

Recognition of Nonclassical HLA Class I Antigens by $\gamma\delta$ T Cells During Pregnancy¹

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The healthy trophoblast does not express classical HLA-A and HLA-B products; therefore, an MHC-restricted recognition of trophoblast-presented Ags is unlikely. In the decidua and also in peripheral blood of healthy pregnant women, $\gamma\delta$ T cells significantly increase in number. We investigated the possible role of $\gamma\delta$ T cells in recognition of trophoblast-presented Ags. PBL and isolated $\gamma\delta$ T cells from healthy pregnant women as well as from those at risk for premature pregnancy termination were conjugated to choriocarcinoma cells (JAR) transfected with nonclassical HLA Ags (HLA-E, HLA-G). To investigate the involvement of killer-inhibitory/killer-activatory receptors in trophoblast recognition, we tested the effect of CD94 block on cytotoxic activity of V δ 2⁺ enriched $\gamma\delta$ T cells to HLA-E- and/or HLA-G-transfected targets. Lymphocytes from healthy pregnant women preferentially recognized HLA⁻ choriocarcinoma cells, whereas those from pathologically pregnant patients did not discriminate between HLA⁺ and HLA⁻ cells. Normal pregnancy V δ 2⁺ T cells conjugated at a significantly increased rate to HLA-E transfectants, whereas V δ 2⁺ lymphocytes from pathologically pregnant women did not show a difference between those and HLA⁻ cells. Blocking of the CD94 molecule of V δ 2⁺ lymphocytes from healthy pregnant women resulted in an increased cytotoxic activity to HLA-E-transfected target cells. These data indicate that V δ 2⁺ lymphocytes of healthy pregnant women recognize HLA-E on the trophoblast, whereas V δ 1 cells react with other than HLA Ags. In contrast to V δ 2⁺ lymphocytes from healthy pregnant women, those from women with pathological pregnancies do not recognize HLA-E via their killer-inhibitory receptors and this might account for their high cytotoxic activity. *The Journal of Immunology*, 2002, 168: 2683–2688.

The healthy trophoblast does not express the common pattern of HLA class I Ags. HLA-G and a small amount of HLA-C are the only class I Ags that have been detected on extravillous cytotrophoblast cells (1–5). HLA-G, similarly to HLA-E and HLA-F, belongs to the group of nonclassical class Ib MHC Ags. These show a limited polymorphism, low cell surface expression, and more restricted tissue distribution than classical class Ia MHC Ags (6, 7). HLA-E transcripts have been demonstrated widely in trophoblast cell subpopulations (8), and King et al. (9) have recently demonstrated that trophoblast cells express HLA-E on their cell surface in addition to the previously reported expression of HLA-G and HLA-C.

Since the trophoblast does not express classical HLA-A and -B class I products, $\alpha\beta$ TCR-mediated recognition of fetal Ag is unlikely. $\gamma\delta$ T cells recognize a distinct group of ligands with a smaller receptor repertoire than $\alpha\beta$ T cells, e.g., various naturally occurring and synthetic nonpeptide phosphoantigens (10–12), without classical MHC restriction (13). In the decidua, $\gamma\delta$ TCR-positive cells significantly increase in number (14). The number of $\gamma\delta$ T cells in the uterus is higher in allogeneic than in syngeneic pregnancy, and the expression of the $\gamma\delta$ TCR in the pregnant

uterus has been shown to be hormonally controlled (15). Therefore, it seems likely that this population might play a role in recognition of fetal Ags. Decidual $\gamma\delta$ T cells preferentially use the V δ 1 (16), whereas in peripheral blood of healthy adult nonpregnant donors the main $\gamma\delta$ population uses the V γ 9/V δ 2 chain combination (17, 18). In peripheral blood of healthy pregnant women, we have demonstrated a significantly increased ratio of activated $\gamma\delta$ TCR⁺ cells compared with that of pregnant recurrent aborters or nonpregnant individuals (19), and among these, the ratio of the noncytotoxic V γ 4/V δ 1 subpopulation to the V γ 9/V δ 2 subpopulation was 8 times higher than in PBL of nonpregnant individuals.

$\gamma\delta$ T cells express killer-inhibitory receptors (KIR)³ that recognize MHC class I molecules (20–23). Nonclassical MHC class I molecules might thus protect the trophoblast from NK-mediated lysis (24, 25).

Our earlier data revealed the presence of two functionally distinct subpopulations of $\gamma\delta$ T cells in normal pregnancy; the mainly cytotoxic V γ 9/V δ 2 subpopulation and the V γ 4/V δ 1 subpopulation which acts in a Th2-like manner (26). The present study was aimed at investigating the possible role of HLA-G or HLA-E in the activation or inhibition of these two distinct $\gamma\delta$ TCR⁺ subpopulations.

Materials and Methods

Patients

PBL from 109 healthy pregnant women in the second trimester and at the beginning of the third trimester were isolated from heparinized venous blood on Ficoll-Paque gradient (Pharmacia Biotech, Uppsala, Sweden). Eighteen of these were idiopathic recurrent aborters or showed clinical symptoms (bleeding or regular uterine contractions) of threatened premature pregnancy termination. Ninety-one women had a normal pregnancy.

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³ Abbreviations used in this paper: KIR, killer-inhibitory receptor; TBC, target-binding cells.

Monoclonal Abs

The following mAbs were used: mouse anti-human TCR V δ 1 (clone TS8.2¹) and mouse anti-human TCR V γ 4 (V γ 1.4 clone 4A11), both from Serotec (Oxford, U.K.); mouse anti-human TCR V δ 2 (clone 15D¹), V γ 9 (clone 7A5¹), pan TCR $\gamma\delta$ (TCR δ constant region clone 5A6.E9¹), T-cell Diagnostic (Woburn, MA); mouse anti-human CD94 (clone HP-3B1), Serotec; mouse anti-human NKG2A (clone Z199), Immunotech (Marseille, France); mouse IgG2a κ and mouse IgG1 isotype controls, Sigma (St. Louis, MO); mouse anti-human TCR V δ 2-FITC (clone B6), anti-pan $\gamma\delta$ TCR-APC (clone B1.1), anti-CD 69-APC (clone FN50), anti-CD3-APC (clone HIT 3a), anti-IL-10-APC (clone JES3-19F1), anti-IFN- γ -APC (clone B27), BD PharMingen (San Diego, CA); anti-mouse biotin (Amersham-Pharmacia Biotech), Piscataway, NJ).

