

Natural Killer-Like Cells in the Sheep: Functional Characterization and Regulation by Pregnancy-Associated Proteins

ŞABAN TEKIN^{1,2} AND PETER J. HANSEN^{1,3}

¹Department of Animal Sciences, University of Florida, Gainesville, FL 32611-0910; ²Department of Biology, University of Gaziosmanpaşa, Tokat, Turkey

Natural killer (NK) cells represent an important component of the innate immune system. In ruminants there are few reports regarding presence or characterization of NK cells. Although absence of expression of major histocompatibility complex proteins on ovine trophoblast makes it potentially a target for NK cells, little is known about regulation of NK cells by products of pregnancy in sheep. Objectives of the present study were to determine whether cells with characteristics of NK cells exist in preparations of ovine peripheral blood lymphocytes (PBL) and endometrial epithelial cells (EEC) and to determine regulation of such cells by two pregnancy-associated molecules with immunoregulatory properties (ovine uterine serpin [OvUS] and interferon- τ [IFN- τ]). Ovine PBL and EEC lysed a putative NK target cell, the BHV-1 infected D17 cell, and lysis by both types of cells was neutralized by antibody against a molecule called function-associated molecule (FAM) expressed on NK cells of several species. Moreover, inhibitors that interfere with perforin-mediated lysis blocked NK-like activity of PBL. The NK-like lytic activity of PBL and EEC was inhibited by OvUS, whereas ovine and bovine IFN- τ significantly enhanced NK-like activity of PBL. In conclusion, NK-like activity present in preparations of ovine PBL and EEC is mediated by FAM⁺ cells, is dependent on processes that involve perforin processing, and is regulated by OvUS and IFN- τ . Inhibition of NK-like activity of PBL and EEC by OvUS is consistent with a role for OvUS in protecting the conceptus from maternal cytotoxic lymphocytes. Stimulation of lysis by IFN- τ implies the existence of other inhibitory mechanisms during early pregnancy to prevent NK cell-mediated destruction of the conceptus. *Exp Biol Med* Vol. 227:803–811, 2002

Key words: NK; lymphocytes; ovine; serpin; interferon.

Natural killer (NK) cells participate in innate immunity by exerting nonspecific lysis without prior exposure to antigen against infected or abnormal cells such as tumor cells (1). Unlike cytotoxic T cells, NK cells do not require presence of major histocompatibility complex (MHC) class I molecules on target cells for lysis (1,2). Indeed, MHC class I expression on target cells inhibit NK cell function (3,4). To date, there are few reports regarding presence, regulation, or mechanism of action of ovine NK cells. Bovine herpes virus-1 infected D17 cells have been used as an NK target in cattle (5) and sheep (6). Exposure to bovine herpes virus-1 (BHV-1) increases target sensitivity because it downregulates expression of MHC class I molecules (7,8). Sheep peripheral blood lymphocytes (PBL) can also exhibit NK-like lytic activity against target cells including K562 cells (9) and YAK cells (10). Such killing differs from killing by human and murine NK in that prolonged incubation with target is required (4 hr vs 20 hr). In addition, NK activity has been reported against ovine pre-attachment conceptuses (11). The monoclonal antibody NK5C6 (also called anti-Function Associated Molecule; anti-FAM) produced against nonspecific cytotoxic cells of fish reacts with NK cells from humans and rodents (12,13) and has been reported to react with a subset of lymphocytes in sheep and cattle (14).

One potential target for NK cells is the placenta. In many species, including sheep (15), expression of MHC class I and II genes is downregulated on trophoblast, making these cells potential NK targets. NK cells have been found in uterine tissues of humans, mice, and pigs (16–18) and implicated in various models of abortion (10,11,19–21). That NK-mediated destruction of the conceptus does not usually occur is probably the result of several mechanisms including increased resistance of trophoblast to NK-mediated lysis (6,22) and production of molecules at the conceptus-maternal interface that downregulate NK cell function.

Several regulatory molecules produced during pregnancy have been implicated in regulation of immune function in sheep. The best studied of these, ovine uterine serpin

This study was supported in part by USDA NRICGP grants #96-35203-3304 and #2001-35204-10797. This is Journal Series No. R-08622 of the Florida Agric. Res. Sta.

³ To whom correspondence and reprint requests should be addressed at PO Box 110910, Department of Animal Sciences, University of Florida, Gainesville, FL 32611-0910. E-mail: Hansen@animal.ufl.edu.

