Human Choriocarcinoma Cell Resistance to Natural Killer Lysis Due to Defective Triggering of Natural Killer Cells

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ABSTRACT

The trophoblast, the outermost layer of the human placenta, lacks expression of the classical human leukocyte antigen (HLA) class I molecules. This prevents allorecognition by T cells but raises the question of what protects the trophoblast from natural killer (NK) cells. In a previous study, we have shown that choriocarcinoma cell (CC) resistance to NK lysis was mainly independent of HLA class I molecules. In the present study, we postulated that CC may prevent activation of NK cells by failing to stimulate their triggering receptors (TR). To test this hypothesis, we evaluated the lysis of JAR and JEG-3 CC after effective cross-linking and activation of NK cells by means of lectins or antibodies. Our results show that NK-resistant CC were sensitive to lysis by unstimulated peripheral blood lymphocytes in the presence of phytohemagglutinin (PHA), to antibody-dependent cell cytolysis in presence of anti-Tja antibodies, and to monoclonal antibody redirected killing using anti-TR antibodies anti-CD16 and anti-CD244/2B4. Finally, CC fail to express CD48, the ligand for CD244/2B4. These results indicate that the resistance of CC to lysis results primarily from defective NK cell activation, at least partially due to the lack of expression of ligands, such as CD48, involved in the triggering of NK cells.

INTRODUCTION

During pregnancy, the fetus, a semiallograft by its genotype, survives without immunological rejection. One explanation for this maternal tolerance is that trophoblast cells, which in humans are in direct contact with the maternal immune system, lack expression of the classical human leukocyte antigen (HLA) class I and II molecules, except for HLA-C in the extravillous trophoblast during the first trimester of pregnancy [1]. This prevents allorecognition and lysis by maternal T lymphocytes. However, cells expressing low levels of surface class I molecules are usually susceptible to cytolysis by natural killer (NK) cells [2, 3]. Nevertheless, both trophoblast cells and choriocarcinoma cells (CC), which are derived from tumors of trophoblast origin, are resistant to lysis by unstimulated effector cells (i.e., NK lysis) [4–6].

What protects trophoblast cells from NK lysis in these conditions? It has been proposed that the nonclassical HLA class I molecule, HLA-G, may provide the protective effect. Indeed, HLA-G is predominantly expressed on the extravillous cytotrophoblast [7]; in other words, it is in contact with decidual NK cells. Moreover, inhibitory receptors (IR), such as CD85/ILT2/LIR-1 or CD158d/KIR2DL4, which can sense HLA-G directly, or CD94/NKG2A, which can sense HLA-E with bound leader peptides derived from HLA-G, are widely expressed on peripheral blood lymphocytes (PBL) and decidual NK cells during pregnancy [8]. Finally, transfection of LCL.721.221 [9], K562 [10], and M8 melanoma [11] cell lines with the HLA-G gene partially protects these cells from NK lysis. However, HLA-negative JAR CC are resistant to NK lysis by PBL [12]. Moreover, down-regulating or masking HLA molecules on HLA-G-positive JEG-3 CC [12, 13] did not affect their resistance to lysis. Given that CC are tumor cells, these observations may have limited significance for placental physiology. Nevertheless, fetal development was not obviously affected in a human with a homozygous HLA-G-null mutation [14]. In addition, the syncytiotrophoblast that lines the placental villi escapes lysis by maternal peripheral blood NK cells, although it is totally devoid of HLA class I molecule expression [7]. Finally, decidual NK cells were unable to kill freshly isolated extravillous trophoblast cells even when major histocompatibility complex (MHC) class I molecules were blocked by monoclonal antibody (mAb) or down-regulated by acid treatment [15]. Taken together, these observations indicate that CC as well as normal trophoblast cells need additional mechanism(s) to escape NK lysis.

The functions of NK cells depend on a balance between inhibitory signals mediated by HLA-specific IR and activating signals mediated by triggering receptors (TR). Most of these TR have been identified very recently. These include Nkp46 and Nkp30, which are both expressed on resting or activated NK cells, and Nkp44, which is confined to interleukin (IL)-2-stimulated NK cells [16]. Other TR able to (co)stimulate NK cells, such as NKG2D [17], Nkp80 [16], CD244/2B4 [18, 19], CD2 [20], and the FcγRIIIa/CD16a involved in antibody-dependent cell cytotoxicity (ADCC) [21], are also expressed on other cell types, such as T lymphocytes or monocytes. Triggering of NK cells is the result of multiple engagement of these different TR (and of still-undefined ones) by their ligands on target cells. We formulated the hypothesis that CC resistance to lysis may be caused by defective triggering of NK cells.

