
APC	antigen-presenting cells
-APC	allophycocyanin
Ag	antigen
BM	bone marrow
BMDC	bone marrow-derived dendritic cells
BMDM	bone marrow-derived macrophages
BSA	bovine serum albumin
BUN	blood urea nitrogen
cAMP	cyclic adenosine monophosphate
CCL	CC chemokine ligand
CD	cluster of differentiation
CFSE	carboxyfluorescein diacetate succinimidyl ester
Cpm	counts per minute
DC	dendritic cells
DMEM	Dulbecco's Modified Eagle's Medium
E_2	estrogen
ELISA	enzyme-linked immunosorbent assay
FACS	fluorescence activated cell sorter
FCS	fetal calf serum
-FITC	fluorescein isothiocyanate
GM-CSF	granulocyte/macrophage colony-stimulating factor
hCG	human chorionic gonadotropin
IDO	indoleamine 2,3-dioxygenase
IFN-γ	interferon gamma
IL	interleukin
LPS	lipopolysaccharide
MCP-1	monocyte chemotactic protein-1
M-CSF	macrophage colony-stimulating factor
Mφ	macrophages
MFI	mean fluorescence intensity
MHC	major histocompatibility complex
MIF	macrophage migration inhibitory factor
MIP-1α	macrophage inflammatory protein-1 alpha
MLR	mixed lymphocyte reaction
MM6	MonoMac-6 (monocytic cell line)
MMP	macrophage metalloelastase
MPS	mononuclear phagocyte system
mRNA	messenger ribonucleic acid
MS	multiple sclerosis
NF-κB	nuclear factor-kappa B
NO	nitric oxide

P_4	progesterone
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
-PE	phycoerythrin
-PerCP	peridinin chlorophyl protein
PHA	phytohaemagglutinin
PKA	protein kinase A
PMN	polymorphonuclear neutrophils
PTX3	pentraxin 3
RA	rheumatoid arthritis
RANTES	regulated on activation, normal T cell expressed and secreted
ROS	reactive oxygen species
RPMI-1640 medium	Roswell Park Memorial Institute-1640 medium
RT-PCR	reverse transcription polymerase chain reaction
SLE	systemic lupus erythematosus
TG	thioglycollate
Th	T helper
TNF-α	tumor necrosis factor-alpha
Treg	regulatory T cells

Chemokine nomenclature

MCP-1	CCL2
MIP-1α	CCL3
RANTES	CCL5

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Dearest all:

It was like a dream, the day I came to The Netherlands; it was raining. The day I left, it was raining too. Too happy to see me and unhappy to say goodbye, or the other way around? I just could not interpret the tears from the Dutch sky, its mood swings are too frequent. It was a pleasant experience to work at the department of Immunology, Erasmus MC, Rotterdam. It was also a pleasure to know you all and working together for almost 5 years. As a foreigner, I did not find it too hard to adapt to a completely new life style, maybe because I kept an open mind, plus you all are nice. I was curious for sure, I knew everything would be different, language, social relation, food…etc, but still I did take pictures of some weird-dressed people in the street and mailed the pictures back home. It turned out that my parents think that all Dutch people look like that, so I spent some time explaining that most are as normal as we are, only a few appeared "remarkable" of whom I took the pictures. But I also realized that people get biased ideas easily, just like on the TV; one (selected) person was interviewed, the audience might think that all the rest are the same. The conclusion is physically being here is so needed. Pity that I still don't know exactly where is where in Rotterdam. I am blind in directions and very bad at reading maps. But the good thing is that I always bumped into beautiful places, which are not on the map. I have to say, Rotterdam is beautiful, such as sunset near a dam, nice tropic fruit market somewhere, or stylish architectures gathered somewhere. I did try to improve my ability of travelling alone in completely new cities. It was nice, but most of the time I worried that I may end up nowhere but the police station. There are definitely much more things I like than dislike, that is the reason Rotterdam gives me a good memory. Imagine, one day when I am old, sitting in a wheelchair and looking at the picture we had taken in front of our "white tower"; *this guy was kind, I was at that girl's wedding, that is Rob I always ran after, she was in my office, he was the cum laude boy* (etcetera, etcetera....).

