

# Inhibition of Septic Shock in Mice by an Oligopeptide From the $\beta$ -Chain of Human Chorionic Gonadotrophin Hormone

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**ABSTRACT:** Human chorionic gonadotrophin (hCG) is a heterodimeric placental glycoprotein hormone required in pregnancy. In human pregnancy urine and in commercial hCG preparations (c-hCG) it occurs in a variety of forms, including breakdown products. Several reports have suggested modulation of the immune system by intact hormone, but such effects of breakdown products have not been reported. In a related article (*Hum Immunol* 62:1315, 2001), it is reported that a 400–2000 Dalton (Da) fraction from c-hCG and from human pregnancy urine inhibits Th1-mediated diabetes in NOD mice. The active component(s) were called natural (immuno)modulatory pregnancy factor(s) (NMPF). This study reports that a single treatment with the same low molecular weight NMPF fraction up to 24-h after high dose lipopolysaccharide (LPS) injection inhibited septic shock in mice. This counteracting effect of NMPF paralleled the downregulation of the effects of LPS on the

production of macrophage migration inhibitory factor (MIF) by spleen cells, on the plasma level of liver aminotransferase, and on the expression of several splenic lymphocyte and macrophage surface markers. Based on the primary structure of the  $\beta$ -chain of hCG a synthetic hexapeptide Valine-Leucine-Proline-Alanine-Leucine-Proline (VLPALP) was designed, which demonstrated it to have the same protective effects as the 400–2000 Da NMPF fraction. These results indicate a new strategy for the treatment of septic shock and the potential of therapeutic use of this synthetic oligopeptide. *Human Immunology* 63, 1–7 (2002). © American Society for Histocompatibility and Immunogenetics, 2002. Published by Elsevier Science Inc.

**KEYWORDS:** pregnancy/urine; sepsis; gonadotrophins chorionic/peptide; immunomodulation

## ABBREVIATIONS

NMPF natural (immuno)modulatory pregnancy factor  
hCG human chorionic gonadotrophin

MIF macrophage migration inhibitory factor  
NK natural killer

## INTRODUCTION

Human chorionic gonadotrophin (hCG) is heterodimeric glycoprotein hormone produced in pregnancy by the placental trophoblast. It maintains the steroid secretions of the corpus luteum and thereby maintains the lining of the uterus for development of the embryo after its implantation [1]. Clinical symptoms of Th1-mediated autoimmune diseases regress in many patients during preg-

nancy [2, 3]. A related article [4] reported, for the first time, the inhibition of Th1-mediated autoimmune diabetes in NOD mice by a fraction from hCG preparation that contains one or more 400–2000 Dalton (Da) natural (immuno)modulatory pregnancy factors (NMPF). It is postulated that this NMPF, possibly in concert with other immunomodulatory factors, accounts for an adjustment of the immune system during pregnancy. This adjustment is not only leading to clinical improvement of Th1 autoimmune diseases, but also to the maintenance of normal immune competence against microorganisms and suppression of the Th1-mediated immune response to the paternal antigens of the developing fetus.

This present study investigated whether the 400–

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2000 Da NMPF was not only able to suppress the ongoing insulinitis and to reverse the development of diabetes in NOD mice [4], but also inhibit septic shock in mice. A high-dose endotoxin shock in BALB/c mice was used as a model of acute inflammation and human septic shock syndrome [5, 6]. In this model intraperitoneal injection of a high dose of lipopolysaccharide (LPS) triggers an acute inflammatory response characterized by the rapid increase of the plasma level of liver aminotransferase and the serum level of proinflammatory cytokines, such as macrophage migration inhibitory factor (MIF) [7]. This study illustrates that the 400–2000 Da NMPF fraction from hCG is able to prevent, and even to suppress, mortality in mice due to LPS-induced septic shock.

The hormone hCG occurs in various forms in blood and urine during pregnancy [1, 8]. Apart from its heterogeneity of carbohydrate content, nicks at various regions of hCG, particularly in the  $\beta$ -chain, are responsible for these different forms. A synthetic oligopeptide was designed (*i.e.*, VLPALP, corresponding to residues 48–53) from the primary structure of the  $\beta$ -chain of hCG with similar anti-shock activity in the endotoxin shock model as the 400–2000 Da NMPF fraction.

