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# Inhibitory receptors sensing HLA-G1 molecules in pregnancy: Decidua-associated natural killer cells express LIR-1 and CD94/NKG2A and acquire p49, an HLA-G1-specific receptor

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**ABSTRACT** **Trophoblastic cells lack classical HLA class I and class II molecules but express HLA-G1. Although this may prevent allorecognition by maternal T cells, it renders trophoblastic cells potentially susceptible to lysis by natural killer (NK) cells. As shown here, only a fraction of peripheral-blood NK cells in pregnant women express the HLA-G1-specific CD94/NKG2A and/or LIR-1 receptors. However, all NK cells isolated from maternal decidua during the first trimester expressed either one or both of these receptors. Perhaps more importantly, a fraction of cells expressed p49, an HLA-G1-specific inhibitory receptor, undetectable in peripheral-blood NK cells. p49 was expressed on virtually all NK cells isolated from placenta at term. Functional analyses revealed that the HLA class I-negative 221 lymphoblastoid cell line transfected with HLA-G1 was only partially protected from lysis by peripheral-blood NK cells isolated from pregnant women, whereas it was fully protected from decidual NK cells. As indicated by the addition of specific antibodies to cytolytic tests, all the above receptors contributed to HLA-G1 recognition by decidual NK cells, although p49 would appear to play a predominant role.**

The trophoblastic cells in human placenta lack classical HLA class I and class II antigens, with the exception of the expression of HLA-C during the first trimester (1, 2). Although this condition prevents allorecognition and lysis by maternal T lymphocytes, it poses the problem of how trophoblastic cells can escape lysis mediated by natural killer (NK) cells. It is conceivable that the expression of the nonclassical HLA class I molecule HLA-G1, characterized by a limited polymorphism, may play a role in the protective effect (3–9). It is now well established that human NK cells express different HLA class I-specific inhibitory receptors termed "killer inhibitory receptors" (KIRs) (10). These include several members of the Ig superfamily, recognizing defined groups of HLA class I alleles. Thus, p58.1 and p58.2 function as receptors for two groups of HLA-C alleles (11), p70 functions as receptor for the HLA-B alleles belonging to the Bw4 supertypic specificity (12), and p140 recognizes certain HLA-A alleles (13, 14). Although none of these KIRs recognizes HLA-G1 (15–17), LIR-1, another recently identified member of the Ig superfamily, recognizes some HLA class I molecules including HLA-G1 (18, 19). Another NK receptor thought to recognize HLA-G1 is the complex formed by CD94 and NKG2A, both type II membrane proteins belonging to the C-type lectin family (20–24). Although recent data have shown that CD94/NKG2A actually recognizes HLA-E molecules, the latter are surface expressed only in association with defined HLA class I alleles, including HLA-G1 (25). Therefore, operationally, CD94/NKG2A$^+$ cells can sense HLA-G1 molecules expressed on trophoblastic cells (26). Another putative receptor for HLA-G1 is the molecular product of the cl.15.212 cDNA. This molecule, termed p49, is characterized by two Ig-like domains and is homologous to KIRs belonging to the Ig superfamily. Importantly, the use of p49 in soluble form allowed to demonstrate binding to HLA-G1 cell transfectants (27).

In a recent study, the expression of some KIRs, including p58.1, p58.2, and CD94, has been evaluated in decidua-derived NK cells and compared with those isolated from peripheral blood of pregnant women (28). A relatively high expression of these molecules has been observed, thus suggesting that the HLA-C-specific p58 receptors may prevent (during the first trimester) the attack to cytotrophoblast at least by those NK cells expressing p58. However, no information has been provided so far on the expression of receptors capable of detecting the HLA-G1 expression during pregnancy.

The present study was designed to analyze the expression of the known HLA-G1-specific receptors both in maternal decidua and in peripheral blood from pregnant women. We show that in pregnant women there is a general up-regulation of KIR expression in peripheral-blood lymphocytes. Moreover, most NK cells isolated from maternal decidua expressed CD94/NKG2A and LIR-1. Perhaps more importantly, p49 was detected in decidual-associated but not in peripheral-blood NK cells. Functional studies revealed that p49 may play a relevant role in HLA-G1 recognition by decidual NK cells. Therefore, it appears that decidua-associated NK cells express different HLA-G1-specific receptors that are likely to prevent lysis of trophoblastic cells.

