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*J Immunol* 2008; 181:3009-3017; doi: 10.4049/jimmunol.181.5.3009

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Critical and Differential Roles of NKp46- and NKp30-Activating Receptors Expressed by Uterine NK Cells in Early Pregnancy

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In early human pregnancy, uterine decidual NK cells (dNK) are abundant and considered as cytokine producers but poorly cytotoxic despite their cytolytic granule content, suggesting a negative control of this latter effector function. To investigate the basis of this control, we examined the relative contribution to the cytotoxic function of different activating receptors expressed by dNK. Using a multicolor flow cytometry analysis, we found that freshly isolated dNK exhibit a unique repertoire of activating and inhibitory receptors, identical among all the donors tested. We then demonstrated that in fresh dNK, mAb-specific engagement of NKp46-, and to a lesser extent NKG2C-, but not NKp30-activating receptors induced intracellular calcium mobilization, perforin polarization, granule exocytosis and efficient target cell lysis. NKp46-mediated cytotoxicity is coactivated by CD2 but dramatically blocked by NKG2A coengagement, indicating that the dNK cytotoxic potential could be tightly controlled in vivo. We finally found that in dNK, mAb-specific engagement of NKp30, but not NKp46, triggered the production of IFN-γ, TNF-α, MIP-1α, MIP-1β, and GM-CSF proinflammatory molecules. These data demonstrate a differential, controlled role of NKp46- and NKp30-activating receptors expressed by dNK that could be critical for the outcome of pregnancy and the killing of uterine cells infected by pathogens. The Journal of Immunology, 2008, 181: 3009–3017.

Natural killer cells, a major component of innate immunity, not only are present in the circulating blood, lymphoid and nonlymphoid organs, and thymus (1, 2) but also in nonpregnant and pregnant human endometrium (3–5). At the site of implantation, endometrium differentiates into decidua basalis (6). The hallmark of this decidua during early gestation is the high number of NK cells (50–70% of leukocytes present locally) that exhibit a unique phenotype and specific functions (4, 7–10). A large number of differentially expressed genes have been identified in decidual NK cells (dNK)3 and peripheral blood (PB)-NK (11). The major dNK subset is CD56bright CD16neg, which contrasts with PB-NK whose major subpopulation is CD56dim CD16pos (12, 13).

Although dNK express a number of activating receptors as well as high levels of perforin and granzyme A and B, different reports indicated that their lytic granules were not released in normal pregnancy and that they display limited cytolytic activity (11, 13, 14). Two major mechanisms have been described to explain this lack of NK effector function. Kopnow et al. (15) found that dNK failed to polarize their microtubules and perforin granules to the immunological synapse. Vacca et al. (13) reported inhibitory function of the 2B4 receptor following its specific CD48 ligation with trophoblast cells. However, most of these studies have used long term IL-2 cultured clonal or polyclonal dNK cells. Much less is known about the requirement for activation of cytotoxic function in freshly isolated dNK cells. To our knowledge, no study to date has evaluated the respective functions and control of single receptors expressed by dNK. In this study, we show that fresh dNK exhibit significant cytotoxic activity after specific engagement of NKp46 activating receptor, and that NKG2A coengagement dramatically blocked this effector function. In contrast, NKp30- but not NKp46-activating receptor engagement in dNK resulted in the production of proinflammatory cytokines and chemokines.

Materials and Methods

Purification of dNK and PB-NK

After informed consent, samples of first trimester decidua (8–12 wk gestation) from healthy women undergoing vaginal elective termination of pregnancy were obtained. Decidual tissue was washed extensively, minced in phosphate buffered saline (PBS), and minced in 70% ethanolic HEPES, penicillin 100 IU/ml, streptomycin 100 μg/ml, and fungizone 25 μg/ml. After digestion with collagenase and elastase, and 2 hr of shaking, the cells were centrifuged at 1500 g for 10 min. The pellet was resuspended in 15 ml of buffer (RPMI 1640, 1 M HEPES, penicillin 100 IU/ml, streptomycin 100 μg/ml, and fungizone 25 μg/ml).
NKp46- AND NKp30-MEDIATED dNK EFFECtor FUNCTIONS

IU/ml) and loaded onto Ficoll-Hypaque density gradient (Amersham Bio-tech) to purify the lymphocyte population. The mononuclear cell layer was collected, washed, suspended in the same buffer supplemented with 10% FCS, and allowed to adhere to tissue culture plate overnight at 37°C in a humidified 5% CO2 incubator. The nonadherent cell fraction was collected and NK cell population subsequently purified by MACS negative selection (Miltenyi Biotech). These dNK were more than 96% CD3neg/CD56pos, as determined by flow cytometry. PB-NK were isolated and purified as previously described (16).