## MATERIAL AND METHODS

### Mice, Endotoxin Shock Model, and hCG Treatment

BALB/c mice were purchased from Harlan (Horst, The Netherlands) and maintained as described in a related article [4]. For the induction of sepsis, 8- to 12-week-old female BALB/c mice ( $n = 10$ ) were injected intraperitoneally with 8 mg/kg LPS (*E. coli* 026:B6; Difco Laboratories, Detroit, MI, USA). The experiments were approved by the Animal Experiments Committee of the Erasmus University (Rotterdam, The Netherlands).

To test the effect of NMPF on LPS-induced septic shock, hCG-preparations from several sources were used: commercial hCG preparation (c-hCG; Pregnyl Organon, Oss, The Netherlands [lot numbers 209893, 235863, 248455]), hCG purified from first trimester pregnancy urine (u-hCG), the 400–2000 Da fractions of c-hCG and u-hCG, recombinant hCG (r-hCG; Sigma, St. Louis, MO, USA), its subunits (r- $\alpha$ -hCG and r- $\beta$ -hCG; Sigma), highly purified urinary hCG, and the synthetic oligopeptide VLPALP designed from  $\beta$ -chain of hCG. BALB/c mice were treated with either a dose of 300 IU c-hCG, 5 mg/kg u-hCG, 0.5 mg/kg 400–2000 Da NMPF-fraction from c-hCG or u-hCG, 5 or 15 mg/kg NMPF peptide (VLPALP), or 5 mg/kg r-hCG or its subunits (r- $\alpha$ -hCG or r- $\beta$ -hCG). This treatment was done either 2-h or 24-h after the LPS injection. Control groups were treated with PBS instead of LPS, and/or PBS instead of hCG/NMPF.

Purification and dose titration of preparation of u-

hCG from first trimester human pregnancy urine and of the 400–2000 Da NMPF fractions was done according to the method described in a related article [4].

### Semiquantitative Sickness Measurements

Mice were scored for sickness severity using the following criteria: score 1 was defined as percolated fur, but no detectable behavior differences compared with untreated control mice; score 2 was defined as percolated fur, huddle reflex, responding to stimuli (such as tap on cage), and just as active during handling as untreated control mice; score 3 was defined as a slower response to tap on cage, and passive or docile when handled, but still curious when alone in a new setting; score 4 was defined as a lack of curiosity, little or no response to stimuli, and quite immobile; score 5 was defined as labored breathing, and unable or slow to self-right after being rolled onto back (moribund); score 6 was defined as death.

### Cytokine Assay

Spleen cells were isolated from treated BALB/c mice 24-h after the LPS shock induction as described previously [4]. Splenic cell suspensions were cultured ( $2 \times 10^5$  cells/well) in 96-well flat-bottom plates (0.2 ml) and stimulated with LPS (10  $\mu$ g/ml). RPMI 1640 medium was used supplemented with 25-mM HEPES, 100-IU/ml penicillin, 50- $\mu$ g/ml streptomycin, 1-mM pyruvate, 50- $\mu$ M 2-ME, and 10% heat inactivated fetal calf serum (FCS). All cultures were performed in triplicate or quadruplicate. After incubation for 12 h, supernatants were collected for MIF analysis.

The amount of MIF was measured with a commercially available ELISA kit (Chemicon International, Temecula, CA, USA) according to the protocol provided by the manufacturer. The detection limit was 800 pg/ml.

### Analysis of Liver Enzymes

Hepatocyte damage was assessed 24-h after LPS administration by measuring plasma enzyme activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) using commercial kits (Merck, Darmstadt, Germany) on an automated analyzer (ELAN-analyzer; Eppendorf, Hamburg, Germany).

