Regulatory role of NKp44, NKp46, DNAM-1 and NKG2D receptors in the interaction between NK cells and trophoblast cells. Evidence for divergent functional profiles of decidual versus peripheral NK cells

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Abstract
During the first trimester of pregnancy NK cells represent >50% of the lymphoid cells present in the human decidua where they reside in close contact with trophoblast cells. Because in decidual tissues NK cell activation and function may be induced by this interaction, we analyzed the cellular ligands recognized by activating NK receptors expressed on trophoblast cells. We show that these cells primarily express the NKp44 and DNAM-1 ligands and that interaction between these ligands and their corresponding receptors results in NK cell triggering. While activated peripheral blood NK (pNK) cells lysed the trophoblast cell lines JAR and JEG3, decidual NK (dNK) cells did not. On the other hand, they released VEGF, SDF-1, IP10 and large amounts of IL-8. Interaction with K562 target cells was exploited to induce optimal NK cell triggering, allowing a parallel, quantitative assessment of both cytolytic activity and cytokine production elicited by dNK cells. While dNK cells were unable to kill K562 even at high effector:target (E:T) ratios, they released large amounts of IL-8 also at low E:T ratios, a scenario compatible with dNK trophoblast cells interaction occurring within decidual tissues.

Introduction
The trophoblast, the outermost layer of the human placenta, is in direct contact with the maternal immune system. The lack of classical HLA molecules on trophoblast cells, with the exception of HLA-C in the extravillous trophoblast during the first trimester of pregnancy (1–3), prevents allorecognition by maternal T lymphocytes (4) but poses the problem of susceptibility to killing by NK cells (5). Notably, during the first trimester of pregnancy, NK cells are particularly abundant in the decidua as they represent 50–90% of the total lymphoid cells (6). They are phenotypically similar to the CD56bright peripheral blood NK (pNK) cell subset (7). In general, cells expressing low levels of surface HLA class I molecules are susceptible to lysis by NK cell populations (2, 3, 8–11). Despite the low levels of HLA class I expression, both normal trophoblast cells and choriocarcinoma (CC) cell lines (derived from tumors of trophoblastic origin) were found to be resistant to lysis by freshly isolated pNK cells (12). The actual mechanism responsible for such resistance remains unclear. Notably, trophoblast cells express non-classical HLA class I molecules, including HLA-E and HLA-G, that could be responsible for blocking the NK cell function upon interaction with HLA class I-specific inhibitory receptors (13–16). However, mAb-mediated blocking of either inhibitory receptors on NK cells or HLA-E and HLA-G molecules on trophoblast cells failed to restore the NK-mediated lysis of CC cell lines (17). Thus, it has been proposed that mechanisms different...
from HLA class I-specific receptor-mediated inhibition may play a role in trophoblast protection from pNK cells (18).

NK cell activation resulting in target cell lysis and/or cytokine production is mediated by various activating receptors, including Nkp46, Nkp30 and Nkp44, collectively referred to as natural cytotoxicity receptors (NCRs) (19, 20). NKG2D (21, 22) and DNAM-1 (CD226) (23). NCRs belong to the Ig superfamily and represent crucial receptors for NK cell function as they trigger both cytolytic activity against various target cells and cytokine production. NKP46 and Nkp30 are expressed on both resting and activated NK cells, while Nkp44 is expressed only upon NK cell activation (19, 20, 24–26). The cellular ligands recognized by NCRs are still unknown while viral ligands have been identified (27). In addition, recently, BAT3 has been reported to function as a putative Nkp30 ligand (28). NKG2D (29) is an activating receptor expressed not only by NK cells but also by T lymphocytes; its cellular ligands are represented by MICA/B (stress-inducible molecules MHC class I-related chain A/B) and UL16-binding proteins (ULBPs) (21, 30, 31). DNAM-1 is expressed by virtually all human NK cells and also by subsets of T lymphocytes or monocytes. The major DNAM-1 ligands are the poliovirus receptor (PVR; CD155) and Nectin-2 (PRR-2; CD112), two closely related molecules belonging to the Nectin family (23). While, as mentioned above, both normal and tumor trophoblast cells are resistant to fresh pNK cells, they are killed by IL-2-cultured pNK cells (9, 18, 32). However, the ligands expressed on trophoblast cells that are recognized by IL-2-induced pNK cells are still unknown.