MiniMACS $\gamma\delta$ T cell separation

V δ 2 T cells were separated using MiniMACS immunomagnetic beads, following the instructions of the manufacturer (Miltenyi Biotec, Bergisch Gladbach, Germany). Briefly, cells were washed with PBS and resuspended at a cell count of 1×10^7 /ml in PBS containing 0.5% BSA (Sigma) and 2 mM EDTA. Ten million cells were incubated for 10 min at 4°C with 10 μ g anti-V δ 2 mAb. After incubation, lymphocytes were washed twice and resuspended in 80 μ l buffer, and 20 μ l goat anti-mouse IgG microbeads (Miltenyi Biotec) were added. Cells were incubated for 15 min at 4°C and then washed. Pelleted cells were resuspended in 500 μ l buffer and applied to a MiniMACS column fitted to a magnet. The column was washed six times and then removed from the magnetic separator. The magnetic adherent cells were flushed out of the column into a fresh tube using a plunger included in the MiniMACS kit. Magnetic adherent cells were washed, and 10^5 cells were incubated with an appropriate dilution of FITC-conjugated anti-V δ 2 mAb for 30 min. During incubation, samples were protected from light. After washing, the cells were resuspended in 250 μ l FACS buffer containing 1% paraformaldehyde and stored at 4°C in the dark to be processed for FACS analysis the following day. The purity of the resulting cell suspension was checked by FACS analysis. Usually a 75–80% enrichment of V δ 2 TCR-positive cells was obtained.

Cell lines and transfectants

The following cell lines were used as target cells: HLA class I⁺ human choriocarcinoma cell line JAR; its HLA-G-transfected variant JAR-G1 (which possibly coexpresses HLA-E on its surface); JAR transfected with only HLA-G (JAR-G1m); JAR transfected with only HLA-E (JAR-E); and furthermore a human erythroleukemia cell line, K562. All cells were cultured in RPMI 1640 with 10% FCS and 300 μ g/ml of geneticin (Life Technologies, Gaithersburg, MD). The medium of JAR and JAR-G cells contained 0.1% pyruvate. Ag expression on the transfectants was regularly checked by staining with W6/32 Ab.

The transfectants were donated by P. Le Bouteiller (Institut National de Science et Recherche Scientifique, Unité 395, Purpan Hospital, Toulouse, France): JAR-HLA-G1 (JAR-G) was produced by transfection of the full length HLA-G1 cDNA under the control of human CMV promoter as described by Mallet et al. (27). JAR-HLA-G1m (JAR-G1m) was produced by transfection of pCDNA3/HLA-G1m plasmid, a gift of Dr. M. Lopez-Botet (Department of Immunology, University Hospital la Princesa, Madrid, Spain), in which HLA-G leader sequence was modified as follows: the methionyl residue at position 2 was mutated in threonine; therefore, it could not provide a good signal peptide for the expression of HLA-E (28). JAR-HLA-E (JAR-E) was transfected with HLA-E containing a cd3.14 cosmid encoding HLA-E, a gift of M. Ulbrecht (Institute of Anthropology and Human Genetics, Munich, Germany) (29), in which the HLA-E leader sequence was replaced by that of HLA-A2, providing good peptides for the expression of HLA-E, as described by Lee et al. (30).

Conjugation of PBL or purified V δ 2 TCR⁺ cells to target cells

Lymphocytes and target cells (ratio, 10:1) were centrifuged at 500 rpm for 5 min, allowing close cell to cell contact, and further incubated at 37°C in 5% CO₂ for 10 min. After conjugation, the cells were cytocentrifuged on glass microscope slides. The slides were dried at room temperature, fixed in cold acetone for 5 min, and labeled with anti-TCR Abs. The percentage of the different $\gamma\delta$ subpopulations bound to target cells was determined by microscopic counting of 300 lymphocytes at high power magnification by a blinded observer.

Immunocytochemistry

Endogenous peroxidase activity of the cells was blocked with 1% H₂O₂. Nonspecific protein binding sites were blocked by 1% BSA. All incubations were conducted at room temperature in a humid chamber. The pri-

mary Abs ($\gamma\delta$ mAbs) were diluted 1/50 in Tris-buffered saline containing 0.5% BSA. After 1 h of incubation with the primary Ab, 1/100 diluted HRP labeled anti-mouse Ig was added as a secondary Ab for 30 min. The slides were washed three times in Tris-buffered saline, and the reaction was developed by diaminobenzidine and intensified with silver staining. Control slides without the primary Abs or with isotype Ig were included.

Assessment of conjugation formation by flow cytometry

A total of 1×10^6 target cells were labeled with the green membrane dye PKH-67 (Sigma-Aldrich, Schnellendorf, Germany), following the instructions of the manufacturer. A total of 1×10^6 PBL were stained for CD3, pan TCR $\gamma\delta$, and V δ 2 TCR. Lymphocytes and target cells (ratio, 1:1) were centrifuged at 500 rpm for 5 min, allowing close cell to cell contact, further incubated at 37°C in 5% CO₂ for 10 min, and analyzed for double-stained cells by flow cytometry.