### Flow Cytometric Analysis

Spleen cells were isolated from treated BALB/c mice 24-h after the LPS shock induction and used for the analysis of cell surface markers. Splenic cells ( $2 \times 10^5$ ) were resuspended in PBS containing 1% BSA and 0.1% sodium azide (PBS-BSA-azide). For the staining of surface antigens, spleen cells were incubated with FITC- or PE-conjugated monoclonal antibodies (mAb) against CD3, B220, NK1.1, major histocompatibility complex II (MHC II), and F4/80 (all obtained from PharMingen,

San Diego, CA, USA). After washing twice with PBS-BSA-azide, the cells were resuspended and analyzed on FACScan (Becton Dickinson, San Jose, CA, USA). Then 10- $\mu$ l propidium iodide (0.2 mg/ml) was added to evaluate the viability of the cells. In all samples the viability was higher than 94%. Events ( $10^4$ ) were collected and the expression of the markers analyzed using CellQuest software (Becton Dickinson).

### Statistics

Data was analyzed by Student's *t*-test and differences were considered significant at  $p < 0.05$ .

## RESULTS

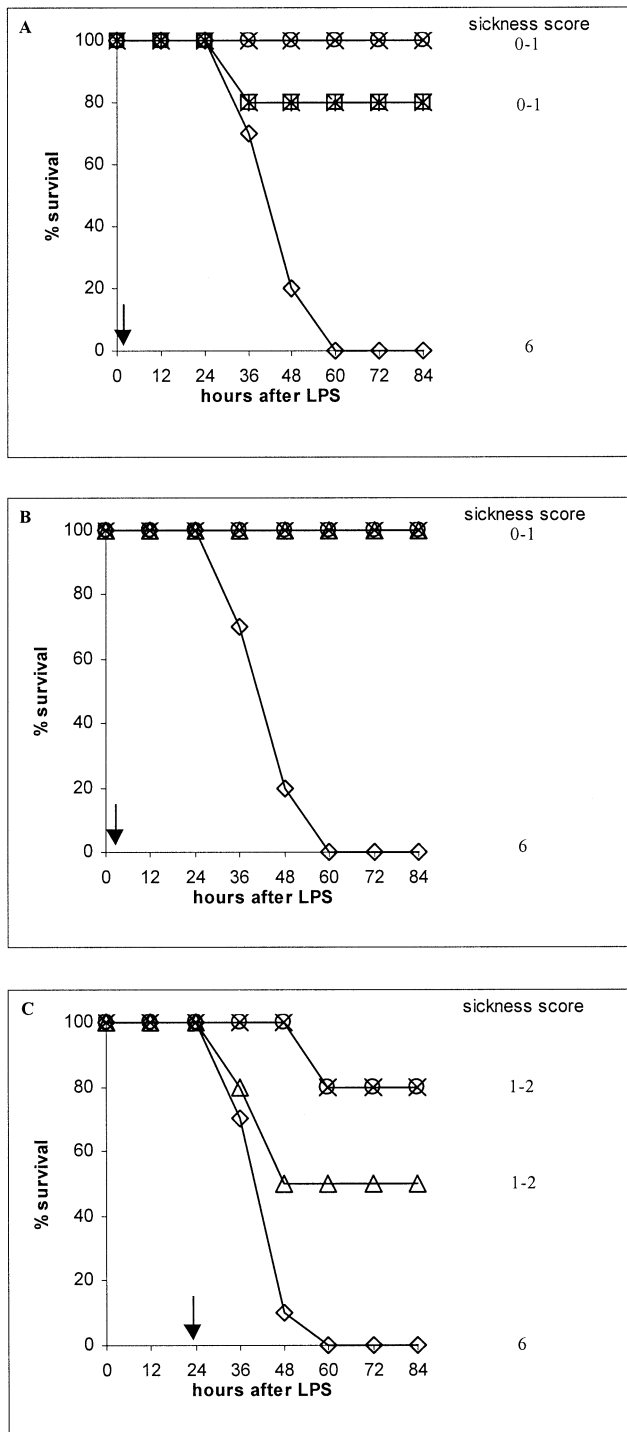
The model for LPS-induced septic shock syndrome in BALB/c mice was standardized by testing various doses of *E. coli* 026:B6 LPS intraperitoneally. Every 12 h the survival and sickness scores were assessed. For the experiments reported here, a treatment of 8-mg LPS per kg body weight (BW) was used, which was lethal for all BALB/c mice by 60 h. The signs of sickness were apparent in all LPS-treated mice, but the kinetics and severity were different between the mice. In order to quantitate the observed differences, a semiquantitative sickness scoring system was used, which was standardized in several shock experiments using large numbers of BALB/c mice ( $n = 30$ ). In these experiments, mice intraperitoneally injected with 8-mg LPS/kg BW achieved a sickness score of about 2 within 12 h (see the Material and Methods section above). At the time point of 24 h, most of the BALB/c mice had reached the sickness score of 3 or 4. Thereafter the mice gradually reached a sickness score of 5 or died (score 6). At the 60-h time point all LPS-injected mice were dead (score 6).

