

Soluble HLA-G influences the release of cytokines from allogeneic peripheral blood mononuclear cells in culture

T.Kanai, T.Fujii¹, S.Kozuma, T.Yamashita, A.Miki, A.Kikuchi and Y.Taketani

Department of Obstetrics and Gynecology, Faculty of Medicine, University of Tokyo, Tokyo, 113-8655 Japan

¹To whom correspondence should be addressed at: Department of Obstetrics and Gynecology, Faculty of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo, 113-8655 Japan. E-mail: fujii-tky@umin.ac.jp

Exquisitely regulated cytokine balance during early pregnancy is thought to be necessary for promoting survival of the fetal allograft. Our previous studies have demonstrated that membrane-bound human leukocyte antigen (mHLA-G) expressed on trophoblasts is one of the key factors in regulating cytokine balance by shifting the Th1/Th2 balance toward Th2 polarization, a favourable milieu for the maintenance of pregnancy. Given that trophoblasts secrete soluble HLA-G (sHLA-G), we examined its biological roles in comparison with mHLA-G. We cultured peripheral blood mononuclear cells (PBMC) with either the HLA-A and -B-deficient B lymphoblast cell line (721.221 cells) or the same cell line transfected with mHLA-G (721.221-G1 cells), in the presence or absence of recombinant sHLA-G. Cytokine concentrations in the culture media were determined by enzyme-linked immunosorbent assay. In contrast to mHLA-G protein, sHLA-G stimulated the release of tumour necrosis factor (TNF)- α and interferon (IFN)- γ , whereas it reduced the release of interleukin (IL)-3, regardless of the presence of the presence of a stimulatory effect of the mHLA-G-expressing cells. Although mHLA-G reduced the release of IL-4, sHLA-G did not have any effect. Conversely, sHLA-G stimulated the release of IL-10 whereas mHLA-G was without effect. These results suggest that sHLA-G regulates the release of cytokines from PBMC chiefly by counterbalancing mHLA-G, and thereby may play a role in maintaining pregnancy.

Key words: cytokines/membrane-bound HLA-G/pregnancy/soluble HLA-G/trophoblast

Introduction

The fetus is a semi-allograft in that half of its histocompatibility antigens come from the father. Pregnancy, therefore, seems to be an immunological paradox in view of the fact that the fetus can survive and develop for as long as 9 months despite the maternal immune system.

Recently, an increasing interest has been focused on the concept of immunotrophism, which suggests that maternal lymphocytes and macrophages actually recognize placental antigens and secrete certain cytokines to regulate the growth and differentiation of trophoblasts (Wegmann *et al.*, 1989).

In recent years, considerable evidence has accumulated to suggest the existence of functionally polarized responses of T cells as determined by the cytokines they produce (Abbas *et al.*, 1996; Romagnani, 1996). More specifically, T helper 1 (Th1) cells produce interferon (IFN)- γ and tumour necrosis factor (TNF)- β , both of which activate macrophages and are involved in cell-mediated immunity. By contrast, T helper 2 (Th2) cells produce interleukin (IL)-4, IL-5, IL-10 and IL-13, which are responsible for antibody production. Pregnancy is assumed to be a phenomenon in which Th1/Th2 balance is shifted to Th2 polarization, a putative mechanism favouring

the survival of the fetal allograft (Kovithavongs and Dossetor, 1978; Wegmann *et al.*, 1993; Szereday *et al.*, 1997; Reinhard *et al.*, 1998; Saito *et al.*, 1999). At present, however, little is known regarding factors modulating Th1/Th2 balance during pregnancy.

In 1987, a novel gene of non-classical class I human leukocyte antigen (HLA), HLA-G, was cloned (Geraghty *et al.*, 1987). This protein is quite different from classical HLA class I antigens, HLA-A, -B and -C, in that it is almost monomorphic and is expressed mainly in the placenta. The unique and restricted pattern of its expression, the extremely low allelic polymorphism, and the fact that the placenta expresses mainly HLA-G instead of the classical HLA class I antigens, HLA-A and -B, argue strongly for the specific role of HLA-G in the maternal-fetal immune relationship during pregnancy (Trowsdal *et al.*, 1980; Ellis *et al.*, 1990; Kovats *et al.*, 1990). In this regard, we have demonstrated that the recognition of membrane-bound HLA-G (mHLA-G) protein on the surface of target cells shifts the balance of Th1 and Th2 cytokines secreted by mononuclear cells from either peripheral blood or decidual tissues to Th2 polarization (Kanai *et al.*, 2001). Furthermore,

in the pre-eclamptic placenta, the expression of HLA-G protein on the trophoblasts is decreased (Hara *et al.*, 1996; Goldman-Wohl *et al.*, 2001), suggesting a physiological role for HLA-G in regulating placental development.