## MATERIALS AND METHODS

**Antibodies and Reagents.** mAbs XA185 (IgG1, anti-CD94), Y9 (IgM, anti-CD94), GL183 (IgG1, anti-p58.2), EB6 (IgG1, anti-p58.1), Z276 (IgG1, anti-p70), Q66 (IgM, anti-p140), Z270 (IgG1, anti-NKG2A), Z199 (IgG2b, anti-NKG2A), JT3a (IgG2a, anti-CD3), HP26 (IgG2a, anti-CD4), B9.4 (IgG2b, anti-CD8), KD1 (IgG2a, anti-CD16), A6136 (IgM, anti-HLA class I), 6A4 ($F(ab')_2$, anti-class I), TOP-2 murine antiserum (IgG1, anti-p49) (all selected in our laboratory; see refs. 11, 14, 22, and 29) were used in this study.

MAb M401 (IgG1, anti-LIR-1) was kindly provided by D. Cosman, Immunex (Seattle, WA). MAbs Leu-19 (IgG1, anti-

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Abbreviations: NK, natural killer; KIR, killer inhibitory receptor; PE, phycoerythrin.

A Commentary on this article begins on page 5343.

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CD56) and Leu-4 (IgG1, anti-CD3) were purchased from Becton Dickinson (San Jose, CA).

Fluorescein isothiocyanate- and phycoerythrin-conjugated anti-isotype goat anti-mouse antibodies were purchased from Southern Biotechnology Associates. The culture medium used was RPMI 1640 supplemented with L-glutamine/10% fetal calf serum (Boehringer Mannheim)/1% antibiotic mixture (5 mg/ml penicillin/5 mg/ml streptomycin/10 mg/ml neomycin stock solution)/human recombinant IL-2, kindly provided by Chiron-Italia (Milan).

**Immunofluorescence Analysis.** The reactivity of mAbs with cell populations was assessed by indirect immunofluorescence and flow cytometric analyses, as described (30). Briefly, $10^5$ cells were stained with the corresponding mAb followed by a fluorescein isothiocyanate-conjugated goat anti-mouse Ig or appropriate fluorescein isothiocyanate- or phycoerythrin-conjugated anti-isotype specific goat anti-mouse antiserum (Southern Biotechnology Associates), as second-step reagents. All samples were analyzed on a flow cytometer (FACSort; Becton Dickinson). Results are expressed as logarithm of green fluorescence intensity (arbitrary units) vs. logarithm of red fluorescence intensity.

**Isolation and Culture of Lymphocytes.** Peripheral-blood lymphocytes were isolated from blood of pregnant women or normal controls by a Ficoll–Hypaque density gradient, as described (30, 31).

In some experiments, peripheral-blood lymphocytes were depleted of $CD3^+$ cells by negative selection using, respectively, Leu-4 and KD1 mAbs and magnetic beads coated with anti-mouse Ig (Immunotech, Luminy, France). Cells were cultured in the presence of irradiated feeder cells and 100 units/ml of recombinant IL-2, as previously described (31).

**Isolation of Decidua-Associated Lymphoid Cells.** Maternal decidual tissues were obtained from elective first trimester terminations of pregnancy (between 8 and 11 wk) by section curettage. Decidual lymphocytes were isolated as described (32).

Lymphoid cells were also isolated from placentas at term. The tissue was dissected mechanically into small pieces and then passed through a stainless mesh to obtain cell suspensions. These were then passed over Ficoll–Hypaque density gradients to enrich the cell suspensions in mononuclear cells.

**Assay for Cytolytic Activity.** Cytolytic activity of polyclonal cell populations was tested in a 4-h $^{51}Cr$-release assay as previously described in detail (31). $^{51}Cr$-labeled human class I negative-transformed cell lines LCL721.221 (thereafter indicated as 221) and 221-G1 transfected (27) were used as target cells. Effector:Target cell ratio ranged between 0.5:1 and 10:1 in a final volume of 200 $\mu$l RPMI 1640. The cytolytic assay was performed in medium alone or in the presence of 50 $\mu$l of culture supernatants of various murine mAbs or sera, including A6136 mAb (IgM), 6A4 $F(ab')_2$, Y9 (IgM), M401 (IgG1), and TOP-2 (IgG1).

**Preparation of Soluble p49-Fc Chimeric Molecule and Binding Assay.** cDNA sequence coding for the extracellular domain of cl.15.212 molecule was amplified by using the set of primers CAG GGG GCG CTA GCG CAC GTG GGT GGT CAG GAC AAG CC (15.212 Ig Up) GAG GTC CCA GGA TCC GCA TGA TGC AGG TGT CTG GCG ATA CC (15.212 Ig down). The PCR fragment was ligated to *Nhe*I-*Bam*HI-cut CD51neg1 expression vector (gift from B. Seed) (33), in frame with the CD5 leader-peptide coding sequence at the 5′ end, and with a genomic sequence for the Fc fragment of human IgG1 at the 3′ end. COS-7 cells were transfected by the DEAE–dextran method and cultured in DMEM supplemented with 10% Ultra-Low IgG Fetal Bovine Serum (GIBCO/BRL). Supernatants were collected on days 4 and 8 after transfection. Soluble chimeric receptors were purified as described (34). 221 cells untransfected or transfected with the HLA-G1 allele-encoding cDNA were incubated with 10 $\mu$g of p49-Fc fusion protein for 30 min at 4°C in culture medium followed by a phycoerythrin (PE)-conjugated goat anti-human antibody (Southern Biotechnology Associates). FACS analysis was carried out as above.