Four-hour single-cell cytotoxicity assay for NK activity

We used the assay originally described by Grimm and Bonavida (31). One hundred microliters of lymphocytes and the same amount of K562 target cells (2×10^6 cells/ml each) were centrifuged at 500 rpm for 5 min and incubated at 37°C in 5% CO₂ for 10 min. The pellets were then resuspended, and 200 μ l of 1% agarose (Serva, Heidelberg, Germany) in RPMI 1640 were added to the mixture. Two hundred microliters of this suspension was spread over microscope glass slides previously coated with 1% agar. Target cells alone were used to detect spontaneous lysis. The gel was allowed to solidify and submerged in RPMI 1640. The slides were incubated for 4 h at 37°C in 5% CO₂. The gels were then stained with 0.5% trypan blue for 1 min. After 2-min washes with PBS, the gels were fixed in 2% formaldehyde for 5 min and desalted in distilled water. The slides were read using a light microscope with $\times 400$ magnification. The proportion of lymphocytes bound to the target cells was expressed as a percentage of total lymphocyte population by counting 100 lymphocytes. Results are expressed as a percentage of target binding cells (TBC). Dead conjugates were scored as a percentage of the total number of conjugates by counting 50 conjugates, and results are expressed as a percentage of dead conjugates (cytotoxic TBC%). The percentage of NK cells was calculated according to the formula NK% = (TBC% \times cytotoxic TBC%)/100. All results for cytotoxic TBC% were corrected for the proportion of target cells that died spontaneously in control plates.

Treatment of enriched V δ 2 TCR⁺ cells

Magnetic bead-separated normal pregnancy V δ 2 TCR⁺ cells were incubated with anti-CD94 mAb or anti-NKG2A at a concentration of 10 μ g/ml for 1×10^6 V δ 2 TCR⁺ lymphocytes for 30 min. After incubation, cells were washed in medium. In all experiments, untreated samples and isotype controls were used. To exclude the possibility that anti-TCR treatment would result in activation, we tested the effect of the treatment on expression of the activation marker CD69 by flow cytometry. The rate of CD69-expressing cells in the anti-TCR-treated population did not significantly differ from that in untreated cells (not shown).

Determination of cytokine expression in anti-NKG2A-treated cells

A total of 1×10^6 lymphocytes were incubated with NKG2A mAb at a concentration of 10 μ g/ 10^6 PBL/ml. In treated and untreated samples, IL-10- and IFN- γ -expressing cells were detected by flow cytometry.

Determination of surface expression of the activation marker CD69

One million lymphocytes were incubated with anti-V δ 2 TCR mAb at a concentration of 10 μ g/ 10^6 PBL/ml for 4 h. Lymphocytes activated for 4 h with ionomycin at a concentration of 1 μ g/ 10^6 PBL/ml or PMA at a concentration of 0.25 μ g/ 10^6 PBL/ml served as a positive control. After incubation, the cells were stained for the activation marker anti-CD69-APC mAb and analyzed by flow cytometric analysis. No lymphocyte activation was seen after treatment with either anti-TCR, or anti-KIR Abs at the concentrations used in this study.

Statistics

The two-tailed Student *t* test and the paired *t* test were used for statistical evaluation of the data. Differences were considered significant if the *p* value was ≤ 0.05 .

Results

Conjugation of peripheral lymphocytes from pregnant women to JAR and JAR-G

For recognition of cell surface-associated Ags, lymphocytes must make close contact with the APC. After adhesion (recognition of different cell surface molecules, markers, or receptors), the lymphocytes will be functionally activated or inhibited. To identify the trophoblast-associated Ag that is recognized, we tested the conjugation capacity of PBL from healthy pregnant women to JAR, JAR-G, and K562 cells.

The conjugation rate of normal pregnancy lymphocytes to HLA⁻ JAR cells was significantly higher than that to HLA-G-transfected JAR ($p < 0.02$) or to K562 cells ($p < 0.001$). Lymphocytes from patients with pathological pregnancies conjugated at a similar rate to JAR or JAR-G cells. Moreover, the rate of lymphocytes conjugated to JAR cells was significantly lower ($p < 0.001$) than that of lymphocytes derived from healthy pregnant women (Fig. 1).

Conjugation of $\gamma\delta$ TCR⁺ peripheral lymphocytes from healthy pregnant women to choriocarcinoma cell lines

$\gamma\delta$ T cells were identified by reaction with specific Abs. The ratio of $\gamma\delta$ T cells that conjugated with JAR-G cells was significantly higher ($p < 0.01$) than of those bound to HLA⁻ JAR cells (Fig. 2). $\gamma\delta$ TCR⁺ lymphocytes from healthy pregnant women conjugated to JAR cells ($p < 0.001$) at a significantly lower rate than PBL did. There was no difference between the PBL and $\gamma\delta$ T cells in conjugation to JAR-G cells (Fig. 2).

Earlier, we identified two major, functionally distinct subpopulations of $\gamma\delta$ TCR⁺ cells in peripheral blood during normal pregnancy: the mainly cytotoxic V γ 9/V δ 2 subset and the non-cytotoxic V γ 4/V δ 1 (V γ 1.4/V δ 1) subset (26). Normal pregnancy is characterized by an increased ratio of V γ 4/V δ 1 to V γ 9/V δ 2 positive cells (26).

In the present study, normal pregnancy $\gamma\delta$ T lymphocytes expressing the V δ 2 chain conjugated at a significantly higher ($p < 0.05$) rate to JAR-G than to JAR cells, whereas V δ 2 lymphocytes from women with pathological pregnancies showed no preference (Fig. 3). Lymphocytes expressing either the V γ 9 or V γ 4 and V δ 1 chains conjugated at the same rate to JAR and JAR-G cells (data not shown).

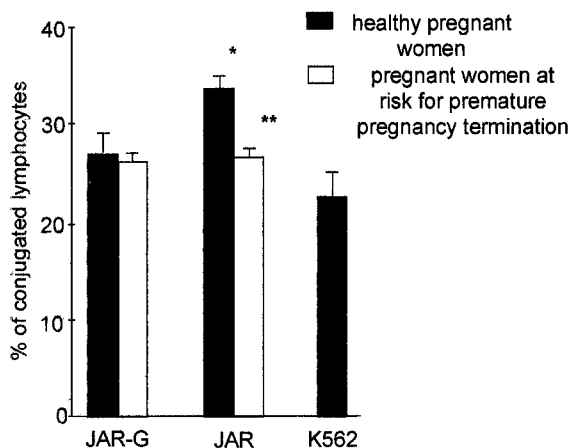


FIGURE 1. Conjugation of PBL from pregnant women to JAR, JAR-G, and K562 cells. *, Significantly different from percent conjugation of normal pregnancy lymphocytes to JAR-G ($p < 0.02$) or to K562 ($p < 0.001$). **, Significantly different from percent conjugation of normal pregnancy lymphocytes to JAR cells. Bars, mean \pm SEM of 10 determinations.