HLA-G is also secreted as a smaller sized and more acidic soluble protein (Kovats *et al.*, 1990). This soluble HLA-G (sHLA-G) protein is encoded by a messenger RNA containing intron 4 (Fujii *et al.*, 1994) and is secreted by trophoblasts. A recent study demonstrated the existence of HLA-G protein in amniotic fluid (Hamai *et al.*, 1999). However, thus far, specific functions for the sHLA-G protein are not well known.

In the expectation that the sHLA-G protein, like mHLA-G, could modulate cytokine release from lymphocytes and macrophages, we examined whether the presence of recombinant sHLA-G protein may alter the ability of peripheral blood mononuclear cells (PBMC) to release cytokines.

Materials and methods

Preparation of PBMC

PBMC were isolated from heparinized venous blood from seven healthy nulligravidous consenting women (28.6 ± 1.8 years old, mean \pm SD) by Ficoll-Paque centrifugation method and frozen in nitrogen until examined.

Cell lines

The HLA-A and -B-deficient B-lymphoblast cell line, 721.221 (.221), was obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). mHLA-G cDNA-transfected cells (.221-G1 cells) were produced by using the retroviral vector, pLNCX, in the laboratory of Dr Daniel E. Geraghty (Fred Hutchinson Cancer Research Center, Seattle, WA, USA), as previously described (Miller and Rosman, 1989; Ishitani and Geraghty, 1992; Miller *et al.*, 1993; Fujii *et al.*, 1994). pLNCX is an improved retroviral vector that does not contain the portions of viral coding regions responsible for the production of viral proteins conferring antigenicity and unpredictable properties on transfected cells (Miller and Rosman, 1989). As for the transfection method, we used an infection method with retrovirus particles, which was not detrimental to the transfected cells when compared with electroporation or calcium phosphate transfection.

Recombinant sHLA-G protein

Recombinant sHLA-G protein was kindly provided by Dr Daniel E. Geraghty (Fred Hutchinson Cancer Research Center). The cDNA that encoded extracellular $\alpha 1$ to $\alpha 3$ domains of HLA-G1 was transfected into *E. coli*. Recombinant sHLA-G protein was purified from the culture media of transformed *E. coli* and refolded with $\beta 2$ -microglobulin and nonamer peptide 'KGPPAALTL' (Lee *et al.*, 1995). The recombinant sHLA-G protein was detected by the enzyme-linked immunosorbent assay (ELISA) using the monoclonal antibody W6/32 (anti-HLA class I protein specific mouse monoclonal antibody, Dako, Copenhagen, Denmark) as a capture antibody and horseradish peroxidase-labelled anti- $\beta 2$ -microglobulin rabbit monoclonal antibody (Dako) as a detection antibody.

Co-culture of mononuclear cell with .221 cells or .221-G1 cells

Either .221 cells or .221-G1 cells (1×10^6 cells) that had been irradiated (100 Gy) before use were co-cultured with or without

PBMC (1×10^6) in 2 ml of Roswell Park Memorial Institute 1640 medium with 10% fetal calf serum, 1 mmol/l minimum essential medium-sodium pyruvate solution supplemented with indicated concentrations of recombinant sHLA-G protein for 48 h in 37°C, 5% CO₂.

The concentrations of TNF- α , IFN- γ , IL-3, IL-4 and IL-10 in the culture media were measured as described below.

Enzyme-linked immunosorbent assay

To measure the concentrations of cytokines, A'NALYZA™ immunoassay kits (all from Genzyme Techné, Minneapolis, MN, USA) for Human TNF- α , Human IFN- γ , Human IL-3, Human IL-4 and Human IL-10 were used.

Statistical analysis

Differences between the two groups were analysed for significance ($P < 0.05$) by Wilcoxon's test for paired samples.

Results

TNF- α , IFN- γ , IL-4, IL-10 and IL-3 were tested for but not detected in RPMI 1640 medium or in the culture medium of either .221 cells or .221-G1 cells alone, regardless of the presence or absence of sHLA-G protein.