Received January 24, 2002
Accepted June 3, 2002

1535-3702/02/2279-0803\$15.00
Copyright © 2002 by the Society for Experimental Biology and Medicine

(OvUS), is a member of the serine proteinase inhibitor (serpin) family. Among its actions, OvUS can inhibit mixed lymphocyte reactions, mitogen-induced lymphocyte proliferation, poly I-poly C induced activation of NK in mice, and poly I-poly C induced abortion in mice (10,23). Another immunoregulatory protein produced during pregnancy in the sheep is interferon- τ (IFN- τ). Although the primary function of this type I interferon is to prevent luteolysis, IFN- τ also possesses immunoregulatory activity and can inhibit mitogen-induced proliferation of lymphocytes (24,25) and increase NK lysis against K562 cells (9). Thus, both OvUS and IFN- τ might be involved in regulation of NK function during pregnancy in sheep although in different ways.

The present study tested whether activities characteristic of NK-like cells can be identified in ovine PBL and preparations of endometrial epithelial cells (EEC), whether lytic function of sheep NK can be blocked by inhibitors of perforin processing, and whether lytic function of sheep NK-like cells is regulated by OvUS and IFN- τ .

Materials and Methods

Materials. Tissue Culture Medium-199 (M-199); Eagle's Minimal Essential Medium (MEM); guinea pig serum; glutamine; penicillin-streptomycin; red cell lysis buffer; β -mercaptoethanol; trypsin-EDTA; Triton X-100; saponin; conconamycin-A; leupeptin; and pepstatin-A were purchased from Sigma Chemical (St. Louis, MO). Ammonium chloride was from Fisher Scientific (Pittsburgh, PA). Fico-Lite 1077 was from Atlanta Biologicals (Norcross, GA). Horse serum was obtained from Hyclone (Logan, UT). Fetal bovine serum and goat serum were from Intergen (Purchase, NY). $\text{Na}^{51}\text{CrO}_3$ (specific activity range between 258 to 598 mCi/mg Cr) was purchased from ICN (Costa Mesa, CA). The D-17 cell line (canine osteocarcinoma) and BHV-1 (tissue culture infectious dose for 0.2 ml bovine turbinate cells, 10 days culture; $\text{TCID}_{50} = 10^{6.5}$) were from ATCC (Rockville, MD). Hi-Trap Protein G columns were from Amersham-Pharmacia (Kalamazoo, MI); T75 cell culture flasks were from Sarstedt (Newton, NC); and 96-well flat bottomed Falcon cell culture plates and cell strainer (100 μm) were from Becton Dickinson (Franklin Lakes, NJ). The FlexyMag Separator was obtained from Sphero-tech, (Schaumburg, IL). Centricon ultrafiltration devices were from Amicon (Beverly, MA). Human recombinant IFN- α was obtained from Genzyme (Boston, MA). Ovine IFN- τ 4 (OvIFN- τ) and bovine IFN- τ (BoIFN- τ) were gifts from Dr. R. Michael Roberts (University of Missouri).

Antibodies. Hybridoma cells producing monoclonal antibodies against sheep CD4 (clone 17D), CD8 (clone 7C2), $\gamma\delta$ T (clone 86D), and CD45R (clone 73B) were purchased from European Type Cell Culture Collection (Salisbury, UK). Hybridoma cells producing monoclonal antibodies against human perforin (clone 2d4) were a gift from Dr. Gillian Griffiths (Oxford University, UK). These monoclo-

nal antibodies were obtained as ascites fluid or as culture supernatants of hybridoma cell cultures prepared by the Hybridoma Core Laboratory of the University of Florida Interdisciplinary Center for Biotechnology Research. Monoclonal antibody NK5C6 (anti-FAM), which is an IgM, was purified by high-performance liquid chromatography and was a gift from Dr. D.L. Evans (University of Georgia Athens, GA). Goat affinity-purified, phycoerythrin (PE)-labeled F(ab) $_2$ fragment against mouse IgG (whole molecule), sheep affinity-purified FITC-labeled F(ab) $_2$ fragment against mouse IgG (whole molecule), mouse ascites fluid (clone NS1), and affinity-purified mouse IgG, guinea pig complement sera were from Sigma Chemical. Affinity-purified mouse IgM was obtained from ICN (Costa Mesa, CA). Goat affinity-purified, PE-labeled antibody against mouse IgM (whole molecule) was purchased from Biomeda (Foster City, CA). Magnetic beads coated with goat anti-mouse IgG (1% w/v, 2×10^8 particles per ml) were from Sphero-tech (Schaumburg, IL).