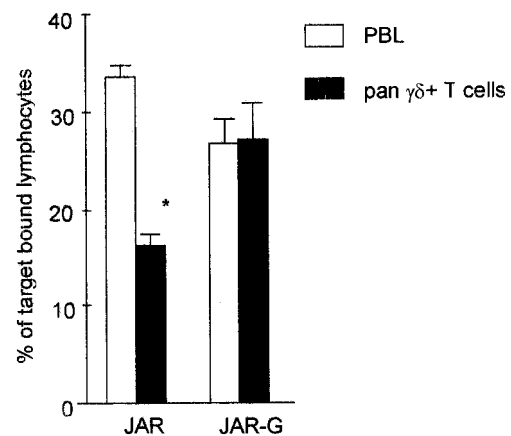


FIGURE 2. Peripheral $\gamma\delta$ TCR⁺ lymphocytes from healthy pregnant women conjugate preferentially to JAR-G cells. *, $p < 0.01$. Bars, mean \pm SEM of nine determinations.

To elucidate whether HLA-G or HLA-E was responsible for the altered conjugation capacity to transfected and untransfected choriocarcinoma cell lines, we tested the conjugation of the V δ 2⁺ population to two additional transfectants, JAR-G1m (HLA-G⁺/HLA-E⁻) and JAR-E (HLA-G⁻/HLA-E⁺). Our results showed a significantly increased ($p < 0.05$) conjugation to JAR-E, whereas no difference could be detected in the conjugation capacity to JAR-G1m compared with untransfected JAR (Fig. 4). These results suggest that HLA-E, rather than HLA-G, were responsible for the increased binding capacity of V δ 2⁺ cells to JAR-G. Flow cytometric analysis of conjugate formation gave similar results. V δ 2 cells from healthy pregnant women conjugated at a higher rate to JAR-G cells (expressing HLA-G and HLA-E) than to HLA⁻ JAR cells, or to HLA-G⁺ JAR-G1m cells (Fig. 5).

Blocking of the CD94 or NKG2A molecules reduces conjugation and increases IFN- γ production as well as cytotoxic activity of normal pregnancy V δ 2⁺ T cells to JAR-G

V γ 9V δ 2 T cells express the inhibitory CD94/NKG2A receptor for HLA class I molecules. Ligand binding of the CD94 receptor induces an inhibitory signal in peripheral V δ 2 T cells (21).

To determine whether the CD94/NKG2A complex of healthy pregnancy V γ 9/V δ 2 TCR⁺ cells is involved in recognition of JAR-G and JAR-E, V δ 2⁺-enriched cells were treated with a blocking concentration (10 μ g/ml/10⁶ V δ 2⁺ T cells) of anti-CD94

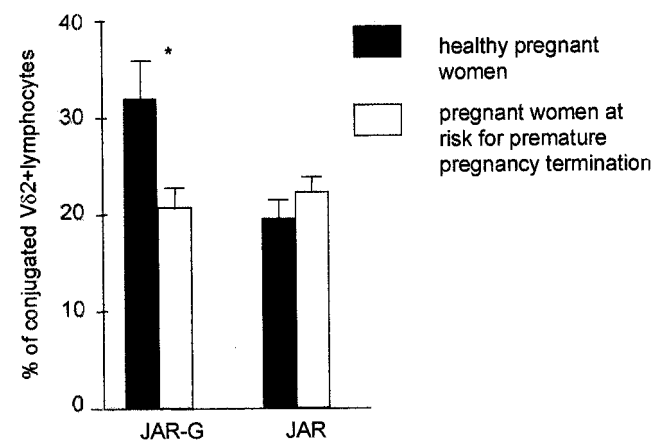


FIGURE 3. V δ 2⁺ lymphocytes from healthy pregnant women conjugate preferentially to JAR-G cells. *, $p < 0.05$. Bars, mean \pm SEM of 13 determinations.

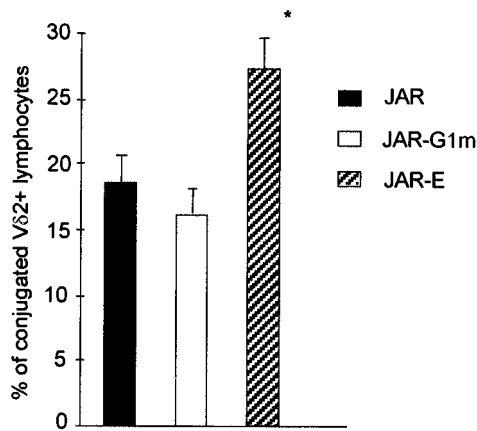


FIGURE 4. V δ 2⁺ cells of healthy pregnant women recognize HLA-E. *, $p < 0.05$. Bars, mean \pm SEM of seven determinations.

mAb. The blocking concentration of the Ab had been determined previously by testing the expression of the activation marker CD69 after treatment of the lymphocytes with different concentrations of the Ab.

Blocking of the CD94 molecule significantly reduced ($p < 0.001$) the conjugation capacity of V δ 2-enriched lymphocytes to JAR-G cells, whereas the same treatment did not alter the conjugation rate to JAR cells (Fig. 6). The effect of CD94 block on cytotoxic activity was tested in a 4-h single-cell cytotoxicity assay. The cytotoxicity of normal pregnancy V δ 2⁺ lymphocytes to JAR-E target cells was significantly lower ($p < 0.01$) than that to nontransfected choriocarcinoma JAR cells or JAR-G1m, transfected with HLA-G (Fig. 7). Anti-CD94 treatment significantly ($p < 0.02$) increased the lytic capacity of the lymphocytes when JAR-E but not JAR-G1m was used as a target (Fig. 7). Similar results were obtained with anti-NKG2A Ab.

We tested the effect of anti-NKG2A treatment on IFN- γ and IL-10 expression of the lymphocytes. Following the treatment, the ratio of IFN- γ -expressing cells significantly ($p < 0.05$) increased (Fig. 8), whereas there was no significant alteration in the percentage of IL-10⁺ cells (data not shown).