TNF- α in culture media

TNF- α concentrations in PBMC culture media in the presence of .221 cells were significantly higher ($P < 0.05$, Wilcoxon's test) compared with those in the presence of .221-G1 cells (Figure 1A and B).

The addition of sHLA-G protein at 1000 ng/ml resulted in a significant increase in TNF- α concentrations in culture media of PBMC co-cultured with .221 cells ($P < 0.05$, Wilcoxon's test) compared with those without sHLA-G protein (Figure 1A). Likewise, sHLA-G protein at concentrations between 250 and 1000 ng/ml produced a significant and dose-dependent increase in TNF- α concentrations in culture media of PBMC co-cultured with .221-G1 cells ($P < 0.05$, Wilcoxon's test) (Figure 1B).

IFN- γ in culture media

IFN- γ concentrations in PBMC culture media in the presence of .221 cells were significantly higher ($P < 0.05$, Wilcoxon's test) compared with those in the presence of .221-G1 cells (Figure 2A and B).

The addition of sHLA-G protein at 500 and 1000 ng/ml resulted in a significant increase in IFN- γ concentrations in culture media of PBMC co-cultured with .221 cells ($P < 0.05$, Wilcoxon's test) compared with those without sHLA-G protein (Figure 2A). The addition of sHLA-G protein at 1000 ng/ml resulted in a significant increase in IFN- γ concentrations in culture media of PBMC co-cultured with .221-G1 cells ($P < 0.05$, Wilcoxon's test) (Figure 2B).

IL-4 in culture media

IL-4 concentrations in PBMC culture media in the presence of .221 cells were significantly lower ($P < 0.05$, Wilcoxon's test) compared with those in the presence of .221-G1 cells (Figure 3A and B).

The addition of sHLA-G protein at 250, 500 and

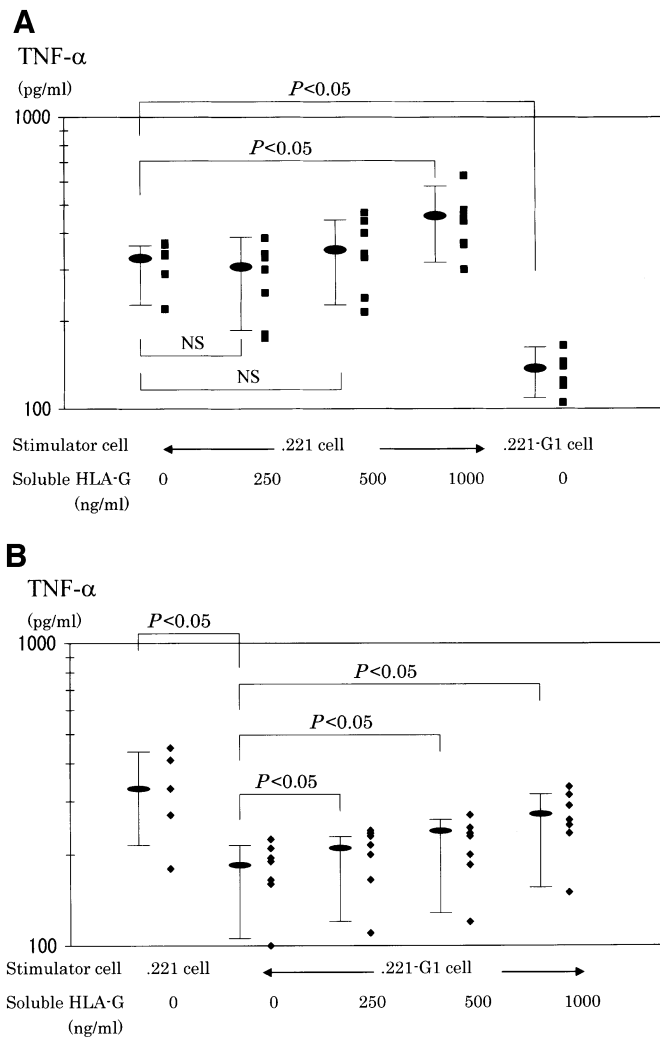


Figure 1. Tumour necrosis factor (TNF)- α concentrations in the culture media. Peripheral blood mononuclear cells (PBMC) from seven healthy nulligravidous women were cultured with either .221 cells (A) or .221-G1 cells (B) for 48 h in the medium supplemented with recombinant soluble human leukocyte antigen (sHLA)-G protein (250, 500, 1000 ng/ml). As controls, PBMC were cultured with either .221 cells or .221-G1 cells without sHLA-G protein (A, B). The concentrations of TNF- α in the culture media were measured. Each bar represents the median concentration and the 5 to 95 percentile range. $P < 0.05$: significantly different by Wilcoxon's test; NS = not significantly different.