Regarding decidual NK (dNK) cells, the surface density of the principal activating receptors, including NKP46, NKP30, NKG2D and DNAM-1 is comparable to that of pNK cells. However, although dNK cells express the CDS6bright surface phenotype, they are substantially different from CDS6bright pNK cells (33). Thus, dNK cells contain both perforin and granzymes at much higher levels than CDS6bright CD16- pNK cells in amounts comparable to those of CDS6-CD16+ pNK cells (33, 34). This observation suggested that dNK cells may be potentially cytotoxic. However, conflicting data exist on this functional capability of dNK cells (35). Recently, freshly isolated human dNK cells have been shown to display low cytotoxicity toward MHC class I target cells as a consequence of their inability to form activating synapses (36). In decidual tissues, dNK cells reside in close cell-to-cell contact with trophoblast cells and produce abundant cytokines. Thanks to the secretion of IL-8, VEGF, IP10 and SDF-1 (that are not released by pNK cells) (37, 38) they may exert a regulatory role on angiogenesis and placental developmental processes, including cell migration, trophoblastic growth, differentiation and invasion (39). Additional studies confirmed that dNK cells are poorly cytolytic (33, 36, 37), while they produce cytokines, chemokines and angiogenic factors (40). However, no direct comparative assessment has been performed on these two functional capabilities during dNK–trophoblast cell interaction.

In this study, we examined trophoblast cells for the surface expression of the ligands recognized by various activating NK receptors. We provide evidence that trophoblast cells primarily express the ligands of NKP44 and DNAM-1 and that dNK cells express functional NKP44 and DNAM-1 receptors. Upon interaction with trophoblast cells, dNK cells released abundant IL-8 even at low dNK/trophoblast cell ratios while no cytolytic activity could be induced. High cytokine production and lack of cytolytic activity was also documented upon interaction of dNK cells with K562, a target cell particularly susceptible to pNK cell-mediated killing.

**Methods**

**mAbs**

The following mAbs were produced in our laboratory: JT3a (IgG2a, anti-CD3), KD-1 (IgG2a, anti-CD16) and the following mAbs were kindly provided by D. Pende or A. Moretta: c218 (IgG1, anti-CD56), BAB281 and KL247 (IgG1 and IgM, respectively, anti-NKP46), Z231 and K338 (IgG1 and IgM, respectively, anti-NKP44), AZ20 and F252 (IgG1 and IgM, respectively, anti-NKP30), F22 and F5 (IgG1 and IgM, respectively, anti-DNAM-1), L95 (IgG1, anti-PVR), L14 (IgG2a, anti-Nectin-2), BAT221 (IgG1, anti-NKG2D), BAM195 (IgG1, anti-MICA), SBO2 (anti-MICB) and A6-136 (IgM, anti-HLA class I). mAbs M295 (IgG1, anti-ULBP1), M310 (IgG1, anti-ULBP2) and M551 (IgG1, anti-ULBP3) were kindly provided by Amgen (Seattle, WA, USA). Anti-CD56–PC5 (Immunotech, Marseille, France), anti-CD3–PE, anti-CD16–FITC (HIT3, IgG2a; BD Pharmingen, San Diego, CA, USA) and anti-IFNγ–PE (IgG1, BD Pharmingen) were also used.

**Isolation and culture of NK cell populations**

Peripheral blood lymphocytes were isolated from peripheral blood from healthy donors using Ficoll-Hypaque density gradient either directly or after enrichment for NK cells using Rosettesep (StemCell Technologies, Vancouver, British Columbia, Canada). Decidual samples were obtained at 9–12 weeks of gestation from singleton pregnancies of mothers requesting termination of the pregnancy for social reasons or who were undergoing evacuation of retained products of conception following spontaneous pregnancy failure. The study was approved by the relevant institutional review boards and all patients gave their written informed consent according to the Declaration of Helsinki. Decidual tissue was separated as previously described (33). NK cells were cultured in the presence of 100 U ml−1 rIL-2 (Proleukin; Chiron, Emeryville, CA, USA) to obtain activation and great expansions of NK cell populations. FACS sorting was performed on FACS Aria (BD Biosciences, San Jose, CA, USA). Post-sorting analysis showed >98% purity.

