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ORIGINAL ARTICLE

Interaction between dendritic cells and natural killer cells during pregnancy in mice

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Abstract A complex regulation of innate and adaptive immune responses at the maternal fetal interface promotes tolerance of trophoblast cells carrying paternally derived antigens. Such regulatory functions involve uterine dendritic cells (uDC) and natural killer (uNK) cells. The existence of a NK and DC "cross talk" has been revealed in various experimental settings; its biological significance ranging from cooperative stimulation to cell lysis. Little is known about the presence or role of NK and DC cross talk at the maternal fetal interface. The present study shows that mouse NK and DC interactions are subject to modulation by trophoblast cells in vitro. This interaction promotes a tolerogenic microenvironment characterized by downregulation of the expression of activation markers on uNK cells and uDC and dominance of Th2 cytokines. NK and DC interactions would also influence uterine cell proliferation and this process would be strongly modulated by trophoblast-derived signals. Indeed; while low proliferation rates were observed upon regular coculture allowing direct contact between uterine cells and trophoblasts, incubation

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in a transwell culture system markedly increased uterine cell proliferation suggesting that soluble factors are key mediators in the molecular "dialog" between the mother and the conceptus during the establishment of mouse pregnancy. Our data further reveal that the regulatory functions of trophoblast cells associated with tolerance induction are impaired in high abortion murine matings. Interestingly, we observed that secretion of interleukin-12p70 by uDC is dramatically abrogated in the presence of uNK cells. Taken together, our results provide the first evidence that a delicate balance of interactions involving NK cells, DC, and trophoblasts at the mouse maternal fetal interface supports a successful pregnancy outcome.

Keywords Natural killer cells · Dendritic cells · Uterus · Tolerance · Pregnancy · Mice

Abbreviations

DC	dendritic cells
NK	natural killer cells
Tro	trophoblast
IDO	indoleamine 2,3 dioxigenase

Introduction

Mammalian pregnancy constitutes a unique situation in which sophisticated immunoregulatory mechanisms are required to provide tolerance towards fetal alloantigens while at the same time allowing effective immunity to protect the mother from infection. It has been suggested that fetal trophoblasts actively avoid recognition through their ability to modulate the nature and constituents of maternal immune responses [1]. However, the immunological mechanisms that determine pregnancy maintenance are still largely elusive, and further progress depends on a better understanding of the functional role of distinct cell subsets within the uterine immune environment.

As in other mucosal surfaces, the outcome of immune responses in the uterus is determined mainly by antigen-presenting cells (APCs). Among the decidual APC populations, dendritic cells appear as candidates to perform regulatory functions in pregnancy given their ability to modulate the nature of immune responses in stimulatory or tolerogenic fashion [2]. Dendritic-cell-associated molecules such as indoleamine 2,3 dioxigenase (IDO) and cluster of differentiation 200 (CD200) have been shown to have pregnancy-protective effects [3, 4]. Moreover, a delicate balance has been suggested between stimulatory (CD86–CD28, intercellular adhesion molecule (ICAM)-1–lymphocyte-function-associated antigen 1) and inhibitory (programmed death 1–programmed death ligand 1) signals in the generation of immune privilege [5–7]. Further evidence for the regulatory role of uterine dendritic cells (uDC) at the fetal maternal interface comes from recent studies in mice performed in our group, revealing that uDC increase their numbers at the time of implantation and exhibit a steady-state phenotype, associated with the induction of tolerance [6, 8].

Both in mice and humans, a prominent feature of the pregnant uterus is the abundance of natural killer cells, which constitute around 70% of resident lymphocytes [9]. Uterine natural killer (uNK) cells may contribute to pregnancy maintenance by responding to novel ligands presented by the decidua and/or trophoblasts. Moreover, it has been suggested that uNK cells regulate maternal mucosal and arterial function [10] and/or trophoblast invasion [11]. In particular, studies in mice have shown that uNK-cell-derived interferon (IFN)-γ is essential in triggering pregnancy-induced spiral artery modification [12]. These findings, together with their distinct phenotype and gene expression profile [13], strongly suggest that uNK cells are pivotal in pregnancy maintenance. During mouse pregnancy, uNK cells accumulate on the mesometrial side of the placenta and play a central role in decidualization. Mouse strains lacking uNK cells show common histopathology, including hypocellularity and edema of the decidua basalis and thickening of the arterial walls with persistence of vascular smooth muscle and luminal narrowing [14, 15]. Interestingly, implantation in these mice is normal and fetal loss (when observed) coincides with the detection of placental anomalies at gestation day (Gd) 10 [16].

From a growing body of evidence on regulatory interactions between these cell subsets, it has recently become apparent that the classical role, as mere innate immune effectors and APCs, attributed to NK cells and DC, respectively, needs to be updated. In vitro studies have described at least three types of NK–DC interactions that may influence the outcome of immune responses: (1) production of interleukin (IL)-15, IL-12–IL-18, and type I interferons by mature DC induces NK cell proliferation, IFN-γ secretion, and cytotoxic functions [17, 18]; (2) activated NK cells are able to induce DC maturation, either directly or in synergy with suboptimal levels of microbial signals [19, 20], and (3) immature DC are selectively susceptible to NK-cell-mediated cytolysis, an effect that appears to be dependent on signals delivered by NKp30 and has also been observed in vivo [21, 22]. Such interactions are highly dependent on cell–cell contact, reflecting the implication of membrane receptor–ligand pairs and/or the need for a synaptic interface at which cytokines can be delivered in high concentrations [19, 23]. Although spatial adjacencies have recently been described for NK cells and DC in human early pregnancy decidua [24], little is known about the physiological aspects of this so-called NK–DC cross talk at the maternal fetal interface.

Therefore, in the present study, we investigated the effect of trophoblasts from low and high abortion murine mating combinations on NK–DC interactions to provide insights on their functional role during pregnancy.

Materials and methods

Animals

Mice were purchased from Charles River (Sulzfeld, Germany) and maintained in an animal facility with a 12-h light–dark cycle. Animal care and experimental procedures were followed according to institutional guidelines and conformed to requirement of the state authority for animal research conduct.

We performed two experiments. Experiment I was carried out in order to obtain trophoblast cells from different murine mating models. We used these trophoblast cells as stimulators in mixed leukocyte reaction assays to measure proliferation and synthesis of cytokines. The CBA/J females were divided into: control pregnancy (mated to Balb/c males $n=10$) and abortion-prone model (mated to DBA/2J males $n=10$). The pregnant females were sacrificed on Gd 14.5; placentas were removed and trophoblast cells were isolated as described below.

Next, experiment II was performed in order to obtain uterine cells used as responders in cell culture. CBA/J-mated CBA/J females with vaginal plugs ($n=30$) were segregated and sacrificed on Gd 8.5 to harvest uterine cells for subsequent use in cell culture.

Preparation of trophoblast cell suspensions

In order to obtain suspensions of trophoblast cells for cell culture, a method described previously [25] was used. Briefly, placentas from 14.5 days pregnancy of CBA/J × Balb/c ($n=10$) and CBA/J × DBA/2J ($n=10$) mice were harvested aseptically by peeling each one off carefully, leaving the maternal decidua behind. The placentas were also separated from the fetal membranes, cut into small pieces, and enzymatically digested with 0.5% dispase (#17105, Gibco, Invitrogen Corporation) containing 0.1% DNase (#1284932, Roche, Mannheim, Germany) in a trypsinizing flask for 45 min at 37°C. The resultant cell suspension was then filtered through a 100-μm net (Becton Dickinson, San Francisco, CA, USA), washed by centrifugation, and resuspended in a 64% Percoll™ solution (#055916001A, Amersham Biosciences, Freiburg, Germany). The trophoblasts were resuspended and irradiated at 30 Gy to block the proliferation. Trypan blue exclusion revealed that cell viability was about 95%. Immunofluorescence examination revealed that >98% of trophoblast cells by this method expressed cytokeratin.