1000 ng/ml did not affect the IL-4 concentrations in culture media of PBMC co-cultured with either .221 cells or .221-G1 cells (Figure 3A and B).

IL-10 in culture media

IL-10 concentrations in PBMC culture media in the presence of .221 cells exhibited no appreciable changes compared with those in the presence of .221-G1 cells (Figure 4A and B).

The addition of sHLA-G protein at 500 and 1000 ng/ml resulted in a significant increase in IL-10 concentrations in culture media of PBMC co-cultured with .221 cells ($P < 0.05$, Wilcoxon's test) (Figure 4A). sHLA-G protein at concentrations between 250 and 1000 ng/ml produced a significant dose-dependent increase in IL-10 concentrations in culture media

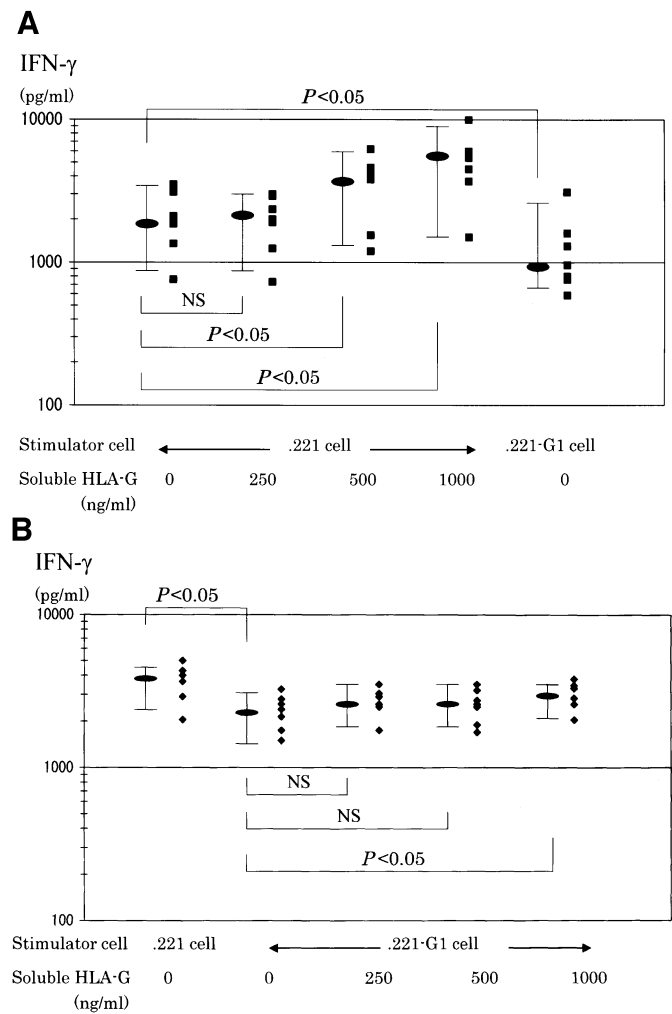


Figure 2. Interferon (IFN)- γ concentrations in the culture media. Peripheral blood mononuclear cells (PBMC) were cultured with either .221 cells (A) or .221-G1 cells (B) for 48 h in the medium supplemented with recombinant soluble human leukocyte antigen (sHLA)-G protein. As controls, PBMC were cultured with either .221 cells or .221-G1 cells without sHLA-G protein (A, B). The concentrations of IFN- γ in the culture media were measured. Each bar represents the median concentration and the 5 to 95 percentile range. $P < 0.05$: significantly different by Wilcoxon's test; NS = not significantly different.

of PBMC co-cultured with .221-G1 cells ($P < 0.05$, Wilcoxon's test) (Figure 4B).

IL-3 in culture media

IL-3 concentrations in PBMC culture media in the presence of .221 cells were significantly lower ($P < 0.05$, Wilcoxon's test) compared with those in the presence of .221-G1 cells (Figure 5A and B).