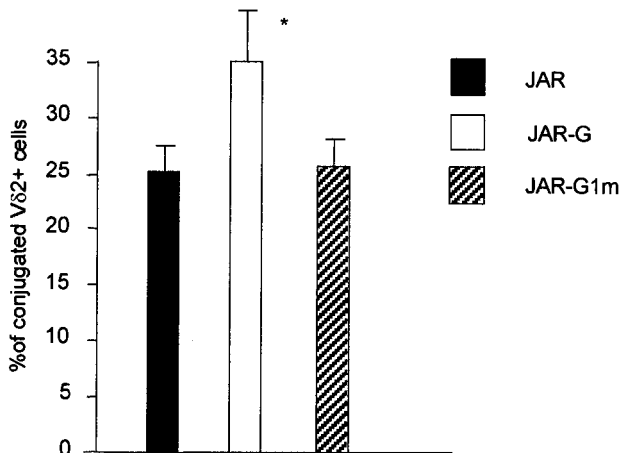


FIGURE 5. Conjugation of V δ 2⁺ lymphocytes of healthy pregnant women to trophoblast cells (flow cytometric analysis). *, $p < 0.05$. Bars, mean \pm SEM of 18 determinations.

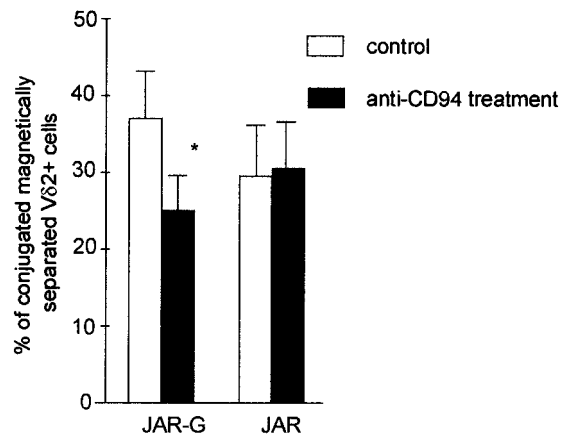


FIGURE 6. Effect of treatment with blocking concentration of anti-CD94 mAb of V δ 2⁺-enriched lymphocytes on conjugation to JAR and JAR-G cells. *, Blocking the CD94 molecule on V δ 2⁺-enriched lymphocytes significantly reduced the rate of conjugation to JAR-G targets ($p < 0.001$). Bars, mean \pm SEM of 10 determinations.

Expression of CD94/NKG2A complex on peripheral lymphocytes from healthy pregnant women and that from women at risk for premature pregnancy termination

There was no difference in CD94 (Fig. 9) and NKG2A (data not shown) expression between lymphocytes of healthy and pathologically pregnant women. This suggests that the decreased recognition capacity of pathologically pregnant V δ 2⁺ cells to JAR-G is not due to the lack or decreased number of surface CD94/NKG2A complexes.

Discussion

Earlier data from our laboratory revealed an increased rate of activated $\gamma\delta$ T cells in peripheral blood of healthy pregnant women (19). These cells similarly to decidual $\gamma\delta$ T cells (16) preferentially use the V δ 1 chain (26). Wen et al. (32) have shown that in contrast to $\alpha\beta$ T cells, once established, the growth of $\gamma\delta$ T cells does not

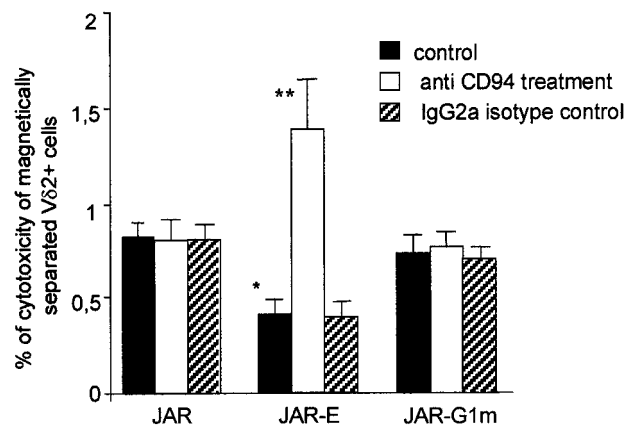


FIGURE 7. Effect of anti-CD94 treatment on cytotoxic activity of V δ 2⁺-enriched cells to JAR, JAR-G, JAR-G1m, and JAR-E targets. *, Cytotoxic activity of normal pregnancy V δ 2⁺ lymphocytes against JAR-E cells is significantly lower than that against JAR cells ($p < 0.01$). **, Cytotoxic activity of normal pregnancy V δ 2⁺ lymphocytes against JAR-E cells after blocking treatment with anti-CD94 mAb is significantly higher than that of the untreated control group ($p < 0.02$). Bars, mean \pm SEM of six determinations.

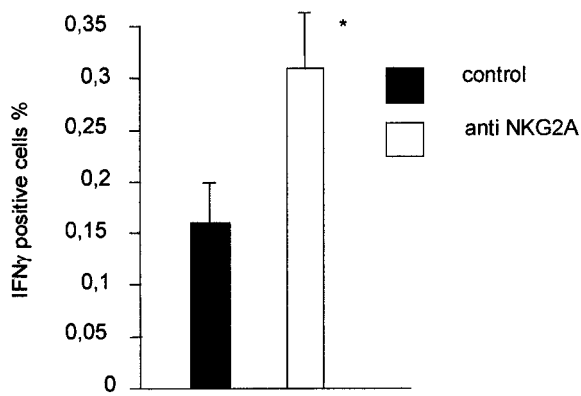


FIGURE 8. Effect of NKG2A treatment on IFN- γ expression of lymphocytes. *, $p < 0.05$. Bars, mean \pm SEM of five determinations.

require the sustained presence of APC. Findings by Mincheva-Nilsson et al. (16) suggest that the human early decidua is a transient site for extrathymic maturation. Taken together, these data allow the hypothesis that these lymphocytes are of decidual origin, which, after activation by trophoblast-presented fetal Ags, appear in the peripheral circulation. Alternatively, circulating shed fetal Ags might activate these cells.