Immunostaining on trophoblast cells

Cytospins were incubated with peroxidase-, avidin-, and biotin-blocking solution (Vector, USA), followed by a protein-blocking agent (Immunotech, Germany). The polyclonal rabbit antigalectin (gal)-1(sc-28248), heme oxygenases (HO) isoform HO-1 (sc-58370), survivin (sc-10811), vascular endothelial growth factor (VEGF; sc-507), phosphorylated signal transducer and activator of transcription (pStat-3 (sc-7993), and transforming growth factor beta 1 (TGF-β1 (sc-146) antibodies were purchased from Santa Cruz Biotechnology, diluted 1:100 in Tris-buffered saline (TBS) containing 1% fetal calf serum (FCS) and 0.3% Triton-X100 and applied for 1 h. As secondary antibody, peroxidase-conjugated goat anti-rabbit diluted 1/100 in TBS/1% FCS/0.3% Triton-X100 was used. As amplification and revealing system, we used peroxidase complex (Vector, USA) 1:100 in TBS for 30 min. The signal was detected by incubating sections with 0.2 mg/ml diaminobenzidine, histo-green, or histo-violet and 0.05% hydrogen peroxide, followed by light counterstaining with 0.1% Mayer's hematoxylin. Negative controls substituting the primary antibody with normal rabbit immunoglobulin G (Vector Laboratories Inc., Burlingame, CA, USA) did not reveal specific immunoreactivity. Slides were examined using a Zeiss Axioscope light microscope (Jena, Germany). Photodocumentation was performed using digital image analysis system (Zeiss KS400, Jena, Germany).

Immunofluorescence analysis

Cytospins and 8-μm sections were stained after the following protocol: Washing in TBS buffer 3×5 min and blocking by incubation with 2% normal serum for 20 min at RT. Primary antibodies (ab) anti-CD49b (#553856) and anti-CD11c (#583800) purchased from BD™ Pharminguen except anti-cytokeratin polyclonal ab (Dako Cytomation, Germany, Z0622) were incubated at previously optimized concentrations over night. After washing in TBS buffer, sections were incubated with tetramethylrhodamine-isothiocyanate- or fluorescein-isothiocyanate-conjugated secondary antibody (Jackson ImmunoResearch, Germany) for 1 h at RT. After washing in TBS buffer, sections were incubated in 4′,6-diamidino-2-phenylindole solution and again washed before mounting of slides. Photo documentation was performed using digital image analysis system (Spot advanced software, version 4.6; Visitron Systems)

Preparation of uterine cell suspensions

In order to obtain suspensions of uterine cells for cell culture, a method described previously [8] was used. Briefly, uteri were collected, washed with sterile PBS, carefully cut into small pieces, collected in tubes containing

sterile Hank's balanced salt solution (HBSS), and digested for 20 min at 37°C under slight agitation in HBSS with 200 U/ml hyaluronidase (# H3506, Sigma, Germany), 1 mg/ml collagenase (type C-2139, Sigma), 0.2 mg/ml DNase I (# 1284932, Boehringer Mannheim GmbH, Germany), and 1 mg/ml bovine serum albumin–fraction V (# A9418, Sigma). Thereafter, the isolated cells were collected in a fresh tube through a 100-μm net (Becton Dickinson, San Francisco, CA, USA) and washed with Roswell Park Memorial Institute (RPMI) 1640–10% FCS. The procedure was repeated twice, with sterile HBSS medium containing no cocktail of enzymes. Cells were resuspended in a 1.0875 g/cm^3 Lympholyte®-M solution (#CL5035, Cedarlane, Linaris GmbH, Germany) and centrifuged at 1,000–1,500×*g* for 20 min at RT. The low density fraction at the interface was collected and washed several times. Trypan blue exclusion revealed that cell viability was about 90%.

Purification of uterine dendritic cells and uterine natural killer cells

In order to obtain DC and NK cells, we isolated the CD11c$^+$ or NK1.1$^+$ cell fraction by magnetic cell sorting (MACS). For collection of cells expressing CD11c or NK1.1 to use in cell culture, uterine suspensions were incubated (30 min, 4°C) with biotinylated hamster anti-mouse CD11c (#553800) diluted 1:100 or biotinylated mouse antimouse NK1.1 (#553163) diluted 1:100 in labeling buffer (PBS supplemented with 2 mM ethylenediaminetetraacetic acid (#L2113)). After washing, cells were incubated (15 min, 4°C) with streptavidin MicroBeads (#130-048-101) and processed using MACS to collect CD11c$^+$ or NK1.1$^+$ cells. Examination by FACS® revealed that >95% of selected cells by miniMACS expressed CD11c or NK1.1.

Cell coculture technique

Uterine cell suspensions from CBA/J-mated CBA/J females obtained on Gd 8.5 were seeded (2×10^5 cells per well) in 96-well or transwell plates (HTS transwell-96 System, with a 0.4-μm polycarbonate filter, # 3381, Corning, Germany) as responders, with DBA/J or Balb/c trophoblast cells as stimulators (stimulator-to-responder ratio was 1:5). In the first experiment after 3 days of coculture, we analyzed the cell proliferation with intracellular fluorescent dye carboxyfluorescein succinimidyl ester (CFSE) by FACS® and cytokine expression in the supernatants by cytometric bead array (CBA). In the second experiment, we analyzed expression of maturation markers by FACS® and cytokine concentrations in the supernatants by CBA after 6, 12, 24, and 48 h in uDC and 2, 4, 8, and 24 h in uNK cells.

All cultures were performed at 37°C in a 5% CO_2 atmosphere in RPMI 1640 supplemented with antibiotic (50 U/ml penicillin and 50 μg/ml streptomycin), 2 g/L sodium bicarbonate, 2 mM L-glutamine, 1 mM pyruvate, and 10% FCS.

Use of the intracellular fluorescent dye CFSE to monitor cellular proliferation

In order to monitor the cellular proliferation, a method described previously [26] was used. Briefly, carboxyfluorescein diacetate succinimidyl ester is commonly referred to as CFSE, but is abbreviated by its supplier as CFDA, SE (#C-1157, Molecular Probes, Eugene OR). Master stocks of CFSE were prepared at 10 mM in tissue-culture-grade dimethyl sulfoxide (Sigma, St. Louis, MO, USA) and stored at −20°C until use. On the day of cell labeling, working stocks of CFSE were prepared at 10 μM in HBSS (Invitrogen, Carlsbad, CA, USA) and prewarmed to 37°C. To prepare CFSE-labeled suspensions, uterine cells were counted and resuspended at 2×10^7/ml in HBSS and warmed to 37°C. An equal volume of 10 μM CFSE was added so that the final concentration was 1 μM and the mixture was incubated for 10 min at 37°C. After labeling, the cells and CFSE were promptly diluted by the addition of at least ten volumes of HBSS, pelleted, washed once more in HBSS, counted, and resuspended in RPMI 1640+ 10% FCS at a density of $2.0–2.5\times10^5$ per well. CFSE diffuses freely into cells and intracellular esterases cleave the acetate groups converting it to a fluorescent, membrane-impermeant dye. CFSE is retained by the cell in the cytoplasm and does not adversely affect cellular function. During each round of cell division, the relative intensity of the dye is decreased by half.

Analysis of secreted cytokines by cytometric bead array

In order to analyze the cytokine concentration in the cell culture supernatants, a method described previously [26] was used. Supernatants from 72-h cell culture were harvested and stored at −80°C until cytokine testing was performed. IL-12, tumor necrosis factor (TNF)-α, IFN-γ, and IL-10 were detected simultaneously using the mouse inflammation cytokine cytometric bead array kit (BD™ PharMingen, San Diego, CA, USA). Briefly, 50 μl of each sample was mixed with 50 μl of mixed capture beads and 50 μl of the mouse inflammation phycoerythrin (PE) detection reagent consisting of PE-conjugated antimouse IL-12, TNF-α, IFN-γ, and IL-10. The samples were incubated at room temperature for 2 h in the dark. After incubation with the PE detection reagent, the samples were washed once and resuspended in 300 μl of wash buffer. Data were acquired on the FACSCalibur (BD Biosciences,

Sunnyvale, CA, USA) and analyzed using CBA software (BD™ PharMingen). Standard curves were generated for each cytokine using the mixed cytokine standard provided by the kit. The concentration for each cytokine in cell supernatants was determined by interpolation from the corresponding standard curve. The range of detection was 10–5,000 pg/ml for each cytokine measured.

Cytotoxicity assay

Cytotoxic activity of freshly isolated uNK cells against DC was measured using a standard protocol. Target cells were labeled with 100 mCi of ^{51}Cr for 1 h at 37 C, and the labelled cells were then washed and resuspended. Effector cells were placed in the wells of round-bottomed microtiter plates at various effector-to-target ratios (E:T). Labeled target cells were then added to each well at a concentration of 3×10^3 cells per well for a total volume of 0.2 ml per well. After 4 h incubation, release of ^{51}Cr into the supernatant was quantified with an automated gamma counter. The percentage of cytotoxicity was calculated as the percent ^{51}Cr release using the equation: (experimental release − spontaneous release / maximum release − spontaneous release)×100.