Flow cytometry

The following mAbs were used: anti-CD56-allophycocyanin, anti-CD3-PE-Cy7, anti-CD2-PE, anti-CD16-PE, anti-KIR2DL1-PE, anti-NK G2D-PE, anti-CD69-PE, IgG1-PE, IgG1-allophycocyanin, IgG1-PE-Cy7, IgM-PE, and rat anti-mouse IgG1-FITC (BD Pharmingen). Anti-KIR2DL2/L3-PE, anti-NKp30-PE, anti-NKp44-PE, anti-NKp46-PE, anti-ILT2-PE, and anti-IgG2a-PE (Beckman Coulter). Anti-NKGC2A-PE, anti-NKGC2C-PE, goat anti-mouse IgM-FITC and goat anti-mouse IgG1-PE (Southern Biotech). Anti-2B4 (CD244) was from Beckman Coulter. Iso- type control mouse IgG (DakoCytomation). Anti-CD160 (BY55) was produced locally (17). Anti-KIR2DL4 (clone 33) was a gift from E.O. Long (National Institutes of Health, Bethesda, MD), anti-KIR3DL2 (Q66) was a gift from A. Moretta (University of Genoa, Genoa, Italy). Histograms shown were obtained by applying a gate on CD56dim CD3neg on purified dNK cells. Data were analyzed using CellQuest (Becton Dickinson).

Calcium mobilization analysis

dNK cells were stained with indo-1 AM (5.10^-3 μM) for 45 min at 37°C in medium (RPMI 1640, 1 M HEPEs, penicillin 100 IU/ml, streptomycin 100 IU/ml, 5% FCS), then washed with PBS/0.1% BSA. Cells were pelleted and incubated with the appropriate mAbs for 20 min at 4°C. Cells were washed with PBS/0.1% BSA and resuspended on ice with medium just before a few minutes of incubation in a water bath at 37°C. Samples were vortexed and analyzed by flow cytometry (LSRII, Becton Dickin- son). After 10–40 s, tubes were briefly removed, goat anti-mouse F(ab')2 Ab (10 μg/ml) was added, samples vortexed, and placed back on the flow cytometer for further analysis. Data were analyzed with FlowJo software (Tree Star).

Confocal microscopy

dNK were analyzed in a redirected cell lysis assay (E:T = 3:1). P815 were stained with 10 mM 5-(and-6)-chloromethyl benzoyl aoxy tetramethyl-ylrhodamine fluorescent dye (Molecular Probes) for 45 min at 37°C, then washed and used in a redirected killing assay in the presence of 10 μg/ml anti-NKp46, anti-NKp30 or anti-NKGC2A mAbs. Cells were then washed in PBS/0.1% FCS, laid on poly-L-lysine-coated slides for 5 min at 37°C, fixed with 3% paraformaldehyde, permeabilized with 0.1% saponine and stained with anti-perforin mAb or isotype control mouse IgG1 (Diaclone). Primary mAbs were revealed using rat anti-mouse Ab-FITC (BD Pharmingen). The samples were examined on a LSM 510 confocal microscope (Carl Zeiss). One hundred conjugates from randomly selected fields on different slides were counted to estimate the percentage of perforin polarization in each specific condition. Percentages presented are the mean of three to six independent experiments.

Redirected killing assay and annexin V expression

P815 were used as target cells of dNK (E:T = 10:1) in the presence of the following mAbs specific to activating and/or inhibiting receptors or IgG isotype controls at 10 μg/ml: anti-NKp46, anti-NKp30 or anti-NKGC2A mAbs. These bind to activated dNK. Cells were then washed in PBS/0.1% FCS, laid on poly-L-lysine-coated slides for 5 min at 37°C, fixed with 3% paraformaldehyde, permeabilized with 0.1% saponine and stained with anti-perforin mAb or isotype control mouse IgG1 (Diaclone). Primary mAbs were revealed using rat anti-mouse Ab-FITC (BD Pharmingen). The samples were examined on a LSM 510 confocal microscope (Carl Zeiss). One hundred conjugates from randomly selected fields on different slides were counted to estimate the percentage of perforin polarization in each specific condition. Percentages presented are the mean of three to six independent experiments.