**Generation of Anti-p49 Antiserum and COS-7 Cell Transfection.** A 5 wk-old BALB/c mouse (Charles River Breeding Laboratories) was immunized with a first injection of 100 $\mu$g of p49-Fc chimeric protein mixed 1:1 (vol/vol) with complete Freund's adjuvant (Sigma), behind the neck. One month later a booster immunization was given, followed after 2 wk by a final injection with 100 $\mu$g of p49-Fc mixed with complete Freund's adjuvant. Three days later, a test sample of blood was taken and analyzed by ELISA, by using the p49-Fc chimeric protein in the coating step and horseradish peroxidase-labeled goat anti-mouse IgG as secondary antibody. This antiserum (termed TOP-2) was extensively adsorbed by using erythrocytes and then untransfected COS-7 cells. To analyze the specificity of TOP-2 for p49 molecules, COS-7 cells were transfected with the cl.15.212-VR1012 construct (Vical, San Diego) or with an irrelevant cDNA by using the DEAE–dextran method. After 48 hr, transfected cells were stained with TOP-2 or with sera derived from unimmunized mice (1:100 final dilution) followed by anti-mouse IgG1 PE-conjugated antiserum and were analyzed by cytofluorimetry.

## RESULTS

**KIR Expression in Peripheral-Blood Lymphocytes of Pregnant Women.** The expression of various HLA class I-specific KIR has been analyzed in peripheral blood of pregnant women at different weeks of gestation. KIRs analyzed included p58.1, p58.2, p70, p140, CD94/NKG2A, and LIR-1. During the first trimester, there was an overall increase of $KIR^+$ cells in the peripheral blood, as compared with nonpregnant women. These increases were maximal within the third month of gestation and subsequently declined and reached basal levels by the end of gestation. Perhaps more remarkable was the general increase in the fluorescence intensity of the various expressed KIRs. Table 1 summarizes the results obtained in eight pregnant women analyzed at a gestational age ranging

Table 1. Increased proportions of $KIR^+$ lymphocytes and up-regulation of KIR expression at the cell surface during early pregnancy

| | p58.2 | | NKG2-A | | LIR-1 | |
|---|---|---|---|---|---|---|
| Donors | Positive cells %* (±SEM) | Mean FI (±SEM) | Positive cells %* (±SEM) | Mean FI† (±SEM) | Positive cells %* (±SEM) | Mean FI† (±SEM) |
| Pregnant women ($n = 8$) | 8 (0.90) | 460 (73)‡ | 11 (1.9) | 250 (40.7)‡ | 24 (3.5) | 122 (25.7)‡ |
| Control women ($n = 8$) | 5 (0.59) | 253 (61.8) | 10 (1.61) | 145 (18.4) | 17 (1.7) | 58 (9.4) |

FI, fluorescence intensity.
*The percentage of cells expressing the indicated KIR were evaluated by cytofluorimetry.
†Numbers indicate the mean FI as evaluated by cytofluorimetric analysis.
‡$P < 0.05$ two-tailed Student's $t$ test vs. control group.

between 8 and 13 wk. Only those receptors that recognize HLA class I molecules expressed on trophoblastic cells during the first trimester (i.e., HLA-C and HLA-G1), including p58.2, NKG2A (revealed the inhibitory CD94/NKG2A receptor complex) and LIR-1, are illustrated. Higher percentages of the various surface receptors could be detected in pregnant vs. nonpregnant women. Perhaps more importantly, the mean fluorescence intensity of the various KIRs was considerably higher (Table 1), thus supporting the notion of a general up-regulation of the expression of different KIRs during pregnancy. Although the higher percentages of circulating $KIR^+$ cells primarily reflected an increase of $KIR^+$ T lymphocytes, the increases in fluorescence intensity of the various KIRs analyzed were equally detectable in NK and T lymphocytes (not shown). In some experiments, KIR expression could be analyzed in the same women during pregnancy and after delivery. It was found that, after delivery, both the proportions of cells expressing different KIRs and the fluorescence intensity were consistently lower than during the first trimester (not shown).