To test this hypothesis, we evaluated the susceptibility of JAR and JEG-3 CC to phytohemagglutinin (PHA)-dependent lysis, to ADCC, and to redirected killing after transfection of JAR CC with the human CD32/FcγRIIa cDNA

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(JAR-CD32). In all cases, effector cells were unstimulated PBL.

MATERIALS AND METHODS

Lectin, Sera, mAbs, and Cell Culture

The PHA was obtained from Murex (Dartford, U.K.). Human AB serum was prepared by pooling of AB-group healthy donor plasma. Human anti-Tja serum was obtained from Diamed (Cressier sur Morat, Switzerland). The 679.1Mc7 (mouse immunoglobulin [Ig] G1) and MOPC-195 (mouse IgG2b) control irrelevant mAbs, T11.1 (mouse IgG1) specific for CD2, UCHT-1 (mouse IgG1) specific for CD3, J4.57 (mouse IgG1) specific for CD48, T119 (mouse IgG1) specific for CD56, NKH-1 (mouse IgG1) specific for CD56, and C1.7 (mouse IgG1) specific for CD244/2B4 were purchased from Beckman Coulter (Villepinte, France). The C1K5M (mouse IgG1) specific for CD32 was obtained from Bioproducts (Gagny, France), and the IV.3 (mouse IgG1) specific for CD244/2B4 were purchased from Beckman Coulter (Villepinte, France). The C1K5M (mouse IgG1) specific for CD32 was obtained from Medarex (Annandale, NJ).

The JEG-3, JAR, and JAR-CD32 CC were cultured as described previously [12].

Expression Cloning of FcyRIIa cDNA and Transfection

The human CD32/FcγRIIa cDNA, clone 16.2, was generously provided by Dr. Kevin Moore (DNAX, Palo Alto, CA). The entire cDNA insert was cloned as a 1.7-kilobase XhoI fragment into the episomal mammalian expression vector pREP4 (Invitrogen, Leek, The Netherlands). Wild-type JAR cells were then transfected with pREP4-CD32 by using the calcium phosphate precipitation technique [22]. After 48 h the cells were placed in selective culture medium with 30 μg/ml of hygromycin (Calbiochem, San Diego, CA). After 3 wk, surviving clones were tested for CD32 expression by immunofluorescence staining with IV.3 mAb. A growing CD32-expressing JAR clone (i.e., JAR-CD32) was finally expanded and used in the experiments described below.

Total cellular mRNA was isolated from 1 × 10⁶ peripheral blood mononuclear cells (PBMC), JAR cells, and JAR-CD32 cells by using the Dynabeads mRNA Direct kit (Dynal, Compiegne, France) and then reverse transcribed with oligo(dT)25 oligonucleotides used as primers. The resulting cDNA mixtures were subjected to 35 cycles of polymerase chain reaction (PCR) amplification with oligonucleotides from the human CD32 cDNA as primers (sense primer: 5'-CTCTGAGAGCGACTCATATT-CAGTTGTTCC-3', position 220–248; antisense primer: 5'-GAA-GAATTGTGACCTGAGACCTGTC-3', position 459–490; Eurobio, Les Ulis, France) or with oligonucleotides from the human glyceraldehyde phosphate dehydrogenase (GAPDH) cDNA as primers (sense primer: 5'-ACAGTCCATGGCCACTCAG-3', position 502–522, antisense primer: 5'-GCTGTGTCACACCACTTCTTGT-3', position 807–827; Eurobio). After PCR amplification, 10 μl from each PCR product were electrophoresed on 1.6% agarose gel, and an amplified band was detected by ethidium bromide staining. The 5′X174 DNA digested by HindII was used as molecular weight marker (Eurobio).