Annexin V expression and cytokine release

Among the inhibitory receptors tested, all dNK expressed ILT2, and lower levels of ILT3 and ILT4. The majority of dNK expressed NKG2D, with a lower percentage of NKG2A. Among the activating receptors tested, NKG2C was the highest, followed by NKp46, NKp30, and NKp44.

IFN-γ measurement

Cross-linking of NKp30, NKp46, or NKG2A receptors was performed at different concentrations of specific mAbs on fresh dNK cultured with IL-2 (100 IU/ml) for 48 h. IgG1 isotype controls were also used at the same conditions. Supernatants were analyzed for IFN-γ secretion using the CBA Kit (BD Biosciences), according to the manufacturer’s instructions. Analysis was on fresh dNK cells cultured with CellQuest software (BD Biosciences). The mean fluorescence was compared with standard curves and IFN-γ concentration (pg/ml) was calculated by using the provided FCAP array software (BD Biosciences).

Determination of cytokine and chemokine concentrations with bead-based multiplex sandwich Luminex immunoassay

Cross-linking of NKp30 or NKp46 receptors was performed at different concentrations of specific mAbs on fresh dNK cells cultured with IL-2 (100 IU/ml). IgG1 isotype controls were also used at the same conditions. Supernatants were analyzed for cytokine and chemokines release using a human cytokine multiplex-22 bead assay kit (Luminex X MAP Technol- ogy). Fifty microliters of each individual sample was added to 50 μl of Ab-conjugated beads directed against human IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-1RA, IL-10, IL-12, IL-13, IL-17, TNF-α, G-CSF, GM- CSF, IFN-α, IFN-β, IFN-γ, IL-1α, IL-1β, IL-1RA, IL-6, IL-8, TNF-α, MIP-1α, MIP-1β, RANTES, MCP-1, vascular endothelial growth factor, hepatocyte growth factor, and Eotaxin (R&D Systems) in a 96-well filter plate (R&D Systems). After 30 min incubation, the plates were washed and biotinylated anti-cytokine Ab solution added to each well for 30 min incubation. The plates were washed and 50 μl of streptavidin-PE was added to each well. After a final wash, each well was filled in with 125 μl of assay buffer (R&D Systems) and analyzed by the Luminex array system. Cytokine concentrations were calculated by using a standard curve established from various concentrations of each cytokine standard used in the assay.

Statistics

Statistical analyses were performed using unpaired Student’s t test, Wilcoxon’s signed rank test, Tukey’s multiple comparison test, or two-way ANOVA test using GraphPad Prism 4 project software.

Results

A unique, identical expression of activating and inhibitory receptors on dNK cells

Freshly isolated dNK from the first trimester pregnancy were analyzed for the expression of various receptors as well as CD69 activation marker (Fig. 1). In addition to the major CD160neg subset, we also found a minor subpopulation expressing CD16 (Fig. 1), which contrasted with PB-NK cells from the same donor (data not shown). The whole dNK population expressed high levels of NKp46, NKGC2D, -B4-, and CD2-, and lower levels of NKp30, -pKp44-, and NKG2C-activating receptors (Fig. 1). Interestingly, most of dNK were CD160neg whereas a minor subset appeared CD160pos (Fig. 1), contrasting again with PB-NK (19). Among the inhibitory receptors tested, all dNK expressed NKG2A: a complete shift of the whole dNK population was in- deed observed, when compared with a negative control such as the CD160neg subset (Control mean fluorescence intensity: 3, NKG2A positive (data not shown). Similarly, most dNK expressed KIR2DL4, whereas a distinct subset exhibited KIR2DL1, whereas a distinct subset exhibited KIR2DL1, KIR2DL2/L3, ILT2, and ILT2. Finally, freshly isolated dNK, including the CD56dim subset, expressed CD69, an early marker of leukocyte activation, indicating that dNK have already become activated in situ. Expression of CD69 further indicates that the
dNK analyzed in this study did not contain any maternal blood cell contaminant as the phenotype of CD56dim PB-NK is CD69neg (20). NKp44 expression on dNK, although at a low level (Fig. 1), is a further indication of their activated status, because NKp44 is only expressed on activated PB-NK cells (21). Fresh dNK thus clearly differ from PB-NK as the majority of dNK do not express CD16 and CD160 but are NKp44pos and CD69pos and express much higher levels of ILT2 (Fig. 1). We further found that, in contrast to PB-NK, this unique repertoire of receptors, including KIRs, exhibited by dNK was identical in all donors tested. This study thus demonstrates the presence of a major CD56bright dNK subpopulation that is also CD16neg, CD160neg, CD69pos, NKp44pos, and ILT2pos and a minor CD56dim dNK subset that is CD16pos, CD160neg, CD69pos, NKp44pos, and ILT2neg. The use of the above markers clearly discriminates between freshly isolated dNK (Fig. 1) and PB-NK of the same donor (data not shown).