Purification of Monoclonal Antibodies. Monoclonal antibodies were either directly used as culture supernatant or ascites fluid or were used after purification from these fluids by affinity chromatography with a Hi-Trap protein G column prewashed with binding buffer (1.5 M glycine, 1 M NaCl, pH 8.9). Ascites fluid or culture supernatant diluted 1:10 with binding buffer was loaded on the column, and IgG was eluted with 1 M citrate-phosphate buffer, pH 5.0. Protein concentration was determined using the Bradford procedure (26) with mouse IgG as standard.

Purification of Ovine Uterine Serpin. Uterine fluid accumulating in the ligated horn of unilaterally pregnant ewes at day 140 of pregnancy (27) was used to purify OvUS. Purification, which involved cation exchange chromatography using carboxymethyl Sepharose and gel filtration chromatography with Sephacryl S-200, was performed as described previously (28). After purification, OvUS was dialyzed against Dulbecco's phosphate buffered saline (DPBS) and concentrated using Centricon ultrafiltration devices. Purity of protein as assessed by sodium dodecyl sulfate-polacrylamide gel electrophoresis was >90%. Protein concentration was determined using the Bradford (26) procedure with bovine serum albumin (BSA) as standard.

Preparation of Cells for $^{51}\text{Chromium}$ Release Assay. Peripheral Blood Lymphocytes. Mononuclear cells were purified from the buffy coat fraction of heparinized peripheral blood by density gradient centrifugation on Fico-Lite-1077 at 450 g for 30 min. Unless otherwise stated, blood was obtained from nonpregnant ewes without reference to cyclic status. After removing red blood cells by incubation in red cell lysis buffer, cells were resuspended in modified M-199 (M-199 containing 5% [v/v] horse serum, 200 U/ml penicillin, 0.2 mg/ml streptomycin, 2 mM supplemental glutamine, and 10^{-5} M β -mercaptoethanol) and incubated at 37°C and 5% (v/v) CO_2 for 1 hr in T75 cell culture flasks to remove adherent cells. Flasks were shaken vigorously and cells were collected in a 50 ml sterile culture

tube. Cells were centrifuged at 110g for 5 min, resuspended with modified M-199, and counted with a hemacytometer.

Endometrial Epithelial Cells. Endometrial epithelial cells were prepared from uteri collected at slaughter from cyclic ewes (collected at random stages of the estrous cycle), unilaterally pregnant ewes at day 140 of gestation, or from a pregnant ewe at day 91 of gestation. For cyclic ewes, tracts were classified as being in the luteal phase or nonluteal phase based on the presence of a corpus luteum. There were no differences due to this classification, and data are pooled for both types of ewes. Unilateral pregnancies were established as described elsewhere (27).

After recovery of the reproductive tract, uterine horns from cyclic ewes were opened longitudinally along the antimesometrial border and luminal epithelium (which contains a mixture of luminal epithelial cells and uterine intraepithelial lymphocytes) was removed from intercaruncular areas of the endometrium by mechanically scraping the inner surface of the endometrium with a sterile surgical blade. For pregnant animals, scraping was preceded by removal of the conceptus and washing the endometrium with sterile DPBS to remove blood contamination. Cell scrapings were collected into a 50 ml sterile culture tube containing 5 ml M-199 supplemented with 2 mM EDTA. Cells were incubated at 37°C for 1 hr. Cells in suspension were then triturated vigorously for 5 min to break cell clumps, filtered through a sterile 100 µm cell strainer into a 50 ml sterile culture tube, and centrifuged at 110 g for 5 min. The cell pellet was resuspended with 5 ml modified M-199 and cell number was determined using a hemocytometer.

D17 Target Cells. D17 cells were cultured continuously in Eagle's MEM supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 200 U/ml penicillin, and 2 mg/ml streptomycin. At confluence, D17 cells were trypsinized, mixed with an equal amount of MEM, centrifuged for 5 min at 110g, resuspended in modified MEM, counted, and adjusted to 1×10^6 cells/ml in modified MEM.