The addition of sHLA-G protein at concentrations between 250 and 1000 ng/ml resulted in a significant and dose-dependent decrease in IL-3 concentrations in culture media of PBMC co-cultured with .221 cells ($P < 0.05$, Wilcoxon's test) (Figure 5A). The addition of sHLA-G protein at 500 and 1000 ng/ml resulted in a significant decrease in IL-3 concentrations in culture media of PBMC co-cultured with .221-G1 cells ($P < 0.05$, Wilcoxon's test) (Figure 5B).

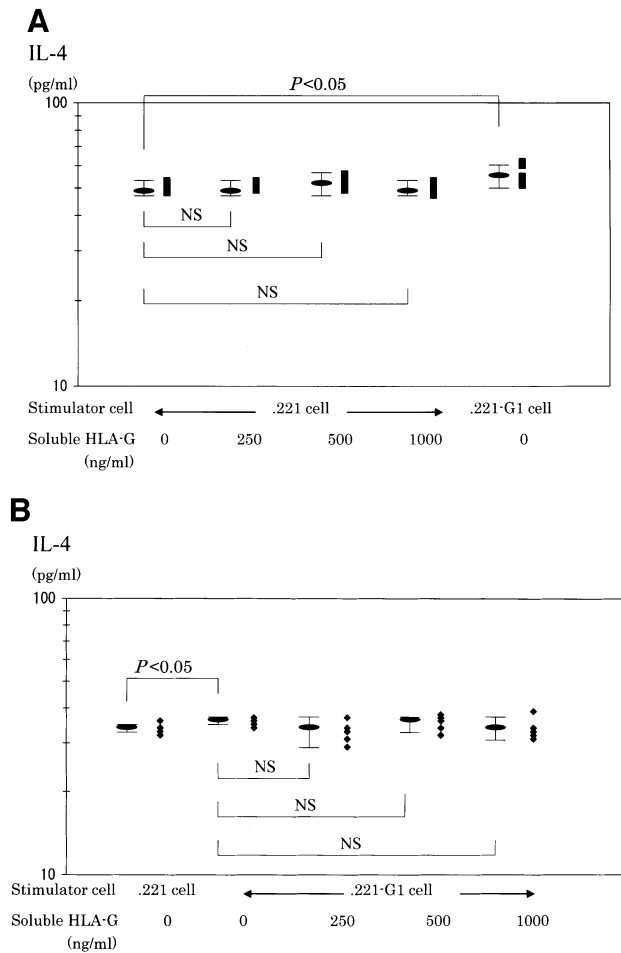


Figure 3. Interleukin (IL-4) concentrations in the culture media. Peripheral blood mononuclear cells (PBMC) were cultured with either .221 cells (**A**) or .221-G1 cells (**B**) for 48 h in the medium supplemented with recombinant soluble human leukocyte antigen (sHLA)-G protein. As controls, PBMC were cultured with either .221 cells or .221-G1 cells without sHLA-G protein (**A**, **B**). The concentrations of IL-4 in the culture media were measured. Each bar represents the median concentration and the 5 to 95 percentile range. $P < 0.05$: significantly different by Wilcoxon's test; NS = not significantly different.

Discussion

HLA-G has been reported to affect the functions of immune cells. For instance, HLA-G expression on .221 cells can protect these cells from the attack of natural killer (NK) effectors isolated from decidua or peripheral blood (Chumbley *et al.*, 1994). This effect might be mediated by the interaction of HLA-G molecules with the NKAT3 receptors (Munz *et al.*, 1997). HLA-G has also been shown to inhibit the transendothelial migration of NK cells (Dorling *et al.*, 2000). Regarding cytotoxic T lymphocytes (CTL), HLA-G can control the allo-CTL response (Kapasi *et al.*, 2000). HLA-G has also been reported to trigger apoptosis in activated CD8⁺ T lymphocytes.