Immunofluorescence Assay

The CC were removed from the tissue-culture flasks by trypsin-EDTA treatment, then resuspended in PBS at 1 × 10⁷ cells/ml. Cells (1 × 10⁶) were then incubated with optimal concentrations of phycoerythrin (PE)-conjugated control irrelevant mAbs, PE-conjugated C1K5M5 AB, human AB serum, or anti-Tja serum for 30 min at 4°C. Cells incubated with human sera were washed and incubated for a further 30 min at 4°C with a fluorescein isothiocyanate (FITC)-conjugated goat F(ab)₂, specific for human IgG (Cappel; ICN, Orsay, France). After washing in PBS, pellets were fixed in 0.5 ml of 0.5% paraformaldehyde PBS solution. Phenotypic analysis of PBL was performed according to standard whole-blood procedures. In brief, 100 μl of blood were incubated with 20 μl of FITC-conjugated UCH1 and 5 μl of R11-conjugated NKH1 mAbs for 15 min at 18–20°C. Then, 100 μl of Optilyse B solution (Beckman Coulter) were added. After 10 min, 1 ml of distilled water was added, and after an additional 10 min, cells were resuspended in 0.5 ml of 0.5% paraformaldehyde PBS solution. Cell surface molecule expression was analyzed by flow cytometry using a FACStar plus flow cytometer (Becton Dickinson, Le pont de Claire, France) as described previously [23].

Cytotoxicity Assay

The PBL were prepared as described previously [12] from healthy donors (age, >18 yr; no pregnant woman). The male:female sex ratio was 54:3 (JAR) and 2:4 (JEG-3) in experiments with PHA, 2:2 in ADCC experiments with anti-Tja, 4:11 in the first set of redirected killing experiments, and 6:7 in the second set of redirected killing experiments. To deplete NK cells, PBL were incubated with anti-CD16 and CD56 mAbs, then mixed with magnetic beads coated with anti-mouse IgG (Dynal). Rossetted cells were retained by a magnet.

The JEG-3, JAR, and JAR-CD32 CC were labeled with Na⁵¹CrO₄. Cytotoxicity assays were carried out with ⁵¹Cr-labeled target cells, and the results were calculated as described previously [12]. In experiments with PHA and mAb redirected killing assay, culture medium that was added contained 10 μg/ml of PHA and 5 μg/ml of mAbs, respectively. In the ADCC assay, culture medium was supplemented with 25% heat-inactivated human AB or anti-Tja sera. The PHA, sera, and mAbs were present throughout the cytotoxicity assay.

Statistics

Values represent the mean ± SD of n different experiments. Student t-test was applied using a two-tailed distribution of two samples of unequal variances on groups of data obtained in experiments with PHA. Data obtained in experiments with antibodies were analyzed in the framework of mixed models in which antibody is a fixed effect and the subject effect is random. Pairwise comparisons were adjusted for multiple comparisons by a simulation approach [24]. The significance level was P < 0.05.

RESULTS

Sensitivity of JAR and JEG-3 Cells to Lysis by Unstimulated NK Cells in the Presence of PHA

The PHA is an effective tool for cross-linking of effector and target cells and activating the effector cells [25]. Therefore, the susceptibility of JAR and JEG-3 CC to lysis was first assessed in the presence of PHA (Fig. 1). As previously shown [12], CC were resistant to lysis by freshly isolated, unstimulated PBL (1.3% ± 1.7%, n = 5, and 0.0% ± 0.0%, n = 6, at the 50:1 Effector:Target (E:T) ratio for JAR and JEG-3 CC, respectively). By contrast, they were lysed by PBL in the presence of PHA (26.3% ± 15.0%, n = 7, and 13.3% ± 4.4%, n = 6, at the 50:1 E:T ratio for JAR and JEG-3 CC; P < 0.005 each vs. CC in the absence of PHA). Depletion of CD16⁺ and CD56⁺ cells resulted in total inhibition of PHA-induced CC lysis (data not shown).