**Flow cyt fluorimetric analysis and immunofluorescence microscopy**

Surface phenotype of NK cells and CC were assessed by indirect immunofluorescence using the appropriate mAb or fusion protein followed by PE-conjugated isotype-specific goat anti-mouse (Southern Biotechnology, Birmingham, AL, USA) or goat anti-human IgG second reagent (Jackson ImmunoResearch Laboratories, West Grove, PA, USA). For the cytokine assay dNK or pNK cells were incubated with JEG3 or JAR cell lines or normal trophoblast cells in the presence of anti-DNAM mAb and/or anti-NKG2D and/or NKP44-Fc, as control, an isotype-matched irrelevant mAb [effector:target (E:T)]
ratios used was 10:1]. GolgiStop (BD Biosciences) was added. Cells were then washed and the intracellular staining using an anti-IFNγ-PE mAb was performed. Cells were analyzed in a FACSCalibur flow cytometer and the analysis was performed using cell CeliQuest software (BD Biosciences). For confocal microscopy, cells were stained with anti-Nectin-2 mAb or fusion proteins. All washes were performed in PBS with 2% fetal calf serum. After staining slides were mounted and visualized through a x60 oil immersion lens with an inverted Olympus IX-81 microscope. Images were analyzed using Flowlview FV500 and exported as .TIF files.

Preparation of soluble chimeric receptors

The sequences coding for the extracellular portion of NKP46, NKP30, NKP44 and DNAM-1 receptors were amplified starting from the corresponding cDNAs inserted in the pcDNA3.1TOPO-V5 plasmid using the following primers: 5′ CAGGGCATCTCGAGTCTGAGGGAGCTGCAGGGCCAGGG (NKP46 XhoI up) and 5′ GACTAGGATCCGCATGATTC-CTGGCGATTGATCC (NKP46 BamHI dw); 5′ CAGGGCATCTCGAGTCTGAGGGAGCTGCAGGGCCAGGG (NKP30 XhoI up) and 5′ GACTAGGATCCGCATGATTC-CTGGCGATTGATCC (NKP30 BamHI dw); 5′ CAGGGCATCTCGAGTCTGAGGGAGCTGCAGGGCCAGGG (NKP44 XhoI up) and 5′ GACTAGGATCCGCATGATTC-CTGGCGATTGATCC (NKP44 BamHI dw); 5′ ACGCTGACAACTGACGCTTTCAA-ACAG (DNAM-1 SalI up) and 5′ CGGGATCCTGTAGTATCGG-GCTCAGG (DNAM-1 BamHI dw). Amplification was performed with Platinum Taq High Fidelity (Invitrogen, Carlsbad, CA, USA) for 20 cycles (30 s at 95°C, 30 s at 58°C and 1 min at 72°C) except for NKP44 for which the annealing temperature was 65°C. The PCR products were digested with SalI or XhoI and with BamHI restriction enzymes and subcloned in the SalI/BamHI-digested pRB1-2B4Fcmut vector (kindly provided by M. Falco and G. Gaslini, Genova) in frame with the sequence coding for the human IgG1 portion that was mutagenized in order to obtain a mutated Fc that does not bind to Fc receptors. The nucleotide sequence for the extracellular region of the different receptors was checked using a d-Rhodamine Terminator Cycle Sequencing Kit and a 377 Applied Biosystems DNA sequencer.

The different constructs were stably transfected into HEK293 cell line (human embryonic fibroblast) utilizing Fugene6 (Roche, Monza, Italy). Supernatants were collected from the cell transfecants cultured in Dulbecco’s modified Eagle’s medium/10% ultra-low IgG fetal calf serum (Invitrogen) and the soluble Fc molecules were purified by affinity chromatography utilizing Protein A Sepharose 4 Fast Flow (Amersham Biosciences). Purified proteins were checked by SDS-PAGE followed by silver staining and by ELISA utilizing mAbs specific for the different receptors.