Treatment of BALB/c mice with c-hCG (300 IU) or u-hCG (5 mg/kg) 2 h after LPS (8-mg/kg BW) injection increased the survival rate to 80% when compared with 0% of the PBS-treated mice, whereas the 400–2000 Da fraction purified from c-hCG or u-hCG (0.5 mg/kg) prevented mortality and reduced the sickness score in all mice (Figure 1A). The maximum sickness scores observed in c/u-hCG and the 400–2000 Da fraction treated groups during the experiment were 3 and 2, respectively. The score 3 was observed in all (10/10) c-hCG treated mice at 24-h after LPS injection, and the score 2 was observed in all (10/10) 400–2000 Da NMPF fraction treated mice at 24-h after LPS injection. In the hours thereafter the mice steadily recovered, so that at 84-h after the shock induction all treated mice had a sickness score not higher than 1. Two days later all mice had completely recovered. Similar hCG treatments using highly purified urinary hCG, r-hCG, r- $\alpha$ -hCG, and r- $\beta$ -hCG were ineffective (data not shown).

The hormone hCG occurs in various forms during pregnancy. It is known that hCG and its uncombined subunits, especially  $\beta$ -hCG, are prone to proteolytic cleavage resulting in different forms of “nicked” hCG [8]. In order to determine whether such fragments or other breakdown products of hCG could be responsible for the above described anti-shock activity, a synthetic oligopeptide with the amino acid sequence of valine-leucine-proline-alanine-leucine-proline (VLPALP) was designed from the primary structure of  $\beta$ -hCG and tested it for its anti-shock activity in the same model. Similar to the c/u-hCG fractions, treatment of BALB/c mice with VLPALP (5-mg/kg BW) 2-h after LPS (8-mg/kg BW) injection completely inhibited mortality due to septic shock. The maximum sickness score observed in the VLPALP-treated mice was 2 (at 24 h) decreasing to score 1 (at 60 h), with complete recovery of the majority of the mice at 84 h (Figure 1B).

Because different pathophysiologic processes are involved in septic shock syndrome, this study determined whether the NMPF fractions and VLPALP have the ability to inhibit the septic shock in the later stages of the disease as well. Therefore, the 400–2000 Da NMPF fraction and the VLPALP treatment were postponed until 24-h after the septic shock induction by LPS. In these experiments the same dose of NMPF fraction (5 mg/kg) was also effective in reducing the mortality, although this delayed treatment was not equally as effective as treatment at 2-h after LPS injection. This delayed treatment manifested an 80% survival (Figure 1C); most of the mice demonstrated improved sickness scores already 24-h after the delayed NMPF treatment. In this set-up of delayed NMPF treatment, VLPALP was able to reduce the mortality to 50%, provided a threefold higher dose (15 mg/kg) was used as in the experiments employing VLPALP-treatment 2-h after LPS injection. In this experiment the sickness scores of the mice of all four experimental groups ranged from 3 to 5 at 24-h after LPS injection, in both NMPF fraction treated groups and the VLPALP-treated group, which decreased to a score 1 or 2 after 84 h. At 96-h after LPS injection all mice had completely recovered.