Sensitivity of JAR Cells to ADCC in the Presence of Anti-Tja Serum

The fact that CC were killed by CD16⁺ PBL in the presence of PHA suggested that they could be a potential target for NK cells by an ADCC mechanism. Human antitrophoblast alloantibodies of the IgG isotype that bind to CC were necessary to test this hypothesis. The human blood group P-system contains three glycophaspholipid antigens (Pk, P, and P1). Women with the very rare group p (defined as anti-Tja serum, contains a mixture of anti-P, anti-Pk, and anti-P1 antibodies, principally of the IgG class [26]. The trophoblast expresses paternal P and P₁ antigens [26]. We therefore investigated whether anti-Tja serum induced CC lysis by ADCC. The JAR CC were first analyzed by flow cytometry after incubation with AB or anti-Tja serum and then stained with a FITC-conjugated goat anti-human IgG. Fixation of IgG was detected with the anti-Tja serum but not with the AB serum (Fig. 2). The JAR CC were then used as targets in the cytotoxicity assay. As shown in Table 1, they were lysed by freshly isolated, unstimulated PBL in the presence of anti-Tja serum, whereas they were totally resistant to lysis in the presence of fetal calf serum (0.2% ± 0.4%, n = 5, at the 50:1 E:T ratio) or AB serum (2.4% ± 3.5%, n = 3, at the 50:1 E:T ratio). In agreement with the low surface levels of IgG binding, the...
percentage lysis in the presence of anti-Tja was moderate (15.0% ± 7.9%, n = 4, at the 50:1 E:T ratio). We verified that anti-Tja serum did not induce lysis in the absence of PBL (data not shown). Similar results were obtained with JEG-3 cells (data not shown, n = 2). These results show that CC are sensitive to ADCC.

**Sensitivity of JAR Cells to Lysis Induced by NK Cell Triggering via FcγRIIa/CD16, CD2, and CD244/2B4**

During ADCC, the IgG alloantibodies simultaneously cross-link NK cells to target cells and activate them via FcγRIIa/CD16a. The sensitivity of CC to anti-Tja-mediated ADCC suggested that their resistance to NK lysis may result from defective cross-linking or activation of NK cells. To further define the mechanism involved, we tested the sensitivity of CC in a mAb redirected killing assay. In this assay, NK-resistant FcγR-positive cells, which can bind murine IgGs by their Fc portion, are used as targets in the presence of mAbs directed to NK cell TR [20]. To study CC lysis in a mAb redirected killing assay, JAR cells were transfected with human CD32/FcγRIIa cDNA. Indeed, human CD32/FcγRIIa binds mouse IgG, and cells transfected with this cDNA have been shown to induce mAb-dependent lymphocyte activation [27]. As shown in Figure 3A, a band corresponding to CD32 mRNA was detected by reverse transcription-PCR on PBMC used as a positive control and on transfected JAR CC (i.e., JAR-CD32), but not on untransfected JAR CC. The membrane expression of the protein was verified by flow cytometry after labeling JAR and JAR-CD32 CC with the PE-conjugated anti-CD32 C1KM5 mAb (IgG1) (Fig. 3B). Cells were also incubated with saturating amounts of mouse IgG1, stained with a FITC-conjugated goat anti-mouse IgG F(ab')2, and analyzed by flow cytometry. Substantial fixation of mouse IgG1 (Fig. 3B) was observed on JAR-CD32, whereas no fixation was detectable on wild-type JAR CC.

In a first set of experiments, JAR and JAR-CD32CC were used as targets in the redirected killing assay in the presence of irrelevant mAb, anti-CD56 mAb, anti-CD16 mAb, and anti-CD3 mAb (all IgG1). As shown in Figure 4 left, wild-type JAR CC were totally resistant to lysis by freshly isolated, unstimulated PBL in the presence of all mAbs tested (4.5% ± 5.3%, n = 12; 3.7% ± 6.6%, n = 9; 3.9% ± 5.1%, n = 13; and 8.3% ± 4.6%, n = 3, at 50:1 E:T ratio for the irrelevant mAb, the anti-CD56 mAb, the anti-CD16 mAb, and the anti-CD3 mAb, respectively). The JAR-CD32 cells (Fig. 4 right) were also resistant to lysis.