⁵¹Chromium Release Assay. A 1 ml aliquot of 1×10^6 D17 cells was centrifuged at 110 g for 5 min, resuspended with 100 µl modified MEM and 100 µCi Na⁵¹CrO₃, and incubated at 37°C and 5% (v/v) CO₂ for 1 hr. The labeled cells were washed twice with medium and resuspended to 1×10^5 cells/ml. Labeled target cells were resuspended to 1×10^5 cells/ml and 100 µl cells pipetted into individual wells of a 96-well flat-bottomed culture plate. Into half of the wells was added 20 µl working BHV-1 solution (a 1:10 dilution of the original BHV-1 solution with modified MEM). Plates were incubated at 37°C and 5% (v/v) CO₂ overnight. Cells were then washed twice with 100 µl modified MEM by centrifugation at 80 g for 3 min and resuspended with 50 µl modified MEM.

An aliquot of 100 µl effector cells (1×10^6 , 5×10^5 , or 2.5×10^5 PBL or EEC in modified M-199) was pipetted into wells containing 1×10^4 ⁵¹Cr labeled target cells (50 µl) in a flat-bottom 96-well plate to produce effector:target cell ratios of 100:1, 50:1, and 25:1. Wells to measure spontane-

ous release contained 100 µl target cells and 100 µl modified MEM or M-199 only, whereas wells to measure maximum release contained 100 µl target cells and 100 µl 2% (v/v) Triton X-100. Plates were then centrifuged at 80 g for 3 min to facilitate contact between effector and target cells before incubation at 37°C and 5% (v/v) CO₂ for 20 hr. The assay was terminated by centrifugation of plates at 350 g for 15 min at room temperature. A 100 µl aliquot of each supernatant was removed and counted for radioactivity using a gamma counter (Cobra Auto-Gamma, Packard Instrument Inc., Downers Grove, IL). Results of triplicate wells were averaged and expressed as percent lysis using the formula: percentage lysis = (sample DPM - spontaneous DPM) / (maximum DPM - spontaneous DPM) × 100.

Depletion of Lymphocytes by Complement-Mediated Lysis. Freshly isolated PBL (1×10^7 cells in 250 µl) were incubated with 10 µl ascites fluid containing anti-CD8 or anti-γδT monoclonal antibodies in 12 × 75 cell culture tubes. Control mouse ascites fluid was used as control. After incubation on ice for 45 min, cells were centrifuged, resuspended, and incubated with 200 µl guinea pig complement serum at 37°C for 30 min. Cells were washed once with 2 ml DPBS and resuspended to 1×10^7 cells/ml with modified M-199. The ⁵¹Cr release assay was performed as described above using effector:target ratios of 50:1 and 25:1. The experiment was repeated three times using PBL from three separate nonpregnant ewes.

Immunomagnetic Depletion of Lymphocytes. Magnetic beads coated with goat anti-mouse IgG (2×10^7 particles) in 250 µl were mixed with 125 µl of either ascites fluid containing anti-CD8 or anti-γδT or with culture supernatant containing anti-CD4. Beads were incubated with antibody in sterile 12 × 75 cell culture tubes at 4°C for 30 min on a tube rotator at low speed. Controls included incubation of beads with DPBS and control mouse ascites fluid. Beads were then washed twice with 2 ml DPBS for 5 min, resuspended with 250 µl modified M-199, and mixed with 100 µl (1×10^7 cell) PBL or EEC on a tube rotator at low speed for 30 min at 4°C. Magnetic beads were then separated using a FlexiMag Separator. The supernatants containing unbound cells were transferred to 12 × 75 mm cell culture tubes, centrifuged, resuspended with 1 ml modified M-199 (1×10^7 cell/ml final), and tested for lysis of D17 target cells at effector:target ratios of 100:1, 50:1, and 25:1. The experiment was performed by using PBL from five nonpregnant ewes and four pregnant ewes (day 140) and by using EEC from five unilaterally pregnant ewes at day 140 of pregnancy. Each ewe was tested separately but, for EEC from the unilaterally pregnant ewes, EEC were pooled from both horns before analysis.

Immunoneutralization of NK-Like Activity. For PBL, an aliquot of 100 µl effector cells (containing 1×10^6 and 5×10^6 cells in modified M-199) was pipetted into wells containing 1×10^4 ⁵¹Cr labeled BHV-1 infected D17 target cells (50 µl) in a flat-bottomed 96-well cell culture plate to produce effector:target cell ratios of 100:1 and 50:1.