Overwhelming inflammatory responses are essential features of septic shock and play a central role in the pathogenesis of the tissue damage, multiple organ failure, and death induced by sepsis [9]. Cytokines, especially macrophage migration inhibitory factor (MIF), once released, induce the expression of an array of proinflammatory mediators by macrophages and activated T cells, which strongly promote the characteristic inflammatory response [7]. Therefore, 24-h after the shock induction, splenocytes were isolated from BALB/c mice that were treated 2-h after LPS (8 mg/kg BW) injection with NMPF fraction or VLPALP. These splenocytes were



**FIGURE 1** Effect of commercial hCG preparation (c-hCG), hCG from first trimester pregnancy urine (u-hCG), 400–2000 Da c-hCG (c-hCG fraction), 400–2000 Da u-hCG (u-hCG fraction), and VLPALP on the survival of BALB/c mice subjected to lipopolysaccharide-induced (LPS-induced) septic shock. (A) Survival percentages and sickness scores (at 84 h) of PBS (◇), c-hCG (□), u-hCG (○), c-hCG fraction (x), and u-hCG fraction (△) treated mice. Treatment was performed 2 h (arrow) after the LPS injection. (B) Survival percentages and

*in vitro* restimulated with LPS (10  $\mu$ g/ml) and, after 12 h of culture, the MIF levels were determined in the supernatants.

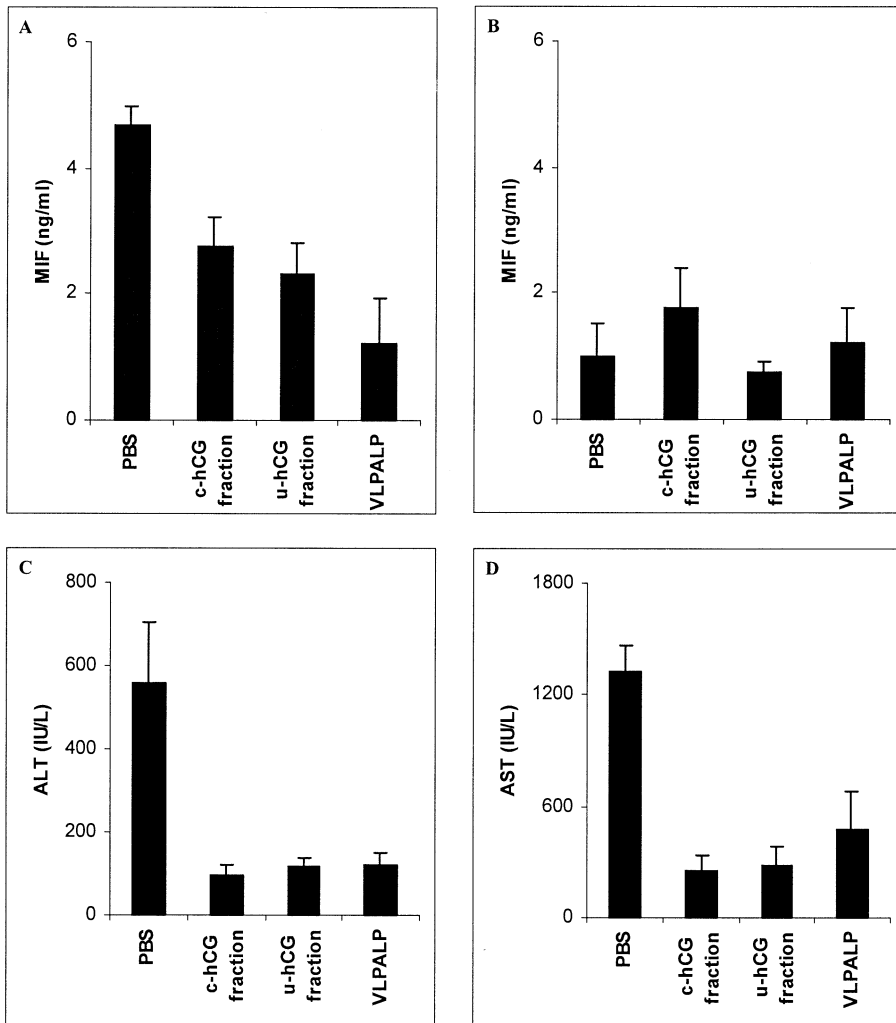
These experiments revealed that the *in vivo* treatment with either NMPF fraction or VLPALP significantly inhibited the MIF production when compared with PBS-treated mice subjected to high dose LPS administration (Figure 2A). This study did not find significant differences in MIF levels produced by LPS-stimulated splenocytes obtained from mice not previously injected with LPS, but only treated with NMPF fractions or VLPALP when compared with PBS (Figure 2B).