**TABLE 1. Lysis of JAR CC by ADCC.**

| E:T ratio | Serum | PBL 1 | PBL 2 | PBL 3 | PBL 4 | PBL 5 |
|-----------|-------|-------|-------|-------|-------|-------|
| 50:1      | FCS   | 1     | 0     | 0     | 0     | 0     |
|           | AB    | 6     | 1     | ND    | 0     | ND    |
|           | anti-Tja | 22   | 4     | 16    | ND    | 19    |
| 25:1      | FCS   | 0     | 1     | 0     | 0     | 1     |
|           | AB    | 3     | 0     | ND    | 0     | ND    |
|           | anti-Tja | 9    | 0     | 12    | ND    | 21    |
| 12:1      | FCS   | 2     | 0     | 2     | 0     | 2     |
|           | AB    | 5     | 0     | ND    | 0     | ND    |
|           | anti-Tja | 0   | 1     | 12    | ND    | 19    |
| 6:1       | FCS   | 2     | 0     | 0     | 0     | 3     |
|           | AB    | 3     | 0     | ND    | 0     | ND    |
|           | anti-Tja | 0   | 4     | ND    | ND    | 10    |

*The ⁵¹Cr-labeled JAR CC were incubated for 4 h at 37°C with freshly isolated PBL in the presence of 25% heat-inactivated fetal calf serum (FCS), human AB serum (AB), or anti-Tja serum (anti-Tja). Cytotoxicity is expressed as the mean percentage of specific lysis of each triplicate. Experiments were performed with PBL from five different donors. ND, Not determined.*
A) Total cellular mRNA of PBMC (lanes 2 and 5), JAR (lanes 3 and 6), and JAR-CD32 CC (lanes 4 and 7) was extracted, reverse transcribed, and then amplified with specific CD32 primers (lanes 2–4) or GAPDH primers (lanes 5–7). Lane 1: \( \varphi X174 \) DNA digested by HincII.

B) JAR and JAR-CD32 were stained with a PE-conjugated isotypic irrelevant mAb (dotted histograms) or PE-conjugated anti-CD32 CIKM5 mAb (shaded histograms) and then analyzed by flow cytometry.

FIG. 3. Expression of CD32 and fixation of mouse IgG1 by JAR CC transfected with the human CD32/FcRIIa cDNA.

In a second set of experiments, lysis of CC by PBL from 13 additional donors was studied (at the 50:1 E:T ratios) in the presence of anti-CD16 mAb in relation to the percentage of NK cells in PBL. As shown in Table 2, low levels of lysis (<15% at the 50:1 E:T ratio) were observed with PBL having less than 10% of NK cells (donors 1–4). In addition, lysis of JAR-CD32 cells by PBL was studied in the presence of anti-CD2 mAb and anti-CD244/2B4 mAb (IgG1). Whereas JAR-CD32 cells were lysed significantly by PBL from only 1 of 12 donors in the presence of anti-CD2 mAb, they were lysed by PBL from 7 of 13 donors in the presence of the anti-CD244/2B4 C1.7 mAb (12.4% ± 10.8%, n = 13, at the 50:1 E:T ratio; \( P = 0.011 \) vs. JAR-CD32 in the presence of anti-CD56 mAb). The percentage lysis in the presence of the anti-CD244/2B4 was correlated to the percentage lysis in the presence of the anti-CD16 mAb \( (r = 0.81, n = 13) \) and, on average, was 2.4-fold less as compared to the latter (Table 2).

Expression of CD48 on JAR Cells

CD48 has been shown to bind CD244/2B4 [28] and to activate NK cells [29] as efficiently as an anti-CD244/2B4 mAb [19]. The JAR CC were therefore analyzed by flow cytometry after labeling with an anti-CD48 mAb. As shown in Figure 5, PBL used as a positive control expressed high levels of CD48, whereas no expression was detected on JAR CC or on JEG-3 cells (data not shown).

DISCUSSION

In the present study, we have shown that NK-resistant CC were sensitive to lysis by freshly isolated, unstimulated CD16+PBL in the presence of PHA. We also have shown that CC were sensitive to ADCC after fixation of anti-P1PPk IgG antibodies contained in anti-Tja serum from blood group p women. Because of the expression of paternal P and Pk antigens on the surface, the trophoblast is a potential target for these antibodies [26]. It is noteworthy that women belonging to the p group have a very high incidence of abortion [30]. This probably results from anti-P1PPk antibodies, because removing these antibodies during the pregnancy leads to normal childbirth [30]. Our results suggest that in addition to complement-mediated lysis, ADCC might be a potential trophoblast-destruction mechanism leading to abortion in group p women.