Immunohistochemistry

For immunohistochemical analysis, CC cell lines were formalin fixed and paraffin embedded. The 6-μm sections were deparaffinized and subsequently exposed to 0.3% hydrogen peroxide-methanol solution to quench the endogenous peroxidase activity. The sections were layered for 30 min with the fusion proteins NKP44-Fc and NKP30-Fc (final dilution 10 μg ml⁻¹). HRP-conjugated goat anti-human antisera (Dako-Envision) and 3-amino-9-ethylcarbazole (Novocastra) were subsequently added. Finally, specimens were counterstained in Mayer’s haemalum (blue in lithium carbonate, dehydrate, clear, mount in balsam) and then coverslipped. All incubations were performed at room temperature. As negative control, primary antibody was omitted or replaced with an isotype-matched control mAb of irrelevant specificity. The results were interpreted under light microscope by two independent observers.

Cytolytic activity

NK cell populations (dNK and pNK) were tested for cytolytic activity in a 4-h chromium 51 (51Cr) release assay, as previously described (24). JEG3 and K562 cell lines were used as targets. Briefly, JEG3 was detached with trypsin/EDTA solution, labeled with 51Cr and plated in 96-well V-bottom microplates at different E:T ratios. All experiments were performed in duplicates. Data are expressed as percentage of lysis of target cells.

Measurement of cytokine production following mAb-mediated cross-linking of activating receptors on dNK cells

The experiments were performed using as target cells JEG3 or normal trophoblast cells as target incubated with fresh purified dNK cells in the presence or in the absence of mAbs-mediated masking (IgM isotype). After overnight incubation, supernatants were collected and analyzed by ELISA assay specific for in vitro quantitative determination of IL-8 (Invitrogen) according to the manufacturer’s instructions.

Culture supernatants derived from pNK and dNK cells stimulated overnight with phorbol-myristate-acetate (PMA) (50 ng ml⁻¹) and ionomycin (25 ng ml⁻¹) (Sigma–Aldrich, St Louis, MO, USA) were analyzed for the release of a large panel of human T₃/T₄ cytokines and chemokines. To this end, we performed cytokfluorometric analysis on culture supernatants collected using ELISA Multiplex kits (Bioclarma, Turin, Italy) and Flow Cytomix kit T₃/T₄ immunoassay (Bender Med System, Wien, Austria) according to the manufacturer’s instructions.

Results

Trophoblast cells express ligands recognized by activating NK receptors

In order to define whether CC cell lines (JEG3 and JAR) express ligands recognized by activating NK receptors, cytokfluorometric analysis was performed using mAbs specific for known ligands of such receptors. Figure 1(A and B) shows the levels of expression of the DNAM-1 and NKG2D ligands. JAR cell line expressed both PVR and Nectin-2 (DNAM-1 ligands), while JEG3 cell line expressed only Nectin-2. ULBP5 (NKG2D ligands) were detected in low amounts on JEG-3 cell line. Analysis of surface HLA class I molecules showed, in agreement with previous reports (16), that JEG3 cell line constitutively expressed HLA-G1 molecules, while JAR was HLA class I negative (data not shown). In order to further analyze the surface expression of the ligands...
recognized by NKp30, NKp44 and NKp46 (or DNAM-1 as control), soluble fusion proteins were used. Binding of NKp44*Fc and DNAM-1*Fc, but not of NKp30*Fc and NKp46*Fc, could be detected (Fig. 1A and B). In parallel, the presence of typical trophoblast markers, such as cyto-keratin 7, was determined (data not shown). Consistent results were obtained on JEG3 cell line by confocal microscopy (Fig. 1C). In order to directly assess whether also normal trophoblast cells present in the decidua expressed PVR, Nectin-2 and NKp44 ligand, adherent (i.e. highly enriched in trophoblast cells) and non-adherent decidual cell fractions were stained with specific mAbs and soluble fusion protein (37). Figure 1(D) shows that adherent cells expressed high levels of PVR, Nectin-2 and NKp44 ligand and were HLA-G+ (data not shown).

Immunohistochemical analysis on paraffin-embedded samples confirmed binding of NKp44*Fc on CC cell lines. Figure 1(E–H) shows either scattered positive cells or small clusters. NKp44*Fc stained the cell surface and displayed also a weak diffuse cytoplasmatic reactivity (Fig. 1E–G). NKp30*Fc did not stain these CC cell lines (Fig. 1F–H).