**mAb specific engagement of NKp46 triggers dNK cell-mediated cytolytic activity**

As dNK express activating receptors, we evaluated whether their specific engagement could trigger calcium mobilization, one of the early steps leading to perforin-dependent NK-cell cytotoxicity. Freshly isolated dNK were loaded with the Ca2+/H11001-sensitive fluorescent dye Indo-1 AM, incubated with either NKp46-, NKp30-, NKG2C-, or CD16-specific, soluble mAbs or Ig isotype controls, and analyzed by flow cytometry. After acquisition of the baseline fluorescence, secondary goat F(ab')2 anti-mouse Ab was added to cross-link the receptors. As shown in Fig. 2A, mAb-cross-linking of NKp46 induced a rise in intracellular Ca2+ in comparison to baseline observed with Ig isotype control-treated dNK. Cross-linking NKG2C induced a very small rise in Ca2+ flux, whereas cross-linking of NKp30 (Fig. 2A) or CD16 (data not shown) did not produce any significant detectable rise in Ca2+ response.

To further establish the involvement of NKp46 in the dNK killing process, we analyzed perforin polarization on dNK in a redirected, mAb-dependent cell lysis assay against P815 target cells. As shown in Figs. 2B and 2C, perforin polarization occurred after NKp46 mAb specific engagement of dNK (mean 66.8% of conjugates with polarized perforin at the E:T interface). In contrast, dNK failed to polarize specific cytolytic perforin-containing granules after NKp30 engagement (mean 32.1% of conjugates with polarized perforin as compared with 35.0% with anti-CD56 negative control mAb). We also found that coengagement of NKp46 and NKG2A inhibitory receptor significantly impaired perforin polarization (Fig. 2C).
FIGURE 2. Engagement of NKp46 receptor in fresh dNK induces intracellular Ca\textsuperscript{2+} uptake, perforin polarization, degranulation, and cytotoxicity. A, NKp46 engagement activates Ca\textsuperscript{2+} flux. dNK cells were loaded with indo1-AM for 45 min at 37°C and incubated on ice with mAbs to indicated receptors or control Ig, washed, and prewarmed at 37°C. After 35 s, F(ab’\textsubscript{2}) goat anti-mouse IgG was added to each sample, cells analyzed by flow cytometry and events acquired for 150 s. Data are representative of five independent experiments. B, dNK polarizes perforin granules toward the P815 target cell contact after specific mAb-mediated NKp46 engagement. Left panel, Phase contrast images. Right panel, Confocal images of perforin granules (green) in dNK and 5-(and-6)-4-chloromethyl benzoyl amino tetramethylrhodamine fluorescent dye (red) in P815 target cells. A typical dNK-P815 conjugate with polarized perforin granules was obtained after NKp46 (upper panel) but not NKp30 receptor engagement (lower panel). Data are representative of six independent experiments. C, Quantification of perforin polarization on dNK/P815 conjugates (E:T cell ratio: 10:3) was evaluated in the presence of the indicated mAbs. The graph displays the percentage of conjugates with polarized perforin. Values represent mean ± SD of three to four separate experiments and were determined by evaluation of 100 conjugates. ***, p < 0.001, Tukey’s multiple comparison test. D, Degranulation of dNK induced by NKp46 engagement. dNK were incubated for 4 h with P815 (E:T cell ratio: 1/1) and mAbs or control Ig, as indicated. Thereafter, cells were stained with anti-CD56-APC, anti-CD3-PeCy7 and anti-CD107a-PE. CD107a expression was analyzed by flow cytometry on the CD56\textsuperscript{+}/CD3\textsuperscript{+} gated subsets. Numbers indicate the percentage of CD107a-positive cells. Upper and lower panels are from two different individuals. Data are representative of six independent experiments. E, Cytolytic activity of dNK cells after NKp46 engagement. Freshly isolated dNK were analyzed for cytolytic activity in a redirected cell lysis assay (E:T cell ratio: 10:1) in the presence of mAbs specific for the indicated NK cell receptors or control Ig (Ctrl Ig). Apoptotic P815 cells (gated by appropriate forward light scatter threshold setting) were stained with annexin V-FITC and analyzed by flow cytometry. Values represent mean specific lysis ± SD of six donors. *, p < 0.05; **, p < 0.01; ***, p < 0.001, Tukey’s multiple comparison test. Purity of dNK was >96%.
Exocytosis of secretory lysosomes, which are lytic granules, is required for the cytotoxicity. We quantified degranulation on fresh dNK based on cell surface expression of the lysosomal protein CD107a (22). Specific expression of CD107a was reproducibly detected on dNK cells after incubation with anti-NKp46 mAb and P815 target cells, as compared with control Ig (Fig. 2D, upper panel). After incubation with anti-NKG2C mAb, CD107a expression was slightly increased. In contrast, mAb ligation of NKp30 did not induce degranulation. Of note, anti-CD16 mAb triggered a slight degranulation specifically attributed to the CD56dim CD16+ minor dNK cell subset (Fig. 2D, lower panel). Therefore, both perforin polarization and granule exocytosis are induced by NKp46 ligation and correlated with the mobilization of Ca^{2+}.