Consistent with the clinical improvement induced by NMPF fraction or VLPALP treatment, and the MIF release reducing effects of these treatments, NMPF fraction and VLPALP reduced the plasma levels of both ALT and AST liver enzymes in the LPS-injected mice (Figures 2C and 2D). The plasma levels of these enzymes in mice treated with NMPF fraction or the oligopeptide VLPALP only (and not previously with LPS) were not significantly different when compared with PBS-treated mice (data not shown).

Activation of cellular immune responses requires the expression of MHC molecules on cells of the innate (macrophages) and adaptive (B cells) immune systems [10]. In sepsis the upregulation of MHC class II (MHC II) molecules on macrophages and B cells is a hallmark of the overactivated immune system [11]. Therefore, we also examined the influence of VLPALP treatment on MHC II expression on F4/80<sup>+</sup> (macrophage marker) and B220<sup>+</sup> (B-cell marker) cells during septic shock. In these experiments the LPS-induced MHC II expression on both splenic cell populations was significantly decreased in mice treated with VLPALP (Figures 3A and 3B). Mice treated with VLPALP alone (and not previously with LPS) did not demonstrate a significant change in MHC II expression.

Remarkably, mice treated with LPS and VLPALP revealed an increased percentage of splenic NK1.1<sup>+</sup>CD3<sup>+</sup> cells (Figure 3C). Treatment of mice with LPS or VLPALP alone did not manifest such differences ( $p = 0.44$  and  $p = 0.15$ , respectively) when compared with PBS-treated mice (Figure 3C).

sickness scores (at 84 h) of PBS (◇), c-hCG fraction (x), u-hCG fraction (○), and VLPALP (△) treated mice. Treatment was performed 2 h (arrow) after the LPS injection. © Survival percentages and sickness scores (at 84 h) of PBS (◇), c-hCG fraction (x), u-hCG fraction (○), and VLPALP (△) treated mice. Treatment was performed 24 h (arrow) after the LPS injection. The results illustrated are from a single experiment and representative of at least three independent sets of experiments (each group:  $n = 10$ ).