Going back to science, there are a lot of people I need to address my thanks to. I couldn't have finished, and I couldn't have invited you all for my defense, if you wouldn't have helped me.

Rob, thank you very much for giving me the chance to see the world outside of China. I still remember the day I arrived at Schiphol, my plane was one hour earlier. All the passengers were gone, only I was left, calculating the possibility of sleeping on the street that night. Then you walked towards me with a board with my name. On the way we talked a lot, my first impression of Dutch people was perfect! Then you took me to the department, showing me around. I was a bit tired. My family got up at 5:00 o' clock in the morning, and we drove from my city to the capital, then to the airport. We rested for a while, and at 11:00 o'clock I entered the gate for numerous checks and forms. Finally at 13:00 o'clock I got on the plane. After a 10 hour flight, I arrived in The Netherlands, and then we drove to Rotterdam. I had been awake for more than 20 hours and strongly missed my bed, but you were so enthusiastic to tell me about Erasmus…☺ I love this memory, somebody is happy to see me, it feels good. Concerning science, you were definitely a bridge guiding me to the field of Immunology. But I think my staying in The Netherlands was more than just work. I compared a lot of The Netherlands with China, and the conclusion is that they are not comparable. From the outside, they are pretty much the

same. There are definitely poorer villages and richer cities in China but I've never seen them. From the inside we are different, we are facing different problems, react differently and thus solve them in our own ways. It is difficult to tell what is good and bad, but there are definitely things to learn.

Marjan, thank you for your work, especially correcting all the papers at the end. You and your family gave me such a warm welcome for my first days in The Netherlands. I still remember you cooked Stampot with blocks of cheese inside. The whole family of your friend and their huge dog came to see me because they had never seen a Chinese before. I really felt honoured. I talked too much that night, because I read somewhere in the news that western people are open and I didn't want to be different. Really quickly, five years have passed. In the end, we corrected my manuscripts again and again. It was a hard period, but was a good experience nonetheless.

Hemmo, thank you for the discussions, on science and on life. As a person involved in the history of research, your knowledge is useful for all of us. Your clear messages really made me think deeper. We definitely had disagreements, but more agreements for sure.

Rebecca, thank you for all the input you gave for my Ph.D. study. All your suggestions were helpful. Now I am working as a Postdoc, I don't know exactly how to do it, but I have your example. I just try to help others in the way that you helped me. Keep in contact, not only as colleagues, but also as a friend.

Hi, Pieter, my "step-boss". Thank you for allowing me to just bump into your office for help. Maybe you did not officially agree on that, but anyway, thanks a lot for your contribution to my work. I will try hard to stay in this field, which means the chance I may bother you in the future is high. Be prepared!

My dearest paranimphs, Corine and Jo, first of all, thank you for accepting to be my paranimphs and agree to dress darker than me, so that my light coloured clothes makes it obvious that I am defending. Second, I greatly appreciated the practical work you did for/with me. We are an excellent team, you know, without you two, the mouse work could have been too much for me. We three small girls had such a happy time together.

Manon, thank you for doing your PhD together with me, although neither one of us planned it. I felt lucky to meet you. We are so different, and that made me learn things which I was missing in myself. But as it turned out, we stay who we are. I must say I admire you, and when I encounter certain problems I often think to myself 'If I was like that, I would..."

Roos and Joey, thanks for being in the same office with me. We had a nice atmosphere to work in. Joey is funny, making noise all the time, but fun is noisy, right?

Tar and Wendy, without you both, my thesis wouldn't look so organized and readable…☺

The lab of Ee 987, A3, Berlinda, Harm, Lonneke, Vinod, Marcel, Esther, Patricia, Gerben, Tatjana, Sacha, …as well as many students, you are all part of my good memory of Rotterdam. To the Ph.D. students and some already Postdocs: the AIO weekend was a nice experience with you all. Rudi, Frank, and Wim, thanks for organizing the journal club as well as other Ph.D. activities, all these activities made my Ph.D. life colourful.

I did not want to copy our employee list, but if I forgot your name here, that doesn't mean you are not important; it only means I forgot!

Last but not the least, this thesis is also dedicated to my parents and my brother in China.