Finally, freshly isolated dNK were tested for their cytolytic activity in a redirected, Ab-dependent cytotoxicity assay against P815 target cells. mAbs specific for NKp46, NKp30, NKG2A, and CD16 NK cell receptors were tested individually. As shown in Fig. 2E, mAb specific engagement of NKp46, but not NKp30, induced lysis of P815 by dNK, as compared with the IgG isotype control. Absence of NKp30-mediated cytolytic activity on dNK was not due to the mAb used in this assay, because the same mAb did trigger cytolytic function of dNK cultured with IL-2 (data not shown) as well as cytokine secretion (see below). The mAbs to NKG2C also induced lysis of P815 but always at lower extent. Almost no killing was observed with mAb to CD16 (Fig. 2D). By comparison, NKp46-specific engagement in fresh PB-NK cells did not induce any significant cytotoxicity, in contrast to CD16 mAb specific ligation of PB-NK that mediated a high level of cytotoxicity (data not shown).

We conclude that freshly isolated dNK, without IL-2 stimulation in vitro, are capable of killing target cells after NKp46 mAb-specific engagement.

**Coactivation of dNK by pair-wise combination of both NKp46 and CD2**

Apart from CD16, most NK triggering receptors present on PB-NK require coengagement of another activating receptor for a more efficient activation (23). Having shown that dNK clearly expressed CD2 (Fig. 1), we investigated whether this coreceptor could modulate NKp46-mediated cytolytic activity. mAb-mediated co-cross-linking of NKp46 and CD2 indeed enhanced the peak of intracellular Ca^{2+} uptake, and the kinetics was faster than the response elicited by NKp46 mAb and control Ig (Fig. 3A).
Cross-linking of CD2 with control Ig did not produce any detectable rise in intracellular Ca\(^{2+}\). Likewise, we found that when dNK were incubated with a combination of anti-CD2 and anti-NKp46 mAbs, CD107a surface expression almost doubled when compared with NKp46 activation with Ig control (Fig. 3B), correlating with Ca\(^{2+}\) uptake. Using a redirected cell lysis assay, we finally showed that CD2 coengagement induced a reproducible and significant increase of NKp46-mediated cytotoxicity against P815, as compared with NKp46-mediated lysis in the presence of IgG1 isotype control (Fig. 3C). By comparison, mAb-specific engagement of CD2 alone did not induce any rise in the NKp46-mediated cytotoxicity (Fig. 3C). Taken together, these data demonstrate an additive response in the cytolytic activity of fresh dNK after coengagement of both NKp46 and CD2 receptors.

**NKp46-mediated dNK cytotoxicity is negatively controlled by specific coengagement of NKG2A inhibiting receptor**

 Tight regulations of the dNK cytolytic potential by inhibitory receptors are likely to occur in situ in the healthy pregnant uterus to prevent potentially harmful lysis of embryo-derived trophoblast cells present in the decidua basalis. Positive intracellular signals mediated by activating receptors can be blocked by dominant negative signals triggered by inhibitory receptors (24). Because the HLA-E physiological ligand of NKG2A is expressed by trophoblast in contact with dNK (25), we investigated whether NKG2A might exert such a negative control. We found that co-cross-linking of NKp46 and NKG2A totally prevented the peak of Ca\(^{2+}\) induced by NKp46 mAb and control Ig, whereas coengagement of NKG2A and control Ig had no effect on Ca\(^{2+}\) uptake (Fig. 4A).