Additionally, purified monoclonal anti-CD8 (10 μ g/well), anti- γ δ T (10 μ g/well), anti-FAM (100 μ g/well), or cell culture supernatant for anti-CD4 (10 μ l/well) were pipetted into designated wells. Isotype controls were mouse IgG (10 μ g/well), and mouse IgM (10 μ g/well). The experiment was repeated with PBL from seven separate nonpregnant ewes.

For EEC, 100 μ l effector cells containing 1×10^6 and 5×10^6 cells in modified M-199 were pipetted into wells containing target cells as described above. Effector:target cell ratios were 100:1 and 50:1. The EEC were treated with 10 μ l ascites fluid containing monoclonal antibodies against CD8 or γ δ T, 10 μ l cell culture supernatant containing monoclonal antibodies against CD4, 10 μ g/well anti-FAM, control mouse ascites fluid (10 μ l/well), or mouse IgM (10 μ g/well). The 51 Cr release assay was carried out as described earlier. The experiment was replicated using EEC from nine cyclic ewes at random stages of the estrous cycle and one pregnant ewe at day 91 of gestation.

Flow Cytometry. To determine expression of FAM, 100 μ l aliquots of PBL (5×10^5 cells in modified-M199) were placed into 13 \times 100 mm polyethylene tubes, washed twice with 2 ml of staining buffer (PBS containing 1% [w/v] BSA and 2% [v/v] fetal calf serum), and resuspended with 100 μ l staining buffer. Cells were incubated with 5 or 10 μ g anti-FAM or mouse IgM (isotype control) on ice for 30 min, washed twice with 2 ml staining buffer, and resuspended with a mixture of 100 μ l staining buffer and 10 μ l anti-mouse IgG-PE. After incubation for 30 min on ice in the dark, cells were washed twice with 2 ml staining buffer and resuspended with 500 μ l staining buffer. The flow cytometry profiles were obtained on a FACSort flow cytometer (Becton-Dickinson) by using CELLQuest flow cytometry software. The cell populations analyzed were gated on the basis of forward and side (90° angle) scatter to avoid contamination by dead cells and debris. The analysis was repeated using PBL from three separate nonpregnant ewes.

For perforin expression, 5×10^6 cells in 100 μ l were washed twice with 1 ml staining buffer, and fixed using 2 ml 1% (w/v) paraformaldehyde in PBS for 15 min at room temperature. Subsequently, 5×10^5 cells in 200 μ l were placed into 13 \times 100 mm polyethylene tubes, washed twice with 1 ml staining buffer, and incubated with 1 ml saponin buffer (PBS containing 0.3% [w/v] saponin and 2% [v/v] goat serum) for 20 min at room temperature. Cells were washed twice with 1 ml saponin buffer and incubated in a total volume of 100 μ l saponin buffer containing either 5 μ l cell culture supernatant containing monoclonal antibody against human perforin or 5 μ l control mouse ascites fluid for 30 min at room temperature. Cells were washed twice with 2 ml saponin buffer and incubated with 10 μ l anti-mouse IgG-PE in a total volume of 100 μ l saponin buffer for 30 min at room temperature. Cells were washed twice with 2 ml saponin buffer and resuspended with 500 μ l staining buffer. The flow cytometry profiles were obtained and cell populations analyzed as described earlier. The analysis

was repeated using PBL from four separate nonpregnant ewes.

To confirm the effectiveness of magnetic bead depletion, PBL from one ewe (1×10^6 cells in modified-M199) that had been depleted by using magnetic beads coated with anti-CD8, anti- γ δ T, and mouse IgG were transferred into 13 \times 100 mm polyethylene tubes, washed twice with 2 ml staining buffer (PBS containing 1% [w/v] BSA and 2% [v/v] goat calf serum), and resuspended with 100 μ l staining buffer. Cells were incubated with 10 μ l anti- γ δ T, CD8, and mouse IgG (isotype control) on ice for 30 min, washed twice with 2 ml staining buffer, and resuspended with a mixture of 100 μ l staining buffer and 10 μ l anti-mouse IgG-FITC conjugate. After incubation for 30 min on ice in the dark, cells were washed twice with 2 ml staining buffer and resuspended with 500 μ l staining buffer, and flow cytometry analysis of duplicate or triplicate samples for each treatment were performed as described earlier.