Analysis of the activating or inhibitory molecular interactions involved in the recognition of CC cell lines by pNK cells
While both human trophoblast cells and CC cell lines were shown to be resistant to cytotoxicity mediated by fresh pNK cells, they were lysed by pNK cell populations or pNK cell clones expanded in IL-2 (9, 12, 32). In this set of experiments, effector cells were represented by highly purified pNK (derived from different healthy donors) or dNK cells that had been cultured in IL-2 for 10–20 days. Target cells were represented by JEG3 cell line. Figure 2 shows a comparative analysis of the cytolytic activity mediated by dNK cells (gray line) or pNK cells (black line). It can be seen that pNK cells lysed JEG3 cell line, while dNK cells did not. In addition, no increases of cytolytic activity were detected in tests performed in the presence of the anti-HLA class I mAb (A6.136). Because this mAb reacts with both classical and non-classical (including HLA-G and HLA-E) HLA class I molecules, these data support the notion that HLA-G and HLA-E molecules do not protect JEG3 cell line from dNK cell-mediated lysis (9, 18). Similar results were obtained in five independent experiments using IL-2-activated pNK cells or IL-2-activated dNK cells from five different healthy donors.
Molecular interactions between NK and trophoblast cells

Fig. 2. Cytolytic activity against trophoblast cell lines by IL-2-activated dNK cells compared with pNK cells. IL-2-activated pNK cell populations from a representative healthy donor (black lines) and IL-2-activated dNK cell populations (gray lines) were analyzed for their cytolytic activity against JEG3 cell line at different E:T ratios. Open symbols refer to the same effector/target cell combinations in the absence of blocking mAbs. mAbs specific for one or another activating NK receptor were used alone or in combination (9). Figure 3(A and B) shows the results of a representative experiment out of eight performed, in which IL-2-activated pNK cells were obtained from five different donors and JEG3 or JAR target cells were used. Anti-2B4 mAb was used as negative control, since neither JEG3 nor JAR cells express CD48 (the 2B4 ligand). mAb-mediated masking of NCRs (NKp30, NKp44, NKp46) resulted in partial inhibition of lysis which was maximal using anti-NKp44 mAb ($P < 0.0286$), intermediate with anti-NKp46 ($P < 0.05$) and minimal or absent with anti-NKp30 ($P > 0.05$ not statistically significant). Blocking of DNAM-1 resulted in a strong inhibition of lysis of both target cells ($P < 0.02$). Notably, the combined use of NKp44 and DNAM-1 virtually abrogated target cell lysis ($P < 0.0079$). This implies that these receptors exert a primary role in the recognition and lysis of JEG3 and JAR cell lines by IL-2-activated pNK cells. mAb-mediated masking of NKGD2 resulted in partial inhibition of lysis in the case of JEG3, which express ULBPs ($P < 0.05$), but not of JAR target cells. Our data are in line with a previous study showing that NKp44 has a major involvement in CC cell killing (9). In order to evaluate our data, a non-parametric $t$-test parallel analysis was used to compare cytotoxicity induced by different NK receptors. Differences were considered significant when $P$-values were $<0.05$.

We further analyzed whether the same NK activating pathways were capable of inducing IFN-$\gamma$ production by NK cells following their interaction with CC cell lines. IL-2-activated polyclonal pNK cell populations were incubated with JEG3 or JAR cell line for 4 h in the presence of monensin (GolgiStop). As shown in Fig. 3(C), IL-2-activated pNK cells cultured in the absence of stimulating CC cells were negative for intracellular staining, while those exposed to JEG3 or JAR cell line were positive for IFN-$\gamma$ production. The effect of addition of specific mAbs-mediated masking for different activating NK receptors is shown in Fig. 3(D). In cultures containing anti-2B4 mAb, the proportions of IFN-$\gamma$-producing cells were identical to those of dNK cells incubated with JEG3 or JAR cell line in the absence of mAb. Decreased proportions of IFN-$\gamma$-producing cells were detected in the presence of mAbs to NKp44, NKp46, DNAM-1 or NKG2D ($P < 0.005$); in co-culture with JAR cell line, anti-NKG2D mAb had no effect. These data provide evidence that the interactions between NKp44, NKp46, DNAM-1 and, in part, NKG2D ligands are involved not only in the NK-mediated cytolytic activity but also in the induction of IFN-$\gamma$ production by IL-2-activated pNK cells. Similar results were obtained in seven independent experiments performed with cells from seven different healthy donors.