Such dramatic inhibition was reproducible in each donor tested. NKG2A coengagement also blocked dNK perforin polarization mediated by NKp46 mAb (Fig. 2C) and impaired the NKp46-induced degranulation of dNK (Fig. 4B), correlating with Ca\(^{2+}\) uptake data. Consistent with these observations, coengagement of both NKp46 and NKG2A by specific mAbs induced a dramatic diminishment of the cytolytic activity of dNK, as measured by Annexin V staining of P815 target cells (Fig. 4C). Taken together, these results demonstrate that NKG2A co-cross-linking clearly inhibited the NKp46-mediated cytolytic activity of dNK cells.

**Engagement of NKp30 but not NKp46 receptor induces IFN-γ, TNF-α, MIP1-α, MIP1-β, and GM-CSF secretion by dNK**

To evaluate whether engagement of NKp46 with mAb could also induce cytokine secretion in dNK, these cells were incubated for 48 h with mAbs against NKp46, NKp30 or control Ig in the presence of IL-2. IFN-γ secretion in the supernatants was then quantified by CBA measurement. mAb to NKp46 did not induce IFN-γ secretion, with a clear dose effect. When mAbs to NKp30 and NKG2A were added together, a dramatic decrease in IFN-γ secretion (\(p < 0.0031\)) was observed (Fig. 5A), demonstrating that NKG2A also exerted an inhibitory effect on NKp30-mediated IFN-γ secretion in dNK. Simultaneous measurement of other cytokines was then performed on the dNK supernatants, using a multiplex bead array cytokine assay and Luminex technology. We reproducibly found that the concentrations of TNF-α, MIP-1α, MIP-1β, and GM-CSF were significantly
above the lowest cut-off levels when dNK were stimulated by increasing concentrations of NKp30 mAb (Fig. 5, B–E, respectively). All other cytokines and chemokines tested by Luminex were either not detected (IL-1β, IL-4, IL-5, IL-8, IL-10, IL-12, IL-13, IL-15, IL-17, eotaxin, hepatocyte growth factor) or produced at nonsignificant levels after specific NKp30 mAb engagement (data not shown). To rule out the possibility that anti-NKp46 mAb was nonfunctional in these experiments, we showed that it triggered IFN-γ production from IL-2-activated PB-NK, as NKp30 mAb did (Fig. 5F). We also found a similar production of IFN-γ, TNF-α, MIP-1α, MIP-1β, and GM-CSF in dNK cell culture supernatants after IL-15 stimulation (100 IU/ml) for 48 h in the presence of NKp30 mAb but not NKp46 mAb or control Ig (data not shown).

Discussion

The observations that dNK exhibit an identical repertoire of activating and inhibitory receptors among all donors tested strongly suggest that dNK have been selected for tissue-specific, specialized functions. In this study, we showed that NKp46- and NKp30-activating receptors, both expressed on dNK, exert a differential role in the early pregnant uterus.

We found that freshly isolated human dNK from early pregnancy have the potential to kill target cells, and this can be induced by specific engagement of the NKp46- and, to a lesser extent, NKG2C-activating receptors. We demonstrated that mAb-specific engagement of NKp46 in fresh dNK cells, without coengagement with another activating receptor, is able to increase calcium flux, recruit perforin, express CD107a, and trigger apoptosis of P815 target cells. This contrasts with freshly isolated PB-NK cells, which do not efficiently kill target cells after NKp46-mAb ligation (26). One possible explanation could be that, in situ, dNK are already activated by cues in the local uterine environment (mainly IL-15) (27). Expression of the CD69 and NKp44 activation markers by dNK cells (Fig. 1) indicates that this is the case. Indeed the cytotoxic assays that we describe in this study were performed just a few hours after recovery of the decidua basalis from elective terminations. This brief interval is expected to be too short to lose the activated status of dNK.