Although not shown, the expression of both CD94/NKG2A and LIR-1 has been evaluated by double immunofluorescence in the $CD16^+$ (i.e., NK) cell population of PB lymphocytes. Consistently, both of these receptors capable of sensing the HLA-G1 expression were found in high proportions of $CD16^+$ cells. However, a fraction (up to 50%) of $CD16^+$ cells did not express these HLA-G1-specific receptors.

**Expression of HLA-G1 Confers Only a Partial Protection from NK Cell Populations Isolated from Peripheral Blood of Pregnant Women.** We next analyzed whether surface expression of CD94/NKG2A and LIR-1 in peripheral-blood NK cells from pregnant women could prevent lysis of $HLA\text{-}G1^+$ cells. Because fresh peripheral-blood NK cells displayed a low cytolytic activity against the class I-negative 221 cells used in this study (see below), we analyzed cultured NK cell lines (obtained by depleting $CD3^+$ cells). NK cell lines were derived from eight pregnant and three nonpregnant women as control. Fig. 1*A* shows the FACS profiles of informative markers expressed by a NK cell line derived from a woman at week 10 of gestation. High proportions of cells expressed CD56 and CD16. The majority of cells expressing CD16 coexpressed NKG2A, indicating that they were equipped with the inhibitory CD94/NKG2A receptor complex. In addition, about half of $CD16^+$ cells coexpressed LIR-1. Thus, CD94/NKG2A and LIR-1, two receptors that can sense HLA-G1 molecules (26, 27), were expressed in high percentages in this cell line. A similar pattern of expression of NKG2A and LIR-1 was detected in the majority of NK cell lines derived from pregnant women.

These cultured NK cells were tested in a cytolytic assay against either untransfected or HLA-G1-transfected 221 target cells. As shown in Fig. 1*B*, cells (corresponding to those analyzed in *A*) lysed efficiently 221 cells, while 221-G1 cells were partially protected from lysis. Addition of mAb specific for either CD94 or LIR-1 reverted the protective effect, at least in part. The simultaneous addition of both mAbs resulted in a virtually complete restoration of lysis of 221-G1 target cells.

Analysis of $CD16^+$ cell lines derived from nonpregnant women indicated that the protection conferred by HLA-G1 was generally lower than that detected with NK cell lines derived from pregnant women. It is of note that, in all these NK cell lines, a lower fraction of cells expressed NKG2A, while only a small percentage expressed LIR-1 (not shown).

Taken together, these data indicate that NK cells isolated from peripheral blood of pregnant women may express receptors potentially able to sense HLA-G1 molecules on placental trophoblastic cells. However, it is also evident that (*i*) a fraction of fresh (or cultured) peripheral-blood NK cells do not express CD94/NKG2A or LIR-1, and (*ii*) even when these receptors are expressed by a large fraction of cultured NK cell lines (as

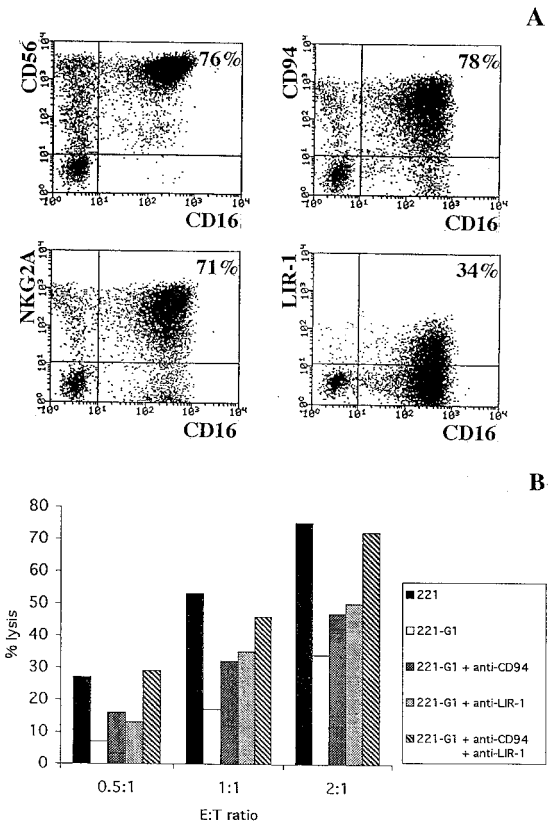


FIG. 1. Expression of CD94/NKG2A and LIR-1 in a NK-enriched cell line derived from a pregnant woman and partial protection from lysis mediated by HLA-G1 expression on target cells. (*A*) Double-fluorescence analysis of a CD3-depleted (NK-cell enriched) lymphocyte population cultured for 8 days in the presence of rIL-2. Cells were stained with different combinations of the following mAbs: anti-CD56, anti-CD16, anti-CD94, anti-NKG2A, and anti-LIR-1 (*x* axis, green fluorescence; *y* axis, red fluorescence). (*B*) The same NK-enriched cultured lymphocyte population was analyzed for the ability to lyse 221 cells or 221-G1 cell transfectants. In addition, the cytolytic activity against 221-G1 was assessed also in the presence of Y9 (anti-CD94) mAb, M401 (anti-LIR-1), or a combination of the two mAbs. Each histogram represents the mean of triplicate experiments.