Heyborne et al. (33) have shown that murine decidual V γ 1⁺ cells recognize a conserved mammalian molecule on the trophoblast. In our hands, V δ 2 cells of healthy pregnant women recognized HLA-E, whereas V δ 1⁺ cells did not preferentially conjugate to trophoblast cells expressing nonclassical HLA Ags. The latter population possibly recognizes other than HLA Ags on the trophoblast in a non-MHC-restricted manner.

Earlier, we showed that in peripheral blood of pregnant women, there are two functionally distinct subpopulations of $\gamma\delta$ T cells. Activation of peripheral $\gamma\delta$ cells via the V γ 4V δ 1 receptor resulted in increased IL-10 production and decreased cytotoxic function, whereas activation via the V γ 9V δ 2 receptor results in decreased IL-10 synthesis and increased cytotoxic activity (26). Wen et al. (32) have isolated $\gamma\delta$ clones that confirm the Th1/Th2 classification both by cytokine expression and by functional activities. Human and murine $\gamma\delta$ T cells have been demonstrated to provide B cell help (34, 35) and in association with this were shown to produce IL4 (35, 36). There are data suggesting that a Th1/Th2 classification can be established in primary T cells in

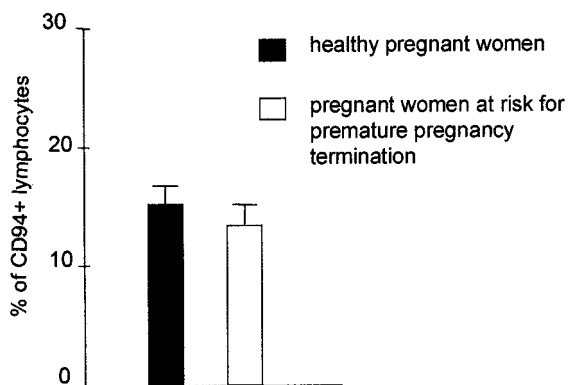


FIGURE 9. Expression of CD94 molecule on peripheral lymphocytes from healthy pregnant women and those from women at risk for premature pregnancy termination. There is no significant difference in the CD94 expression on peripheral lymphocytes from healthy and pathologically pregnant women. Bars, mean \pm SEM of 10 determinations.

the absence of specific peptide presentation by conventional class I/class II MHC (32).

Our data revealed that the potentially cytotoxic V δ 2⁺ lymphocytes recognize HLA-E on the trophoblast. It has been shown that NK cells can interact with HLA-E, complexed with specific peptides on target cells, and that this recognition is mediated, at least partially, if not solely, by the CD94 molecule of the lectin-like CD94/NKG2-inhibitory receptor (37–40). Expression of CD94 was found not only on NK cells but also on the majority of circulating human $\gamma\delta$ TCR⁺ cells. The distribution of CD94 on $\gamma\delta$ T cells is considerably higher (~80%) than that found in $\alpha\beta$ TCR⁺ cells (~4.2% from the same donor) and is closer to that found on NK cells, where virtually all express CD94 (20, 41). This suggests that the regulation of $\gamma\delta$ T cell function is likely to be different from that found in most $\alpha\beta$ T cells, involving activation (or inhibition) by signaling through both the TCR and the NK cell receptor (23). Most peripheral $\gamma\delta$ T cells express the inhibitory form of CD94 (23). In our hands, blocking of the CD94 molecule by specific Ab resulted in a reduced conjugation capacity of V δ 2⁺ normal pregnancy lymphocytes to JAR-G, but not to other transfectants, and at the same time cytotoxicity to JAR-G increased. The above effects could not be observed with JAR-G1m but with JAR-E, suggesting that anti-CD94 treatment affects the CD94-HLA-E interaction. Increased cytotoxic activity despite the reduced conjugation capacity can possibly be explained by an altered cytokine production resulting from inhibition of the KIR HLA-E interaction (42). The anti-CD94 mAb inhibits the V γ 9V δ 2 T cell proliferation in response to mycobacterial phosphoantigens and also the HIV-induced V γ 9V δ 2 T cell expansion (22). V γ 9V δ 2 T cells stimulated via the $\gamma\delta$ TCR with nonpeptidic mycobacterial Ags produce IFN- γ and TNF- α . Signaling through the CD94 receptor induces an inhibitory signal in peripheral V δ 2 T cells thus might inhibit IFN- γ and TNF- α production of V δ 2 cells (22). In this study, we found an increased IFN- γ production by anti-NKG2A-treated lymphocytes.

We have observed an increased cytotoxic activity of lymphocytes treated with a blocking concentration of anti-CD94 against HLA-G/HLA-E-transfected JAR cells, but not against JAR cells not expressing HLA-G or HLA-E. Because the rate of conjugation of anti-CD94-treated cells to JAR-G was lower than that of untreated cells, whereas anti-CD94 treatment did not influence cytotoxicity to JAR cells, we assume that the low cytotoxic activity to HLA-E-expressing cells was due to activation via the KIR receptor. Owing to the lack of the CD94-ligand interaction, an excessive Th1-type cytokine production might have occurred, accounting for the increased cytotoxicity.

According to our hypothesis, on recognition of nonclassical MHC molecules via CD94, a known KIR, the potentially cytotoxic V γ 9/V δ 2 T cell population is inhibited. V δ 2⁺ lymphocytes from pregnant women at risk for premature pregnancy termination, in contrast to those of healthy pregnant women, do not recognize HLA-E, which might result in a lack of inhibition of cytotoxicity. This cannot be due to a decreased expression of the CD94/NKG2A KIR on these cells, because we found no difference in the expression of this KIR between healthy pregnant women and recurrent aborters. This phenomenon may play a part in the inadequate maternal antifetal immune response observed in failed pregnancies and might also underlie the role and importance of $\gamma\delta$ T cells during pregnancy.