Using the same cytotoxic assay, it has been reported that specific mAb engagement of NKp46, but also NKp44 and NKp30, did mediate cytolytic activity of dNK (13). However, in this latter study, dNK were cultured in the presence of IL-2. We also observed that after such IL-2 activation, activating receptor mAb-mediated dNK lytic activity in a redirected cell lysis assay was strongly enhanced (data not shown), when compared with unstimulated fresh dNK (this study). We have further demonstrated that CD2 coengagement further increased the NKp46-mediated cytotoxic potential of fresh dNK. This is consistent with a previous study showing that CD2 was unique in its exclusive synergy with NKp46 in resting PB-NK cells (26). Our observation that a major dNK subset did express high levels of CD2 (Fig. 1) strongly suggests that the presence of this coactivating receptor could have important physiological consequences in the decidua basalis. Interestingly, CD58 (natural ligand of CD2) expressed on T cells was shown to ligate CD2 on dendritic cells, leading to dendritic cell maturation (28). A parallel interaction between dNK and dendritic cells is also possible and might favor a local antiviral immune response (29).

Why are dNK poorly cytotoxic in normal pregnancy despite their cytolytic NKp46-mediated potential? The first explanation could be that this dNK effector function is negatively controlled. We indeed demonstrated that NKp46-mediated cytotoxic activity of dNK was dramatically inhibited by coengagement of the NGGA inhibitory receptor, suggesting balanced signaling machinery in normal pregnancy, likely due to disruption of the actin network at the immunological synapse, as recently demonstrated (30). It is of note that, unlike PB-NK, the vast majority of dNK express CD94/NKG2A (31). Furthermore, we found that 100% of dNK cells expressing NKp46 also express NKG2A (data not shown). Such negative control of NKp46 cytolytic potential is likely to occur in normal healthy pregnancy as the HLA-E specific ligand of CD94/NKG2A is expressed by the invading extravillous trophoblast, the only "nonsfl" target cells present in the decidua basalis and in direct contact with dNK (32–34). The second explanation could be that in normal pregnancy there is no NKp46 ligand expressed on local maternal uterine cells, as recently demonstrated (35). It has been shown that fresh dNK exert reduced lytic activity on K562 or 721.221 target cells, unless they were incubated with IL-15 (15). One possible explanation might be that the as-yet undefined non-viral ligand of NKp46 is not sufficiently expressed on these target cells.

Could NKp46-mediated cytolytic function of dNK be exerted in some pathologic conditions of pregnancy, including recurrent spontaneous abortion (36), or uterine viral infection (37)? Prevention of uterine infection is critical to the establishment of human pregnancy and appropriate fetal development. Although the nature of NKp46 cellular ligands is not yet fully understood, such ligands to NKp46 may be expressed primarily as a consequence of viral infection (38). Thus, a likely explanation is that dNK cells might be an important component of the local innate immune response to uterine virus infection, through specific ligand triggering of NKp46 (and possibly other coactivating receptors) that would be dominant over NKG2A-mediated inhibitory signals. NK cell inhibitory receptors are indeed targeted by viral immune evasion strategies (23, 39), which may lead to the absence or diminished expression of HLA-E-specific ligand (40). Some virus-infected cells might also be recognized by the NKp46 receptor through the binding of viral hemaglutinin (41) or as-yet undefined protein ligand, leading to the killing of these infected target cells. The recent observations that NCR1, a murine ortholog of NKp46, recognized influenza virus-infected cells and that such infection was lethal in the absence of NCR1 (42) favor such hypothesis. One can speculate that NKp46 would detect up-regulated specific ligands in virally infected cells (43). A recent report indeed indicated that infection of JEG-3 and JAR trophoblast cell lines with the same virus increased expression of NKp46 ligand and their susceptibility to killing by NK cells (O. Mandelboim, personal communication). The distribution of NKp46-specific ligand in vivo during infection would thus determine the level of cytotoxic effector function. Another nonexclusive possibility is that viral infection may induce an increased expression of specific ligands of NKp46 such that the balance of positive signaling becomes dominant and favors dNK cell activation. This possibility is supported by a recent observation that the immediate early herpes simplex virus gene product ICP0 triggers the up-regulation of cellular ligands for the NCR including NKp46 (43). Some NKG2D or other NCR ligands are also up-regulated in cells infected with cytomegalovirus (39). Expansion of CD94/NKG2C activating receptor has been detected in response to human cytomegalovirus infection (44). Furthermore, NKp46 was recently shown to be important in binding mitotic cells and thus affecting mitotic cell surveillance (45). Therefore, it is reasonable to hypothesize that NKp46 could prevent or limit possible proliferation of locally infected autologous, maternal decidu- al cells, with mitosis being an indicator of potential danger (45).