High-pressure liquid chromatography determination of tryptophan, kynurenine, and anthranilic acid

High-pressure liquid chromatography was carried out as previously described [27] with minor modifications. uDC and uNK cells were seeded into 24-well plates (1×10^6 cells per well) in 1 ml RPMI 1640+10% FCS culture medium containing DBA2J or Balb/c trophoblasts as described before with or without 1,000 U/mL IFN-γ. Culture supernatants of two to three wells were collected and deproteinized by mixing with 2.4 M perchloric acid (10:1, vol/vol). After centrifugation (3,500×*g*, 15 min, 4°C), supernatants were transferred into new tubes, the pH value adjusted to 7, and 100-μL filtered supernatant was injected into a C-18 column (Supelco, Aschaffenburg, Germany). Samples were eluted with PBS buffer over 30 min, and the absorbance of column effluent was monitored at 230 nm for tryptophan and kynurenine (kyn). The peaks of kynurenine, anthranilic acid, and tryptophan were identified by comparison with the retention time of previously determined standard compounds, and quantification was based on the ratios of the peak areas of the compound to the internal standard.

Statistical analysis

All experiments were performed in triplicate and repeated at least three times on different occasions. Significance of differences among groups was determined using the nonparametric Mann–Whitney *U* test. A *p*-value of <0.05 was considered to be significant.

Results

Trophoblast cells from low and high abortion matings differ in the expression of pregnancy-protective molecules

A wealth of molecules expressed by trophoblast cells, such as Gal-1, HO-1, survivin, and VEGF, have been proposed to modulate the innate and adaptive immune response at the maternal fetal interface [26, 28–30]. Thus, the first issue addressed was to identify the expression of such molecules on trophoblast cells derived from low (Balb/c-mated CBA/J females) and high abortion (DBA/2J-mated CBA/J) murine mating combinations by immunocytochemistry (Fig. 1). Cytospin preparations of trophoblast cells obtained from low abortion matings exhibited high amounts of Gal-1-positive cells (left top panels) and were highly positive for the cytoprotective enzyme HO isoform HO-1 (right top panels) compared to trophoblast cells derived from the high abortion combination DBA/2J-mated CBA/J females. Furthermore, expression of the antiapoptotic factor survivin and pSTAT-3 (which has been described as a mediator of survivin response) was higher in trophoblasts from the low abortion mating combination compared to high abortion matings, as shown in left and right middle panels, respectively. Immunostaining for the proangiogenic molecule VEGF revealed that protein expression was higher in preparations obtained from the high abortion mating combination (left bottom panels). Finally, trophoblasts from both murine matings expressed similar amounts of the Th-3 cytokine TGF-β1 (right bottom panels), cytokeratin (left panels), and cell surface CD1 (right panels; Fig. 1b).

Next, pregnant uteri were analyzed by immunofluorescence for expression of CD11c and CD49b, markers of the DC and NK cell populations, respectively. Both CD11c- and CD49b-positive cells (Fig. 1c) were observed at the maternal fetal interface of murine allogeneic matings. Interestingly, uDC were often found in close proximity to uNK cells, suggesting that these two populations interact at the murine maternal fetal interface.

NK cells and DC regulate trophoblast induced uterine cell proliferation

After characterizing murine trophoblasts in terms of pregnancy-protective molecule expression, our next aim was to investigate the effect of trophoblasts from low and high abortion matings on the phenotype and function of uterine NK cells and DC, as well as on NK and DC cross talk. During early pregnancy, proliferation of hormonally

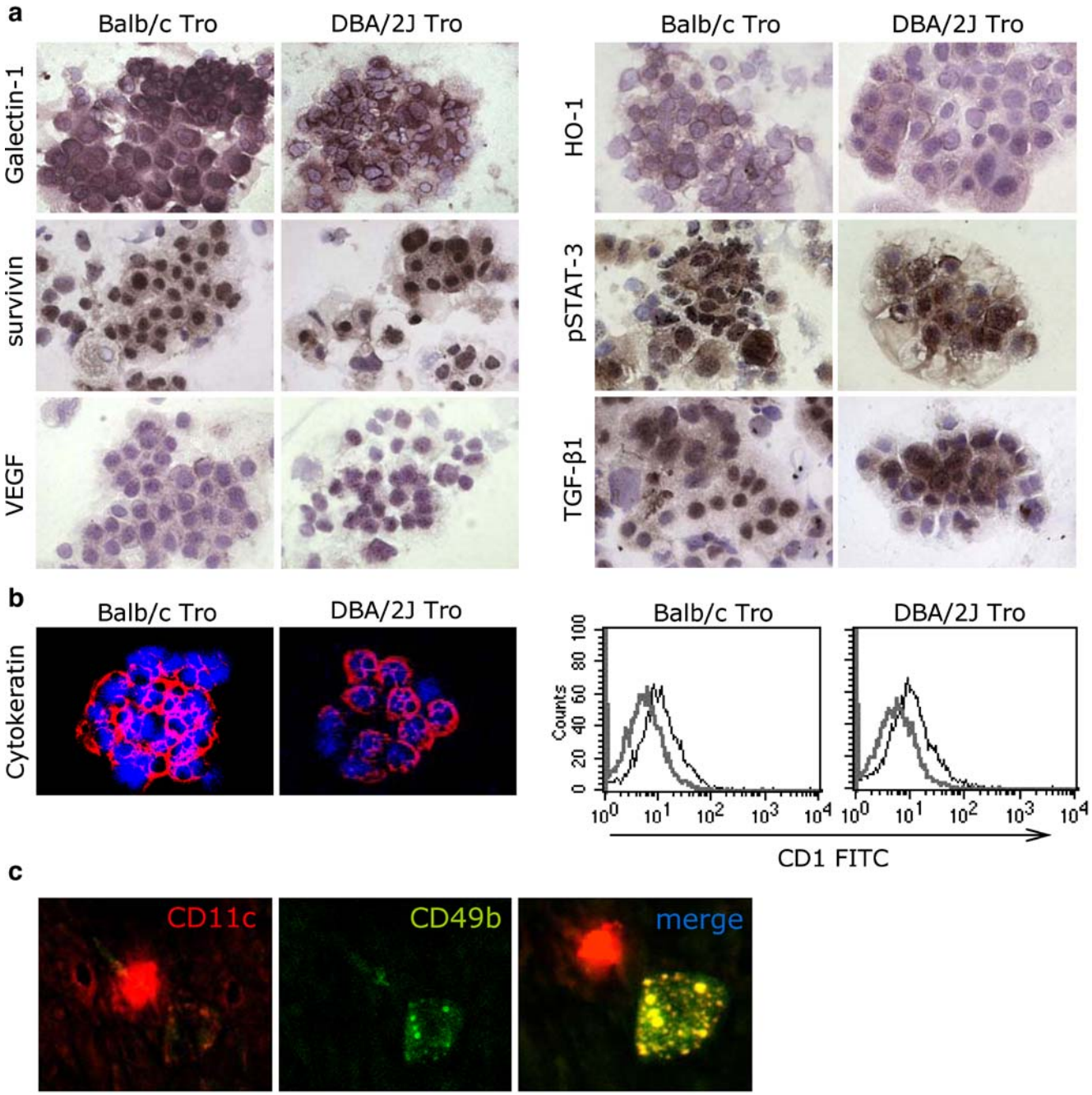

Fig. 1 Molecular characterization of mouse trophoblasts, uterine NK and DC. **a** Cytospins from trophoblast cells isolated on gestation day 14.5 from CBA/J × Balb/c (Balb/c Tro) and CBA/J × DBA/2J matings (DBA/2J Tro), showing the following stainings: (*left panels*) galectin-1, survivin, and vascular endothelial growth factor (*VEGF*); (*right panels*) hemoxygenase-1 (*HO-1*); phosphorylated stat-3 (*pStat-3*) and transforming growth factor beta 1 (*TGF-β1*). **b** Trophoblast cell suspensions obtained from CBA/J × Balb/c and CBA/J × DBA/2J matings were also analyzed by immunofluorescence for cytokeratin expression (*left*) and by flow cytometry for CD1 expression (*right*); representative histogram plots showing analysis of CD1 (*dark line*) and isotype (Rat IgG_{2b}, *light line*) expression on trophoblast cells from low and high abortion-prone matings on gestational day 14.5. **c** (*Left panel*) Uterine dendritic cells as identified by CD11c staining using rhodamine-conjugated streptavidin. (*Middle panel*) Uterine natural killer cells characterized by CD49b expression and (*right panel*) merge image, showing the close topographical relationship between both cell subsets at the maternal fetal interface

primed uterine cells is critical for decidualization and subsequent placentation. Because NK cells comprise the major decidual lymphocyte population at this stage, we aimed to analyze if this subset could influence uterine cell proliferation under the stimulus of trophoblast cells. Figure 2 (left panels) shows that incubation with trophoblasts from low or high abortion murine matings induced poor proliferation of total uterine cells isolated from