Being here alone outside of home, every old Chinese man I saw on the street always reminds me of you, Dad. You know, if you asked me to come back now, I would. But in every telephone call you told me not to worry about you, and fly as far as I want to. I feel like a kite, the thread is in your hand, but I have to check every now and then to make sure the thread is still in your hand so that I won't get lost. My cutest Mum, it does not remind me of you when I saw any Chinese woman here, I was wondering why. I think it is because you are very beautiful, to me. Thank you for giving me your character, always happy and thinking about the positive sides of my life, although I would also like to have your beauty. It was such a pleasure every weekend to talk with you, hours and hours just passed away like a few moments. We gossiped about my Dad and my brother, and try to change them which we know is impossible. Brother, although the quarrels we had did influence my hyper mood, I know you love your "always little" sister. I want you to be happy. I hope you can marry yourself out successfully this year, and have kids asap. To all of you, if you have a good life, I will be at ease and live/work as happy as I can.

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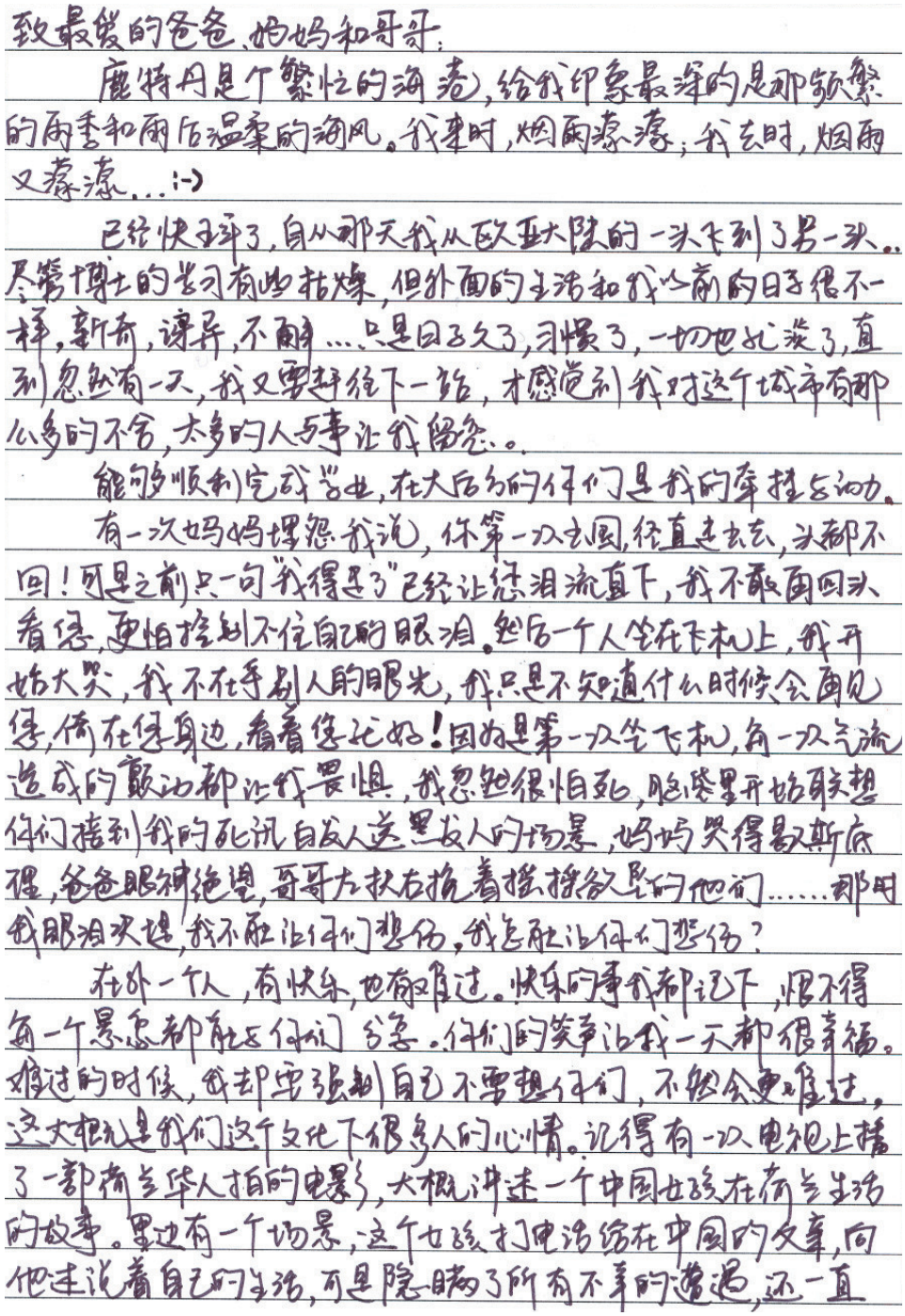