In contrast to NKp46, we found that NKp30 specific engagement on fresh dNK had almost no effect on their cytolytic function but triggered the release of Th1 cytokines including IFN-γ and
TNF-α, whereas none of the Th2 cytokines (IL-4, IL-5, IL-10, IL-13) were produced. By comparison, we found that KIR2DL4 engagement on fresh dNK by specific mAb induced similar IFN-γ and TNF-α secretion (data not shown), as previously described (46). This study has shown that NGK2A coengagement inhibited NKp30-induced IFN-γ production, suggesting that NGK2A was coexpressed on NKp30 positive dNK. Such NKp30 and NGK2A coexpression was indeed demonstrated by using two-color flow cytometry analysis (data not shown). The GM-CSF release, known to stimulate granulocyte, macrophage and eosinophil formation and proliferation (47), could also contribute to inflammation. We also found that after specific NKp30-mediated mAb ligation, dNK cells also released high levels of MIP-1α and MIP-1β chemokines. These findings fit with previous reported observations showing by microarray analysis that MIP-1α and MIP-1β were over expressed in dNK (11). During viral infections, PB-NK cells produce IFN-γ, MIP-1α, and MIP-1β, important to attract and activate inflammatory cells (48). Moreover, it has been shown that the CD16<sup>pos</sup>CD56<sup>bright</sup> PB-NK subset, which resemble the major dNK subpopulation, produced higher amounts of IFN-γ, TNF-α, and GM-CSF than their CD16<sup>pos</sup>CD56<sup>dim</sup> counterparts (49). These secreted molecules may also contribute to the generation of an inflammatory environment after viral local infection by recruiting eosinophils, macrophages, and activated dendritic cells. NKp30 expressed by dNK cells is thus likely to play a role in local antiviral immunity by secreting proinflammatory cytokines.

Because the activities of NKp46 and NKp30 appear distinct and nonoverlapping in dNK, an important question is whether this activity difference could be due to their downstream signaling through different adaptors. Biochemical analysis has indeed revealed that the NKp46 receptor is associated with both CD3ζ and FcεRIα adaptor proteins, which contain ITAMs, whereas NKp30 uses only CD3ζ (50). However, we have shown that NKp46 engagement of IL-2 stimulated PB-NK did induce cytokine secretion. siRNA silencing of CD3ζ in dNK may help to further clarify this issue in the future.

In conclusion, this study demonstrates an unexpected differential role that NKp46- and NKp30-activating receptors expressed by dNK may have in early pregnant uterus. In normal pregnancy, NKp46-mediated cytolytic potential is likely to be controlled by the negative signals mediated by the NKG2A inhibitory receptor/HLA-E-specific interaction as well as by the absence of expression of NKp46-specific physiological ligand. In normal pregnancy, NKp30-mediated cytokine and chemokine secretion by dNK occurs through engagement of as yet uncharacterized ligand expressed by decidual stromal cells, as recently shown by Hanna et al. (35). Such specific interactions lead to secretion of IL-8/IP10 chemokines attracting trophoblast, vascular endothelial growth factor/placental growth factor angiogenic factors which in turn control the uterine vascular remodeling (35), and likely several additional inflammatory cytokines (this study). In the case of uterine infection, NKG2A-mediated negative signals controlling NKp46-mediated cytolytic function might be abrogated by some viral immune evasion mechanisms, leading to the absence or diminished expression of its HLA-E-specific ligand (40) and the up-regulation of the NKp36 specific ligand. Such NKp46-mediated cytotoxic activity together with the NKp30-mediated secretion of inflammatory cytokines by dNK may thus contribute to the drop in the number of uterine infected cells.

Understanding how the NKp46 and NKp30 receptor-ligand interactions of dNK are integrated in vivo during normal and pathological pregnancy remains a challenging goal in human reproduction.

Acknowledgments
We thank Steven P. Schwendeman for critical reading of the manuscript, Sophie Chabot for advice with statistics, Fatima L’Faqih (IFR30 Plateau Technique Cytométrie, Toulouse), and Sophie Allart (IFR30, Plateau Technique Imagerie Cellulaire, Toulouse) for assistance with cytometry and confocal microscopy, respectively. We thank Dr. Lanusse (Hôpital J. Ducuing, Toulouse) for providing human decidua, and Françoise La-Passade for very helpful assistance for the tissue collection at P. de Viguier hospital. The study was approved by a local research ethics committee.

Disclosures
The authors have no financial conflict of interest.

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