Since CC cell lines express DNAM-1 and NKp44 ligands, we further analyzed whether these molecules were indeed involved in the killing of JEG3 cell line. To this end, cytotoxicity assays were performed in the presence or in the absence of blocking mAbs. mAbs specific for one or another activating NK receptor were used alone or in combination (9). Figure 3(A and B) shows the results of a representative experiment out of eight performed, in which IL-2-activated pNK cells derived from five different donors and JEG3 or JAR target cells were used. Anti-2B4 mAb was used as negative control, since neither JEG3 nor JAR cells express CD48 (the 2B4 ligand). mAb-mediated masking of NCRs (NKp30, NKp44, NKp46) resulted in partial inhibition of lysis which was maximal using anti-NKp44 mAb ($P < 0.0286$), intermediate with anti-NKp46 ($P < 0.05$) and minimal or absent with anti-NKp30 ($P > 0.05$ not statistically significant). Blocking of DNAM-1 resulted in a strong inhibition of lysis of both target cells ($P < 0.02$). Notably, the combined use of NKp44 and DNAM-1 virtually abrogated target cell lysis ($P < 0.0079$). This implies that these receptors exert a primary role in the recognition and lysis of JEG3 and JAR cell lines by IL-2-activated pNK cells. mAb-mediated masking of NKGD2 resulted in partial inhibition of lysis in the case of JEG3, which express ULBPs ($P < 0.05$), but not of JAR target cells. Our data are in line with a previous study showing that NKp44 has a major involvement in CC cell killing (9). In order to evaluate our data, a non-parametric $t$-test parallel analysis was used to compare cytotoxicity induced by different NK receptors. Differences were considered significant when $P$-values were $<0.05$.

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Fresh dNK cells produce IL-8 upon interaction with trophoblast cells: role of DNAM-1 receptor

As shown above, IL-2-activated dNK cells were unable to kill CC cell lines (Fig. 2). Therefore, analysis of cytotoxicity against these target cells would clearly be inadequate as a read-out system to study the induction of dNK cell activation. To this end, the ability of dNK cell to release cytokines was analyzed. We first investigated which cytokines were produced by freshly isolated dNK cells upon stimulation with PMA and ionomycin for 8 h. As shown in Fig. 4(A), dNK cells were poor IFN-$\gamma$ producers, while they released large amounts of IL-8, SDF-1, VEGF and IP-10. Notably, these cytokines were not produced or produced at low levels by pNK cells. IL-8 production was further used as a read-out system to analyze dNK cell activation upon interaction with trophoblast cell lines. Fresh dNK cells spontaneously produced IL-8 upon interaction with JEG3 cell line (Fig. 4(B)). Next, the IL-8 release assay was used as a read-out system to analyze the receptor–ligand pairs involved in dNK–trophoblast cell interaction. In these experiments, the role of different NK receptors was evaluated by analyzing the effects of mAbs specific for one or another receptor. Figure 4(C and D) shows the results of a representative experiment out of four performed, using fresh dNK cell populations derived from three different donors and JEG3 cell line (Fig. 4(C)) or fresh trophoblast cells (Fig. 4(D)) as target cells. Anti-CD56 mAb was used as control. Although not shown, anti-CD56 mAb did not exert any inhibitory effect. On the other hand, decreased proportions of IFN-$\gamma$-producing cells were detected in the presence of mAbs to NKp44, NKp46, DNAM-1 or NKG2D ($P < 0.05$). Notably, these cytokines were not produced or produced at low levels by pNK cells. IL-8 production was further used as a read-out system to analyze dNK cell activation upon interaction with trophoblast cell lines. Fresh dNK cells spontaneously produced IL-8 upon interaction with JEG3 cell line (Fig. 4(B)). Next, the IL-8 release assay was used as a read-out system to analyze the receptor–ligand pairs involved in dNK–trophoblast cell interaction. In these experiments, the role of different NK receptors was evaluated by analyzing the effects of mAbs specific for one or another receptor. Figure 4(C and D) shows the results of a representative experiment out of four performed, using fresh dNK cell populations derived from three different donors and JEG3 cell line (Fig. 4(C)) or fresh trophoblast cells (Fig. 4(D)) as target cells. Anti-CD56 mAb was used as control. Although not shown, anti-CD56 mAb did not exert any inhibitory effect. On the other hand, decreased concentrations of IL-8 were detectable upon mAb-mediated masking of NKp46, NKG2D or DNAM-1 (Fig. 4(C)). This effect
was more evident when combinations of masking mAbs were used ($P < 0.05$). These data provide evidence that the interactions between DNAM-1, NKG2D and NKp46 and their respective ligands are involved in the increase of cytokine production by fresh dNK cells upon interaction with fresh normal trophoblast cells and CC cell lines. Notably, a partial inhibitory effect was also noticed with anti-NKp46 mAb suggesting that, despite the inability to detect binding of the fusion protein to trophoblasts, this NCR may also be involved in the response.