in Fig. 1), 221-G1-transfectants were only partially protected from NK-mediated lysis. These data suggest that $HLA\text{-}G1^+$ trophoblastic cells may not be fully protected from NK cells unless all NK cells in placenta express these receptors and/or unless they express additional HLA-G1-specific receptors.

**Generation of an Antiserum Specific for p49, a Putative HLA-G1-Specific KIR.** We recently described a cDNA termed cl.15.212 that encodes a HLA class I-specific receptor (p49) belonging to the Ig superfamily and sharing homology with p58, p70, and p140 KIRs (27). By the use of a soluble chimeric p49-Fc molecule, we could demonstrate selective binding to some HLA class I alleles including HLA-G1 (Fig. 2*A;* see also ref. 27). We immunized mice with the p49-Fc protein to generate a p49-specific antiserum. This antiserum, termed TOP-2, stained COS-7 cells transfected with cl.15.212 cDNA but not COS-7 cells either untransfected or transfected with an irrelevant cDNA (Fig. 2*B*). Control mouse sera (i.e., derived from nonimmunized mice) did not stain p49-COS-7 cell transfectants (not shown). In view of these data, we used the p49-specific TOP-2 antiserum to analyze p49 expression in lymphocytes isolated from pregnant and nonpregnant women.

**Expression of p49, CD94/NKG2A, and LIR-1 in Maternal Decidua-Associated Lymphoid Cells.** We first analyzed the reactivity of the p49-specific TOP-2 antiserum with peripheral-blood lymphocytes isolated from either pregnant or nonpreg-

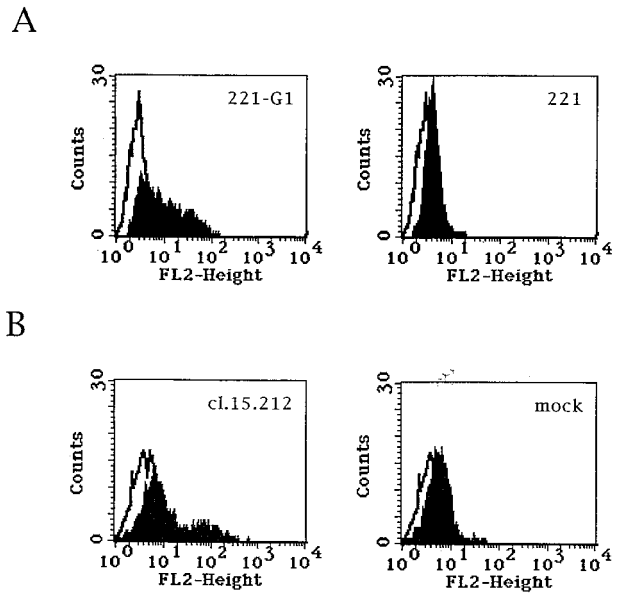


FIG. 2. Specific binding of the anti-p49 TOP-2 antiserum to p49 cell transfectants. (*A*) Binding of p49-Fc soluble molecule to HLA-G1 cell transfectants. p49-Fc has been incubated with HLA-G1 221-transfected cells (*Left*) or with 221 untransfected cells (*Right*) followed by anti-human IgG PE-conjugated mAb. White profiles represent controls stained with the second reagent only. (*B*) Staining of cl.15.212-transfected COS-7 cells by the anti-p49 TOP-2 antiserum. COS-7 cells transfected with cl.15.212-VR1012 construct (*Left*) or with an irrelevant cDNA (*Right*) were stained with TOP-2 antiserum followed by a PE-conjugated anti-mouse IgG1. White profiles represent controls stained with the second reagent only.

nant women. Staining with TOP-2 could be detected neither in 14 of 15 pregnant women analyzed nor in all nonpregnant controls (not shown). Only in a single pregnant woman, at the 28th week of gestation, did a small fraction of peripheral-blood lymphocytes display reactivity with TOP-2, but not with control Mo serum (not shown). On the other hand, both anti-NKG2A and anti-LIR-1 consistently stained a fraction of lymphocytes (see Table 1).