Using a mAb redirected killing assay, we have shown that cross-linking of unstimulated PBL to JAR-CD32 cells by mAbs directed to CD16 resulted in CC lysis, whereas cross-linking by an anti-CD56 mAb did not. The level of lysis in the presence of the anti-CD16 mAb was dependent in the presence of irrelevant, anti-CD56, and anti-CD3 mAbs (1.5% ± 2.3%, n = 13; 2.5% ± 3.8%, n = 11; and 3.1% ± 2.9%, n = 3, at the 50:1 E:T ratio, respectively).

By contrast, they were sensitive to lysis in the presence of the anti-CD16 mAb (29.5% ± 16.9%, n = 15, at the 50:1 E:T ratio; \( P < 0.01 \) vs. JAR-CD32 in the presence of irrelevant or anti-CD56 mAbs), although percentage lysis was dependent on the blood donor.

FIG. 4. CD16-dependent lysis of JAR CC.

CD48 has been shown to bind CD244/2B4 [28] and to activate NK cells [29] as efficiently as an anti-CD244/2B4 mAb [19]. The JAR CC were therefore analyzed by flow cytometry after labeling with an anti-CD48 mAb. As shown in Figure 5, PBL used as a positive control expressed high levels of CD48, whereas no expression was detected on JAR CC or on JEG-3 cells (data not shown).

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on the blood donor; particularly low levels of lysis were observed with PBL having low percentages of NK cells. This is not surprising, because lysis of K562 cells (the usual target of human NK cells) by PBL from different donors is dependent on the percentage of NK cells among PBL. Redirected killing of JAR cells was also observed in the presence of an anti-CD244/2B4 mAb, although the percentage lysis was lower as compared to those observed in the presence of anti-CD16 mAb. In a previous work, Tangye et al. [19] have shown that CD244/2B4-mediated redirected killing of P815 target cells by NK cell lines was similar to that mediated by CD16. Similar results have been obtained by using NKp46<sup>bright</sup> NK clones as effector cells [31]. The lower levels of CD244/2B4-mediated killing observed in the present study may reflect the fact that we used PBL as effector cells. Indeed, in these conditions, the anti-CD244/2B4C1.7 mAb probably cross-links fewer NK cells than the anti-CD16 mAb, because it binds to half of CD8+ TCRαβ+ PBL and to TCRγδ+ PBL, which express CD244/2B4 [18]. Whatever the case, our results thus far show that cross-linking of PBL to JAR CC by mAbs directed to TR (e.g., CD16 or CD244/2B4) abrogated their resistance to lysis, whereas cross-linking by a mAb reacting with a nontriggering surface protein (e.g., CD56) did not. Accordingly, the resistance of CC to NK lysis cells, which is independent of HLA class I-molecules [12, 13], originates primarily from defective triggering of NK cells.

This default of NK cell triggering could result from defective expression of the ligands for TR, as proposed earlier on the basis that trophoblast cells and CC did not inhibit cytotoxic activity against K562 [5, 6]. However, it has been recently shown that redirected killing of P815 target cells via CD244/2B4 requires the coengagement of NKp46 by its ligand [31]. Accordingly, the susceptibility of JAR-CD32 cells to CD244/2B4-mediated redirected killing (Table 2) support the conclusion that CC express the ligand for NKp46. This conclusion is in accordance with the fact that the anti-NKp44 mAb-mediated inhibition of CC lysis by IL-2-stimulated NK cells was increased in the presence of anti-NKp46 mAb [13]. Whereas NK cell triggering mediated by CD244/2B4 is sufficient to induce lysis of JAR CC, these cells fail to express the ligand for CD244/2B4. This result accounts for the finding that lysis of CC by IL-2-stimulated NK cells was not inhibited in the presence of mAb directed to CD244/2B4 [13]. We conclude that the resistance of CC to lysis by unstimulated NK cells results from defective activation of NK cells, which is at least partially caused by the lack of expression of ligands (e.g., CD48) involved in their triggering. This conclusion agrees with the recent finding that MICA (MHC class I-related