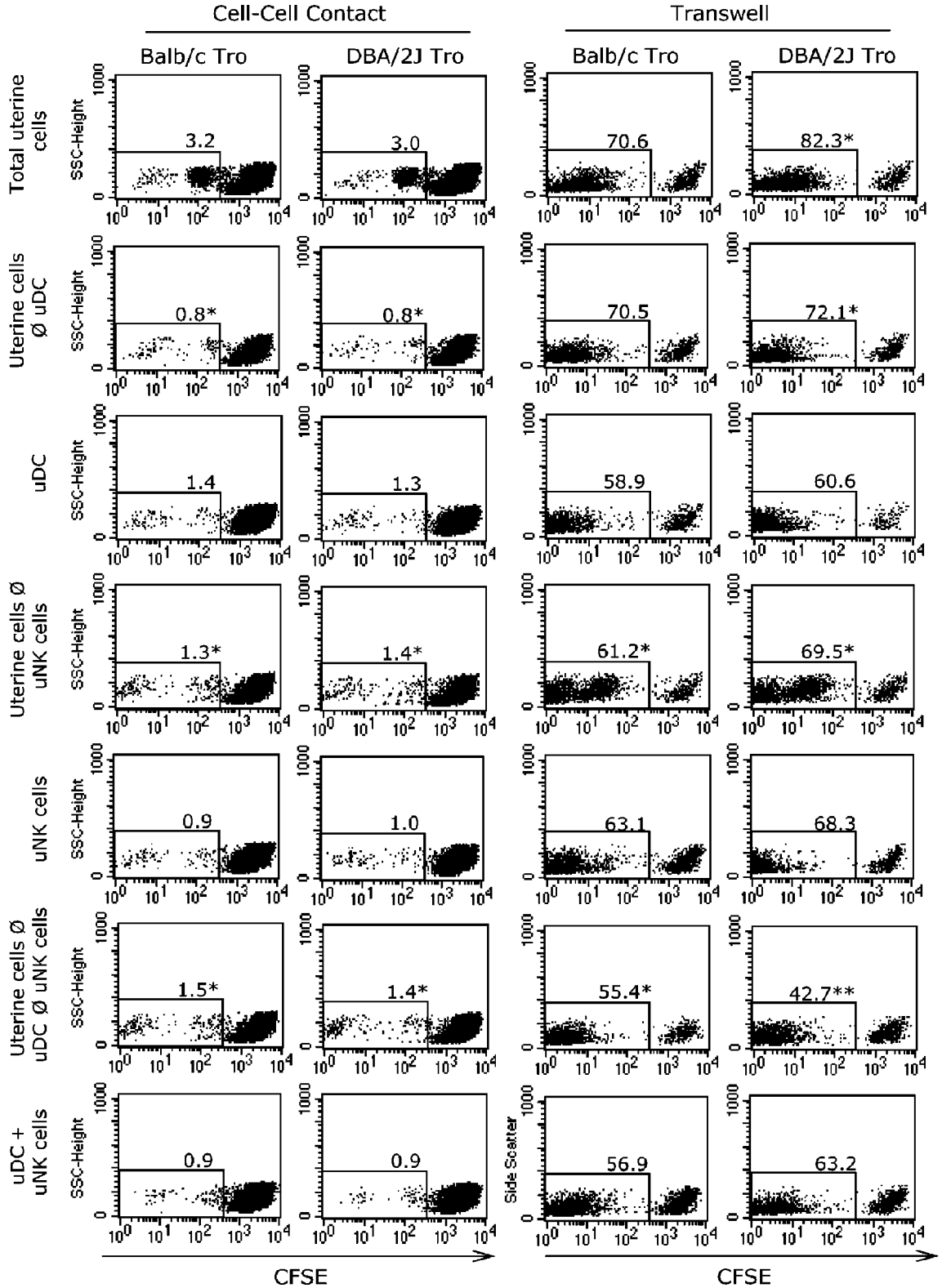

Fig. 2 Uterine cell proliferation in response to murine trophoblasts, as analyzed by flow cytometry. After 72 h of regular coculture ("cell–cell contact") or incubation in a transwell culture system ("transwell") with Balb/c or DBA/2J trophoblasts, uterine cells were labeled using the intracellular dye CFSE to investigate cell proliferation. Ø denotes depletion of either uDC or uNK cells, using magnetic cell sorting. *Boxes* indicate the percentage of proliferating uterine cells, as identified by CFSE dilution. $*p<0.05$ and $**p<0.01$ indicate statistical significant differences between the total uterine cells and uterine cells Ø uDC, uterine cells Ø uNK, or uterine cells Ø uDC Ø uNK, as calculated by Mann–Whitney *U* test

syngeneic matings. We were unable to observe differences between the percentage of proliferating cells after incubation with trophoblast from low and high abortion murine matings (Fig. 2 left panels). Furthermore, the percentage of proliferating cells decreased (threefold) when the DC subset was depleted ($p<0.05$) and twofold upon depletion of NK cells ($p<0.05$). Indeed, this poor percentage of proliferation persisted when both DC and NK subset were depleted. Taken together, these results suggest that NK and DC at the fetal maternal interface cooperate in controlling uterine cell proliferation.

In order to evaluate whether trophoblast cells need direct contact with uterine cells to modulate their proliferation, we performed transwell assays. The results are shown in Fig. 2 (right panels); we observed a strong proliferation after the cell–cell contact was avoided, suggesting that surface molecules present on trophoblast cells negatively regulate the proliferative activity of uterine cells. Furthermore, a significantly higher percentage of proliferating uterine cells could be detected after incubation with trophoblasts derived from high abortion murine matings ($p<0.05$). Interestingly, whereas the percentage of proliferating cells remained constant when DC were depleted after incubation with trophoblast derived from low abortion murine matings, DC depletion induced a significant decrease on the percentage of proliferating cells ($p<0.05$) when transwell assays were performed using trophoblasts from the high abortion combination. Decreased percentages of proliferating uterine cells were also observed when uterine NK cells were depleted from the transwell system, with similar results for both low and high abortion murine matings ($p<0.05$). Moreover, the percentage of proliferating uterine cells strongly decreased also when both uterine DC and NK cells were depleted, having a major impact when trophoblasts derived from high abortion matings were employed ($p<0.05$ and $p<0.01$). These results suggest that soluble factors derived from trophoblasts also regulate the proliferation of decidual cells and the function of DC and NK in the decidualization process.

NK and DC encounter results in inhibition of uterine IL-12p70, TNF-α, and IL-10 production

In addition to cell–cell contact, the dialog between NK cells and DC involves bidirectional cytokine signals. In vitro studies have shown that DC-derived IL-15, IL-12–IL-18, and IFN-α/β could specifically enhance NK cell proliferation, IFN-γ secretion, and cytotoxic function [15, 16]. In turn, activated NK cells release cytokines, particularly IFN-γ, which are able to promote DC maturation. To further establish how this cross talk is accomplished at the maternal fetal interface, we analyzed IL-12p70, TNF-α, IFN-γ, and IL-10 secretion by uterine cells obtained from syngeneic matings cultured in the presence of trophoblasts from low or high abortion matings.