We further investigated whether p49 was expressed by NK cells isolated from maternal decidua obtained from elective termination of pregnancy during the first trimester. Three of seven samples of decidua analyzed yielded sufficient cell numbers to be further investigated for surface staining. A representative experiment is shown in Fig. 3. In agreement with previous data (28), a substantial fraction of cells were $CD56^{bright}$, while cells expressing CD16 were present in low proportions. A large fraction of cells expressed CD94, NKG2A, and, in lower percentage, LIR-1. Although not shown, double fluorescence analysis revealed that all $CD56^+$ (NK) cells expressed CD94/NKG2A. Importantly, a fraction of cells expressed p49. The control mouse serum showed no reactivity (not shown; see also Fig. 4*A*). Cells expressing LIR-1 or p49 molecules were also confined to $CD56^+$ cells. These data suggest that p49 may be selectively expressed by NK cells associated to maternal decidua. Thus, these NK cells not only expressed CD94/NKG2A and LIR-1 but were also equipped with an additional HLA-G1-specific receptor that is usually absent in peripheral-blood NK cells.

To obtain additional information on the various receptors that may sense the HLA-G1 expression in trophoblastic cells, we further analyzed mononuclear cells isolated from samples of placenta at term. As shown in Fig. 4*A*, about half of such cells were $CD56^+$ cells. As opposed to NK cells isolated from maternal decidua during the first trimester, most $CD56^+$ cells were also $CD16^+$. In addition, the majority of $CD16^+$ cells expressed LIR-1, and, in lower percentages, CD94/NKG2A. Finally, virtually all $CD16^+$ cells expressed p49, as assessed by using the TOP-2 antiserum. No reactivity could be detected with the control MO-antiserum.

Taken together, these data show that the majority of NK cells isolated from maternal decidua coexpress different receptors potentially able to sense the HLA-G1 expression in placental trophoblastic cells. However, as opposed to NK cells isolated from maternal decidua during the first trimester, the proportions of NK cells stained by TOP-2 antiserum outnumbered those stained by NKG2A.

**Role of p49 in the HLA-G1 Recognition by NK Cells Isolated from Maternal Decidua.** Because maternal decidua-associated NK cells coexpress p49, CD94/NKG2A, and LIR-1, we analyzed the involvement of these receptors in HLA-G1 recognition and thus in the inhibition of NK cell-mediated cytolysis of $HLA\text{-}G1^+$ target cells. To this end, we tested freshly isolated decidual NK cells isolated from placenta at delivery for the ability to lyse 221-G1 transfectants either in the presence or in the absence of mAbs to one or another receptor. Untrans-

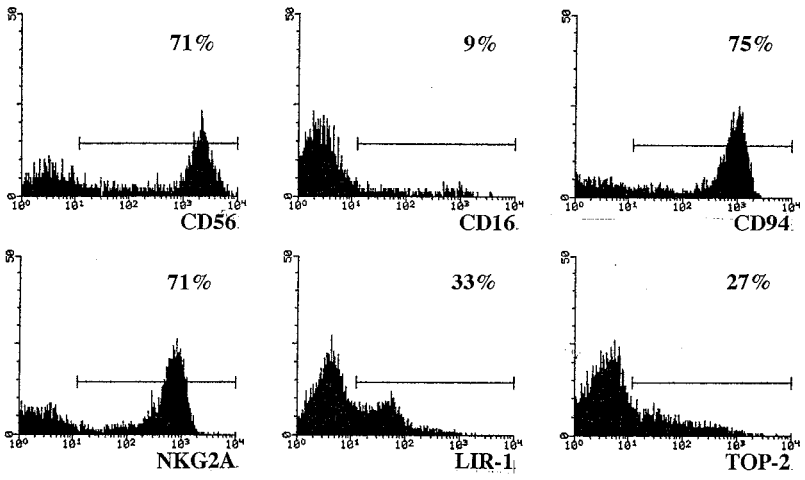


FIG. 3. Expression of CD94/NKG2A, LIR-1, and p49 receptors in lymphoid cells freshly isolated from maternal decidua in early pregnancy. The lymphoid population analyzed was derived from maternal decidua tissue obtained from elective (10th week) termination of pregnancy. Cells were purified on Ficoll–Hypaque gradients and were analyzed for surface expression of informative markers including CD56, CD16, CD94, NKG2A, LIR-1 (by using the specific mAbs indicated in *Materials and Methods* followed by an anti-mouse IgG1), and p49 (by using the TOP-2 antiserum); a PE-conjugated anti-mouse IgG1 was used as second reagent.