Analysis of cytolytic activity and of IL-8 and IFN-$\gamma$ production by freshly isolated or IL-2-cultured dNK cells

In this set of experiments, dNK or pNK cells either freshly isolated or exposed to IL-2 for 18 h were analyzed in parallel for their cytolytic activity against K562 target cells and for their ability to release IL-8 (or IFN-$\gamma$) upon exposure to K562 cells. Notably, K562 cells are well known as a tumor target highly susceptible to NK-mediated lysis. As shown in Fig. 5(A), dNK cells (gray line) did not lyse K562 cells even at high E:T ratios, while these target cells were efficiently killed by pNK cells (black line). Cytolytic activity did not increase in dNK cells incubated in IL-2. In contrast, IL-2-induced pNK cells greatly enhanced their killing capability. Regarding the cytokine production upon interaction with the same target cells, Fig. 5(B) shows that dNK cells (but not pNK cells) produced high amounts of IL-8 (Fig. 5B) even at low E:T ratios. Interestingly, dNK cells exposed to IL-2 for 18 h reduced, at least in part, their capability of producing IL-8. Figure 5(C) shows IFN-$\gamma$ production by dNK and pNK cell populations. Fresh dNK cells produced very low amounts of IFN-$\gamma$ that was up-regulated upon cell exposure to IL-2 for 18 h. As expected, IL-2-induced pNK cells displayed great increases of their IFN-$\gamma$ production. These data clearly indicate that dNK cells isolated ex vivo are not cytolytic even against highly susceptible target cells at high E:T ratios. However, they release abundant IL-8 (Fig. 5B) even at low E:T ratios. Notably, in placental tissues, dNK and trophoblast cells may interact in comparable proportions.

Another relevant question to ask is whether dNK cells represent a functionally stable NK cell subset or whether, as suggested by the experiments shown in Fig. 5(B), external stimuli could modify their function, for example their ability to produce IL-8. To address this question, we analyzed the effect of dNK cell exposure to IL-2 for long-time intervals. We found that the IL-8 production was virtually abrogated in dNK cells cultured for 20 days in IL-2 (Fig. 5D). These data would support the notion that production of high amounts of IL-8 may be a transient functional characteristic of dNK cells, possibly induced by a particular cytokine and/or hormonal milieu.
Discussion

In the present study, we analyzed the molecular interactions occurring between NK cells (isolated either from peripheral blood or decidual tissues) and trophoblast cells (41, 42). First, we show that trophoblast cells express the surface ligands specifically recognized by DNAM-1- and NKp44-activating NK receptors. Recognition of these ligands on CC cell lines induced, in IL-2-activated pNK cell populations, both cytolytic activity and cytokine production. Unlike pNK cells, IL-2-activated dNK cells did not kill trophoblast cell lines. Analysis of dNK cells freshly isolated from decidua revealed an abundant production of cytokines. However, they were not ‘classical’ NK cell cytokines, such as IFN-γ, but rather a set of factors including IL-8, VEGF, IP-10 and SDF-1 which are thought to play a relevant role in placental development. Our data also indicate that this dNK cell function can be induced upon interaction with trophoblast cells. During the first trimester of human pregnancy, fetal trophoblast cells invade the decidua and its blood vessels. Defects in trophoblast invasion result in incomplete spiral artery remodeling (43), causing reduced blood flow to fetoplacental tissues (37). The observation that in early pregnancy dNK cells are in close contact with the invading trophoblast and are thus exposed to ligands recognized by their activating receptors suggests that dNK cells may be triggered in vivo by this cellular interactions and secrete cytokines. Along this line, the observation that fresh dNK cells release large amounts of cytokines such as VEGF, IP10 and SDF-1 is particularly suggestive of an important physiological role played by these cells in modeling placental vessels and tissues (7, 40, 44). More puzzling appears the secretion of abundant IL-8 (37). IL-8 has been known for long time as a major chemoattracting neutrophils and other leukocytes at the site of inflammatory responses. However, more recent data would suggest a wider capability of inducing cell
In particular, it has been reported that endothelial cells can migrate in response to IL-8, thus contributing to building/remodeling new vessels in cooperation with angiogenic factors (e.g. VEGF) (38).