致最爱的爸爸、妈妈和哥哥：

鹿特丹是个繁忙的海港，给我印象最深的是那缺乏的雨季和雨后温柔的海风。我来时，烟雨蒙蒙；我去时，烟雨又蒙蒙…:-)

已经快五年了，自从那天我从欧亚大陆的一头飞到了另一头，尽管博士的学习有些枯燥，但外面的生活和我以前的日子很不一样，新奇，诧异，不解……只是日子久了，习惯了，一切也就淡了，直到忽然有一天，我又要赶往下一站，才感觉到我对这个城市有那么多的不舍，太多的人与事让我留念。。

能够顺利完成学业，在大后方的你们是我的牵挂与动力。

有一次妈妈埋怨我说，你第一次去国，径直走去云，头都不回！可是之前只一句"我得走了"已经让您泪流直下，我不敢回头看您，更怕控制不住自己的眼泪。独自一个人坐在飞机上，我开始大哭，我不在乎别人的眼光，我只是不知道什么时候会再见到您，倚在您身边，看着您老的好！因为是第一次坐飞机，每一次气流造成的颠动都让我畏惧，我忽然很怕死，脑袋里开始联想你们接到我的死讯自发人送黑发人的场景，妈妈哭得歇斯底里，爸爸眼神绝望，哥哥左扶右挽着摇摇欲坠的他们……那时我眼泪决堤，我不敢让你们悲伤，我怎敢让你们悲伤？

在外一个人，有快乐，也有难过。快乐的事我都记下，恨不得每一个景点都献与你们分享。你们的笑声让我一天都很幸福。难过的时候，我却宁愿独自忍受不要想你们，不然会更难过。这大概就是我们这个文化下很多人的心情。记得有一次电视上播了一部荷生华人拍的电影，大概讲述一个中国女孩在荷生活的故事。里边有一个场景，这个女孩打电话给在中国的父亲，向他述说着自己的生活，可是隐瞒了所有不幸的遭遇，还一直

答应看那边的爸爸会好好照顾自己。然后挂了电话，默默流泪。我的外国朋友反复问我好什么，好什么，回过头猛然发现我泪流满面。我该怎样向他解释这撞击心灵的感受呢？从解释"家"开始？还是从解释"爱"开始呢？

想想自己真的很不孝，父母退休正是开始需要我的时候，我离开了。偶尔回去的那次，也没有好好做做家务，陪他们聊聊天。几天前在网络上看到的"不要对父母说的十句话"，主为四.子句我记忆深刻，像"好了，好了，知道，真啰嗦"，"说了你也不懂，别问了！"，"你那一套早就过时了"，"我自己知道，不要充说了，烦不烦"…请原谅我的无知与无知，还好，你们一直都爱我，从未变过。

我也爱你们，而且从未变过。有一次梦里梦到妈妈，她在给我擦窗户，我说"妈，别太累了，别干了。"妈妈说："我面都仔擦一扇…擦完，好再就不见了，我大叫，妈妈，妈妈…惊醒，你知道我有多想你吗？你可爱的笑容，明亮的大眼睛，谢谢你给了我朱此时间，凡事多看积极面，实对人生。在国外的街上，每次看到中国老人，都会让我想起爸爸，你是那么平凡，又那么真切，谢谢你们教导我长大，也谢谢你对我一直心辛时期待、指导。你对我的辛苦，我知道。

亲爱的哥哥，你陪我长大，也要陪我变老，我知道你对我的爱一直都在，不要说，我明白，我只要你快乐！

很久没在中文了，尽管感动还在，却变得有些无声无息，只希望我们都会幸福。我们一定要幸福！

2008年12月12日，于德拜丹。

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