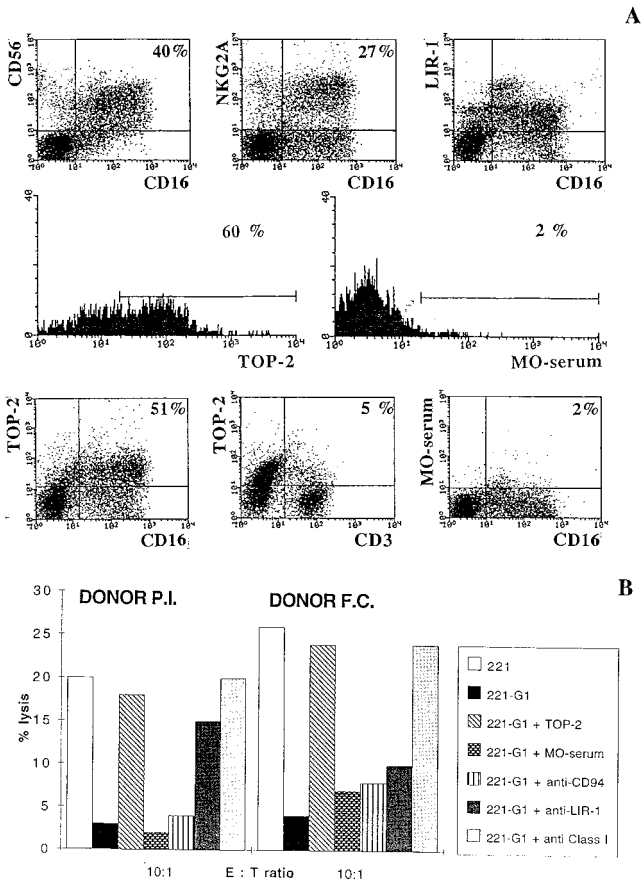


FIG. 4. Surface expression of CD94/NKG2A, LIR-1 and p49 receptors in lymphoid cells freshly isolated from placenta at term and their role in the HLA-G1 recognition. The lymphoid population analyzed was freshly derived from maternal decidua tissues of placenta at term. (*A*) Cells were stained with the indicated combinations of antibodies of different isotype, followed by appropriate isotype-specific second reagents, and were analyzed by double fluorescence analysis (*Upper*). The TOP-2 mouse antiserum (*Lower*) specifically recognizes the p49 receptor, while a control mouse serum (MO-serum) represents a control serum derived from a nonimmunized mouse (*x* axis, green fluorescence; *y* axis, red fluorescence). In this case, an anti-mouse IgG1 (PE-conjugated) has been used as second reagent. Note that, in double fluorescence experiments, both anti-CD16 and anti-CD3 mAbs belonged to the IgG2a subclass. (*B*) Lymphoid populations, freshly isolated from two placentas at term, were analyzed for the ability to lyse 221 cells transfected or not with HLA-G1. The cytolytic activity against 221-G1 was also assessed in the presence of Y9 (anti-CD94) or M401 (anti-LIR-1) mAb, the anti-p49 TOP-2 antiserum, and a MO-serum. A6136 (IgM) mAb in combination with 6A4 $F(ab')_2$, both directed to HLA class I molecules and reacting with HLA-G1, were also used. Note that masking of p49 restored lysis to an extent similar to that obtained by masking HLA-G1. Each histogram represents the mean of triplicate experiments.

fected 221 cells were used as control. Fig. 4*B* shows data from two different donors. It is evident that while untransfected 221 cells were susceptible to lysis, 221-G1 transfectants were not lysed. A partial restoration of lysis was detected in the presence of anti-CD94 or anti-LIR-1 mAb (Fig. 4) or a combination of the two mAbs (not shown). Maximal restoration of lysis occurred in the presence of TOP-2 antiserum (while the control mouse serum had no effect). Therefore, decidua-associated NK cells express different receptors capable of sensing the HLA-G1 expression; however, the newly inducible p49 receptor may play a major role in preventing the NK-mediated lysis of trophoblastic cells, at least during the late phases of pregnancy.

## DISCUSSION

Our present study shows that, during pregnancy, there is substantial increase of KIR expression in peripheral-blood lymphocytes. In particular, CD94/NKG2A and LIR-1 receptors, capable of sensing HLA-G1 molecules expressed by trophoblastic cells, were highly represented. More importantly, decidua-associated NK cells expressed not only CD94/NKG2A and LIR-1, but also an HLA-G1-specific KIR that may play an important role in preventing the NK cell-mediated attack to trophoblastic cells.