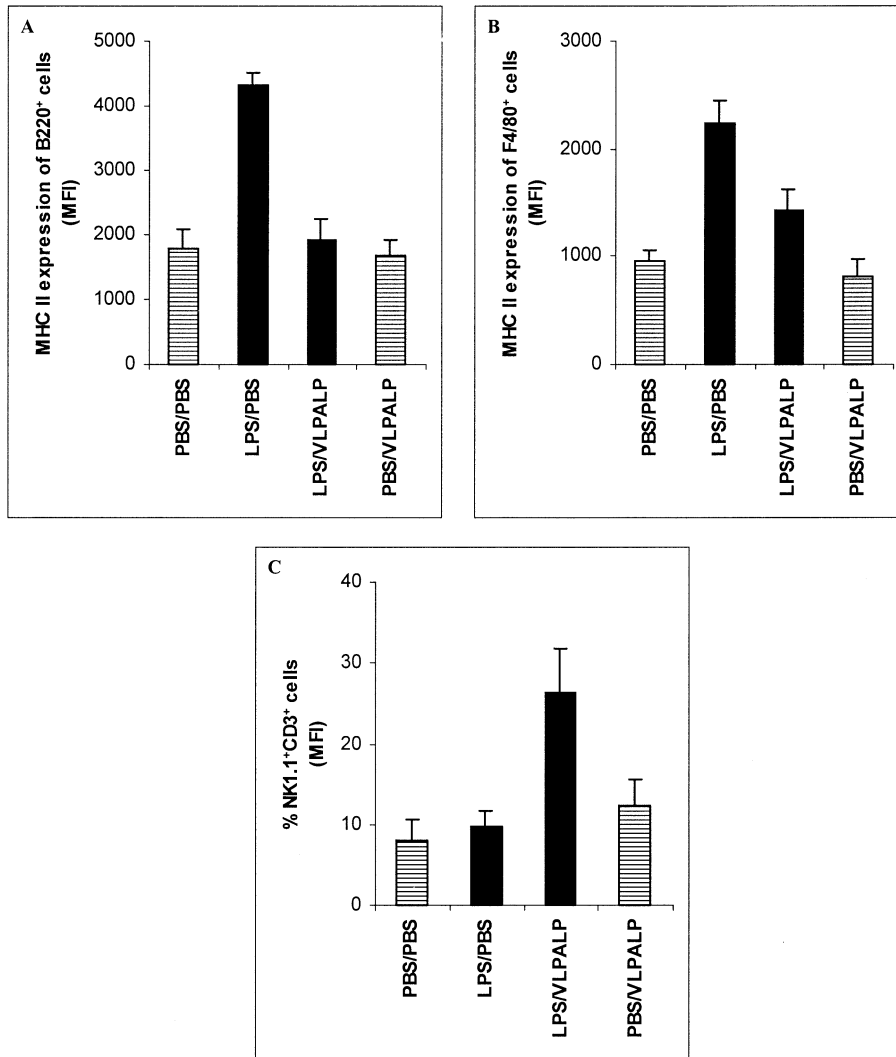
**FIGURE 2** Effect of 400–2000 Dalton (Da) NMPF fraction from commercial hCG (c-hCG fraction), urine hCG (u-hCG fraction), and VLPALP on the *in vitro* macrophage migration inhibitory factor (MIF) production by spleen cells and on the plasma aminotransferase levels of septic shock mice. (A) MIF levels in cultures of splenocytes isolated from lipopolysaccharide-injected (LPS-injected) mice 24-h after LPS injection. The mice were treated with PBS, c-hCG fraction, u-hCG fraction, or VLPALP 2-h after LPS injection. (B) MIF levels in cultures of splenocytes isolated from PBS-injected control mice 24-h after PBS injection. The mice were treated with either PBS, c-hCG fraction, u-hCG fraction, or VLPALP 2-h after PBS injection (*i.e.*, 22-h before harvest of the spleen cells). (C) Plasma alanine aminotransferase (ALT) and (D) plasma aspartate aminotransferase (AST) levels of mice 24-h after LPS injection and treated (at 2-h after LPS injection) with either PBS, c-hCG fraction, u-hCG fraction, or VLPALP. The results presented are from a single experiment and representative of at least three independent sets of experiments ( $n = 6$ ).

## DISCUSSION

In pregnancy the immune system is tightly regulated in order to maintain the coexistence of mother and fetus

[12]. Many women experience less disease severity or even remission of Th1-mediated autoimmune diseases during pregnancy. This epiphenomenon has been attributed at least in part to a shift from a “Th1” to a “Th2” state of the immune system during pregnancy [12], which leads investigators to view that pregnancy is associated with a state of immunosuppression. Considering that pregnant women, in general, do not manifest an increased susceptibility to infections, this supposed immunosuppression must be quite selective in order to provide for the required immunologic homeostasis between mother and fetus. In a previous article [4], it was reported that a 400–2000 Da NMPF fraction from pregnancy u- and c-hCG preparation inhibits Th1-mediated diabetes in NOD mice. The data presented in the present study reveals that the same NMPF fraction is also capable of inhibiting an acute inflammatory response, as is the case in high-dose LPS-induced septic shock in mice.

The pathophysiology of septic shock shares characteristics of systemic inflammatory response syndrome



**FIGURE 3** Effect of VLPALP (and PBS as control) on the expression of several surface markers on spleen cells of BALB/c mice intraperitoneally injected with lipopolysaccharide (LPS) or PBS as a control. VLPALP (and PBS as control) injection was done 2-h after the injection of LPS (and PBS as control). (A) Mean fluorescence intensity (MFI) of major histocompatibility complex II (MHC II) on B220<sup>+</sup> cells; (B) MFI of MHC II on F4/80<sup>+</sup> cells; and (C) percentage of NK1.1<sup>+</sup>CD3<sup>+</sup> cells. Solid bars represent LPS-injected mice and hatched bars represent PBS-injected mice. These results are from one experiment and are representative of at least three independent sets of experiments ( $n = 6$ ).