As shown in Table 1, total uterine cells after a 72-h culture in the presence of Balb/c trophoblasts released minimal amounts of IL-12p70, TNF-α, and IL-10. However, Balb/c trophoblast-stimulated uterine cells markedly increased cytokine secretion after depletion of either the DC or NK populations (Table 1). Similar results were observed when stimulation was performed using DBA/2J trophoblasts (Table 2). Moreover, in both experimental setups, isolated uDC were found to release high amounts of IL-12p70, TNF-α ($p<0.01$), IFN-γ ($p<0.05$), and IL-10 ($p<0.01$; Tables 1 and 2). Interestingly, this effect was dramatically abrogated

Table 1 Cytokine secretion in response to Balb/c trophoblasts after 72 h culture

Groups	Cell–cell contact	IL-12p70 (pg/ml)	TNF-α (pg/ml)	IFN-γ (pg/ml)	IL-10 (pg/ml)
Total uterine cells	+	17.6±3.2	28.0±2.2	3.6±0.9	8.1±3.3
	−	68.5±7.9*	35.1±2.8	9.6±0.7*	279.5±22.6***
Uterine cells Ø uDC	+	34.2±0.8*	48.6±0.3*	6.4±1.5	41.4±7.6*
	−	63.3±2.5	17.4±2.2	8.0±0.6	322±12.7
uDC	+	149.6±22.2***	151.6±21.0***	11.4±1.5*	178.4±29.4**
	−	32.7±5.9**	18.6±1.5***	6.6±0.8	239.4±12.8
Uterine cells Ø uNK	+	32.4±2.6*	106.2±10.1**	6.2±1.1	81.7±9.1**
	−	62.7±4.6	55.8±5.1	4.3±0.9	246.4±14.9
uNK	+	29.4±9.8	90.6±9.5	5.7±0.6	72.9±9.0
	−	51.7±4.1	30.5±2.7	7.1±0.9	279.5±18.3
Uterine cells Ø uDC	+	ND	ND	ND	1.7±0.5
Ø uNK	−	48.6±3.6	21.0±1.8	5.5±0.6	263.7±27.5
uNK–uDC	+	45.8±7.5**	23.4±1.9**	6.0±1.4*	75.4±8.4*
	−	78.4±3.4*	33.5±2.5*	7.5±0.8	384.1±18.9*

Cytokines were analyzed by cytometric bead arrays (CBA). Results are expressed as mean ± SEM ($n=15$).
ND Not detectable, *Ø* denotes depletion of either uDC or uNK cells using magnetic cell sorting
*$p<0.05$; **$p<0.001$; ***$p<0.01$ as evaluated by nonparametric Mann–Whitney *U* test.

Table 2 Cytokine secretion in response to DBA/2J trophoblasts after 72-h culture

Groups	Cell–cell contact	IL-12p70 (pg/ml)	TNF-α (pg/ml)	IFN-γ (pg/ml)	IL-10 (pg/ml)
Total uterine cells	+	2.5±0.8	18.6±3.3	2.3±0.2	23.2±1.2
	−	90.5±8.7*	42.6±2.9**	9.7±0.9**	548.6±48.7***
Uterine cells Ø uDC	+	19.5±0.8*	48.0±8.1**	3.6±0.7	45.5±4.1**
	−	49.3±2.8	23.8±1.9	5.8±0.4	296.5±11.8
uDC	+	75.1±4.6*	149.4±7.1*	8.8±1.6**	194.5±34.3***
	−	35.6±4.9**	14.6±0.8***	4.3±0.5	172.7±16.5
Uterine cells Ø uNK	+	88.5±2.4*	100.4±8.9*	5.8±0.9	93.8±6.9*
	−	65.2±5.9	65.7±7.5	4.7±1.2	178.5±12.3
uNK	+	87.8±1.6	89.9±12.4	5.4±0.8	48.0±13.1
	−	66.7±5.3	31.4±2.7	6.6±0.7	364.1±31.5
Uterine cells Ø uDC	+	ND	ND	ND	2.4±0.9
Ø uNK	−	41.7±3.5	17.7±1.5	5.1±0.9	175.6±5.7
uNK–uDC	+	64.4±1.8	33.4±9.5*	8.2±1.8	94.9±17.7**
	−	67.5±3.7**	38.6±2.1**	7.2±0.9	347.3±13.8*

Cytokines were analyzed by cytometric bead arrays (CBA). Results are expressed as mean ± SEM (n=15).
ND not detectable, *Ø* denotes depletion of either uDC or uNK cells using magnetic cell sorting
*$p<0.01$; **$p<0.05$; ***$p<0.001$ as evaluated by nonparametric Mann–Whitney U test.

when uDC were cultured in the presence of uNK cells. Indeed, we observed a significant decrease on the IL-12p70 ($p<0.01$), TNF-α ($p<0.01$), and IFN-γ and IL-10 ($p<0.05$) release upon Balb/c stimulation. Similar results were found when DBA/2J trophoblast was employed, but we were unable to show any significant difference on the IL-12p70 secretion by uDC in presence of uNK (Table 2).

We next measured cytokine secretion in supernatants from transwell assays in order to determine whether physical interactions with uterine cells are required for the stimulatory function of trophoblast cells. Strikingly, total uterine cells significantly upregulated cytokine secretion when direct cell–cell contact with trophoblasts from both mating combinations was avoided. While both combinations had a similar stimulatory effect on IL-10 secretion (Balb/c trophoblasts 34-fold ($p<0.001$) and DBA/2J trophoblasts 23-fold ($p<0.001$) with respect to regular coculture), DBA/2J trophoblasts were found to exert the most potent effect on the secretion of Th1 cytokines by uterine cells, particularly IL-12p70 (36.2-fold ($p<0.01$) vs. Balb/c fourfold ($p<0.05$)) and IFN-γ (4.2-fold ($p<0.05$) vs. Balb/c 2.6-fold ($p<0.05$)). Furthermore, it is worth noting that in transwell system trophoblasts from either low (Balb/c Tro) or high (DBA/2J Tro) abortion matings fail to stimulate IL-12p70 ($p<0.01$ and $p<0.05$) and TNF-α ($p<0.001$ and $p<0.001$), respectively, secretion by isolated uDC compared to the regular cell–cell contact setup. The effect on IL-12p70 TNF-α and IL-10 secretion by uDC in the presence of uNK were particularly striking in the transwell setup; we observed a significant increase of their release compared with those values in absence of uNK in response to Balb/c or DBA/2J trophoblast stimulation, suggesting that uNK module DC cytokine production (Tables 1 and 2).

Presence of trophoblast cells interferes with maturation–activation of uDC and uNK cells

Increasing experimental evidence shows that immature–steady-state DC are required for the initiation and maintenance of peripheral tolerance, whereas mature DC, which are only found in minimal amounts in decidua, are associated with a Th1 polarization of T cells [6, 31]. This prompted us to investigate the effect of trophoblast stimulation on the expression of cell surface markers of DC maturation. Figure 3 shows that a proportion of isolated uDC was found to coexpress cell surface major histocompatibility complex (MHC) class II molecules, CD80/86 or ICAM-1, reproducing our previous finding that semimature DC are present at the fetal maternal interface in mice [8]. Surprisingly, upon encounter with trophoblast cells from either low or high abortion matings, expression of maturation markers was dramatically downregulated on uDC harvested from syngeneic matings, as demonstrated by the significant inhibition of MHC class II (Fig. 3a; $p<0.01$), CD80/86 (Fig. 3b; $p<0.01$), and ICAM-1 expression (Fig. 3c; Balb/c Tro $p<0.05$ and DBA/2J Tro $p<0.01$). Maximum inhibition was achieved within 6 to 12 h of coculture, and DBA/2J trophoblasts were found to exert the most potent effect on expression of all markers analyzed.

The expression of NK cell activation markers in response to Balb/c or DBA/2J trophoblast stimulation was also analyzed. On Gd 8.5, a small proportion of uNK cells harvested from syngeneic matings was found to spontaneously express CD69, CD25, or B220 (Fig. 3). However, such expression rapidly (i.e., within 2–4 h) downregulated CD69 (Fig. 3d; $p<0.05$), CD25 (Fig. 3e; $p<0.05$), or B220 (Fig. 3f; $p<0.01$) when cells were cultured in the presence