Maternal NK cells are closely associated with trophoblast that does not express classical HLA class I molecules, with the exception of HLA-C (expressed only during the first trimester), although it expresses the nonclassical HLA-G1 molecule. The obvious question arises of why NK cells do not destroy trophoblastic cells, since not all peripheral-blood NK cells express receptors that may sense HLA-G1 molecules. Different mechanisms could account for this phenomenon. NK cell activation during natural cytotoxicity reflects the engagement of different triggering receptors with their ligands expressed on potential target cells. Thus, either down-regulation of triggering receptors on NK cells or the lack of expression of their ligands on cytotrophoblastic cells might explain the phenomenon. In this context, decidua-associated NK cells do not express CD16. However, CD16 does not appear to be involved in natural cytotoxicity but rather in antibody-mediated cellular cytotoxicity (35). In addition, decidua-associated NK cells lyse efficiently NK-susceptible target cells, thus suggesting that triggering receptors involved in natural cytotoxicity are functioning. Regarding the expression of ligands for triggering receptors on trophoblastic cells, the finding that the trophoblastic cell line JAR (36) is susceptible to NK-mediated lysis suggests (but does not prove) that their normal counterpart may also express these ligands.

Although we cannot rule out that down-regulation of triggering receptors in NK cells and/or ligands on trophoblastic cells may indeed occur, our present study indicates that virtually all decidua-associated NK cells express one or more inhibitory receptors capable of sensing HLA-G1 molecules. Importantly, besides the high expression of known receptors such as CD94/NKG2A and LIR-1 (particularly during the first trimester), our data provide evidence that p49, a recently identified HLA-G1-specific receptor, is expressed by decidua-associated NK cells but not by peripheral NK cells isolated from pregnant (or nonpregnant) women. p49 is the molecular product of a previously described gene, termed cl.15.212, which belongs to the KIR family and is characterized by two extracellular Ig-like domains (27). We have recently shown that soluble p49-Fc selectively binds to some HLA class I alleles, including HLA-G1 (27). In the present study, we generated a mouse antiserum (TOP-2) against the soluble p49-Fc molecule. TOP-2 reacted with COS-7 cells transfected with cl.15.212 cDNA but not with COS-7 cells either untransfected or transfected with irrelevant cDNAs. Remarkably, TOP-2 failed to react with peripheral NK cells but stained most NK cells isolated from decidua (especially from placenta at term). In addition, while freshly isolated decidual NK cells did not lyse 221-G1 target cells, lysis of the latter was restored by the addition of TOP-2 to the cytolytic assay (control mouse serum had no effect). These data indicate that the HLA-G1-specific p49 receptor is expressed by NK cells in placenta and suggest that it may cooperate with other receptors to sharply inhibit NK cell function on binding to HLA-G1 molecules expressed on placental trophoblastic cells. Remarkably, while less than half of decidual ($CD56^+$) NK cells during the first trimester expressed TOP-2 (while all expressed CD94/NKG2A), this receptor was the predominant one in NK cells isolated from placenta at term. These data may suggest that different receptors capable of sensing HLA-G1 molecules may

exert a predominant role at different gestational stages. Our data also strongly suggest that (*i*) p49 expression may be induced *ex novo* in the decidual microenvironment, possibly as a result of the effect of cytokine(s) and/or hormone(s), and (*ii*) p49 may be down-regulated once NK cells leave the placenta, as suggested by the fact that no p49 reactivity could be detected in peripheral NK cells from pregnant women (with a single exception). It is possible that the surface expression of p49 may be induced rapidly, to prevent the attack to trophoblastic cells by NK cells trafficking to maternal decidua. If this is the case, one may speculate that p49 may be preformed by NK cells and stored in cytoplasmic compartments. Their prompt release after appropriate stimuli would represent a fail-safe mechanism to avoid NK-mediated damage to trophoblastic cells. Studies to analyze this possibility are in progress in our laboratory. It will also be important to determine whether impaired expression of p49 and/or other HLA-G1 specific receptors may play a role in recurrent abortions.

The finding that most $CD56^+$ cells isolated from decidua express CD94 and NKG2A and, in part, LIR-1, and that there is a general up-regulation of KIR expression also in peripheral-blood NK and T cells suggests that the expression of different KIRs may be induced by still undefined signals delivered within the placental microenvironment. Candidates for the induction of CD94/NKG2A expression may be IL-15 and/or TGF-$\beta$, which are produced in placenta and have been shown to induce surface expression of CD94/NKG2A in both NK and T cells (37–39).

Taken together, our data suggest that an important mechanism to prevent the attack of fetal tissues by the immune system is the induction of expression of inhibitory receptors on cytolytic effector cells (including both NK and cytolytic T lymphocytes). In this context, inhibitory receptors including LIR-1 and other KIR-related molecules of still-undefined specificity (such as p40) have also been identified in macrophages and dendritic cells (18, 19, 40). It is possible that common mechanism(s) of induction may exist for KIRs and related molecules, and that this phenomenon may represent a more general device to inhibit the immune response during pregnancy.

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