Acknowledgments

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References

- Hutter, H., A. Hammer, A. Blaschitz, M. Hartmann, P. Ebbesen, G. Dohr, A. Ziegler, and B. Uchanska-Ziegler. 1996. Expression of HLA class I molecules in human first trimester and term placenta trophoblast. *Cell Tissue Res.* 286:439.
- King, A., C. Boocock, A. M. Sharkey, L. Gardner, A. Beretta, A. G. Siccardi, and Y. W. Loke. 1996. Evidence for the expression of HLA-C class I mRNA and protein by human first trimester trophoblast. *J. Immunol.* 156:2068.
- McMaster, M. T., C. L. Librach, Y. Zhou, K. H. Lim, M. J. Janatpour, R. DeMars, S. Kovats, C. Damsky, and S. J. Fisher. 1995. Human placental HLA-G expression is restricted to differentiated cytotrophoblast. *J. Immunol.* 154:3771.
- Loke, Y. W., A. King, T. Burrows, L. Gardner, M. Bowen, S. Howlett, N. Holmes, and D. Jacobs. 1997. Evaluation of trophoblast HLA-G antigen with a specific monoclonal antibody. *Tissue Antigens* 50:135.
- Pröll, J., A. Blaschitz, H. Hutter, and G. Dohr. 1999. First trimester human trophoblast cells express both HLA-C and HLA-G. *Am. J. Reprod. Immunol.* 42:30.
- Le Bouteiller, P. 1994. HLA class I chromosomal region, genes and products: facts and questions. *Crit. Rev. Immunol.* 14:89.
- Parham, P. 1995. Antigen presentation by class I major histocompatibility complex molecules: a context for thinking about HLA-G. *Am. J. Reprod. Immunol.* 34:10.
- Guillaudeux, T., A. M. Rodriguez, M. Girr, V. Mallet, S. A. Ellis, I. L. Sargent, R. Fauchet, E. Alsat, and P. Le Bouteiller. 1995. Methylation status and transcriptional expression of the MHC class I loci in human trophoblast cells from term placenta. *J. Immunol.* 154:3283.
- King, A., D. S. J. Allan, M. Bowen, S. J. Powis, S. Joseph, S. Verma, S. E. Hiby, A. J. McMichel, Y. W. Loke, and V. M. Braud. 2000. HLA-E is expressed on trophoblast and interacts with CD94/NKG2 receptors on decidual NK cells. *Eur. J. Immunol.* 30:1623.
- Constant, P., F. Davodeau, M. A. Peyrat, Y. Poquet, G. Puzzo, M. Bonneville, and J. J. Fournie. 1994. Stimulation of human $\gamma\delta$ T cells by nonpeptidic mycobacterial ligands. *Science* 264:267.
- Tanaka, Y., C. T. Morita, Y. Tanaka, E. Nieves, M. B. Brenner, and B. R. Bloom. 1995. Natural and synthetic nonpeptide antigens recognized by human $\gamma\delta$ T cells. *Nature* 375:155.
- Burk, M. R., L. Mori, and G. DeLibero. 1995. Human $V\gamma 9/V\delta 2$ cells are stimulated in a cross-reactive fashion by a variety of phosphorylated metabolites. *Eur. J. Immunol.* 25:2052.
- Morita, C. T., E. M. Beckman, J. F. Bukowski, Y. Tanaka, H. Band, B. R. Bloom, D. E. Golan, and M. B. Brenner. 1995. Direct presentation of nonpeptide prenyl pyrophosphate antigens to human $\gamma\delta$ T cells. *Immunity* 3:495.
- Mincheva-Nilsson, L. Baranov, V. Yeung, M. Mo-Way, S. Hammarstrom, and M.-L. Hammarstrom. 1994. Immunomorphologic studies of human decidua-associated lymphoid cells in normal early pregnancy. *J. Immunol.* 152:2020.
- Kimura, M. Hanawa, H. Watanabe, H. Ogawa, and M. Abo, T. 1995. Synchronous expansion of intermediate TCR cells in the liver and uterus during pregnancy. *Cell. Immunol.* 162:15.
- Mincheva-Nilsson, L., M. Kling, S. Hammarström, O. Nagaeva, K. G. Sundqvist, M. L. Hammarström, and V. Baranov. 1997. $\gamma\delta$ T cells of human early pregnancy decidua. *J. Immunol.* 159:3266.
- Lanier, L. L., J. Ruitenber, R. L. Bolhuis, J. Borst, J. H. Phillips, and R. Testi. 1988. Structural and serological heterogeneity of $\gamma\delta$ T cell antigen receptor expression in thymus and peripheral blood. *Eur. J. Immunol.* 18:1985.
- Parker, C. M., V. Groh, H. Band, S. A. Porcelli, C. Morita, M. Fabbri, D. Glass, J. L. Strominger, and M. B. Brenner. 1990. Evidence for extrathymic changes in the T cell receptor $\gamma\delta$ repertoire. *J. Exp. Med.* 171:1597.
- Polgar, B., A. Barakonyi, I. Xynos, and J. Szekeres-Bartho. 1999. The role of $\gamma\delta$ T cell receptor positive cells in pregnancy. *Am. J. Reprod. Immunol.* 41:239.
- Aramburu, J., M. A. Balboa, A. Ramirez, A. Silva, A. Acevedo, F. Sanchez-Madrid, M. O. DeLandazuri, and M. Lopez-Botet. 1990. A novel functional cell surface dimer (Kp43) expressed by natural killer cells and T cell receptor- $\gamma\delta^+$ lymphocytes. *J. Immunol.* 144:3238.
- Rubio, G., J. Aramburu, J. Ontanon, M. Lopez-Botet, and P. Aparicio. 1993. A novel functional cell surface dimer (kp43) serves as accessory molecule for the activation of subset of human $\gamma\delta$ T cells. *J. Immunol.* 151:1312.
- Poccia, F., B. Cipriani, S. Vendetti, V. Colizzi, Y. Poquet, L. Battistini, M. Lopez-Botet, J. J. Fournie, and M. L. Gougeon. 1997. CD94/NKG2A inhibitory receptor complex modulates both anti-viral and anti-tumoral responses of polyclonal phosphoantigen-reactive $V\gamma 9/V\delta 2$ T lymphocytes. *J. Immunol.* 159:6009.