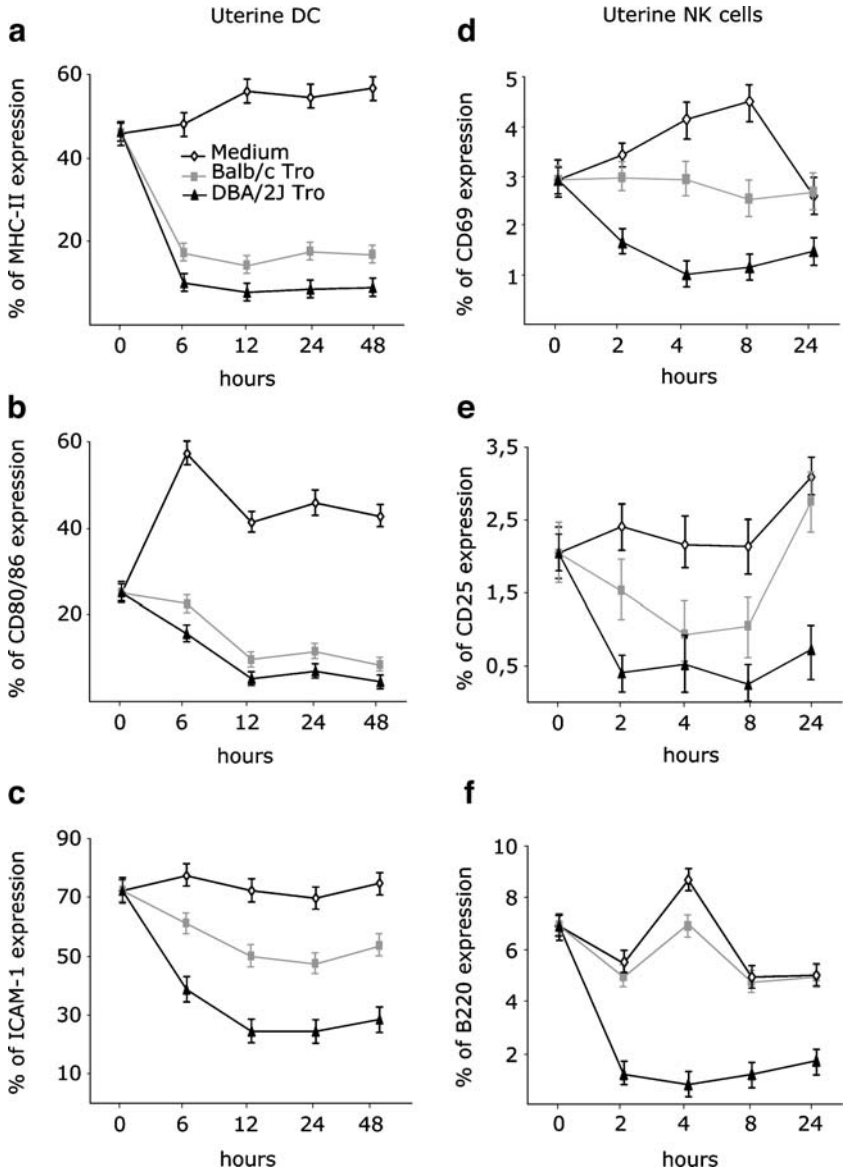

Fig. 3 Downregulation of maturation–activation markers of uDC and uNK cells upon coculture with trophoblasts from normal or abortion-prone matings. The figures show the time course of expression of maturation–activation markers on trophoblast-stimulated uDC (**a**–**c**; MHC-II, CD80–86 and ICAM-1) or uNK cells (**d**–**f**; CD69, CD25, and B220), as determined by FACS®. Results are expressed as percentage of positive cells, set according to isotype controls. Data are representative of three independent experiments. Significant differences were evident between Balb/c- or DBA/2J-treated DC and untreated DC (**a** $p<0.01$), **b** $p<0.01$, and **c** Balb/c Tro $p<0.05$ and DBA/2J Tro $p<0.01$). For CD69, CD25, and B220, significant differences were evident between DBA/2J-treated uNK and untreated uNK (**d** $p<0.05$, **e** $p<0.05$, **f** $p<0.01$)

of DBA/2J trophoblasts. Only limited downregulation of CD69, CD25, and B220 expression were found in uNK cells upon Balb/c trophoblast stimulation, but no significant decrease of those expressions was observed (Fig. 3d–f).

Trophoblasts modulate cytokine secretion by uDC and uNK cells derived from syngeneic murine matings

The finding that uNK cell and uDC maturation state is modified by mouse trophoblasts from low and high abortion matings led us to investigate whether cytokine secretion could also be affected in this system. Concentrations of IL-10, IL-12p70, TNF-α, and IFN-γ in supernatants from uDC or uNK cells cultured in the presence or absence of trophoblast cells are shown in Fig. 4. In our experimental setup, isolated uDC were found to spontaneously release significant levels of cytokines, particularly IL-10 (Fig. 4a), TNF-α (Fig. 4c), and IFN-γ (Fig. 4d). Interestingly, IL-10, IL-12p70, TNF-α, and IFN-γ production was significantly downregulated ($p<0.001$; $p<0.01$; $p<0.001$, and $p<0.001$, respectively) when cells were cocultured with trophoblasts from both mating combinations.

Conversely, minimal spontaneous cytokine secretion was detected in uNK cell culture supernatants. While trophoblasts did not appear to affect IL-10 secretion in the first 8 h of culture, a significant increase of this Th-2/3 cytokine after 24 h of culture in response to Balb/c trophoblasts was observed (Fig. 4e; $p<0.05$). Though to a lesser extent, similar results were observed for IL-12p70 secretion (Fig. 4f; $p<0.05$), with a slight increase after 8 h of coculture with Balb/c trophoblasts. As shown in Fig. 4g, TNF-α secretion was

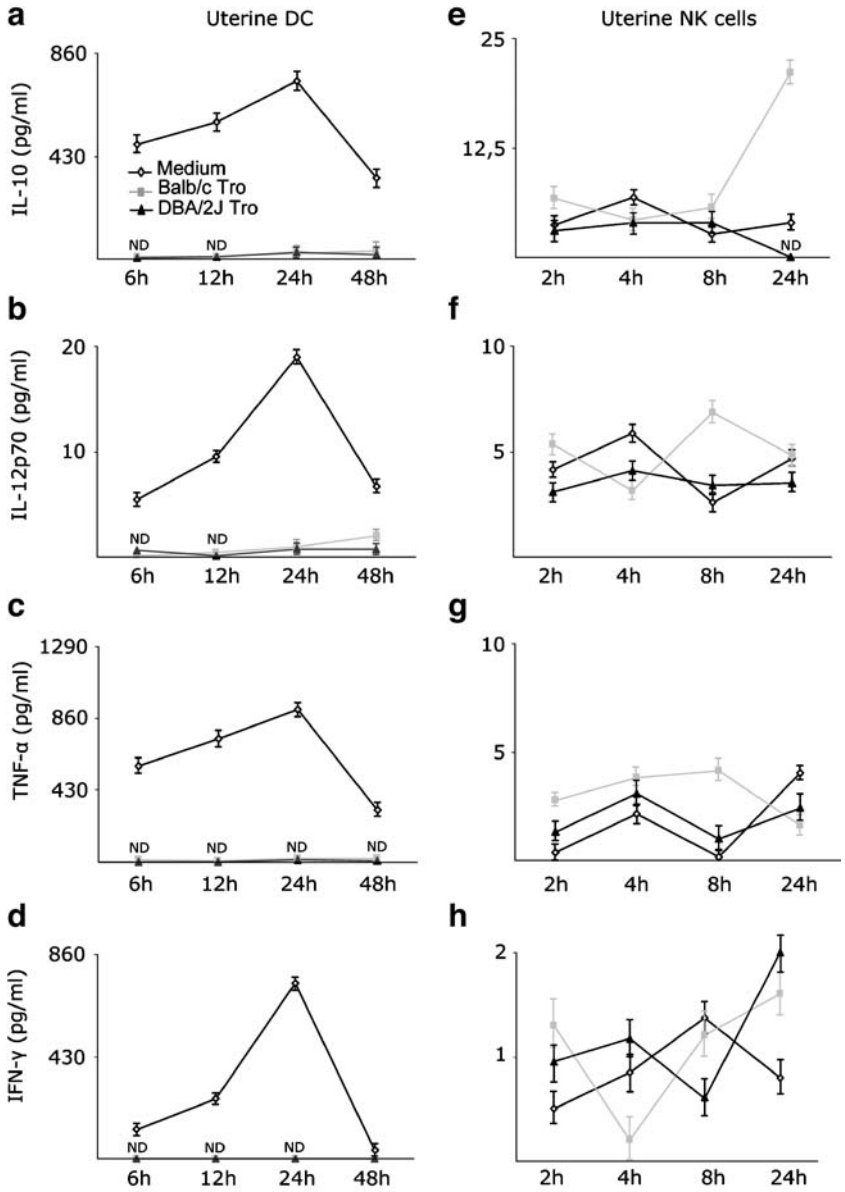

Fig. 4 Isolated uDC and uNK cell cytokine secretion in response to trophoblasts from normal and abortion-prone matings. After culture of uDC and uNK cells in the presence or absence of trophoblasts for the indicated times, concentrations of IL-10 (**a**, **e**), IL-12p70 (**b**, **f**), TNF-α (**c**, **g**), and IFN-ϒ (**d**, **h**) in the supernatants was measured by CBA. Results are representative of three independent experiments with similar results. Data are shown as mean ± SEM. *y* axis represents the IL-10, TNF-α, IL-12p70, or IFN-γ concentration (picogram per milliliter), compared to the incubation time (**h**). Significant differences were evident between Balb/c- or DBA/2J-treated DC and untreated DC (IL-10; $p<0.001$, IL-12p70 $p<0.01$, TNF-α; $p<0.001$ and IFN-γ $p<0.001$). For IL-10 (24 h), Il-12p70 (8 h), and TNF-α (2 h), significant differences were evident between Balb/c-treated uNK and untreated uNK (**e**–**g**; $p<0.05$)

increased when uNK cells were cultured in the presence of trophoblasts from both mating combinations. Indeed, this effect was already detectable after 2 h of coculture and more pronounced when Balb/c trophoblasts were used as stimulators after 8 h of incubation ($p<0.05$). Even in the presence of trophoblasts from the control or the abortion-prone matings, IFN-γ levels remained below the detection limits of the assay during the entire time course analyzed in this experiment (Fig. 4h).