In early pregnancy, NK cells characterized by the CD56brightCD16– surface phenotype represent >50% of decidual lymphoid cells. At present, it is difficult to establish whether dNK cells may represent a peculiar stage of NK cell differentiation or a different cell lineage or be the result of conditioning signals delivered by the placental microenvironment. Because of their CD56brightCD16– surface phenotype, they are reminiscent of the minor CD56bright NK cell population present in peripheral blood. However, important differences exist between these two NK cell subsets (6). Although CD56brightCD16– pNK cells, similar to dNK cells, are poorly cytolytic, they secrete IFN-γ, granulocyte macrophage colony-stimulating factor and tumor necrosis factor-α and do not produce significant amounts of IL-8 (as well as VEGF or...
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We show that both trophoblast cell lines (JEG3 and JAR cell lines), whereas the expression of PVR was confined to fresh trophoblast cells and JEG3 cell line. Immunohistochemical analysis and confocal microscopy confirmed that the Nkp44 ligand is expressed by trophoblast cell lines. In line with previous studies (9), we show that Nkp44 is involved in the process of recognition and killing of CC cell lines by IL-2-activated pNK cells. In addition, we provide direct evidence that also the DNAM-1 receptor plays a relevant role in the interaction between NK and CC cell lines. Blocking experiments using mAbs specific for activating NK receptors revealed that lysis of trophoblast cell lines may also partially depend on Nkp46 (for both JEG3 and JAR cell lines) and on NKG2D for JEG3 cells. The fact that binding of soluble Nkp46 Fc to CC cell lines could not be detected might reflect a level of expression of Nkp46 ligand on these cells under the limit of detection. It should be stressed that Nkp44 is only expressed upon NK cell activation. Different from CD69 that is expressed promptly upon NK cell triggering, Nkp44 expression requires several days of culture in IL-2. In our experiments, freshly isolated dNK cells did not express surface Nkp44 (33), while they did express CD69 (data not shown), thus suggesting that during normal pregnancy, dNK cells may be unable to interact with the Nkp44 ligand expressed at the trophoblast cell surface.

In conclusion, in view of our present findings, one may ask whether lack of appropriate triggering of dNK cells by trophoblast cells might be involved in pathological events such as recurrent miscarriages. In this context, it will be important to evaluate the surface expression of relevant ligands for activating NK receptors on trophoblast cells and of activating receptors on dNK cells. Indeed, the lack of appropriate receptor–ligand interactions may lead to reduced release of the peculiar set of cytokines that are produced by dNK cells upon stimulation. As mentioned above, these cytokines may play an important role in contributing to building/remodeling of new vessels during pregnancy. On the other hand, one may speculate that an excessive dNK cell triggering due to abnormally high numbers of dNK cells and/or over-expression of ligands for activating NK receptors may lead to an overwhelming growth of endometrial tissues. Interestingly, in preliminary experiments, cells resembling dNK cells have been detected in endometriotic tissues (unpublished results). Further studies along this line may offer important clues for a better understanding of pregnancy-associated pathological events and/or endometriosis.

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Abbreviations

CC choriocarcinoma
dNK decidual NK
E:T effector:target
NCR natural cytotoxicity receptor
PMA phorbol-myristate-acetate
PVR poliovirus receptor
ULBP UL16-binding protein

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