### Table 2. CD244/2B4-dependent lysis of JAR CC.

| Donor | % CD3<sup>-</sup>CD56<sup>-</sup> in PBL<sup>a</sup> | Anti-CD56<sup>b</sup> | Anti-CD16<sup>b</sup> | Anti-CD2<sup>b</sup> | Anti-CD244/2B4<sup>b</sup> |
|-------|--------------------------------|----------------|----------------|----------------|----------------------------|
| 1     | 2.7                           | 0              | 6              | 0              | 0                          |
| 2     | 7.8                           | 0              | 10             | 1              | 3                          |
| 3     | 5.1                           | 1              | 14             | 2              | 2                          |
| 4     | 5.1                           | 3              | 15             | 4              | 6                          |
| 5     | 12.1                          | 7              | 21             | 0              | 3                          |
| 6     | 12.2                          | 5              | 32             | 16             | 25                         |
| 7     | 14.1                          | 2              | 33             | 3              | 17                         |
| 8     | 19.9                          | 0              | 35             | 0              | 15                         |
| 9     | 10.6                          | 0              | 37             | 2              | 1                          |
| 10    | 11.1                          | 3              | 39             | 0              | 15                         |
| 11    | ND                            | 0              | 42             | 0              | 15                         |
| 12    | 11                            | 2              | 48             | 9              | 29                         |
| 13    | 13.4                          | 1              | 66             | ND             | 30                         |
| Mean ± SD | 1.9 ± 2.2   | 30.6 ± 17.0<sup>c</sup> | 3.1 ± 4.7 | 12.4 ± 10.8<sup>d</sup> |

<sup>a</sup> Blood samples were incubated for 15 min at 18–20°C with FITC-conjugated anti-CD3 UCHT1 and RD1-conjugated anti-CD56 NKH-1 mAbs. Red blood cells were lysed, and fluorescence of PBL was analyzed by flow cytometry.

<sup>b</sup> The 51Cr-labeled JAR-CD32 CC were incubated for 4 h at 37°C with PBL from 13 donors in the presence of the anti-CD56 T199 mAb, the anti-CD16 3G8 mAb, the anti-CD2 T11.1 mAb, and the anti-CD244/2B4 C1.7 mAb. The donors were classified as a function of the magnitude of JAR-CD32 CC-specific lysis in the presence of anti-CD16 mAb from the lowest to the highest. Cytotoxicity is expressed as the mean percentage of specific lysis of each triplicate at the 50:1 E:T ratio. ND, Not determined.

<sup>c</sup> P < 0.001 versus JAR-CD32 CC in the presence of anti-CD56 mAb.

<sup>d</sup> P = 0.011 versus JAR-CD32 CC in the presence of anti-CD56 mAb.
The CC and normal trophoblast cells display some different properties (e.g., syncytium never develops from CC). Thus, it is not certain that the mechanism of resistance revealed in the present study applies to normal trophoblast. However, CC and normal trophoblast cells are both resistant to lysis by unstimulated PBL or decidual NK cells (4–6) and sensitive to lysis by IL-2-stimulated PBL and decidual NK cells [12, 32]. In addition, down-regulating or masking HLA molecules on HLA-G-positive JEG-3 CC [12, 13] or on freshly isolated extravillous trophoblast cells [15] did not affect their resistance to lysis. Therefore, CC and normal trophoblast cells display very similar patterns of sensitivity/resistance to MHC unrestricted cell cytotoxicity in vitro. That fetal development was not obviously affected in a human with a homozygous HLA-G-null mutation [14] and that the HLA class I-negative syncytiotrophoblast escapes lysis by maternal peripheral blood NK cells [7] indicate that normal trophoblast cells need HLA class I-independent mechanism(s) to escape NK lysis. If our in vitro findings can be considered as representative of events occurring in vivo, they suggest that the lack of expression of the ligand for NK cell TR may represent one of them. However, additional in vivo studies are required to demonstrate that this mechanism is involved in the placental physiology. This mechanism does not exclude that trophoblast could express non-HLA class I ligands interacting with NK cell IR or produce soluble HLA class I molecules, which could anergize NK cells. These mechanisms would be critical at the interface between the HLA class I-negative syncytiotrophoblast and maternal peripheral blood NK cells. Finally, HLA class I molecule down-regulation on tumor cells is a frequent event associated with tumor invasion and development. It is tempting to speculate that the down-regulation of the ligands involved in NK cell triggering is not restricted to cells of trophoblast origin and could be used by tumor cells to escape lysis by NK cells in pathological situations.

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