Trophoblast stimulation fails to induce cytotoxic activity on uDC and uNK isolated from syngeneic matings

The interaction between immature DC and activated NK cells results in either DC maturation or cell death [32]. The mechanisms that determine the outcome between death and maturation depend on a dynamic interplay between the DC-to-NK ratio and on the DC maturation state [33]. Indeed, it has recently been demonstrated that only in conditions of a low DC-to-NK ratio, the DC–NK cell interaction results in NK cell activation [19]. Based on these findings, we decided to investigate the effect of Balb/c and DBA/2J trophoblasts on the induction of cytotoxicity as a result of uDC–uNK interaction. Figure 5a shows that no significant cytotoxic activity was induced in cocultures at different uDC-to-uNK ratios when uDC were preincubated with trophoblasts from both mating combinations. Similarly, minimal cytotoxicity was observed when uDC were cultured with Balb/c or DBA/2J trophoblast-pretreated uNK cells, irrespective of uDC-to-uNK ratio (Fig. 5b).

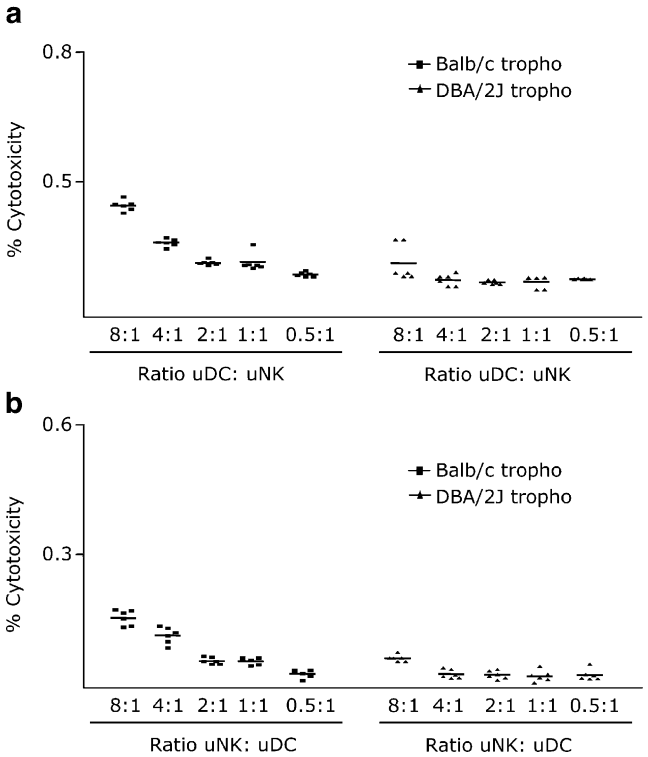

Fig. 5 NK and interaction NK and DC cross talk at the maternal fetal interface does not result in cytolytic activity. Cytotoxicity was measured using a standard ^{51}Cr release assay. **a** After preincubation of uDC with trophoblasts from normal or abortion-prone matings, no significant cytotoxicity was observed in uDC–uNK cocultures, irrespective of the effector-to-target ratio analyzed. **b** shows similar results for the reciprocal experiment, where uNK cells were preincubated with trophoblasts and afterwards used as effectors. The results are means of triplicate cultures from three independent experiments. There were no significant differences between any of the groups analyzed

Trophoblast cells are unable to induce IDO activity on mouse uNK and uDC

Several studies have focused on the role of IDO, a tryptophan (trp)-catabolizing enzyme, in the regulation of immune responses. Upon activation with IFN-γ or CD40L, DC were found to produce functional IDO which resulted in the inhibition of T cell proliferation [34]. In addition, it has been recently reported that besides APCs, NK cells also express IDO, which appears to be necessary for their cytolytic activity against tumor cells [35]. Given that the immunoregulatory functions of IDO seem to be particularly important during pregnancy, our next aim was to test whether incubation with trophoblast cells could influence IDO activity of uNK cells and uDC. Concentrations of trp and its catabolites kyn and anthranilic acid measured as a read out of IDO activity are displayed on Fig. 6. Supernatants from either untreated or IFN-γ-stimulated uDC showed no significant differences in trp, kyn, and anthranilic acid concentrations, though a trend towards decreased trp and increased kyn was detected upon IFN-γ stimulation. Furthermore, stimulation with either Balb/c or DBA/2J trophoblasts was unable to induce significant changes in trp, kyn, and anthranilic acid concentrations. Similarly; supernatants from uNK cells cultured in the presence of IFN-γ or trophoblasts from both mating combinations showed no significant differences in trp, kyn, and anthranilic acid levels with respect to untreated cells.

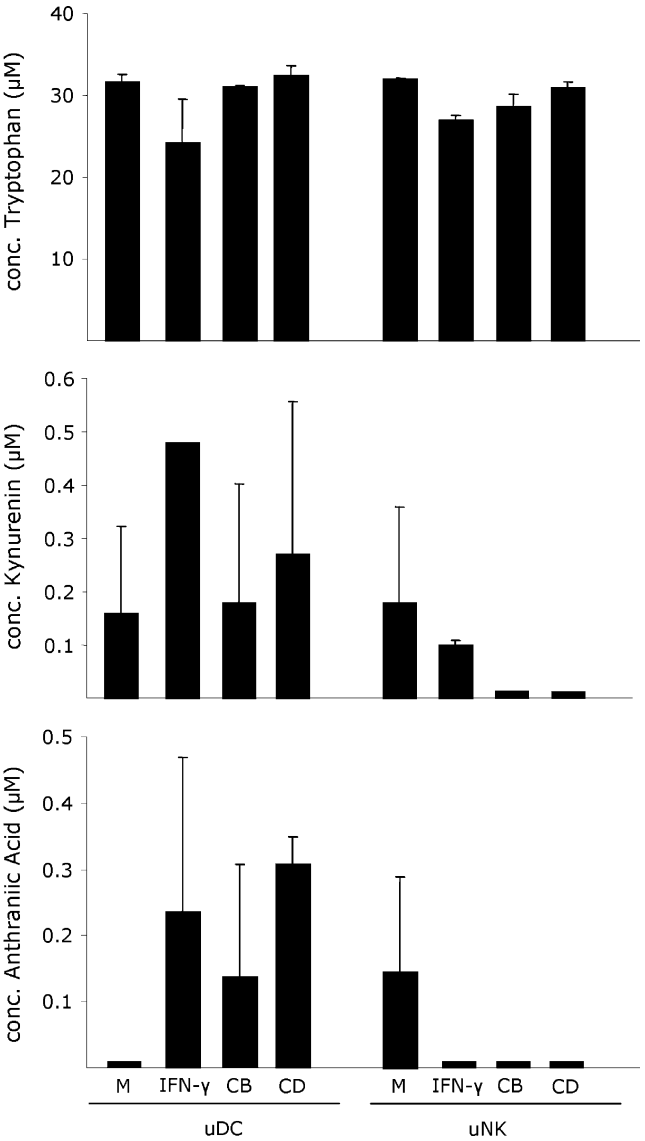

Fig. 6 Trophoblasts fail to induce IDO activity on mouse uDC and uNK cells. Uterine DC or NK cells isolated from syngeneic matings were cultured alone (*M*, medium) or in the presence of IFN-γ (1,000 U/ml), trophoblasts from low (*CB*) and high abortion (*CD*) murine matings for 48 h. Concentrations (micromolar) of tryptophan and its catabolites kynurenine and anthranilic acid in culture supernatants were measured by HPLC. Results are expressed as mean ± SEM. The data shown are representative of two independent experiment

Discussion

As major populations present at the decidualized endometrium during early pregnancy, several lines of evidence suggest a role for uNK cells and uDC in the regulation of maternal immune responses against trophoblast antigens. Although a close topographical relationship between these subpopulations has been described in human decidua [24], most reports have focused on the potential regulatory functions of uNK cells or uDC individually and the question as to whether an interaction between these two cell types could contribute to the mechanisms of pregnancy maintenance remains elusive. In this study, we provide evidence that spatial adjacencies between uNK cells and uDC, which can be observed in mouse pregnancies, have the potential to modulate the outcome of maternal immune responses.