(SIRS) and multisystem organ failure (MOF) involving bacterial products, immunocompetent cells, soluble mediators, and cell–cell interactions between blood cells and endothelium [9, 12]. In the high-dose LPS model for septic shock in mice, hCG-derived NMPF, including the synthetic peptide VLPALP, were found to be capable of inhibiting the disease severity as well as mortality. This effect was not only apparent from the clinical symptoms,

but also from the inhibition of the *in vitro* MIF production and the inhibition of the plasma ALT levels and the cellular characteristics in the spleen. The LPS-induced increase of plasma ALT level is indicative of liver damage, which plays a crucial role in septic shock [6].

Several reports have suggested immunosuppressive and antiproliferative effects of hCG *in vitro* [13, 14]. In these experiments the main sources of the intact hormone were pregnancy u-, c-hCG preparations, and recombinant hCG. In view of the anti-diabetic [4] and the anti-shock effects of the 400–2000 Da NMPF fraction of hCG, and the anti-shock effect of the synthetic oligopeptide VLPALP, it is possible that the previously reported immunosuppressive and antiproliferative effects were due to hCG breakdown products.

It is of interest that the low molecular weight NMPF fractions and VLPALP were also able to decrease the clinical symptoms and mortality due to septic shock when treatment was given as late as 24-h after LPS

administration. Apparently the active component(s) interfered with the inflammatory process and the tissue damage underlying the clinical symptoms. Preliminary data also reveals the inhibition of exotoxin shock induced by toxic shock syndrome toxin (TSST-1) by NMPF (data not shown), which indicates that the anti-shock activities of NMPF are not limited to endotoxin shock. These properties fulfill essential requirements for any anti-sepsis agent, because in patients treatment is always started after the onset of the clinical symptoms of septic shock.

The downregulation of MHC II expression, both on macrophages and activated B cells, may not only be beneficial in controlling acute inflammatory responses, but it is likely also in chronic inflammatory immune reactions, particularly in autoimmunity in which MHC II expression plays a critical role in the presentation of autoantigens to T cells [10]. This suggests that VLPALP might also be effective in the prevention and possibly the suppression of autoimmunity. Figure 3 illustrates that NMPF peptide upregulated the percentage of NK1.1<sup>+</sup>CD3<sup>+</sup> cells. These so-called natural killer T cells (NK T cells) are a subpopulation of T cells that share some characteristics with NK cells [15, 16]. NK T cells are phenotypically, functionally, and developmentally heterogeneous. In general, three distinct subsets are known (CD4<sup>+</sup>, CD8<sup>+</sup>, and CD4<sup>-</sup>CD8<sup>-</sup>). These subpopulations are differentially distributed in a tissue-specific fashion [16]. We do not know yet to which subset(s) the VLPALP-induced NK T-cell subpopulation belongs. NK T cells are believed to be involved in immune responses ranging from suppression of autoimmunity to tumor rejection [16, 17].

Most interesting is the observation that the 400–2000 Da NMPF fractions and VLPALP did not affect the splenic MIF production, the plasma aminotransferase levels, the MHC II expression on splenic macrophages and B cells, and the proportion of NK1.1<sup>+</sup>CD3<sup>+</sup> double-positive cells in the spleen of otherwise untreated mice. This strongly suggests that NMPF do not have a general immunosuppressive effect, but correct disturbances of the immune homeostasis as occurs after high-dose LPS injection and during the development of diabetes in NOD mice [4]. This warrants studies on the therapeutic potential of VLPALP and related NMPF in inflammation, autoimmunity, transplantation, allergies, and related conditions, including pre-eclampsia and tumor rejection.

In summary, this study data reveals that a low molecular weight fraction from first trimester human pregnancy urine and the novel synthetic oligopeptide VLPALP, designed from  $\beta$ -hCG, can inhibit septic shock in BALB/c mice. Moreover, we report for the first time that hCG fragments can regulate the immune balance and, therefore, might be of potential value to correct dysregulation of the immune system in a variety of immune and inflammatory diseases.

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