Pregnancy is thought to be a phenomenon in which the Th1/Th2 cytokine balance is shifted to Th2 polarization. In this study, in agreement with previous reports from our laboratory (Hamai *et al.*, 1998; Kanai *et al.*, 2001), the amounts of TNF- α and IFN- γ (Th1 cytokines) released from PBMC were decreased when co-cultured with mHLA-G expressing cells,

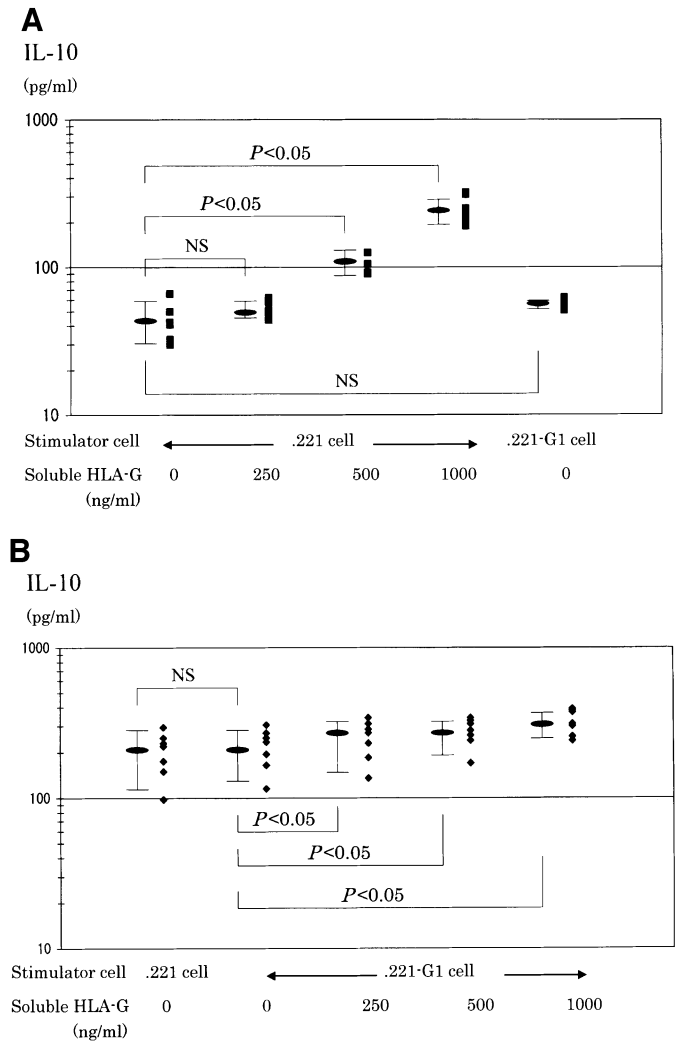


Figure 4. Interleukin (IL-10) concentrations in the culture media. Peripheral blood mononuclear cells (PBMC) were cultured with either .221 cells (**A**) or .221-G1 cells (**B**) for 48 h in the medium supplemented with recombinant soluble human leukocyte antigen (sHLA)-G protein. As controls, PBMC were cultured with either .221 cells or .221-G1 cells without sHLA-G protein (**A**, **B**). The concentrations of IL-10 in the culture media were measured. Each bar represents the median concentration and the 5 to 95 percentile range. $P < 0.05$: significantly different by Wilcoxon's test; NS = not significantly different.

namely .221-G1 cells, while the amounts of IL-4 and IL-3 (Th2 cytokines) from PBMC were increased, with IL-10 release being unchanged (Table I). Viewed in this light, although .221-G1 cells express a low level of HLA-E (Lee *et al.*, 1998) which might slightly affect the cytokine release from lymphocytes, mHLA-G seems to work in sustaining pregnancy, in part by enhancing the Th2 response while decreasing the Th1 response.

In this study, the addition of recombinant sHLA-G protein in the medium increased the amounts of TNF- α , IFN- γ and IL-10 released from PBMC regardless of the presence of mHLA-G-expressing cells. On the other hand, the amounts of IL-3 from PBMC were decreased, and IL-4 release from PBMC was unchanged (Table I). Thus, for TNF- α , IFN- γ and IL-3 release from PBMC, sHLA-G protein seems to antagonize