In mice and humans, several mechanisms by which trophoblast cells actively avoid recognition and rejection by the maternal immune system have been described, including expression of nonpolymorphic MHC class I molecules [9], Fas-L-induced apoptosis of maternal immunocompetent cells [36], and suppression of T cell proliferation–responsiveness by IDO-mediated tryptophan catabolism [37, 38]. It is therefore tempting to speculate that components of the innate immune system, as mediators of the first encounter with fetal antigens, might be preferential targets of the regulatory mechanisms induced by trophoblast cells. In the present study, we show that trophoblasts from low and high abortion murine matings differentially modulate uNK cell and uDC maturation–activation state and cytokine secretion. Indeed, a higher downregulation of maturation marker expressed by uDC and uNK cells was observed upon stimulation with DBA/2J trophoblasts. In addition, while both mating combinations showed a similar effect on uDC cytokine secretion, only trophoblasts from low abortion matings were able to stimulate IL-10 and IL-12p70 secretion by uNK cells, regardless of these differential effects of both mating combinations (which would be due to their distinct pregnancy-protective molecule expression). Our results strongly suggest that interactions between uDC and uNK cells are subject to modulation by trophoblast cells during murine pregnancy.

What are the tissue-specific features of NK and DC "cross talk" at the maternal fetal interface? We first considered the possibility that uNK cells and uDC could be involved in the control of uterine cell proliferation. During early pregnancy, uterine cells close to the implantation sites locally proliferate and differentiate under the influence of steroid hormones to form the decidua [39]. Here, we show that significant uterine cell proliferation was only induced in the presence of both uNK and uDC, suggesting that this process is controlled by the coordinate action of these cell subsets at the murine maternal fetal interface. The involvement of uNK cells in the initiation of the decidualization process has been suggested [40], as these cells are found in increased numbers close to the implantation site and express leukemia-inhibitory factor, a cytokine that has been shown to play a crucial role in implantation [41]. However, no evidence for a direct contribution of uDC to the uterine changes that lead to embryo implantation exist yet. Interestingly, cell proliferation was markedly upregulated when direct physical interactions between uterine cells and murine trophoblasts were avoided by using a transwell culture system. Thus, it appears that soluble factors control decidual cell proliferation and DC–NK cell functions at the murine maternal fetal interface and cell–cell interactions through surface molecules negatively affect this process. Based on these findings, the establishment of pregnancy in mice could be seen as a dual process in terms of the regulation of uterine cell proliferation. At an initial phase, the dialog between the conceptus and the maternal compartment would be mediated by soluble factors that promote uterine cell proliferation and differentiation to form the decidua. Later on, as trophoblast cells invade the uterine stroma leading to the formation of a functional placenta; cell–cell interactions involving surface signalling complexes would result in decreased decidual cell proliferation rates. Our results provide the basis for further studies aimed at defining the nature of cell interactions involved as well as the individual role of each cell subset in the control of uterine cell proliferation during implantation and decidualization in mice.

The balance between Th1 and Th2 cytokines seems to be crucial for successful pregnancy: an inflammatory response mediated by Th1 cytokines (IL-2, TNF-α, IL-12, IFN-γ) is suggested to have deleterious effects on mammalian pregnancy, while anti-inflammatory Th2 cytokines (IL-4, IL-10) appear to be pregnancy protective [6]. In our experimental setup, trophoblasts from low and high abortion matings were found to dramatically abrogate IL-12p70 secretion by uDC. Moreover, in low abortion matings, the synthesis of Th1 cytokines appears to be tightly controlled by the NK cell subset, as demonstrated by the significant downregulation of IL-12p70 and TNF-α levels observed when uDC where cultured in the presence of uNK cells. In line with our previous results reporting a bias towards IL-10 production by DC at the uterine level in unchallenged pregnancies [8], we also found that both uNK cells and uDC produce significant levels of this Th2 cytokine in response to trophoblast stimulation. Interestingly, IL-10 secretion was markedly upregulated when direct contact with trophoblast cells was impaired by using a transwell culture system, suggesting that the stimulatory functions of trophoblast cells are mainly mediated by

soluble factors. On the other hand, uDC appears to be most sensitive to physical interactions involving surface molecules (as demonstrated by the failure to stimulate cytokine secretion observed when direct contact with trophoblasts was avoided). Though establishing the predominance of any of these mechanisms exceeds by far the scope of the present study, our results suggest that modulation of NK–DC cross talk by trophoblast cells at the fetal maternal interface results in the promotion of a tolerogenic microenvironment characterized by Th2 cytokine dominance. The distinct ability of Balb/c trophoblasts to stimulate IL-10 production by isolated uNK cells is also worth noting and could indicate that the regulatory functions of trophoblast cells associated with tolerance induction are impaired in high abortion matings.

The finding that DBA/2J trophoblasts were more effective than Balb/c trophoblasts in downregulating uDC and uNK cell maturation marker expression provides further evidence that regulatory functions are impaired in abortion-prone matings. The biological plasticity of DC in promoting immunity or tolerance appears to depend on their maturation state, with mature DC associated with a Th1 polarization of T cells [31]. Although successful pregnancy is widely recognized as a Th2 phenomenon, it is well established that an inflammatory milieu involving several Th1 cytokines mediates the communication between embryonic cells and maternal uterine cells as part of the physiologic response to implantation [42, 43]. Accordingly, a certain degree of uDC maturation would be required during early pregnancy to provide this transient inflammatory milieu and trophoblast cells failing to induce this process would result in the high abortion rates characteristic of the CBA/J × DBA/2J mouse model.

Another striking finding in the present study is that trophoblasts from either low or high abortion mating combinations failed to induce significant uNK-cell-mediated cytotoxic activity, irrespective of the uNK-to-uDC ratio examined. This represents a unique feature of NK and DC "cross talk" at the maternal fetal interface that so far has not been described in other tissues. In line with this finding, there is no evidence to date that uNK cells can lyse trophoblast, even when activated by IL-2 (which is absent in decidua). Indeed, only in vitro cultured trophoblast can be killed by lymphokine-activated killer cells [44]. Thus, maternal immune responses involving trophoblast cytolysis would result from the activity of other uterine cell subsets rather than NK cells. In this regard, Mellor et al. [45] have reported C3 deposition in occult losses, which has also been reported in Crry knockout mice [46]. C3 and C5 have also been suggested to play a key role in the CBA/J × DBA/2J model, as discussed by Girardi et al. [47], who describe T cell and macrophage molecules that can activate complement system. In fact, thrombin is able to activate C5 [48], so T cells that activate coagulation pathways can coactivate complement. From our results, it seems most likely that cytokine production rather than cytotoxic activity is important for uNK cell function at the maternal fetal interface.

Both NK cells and DC have been suggested to express IDO, which could be involved in the regulation of T cell responses and cytolytic activity, respectively [34, 35]. No significant IDO activity of uNK cells or uDC was observed in response to trophoblast stimulation. Thus, we propose that IDO is not involved in the mechanisms by which NK and DC interactions modulate the outcome of immune responses at the maternal fetal interface. Evidence regarding IDO as a key molecule in the development of maternal tolerance is in fact controversial because pregnancy in mice lacking a functional IDO gene progress normally [49]. Further studies are necessary to provide a better understanding of the mechanisms involved in IDO-induced pregnancy protection.

In summary, our study provides the first evidence that a strong NK and DC interaction takes place at the murine fetomaternal interface, with tissue-specific features that result in a balanced process determining the outcome of maternal immune responses. Notably, like in the present study, most data regarding NK and DC interactions have been generated using in vitro studies. Because studies addressing the relevance of these interactions in the in vivo context are limited, we hope that our findings will encourage future research on the role of NK and DC in pregnancy maintenance in vivo.

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