- Battistini, L., G. Borsellino, G. Sawicki, F. Poccia, M. Salvetti, G. Ristori, and C. F. Brosnan. 1997. Phenotypic and cytokine analysis of human peripheral blood $\gamma\delta$ T cells expressing NK cell receptors. *J. Immunol.* 159:3723.
- Navarro, F., M. Llano, T. Bellon, M. Colonna, D. E. Geraghty, and M. Lopez-Botet. 1999. The ILT2/LIR1 and CD94/NKG2A NK cell receptors respectively recognize HLA-G1 and HLA-E molecules co-expressed on target cells. *Eur. J. Immunol.* 29:277.
- Cantoni, C., S. Verdiani, M. Falco, A. Pessino, M. Cilli, R. Conte, D. Pende, M. Ponte, M. S. Mikaelsson, L. Moretta, and R. Biassoni. 1998. p49, a putative HLA class I-specific inhibitory NK receptor belonging to the immunoglobulin superfamily. *Eur. J. Immunol.* 28:1980.
- Barakonyi, A., B. Polgar, and J. Szekeres-Bartho. 1999. The role of $\gamma\delta$ T-cell receptor-positive cells in pregnancy: part II. *Am. J. Reprod. Immunol.* 42:83.
- Mallet, V., J. Proll, C. Solier, M. Aguerre-Girr, M. DeRossi, Y. W. Loke, F. Lenfant, and P. Le Bouteiller. 2000. The full length HLA-G1 and no other alternative form of HLA-G is expressed at the cell surface of transfected cells. *Hum. Immunol.* 61:212.
- Llano, M., N. Lee, F. Navarro, P. Garcia, J. P. Albar, D. E. Geraghty, and M. Lopez-Botet. 1998. HLA-E-bound peptides influence recognition by inhibitory and triggering CD94/NKG2 receptors: preferential response to an HLA-G-derived nonamer. *Eur. J. Immunol.* 28:2854.
- Ulbrecht, M., T. Honka, S. Person, J. P. Johnson, and E. H. Weiss. 1992. The HLA-E gene encodes two differentially regulated transcripts and a cell surface protein. *J. Immunol.* 149:2945.
- Lee, N., D. R. Goodlett, A. Ishitani, H. Marquardt, and D. E. Geraghty. 1998. HLA-E surface expression depends on binding of TAP-dependent peptides derived from certain HLA class I signal sequences. *J. Immunol.* 160:4951.
- Grimm, E., and B. Bonavida. 1979. Mechanism of cell-mediated cytotoxicity of the single cell level. I. Estimation of cytotoxic T lymphocyte frequency and relative lytic efficiency. *J. Immunol.* 123:2861.
- Wen, L., D. F. Barber, W. Pao, F. S. Wong, M. J. Owen, and A. Hayday. 1998. Primary $\gamma\delta$ cell clones can be defined phenotypically and functionally as Th1/Th2 cells and illustrate the association of CD4 with Th2 differentiation. *J. Immunol.* 160:1965.
- Heyborne, K., Y.-X. Fu, A. Nelson, A. Farr, R. O'Brien, and W. Born. 1994. Recognition of trophoblasts by $\gamma\delta$ T cells. *J. Immunol.* 153:2918.
- Peng, S. L., M. P. Madaio, D. P. Hughes, I. N. Crispe, M. J. Owen, L. Wen, A. C. Hayday, and J. Craft. 1996. Murine lupus in the absence of $\alpha\beta$ T cells. *J. Immunol.* 156:4041.
- Horner, A. A., H. Jabara, N. Ramesch, and R. S. Geha. 1995. Gd T lymphocytes express CD40 ligand and induce isotype switching of B lymphocytes. *J. Exp. Med.* 183:2271.
- Wen, L., S. J. Roberts, J. Viney, F. S. Wong, C. Mallick, R. C. Findly, Q. Peng, J. E. Craft, M. J. Owen, and A. C. Hayday. 1994. Immunoglobulin synthesis and generalized autoimmunity in mice congenitally deficient in $\alpha\beta^+$ T cells. *Nature* 369:654.
- Borrego, F., M. Ulbrecht, E. H. Weiss, J. E. Coligan, and A. G. Brooks. 1998. Recognition of human histocompatibility leukocyte antigen (HLA)-E complexed with HLA class I signal sequence-derived peptides by CD94/NKG2 confers protection from natural killer cell-mediated lysis. *J. Exp. Med.* 187:813.
- Braud, V. M., D. S. Allan, C. A. O'Callaghan, K. Soderstrom, A. D'Andrea, G. S. Ogg, S. Lazetic, N. T. Young, J. I. Bell, J. H. Phillips, L. L. Lanier, and A. J. McMichael. 1998. HLA-E binds to natural killer cell receptors CD94/NKG2A, B and C. *Nature*. 391:795.
- Brooks, A. G., F. Borrego, P. E. Posch, A. Patamawenu, C. J. Scorzelli, M. Ulbrecht, E. H. Weiss, and J. E. Coligan. 1999. Specific recognition of HLA-E, but not classical, HLA class I molecules by soluble CD94/NKG2A and NK cells. *J. Immunol.* 162:305.
- Lee, N., M. Llano, M. Carretero, A. Ishitani, F. Navarro, M. Lopez-Botet, and D. Geraghty. 1998. HLA-E is a major ligand for the natural killer inhibitory receptor CD94/NKG2A. *Proc. Natl. Acad. Sci. USA* 95:5199.
- Moretta, A., R. Biassoni, C. Bottino, D. Pende, M. Vitale, A. Poggi, M. C. Mingari, and L. Moretta. 1997. MHC class I specific receptors on human natural killer and T lymphocytes. *Immunol. Rev.* 155:105.
- Halary, F., M.-F. Peyrat, E. Champagne, M. Lopez-Botet, A. Moretta, H. Vié, J.-J. Fournié, and M. Bonneville. 1997. Control of self-reactive cytotoxic T lymphocytes expressing $\gamma\delta$ T cell receptors by natural killer inhibitory receptors. *Eur. J. Immunol.* 27:2812.