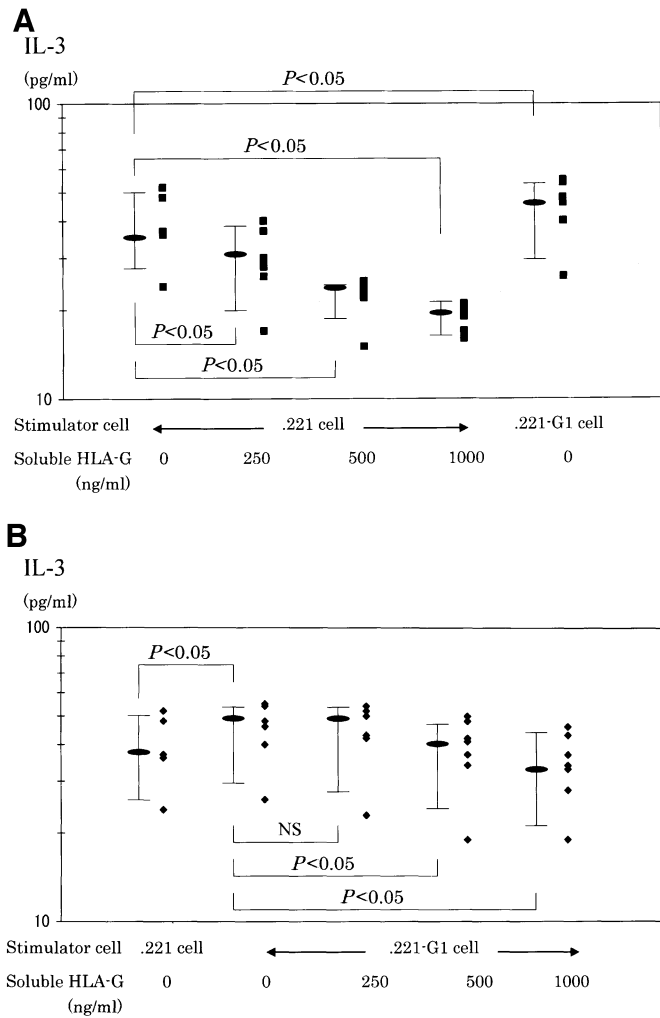


Figure 5. Interleukin (IL)-3 concentrations in the culture media. Peripheral blood mononuclear cells (PBMC) were cultured with either .221 cells (A) or .221-G1 cells (B) for 48 h in the medium supplemented with recombinant soluble human leukocyte antigen (sHLA)-G protein. As controls, PBMC were cultured with either .221 cells or .221-G1 cells without sHLA-G protein (A, B). The concentrations of IL-3 in the culture media were measured. Each bar represents the median concentration and the 5 to 95 percentile range. $P < 0.05$: significantly different by Wilcoxon's test; NS = not significantly different.

Table I. Effect of human leukocyte antigen (HLA)-G protein on the release of cytokines from peripheral blood mononuclear cells

	TNF- α	IFN- γ	IL-3	IL-4	IL-10
mHLA-G	↓	↓	↑	↑	↔
sHLA-G	↑	↑	↓	↔	↑

m = membrane-bound; s = soluble; TNF = tumour necrosis factor; IFN = interferon; IL = interleukin

the effects of mHLA-G. The physiological relevance of these observed effects of sHLA-G is not immediately clear. One tentative explanation could be that sHLA-G might fine-tune the growth of trophoblasts by antagonizing the growth-promoting effects of mHLA-G.

Natural killer receptors (NKR) expressed on lymphocytes might recognize extracellular regions of HLA class I molecules

together with their binding peptide. If this is the case, HLA class I molecules might modulate the ability of lymphocytes to release cytokines. Recombinant sHLA-G molecule is regarded as the extracellular region of mHLA-G molecule. Thus, a possible mechanism for the antagonistic effects of sHLA-G molecules toward mHLA-G molecules could be that the former competes with the latter for binding to NKR on lymphocytes.

The stimulatory effect of sHLA-G on IL-10 release is not understood. IL-10 belongs to Th2 cytokines and enhances the antibody production, thus favouring the maintenance of pregnancy. In view of the fact that mHLA-G could not stimulate the release of IL-10, an intriguing possibility is that sHLA-G might supplant mHLA-G in releasing IL-10.

The authentic sHLA-G protein secreted by trophoblasts has an additional 20 amino acids at the C terminus of the molecule when compared with recombinant sHLA-G used in this study. This leaves the question of whether the observed effects of sHLA-G reflect the biological roles of authentic sHLA-G. However, given that lymphocytes recognize membrane-bound HLA class I molecules by binding to its extracellular $\alpha 1$, $\alpha 2$ and $\alpha 3$ domains, it is therefore reasonable to think that lymphocytes likewise recognize authentic sHLA-G molecules by binding to its $\alpha 1$, $\alpha 2$ and $\alpha 3$ domains. Since the recombinant sHLA-G consisted of $\alpha 1$, $\alpha 2$ and $\alpha 3$ domains, it seems logical to assume that effects of recombinant sHLA-G mimic those of its authentic counterpart.

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