Immunohistochemistry for FAM and CD45R. Immunohistochemical analysis of uterine endometrium was performed on 6 μ m thick sections of snap-frozen tissue samples. Tissue sections were either fixed with 95% (v/v) ethanol or acetone. Procedures for immunohistochemistry were carried out according to manufacturer's (Biomedica) instructions—all steps were performed at room temperature in a humidified chamber and samples were washed with staining buffer (PBS containing 2% [v/v] goat serum) between all steps. Incubation with primary antibody (anti-FAM in staining buffer at concentrations ranging between 1:20 to 1:750 and anti-CD45R at 1:800 dilution) or controls (mouse IgM and control mouse ascites fluid) was overnight. The study was replicated with tissues from four unilaterally pregnant ewes at day 140 of pregnancy. In addition, PBL affixed on slides were used as an additional control tissue.

Effect of Inhibitors on NK-Like Activity of PBL. Aliquots of 100 μ l PBL (containing either 5×10^5 or 2.5×10^5 cells) were placed into wells containing 1×10^4 51 Cr labeled BHV-1 infected D17 target cells (50 μ l) in a flat-bottomed, 96-well cell culture plate to produce effector:target cell ratios of 50:1 and 25:1. In addition, concanomycin-A (100 ng/ml), leupeptin (30 μ g/ml), pepstatin-A (10 μ g/ml), and ammonium chloride (10 mM) were added into designated wells. Control wells included similar volumes of DPBS as used to deliver the inhibitors; final volume was adjusted with modified M-199 to 200 μ l. The killing assay was carried out as described earlier. The experiment was performed using PBL from six nonpregnant ewes.

Effect of OvUS on NK-Like Activity of PBL. Aliquots of PBL (5×10^6 cell/tube in 100 μ l modified M-199) were pipetted into 13 \times 100 mm sterile cell culture tubes. The OvUS was added to designated tubes at a final concentration of 1 mg/ml. Two separate preparations of OvUS were tested. In addition, control tubes included the same volume of DPBS as used for OvUS and the final volume was adjusted with modified M-199 to 400 μ l. The cells were incubated at 37°C and 5% (v/v) CO₂ for 1 hr. Cells were

then centrifuged at 110 g for 5 min, the supernatant was removed, and the pellet was resuspended with 1 ml fresh modified M-199. Aliquots (100 and 50 μ l) of cells were placed with BHV-1 infected D17 cells. The ^{51}Cr release assay was carried out as described earlier. The experiment was performed using PBL from six nonpregnant ewes.

Effect of OvUS on NK-Like Activity of EEC. Aliquots of EEC (1×10^7 cell/tube in 250 ml modified M-199) were pipetted into 13 \times 100 mm sterile cell culture tubes and OvUS and ovalbumin (OVAL) were added to designated tubes at a final concentration of 1 mg/ml. Control tubes included the same volume of DPBS as used to deliver the proteins and the final volume was adjusted with modified M-199 to 1 ml. The cells were incubated at 37°C and 5% (v/v) CO_2 for 1 hr and centrifuged at 110 g for 5 min; supernatant was then removed and the pellet was resuspended with 1 ml of fresh modified M-199. The ^{51}Cr release assay was carried out as described earlier. The experiment was performed using EEC from three ewes at random stages of the estrous cycle.

Effect of IFN- τ on NK-Like Lysis of PBL. Aliquots of PBL (5×10^6 cell/tube in 100 μ l modified M-199) were placed in 13 \times 100 mm sterile cell culture tubes and OvIFN- τ and BoIFN- τ added to designated tubes at a final concentration of 100 ng/ml. Control tubes included the same volume of DPBS as used to deliver the cytokines and the final volume was adjusted with modified M-199 to 500 μ l. The cells were incubated at 37°C and 5% (v/v) CO_2 for 1 hr, centrifuged at 110 g for 5 min, supernatant removed, and the pellet resuspended with 1 ml of modified M-199. Aliquots (100 and 50 μ l) of cells were placed with BHV-1 infected D17 cells. The ^{51}Cr release assay was carried out as described earlier and performed using PBL from six separate nonpregnant ewes.

Effect of Pregnancy Status on Activity of NK-Like cells in PBL. Peripheral blood lymphocytes from nine unilaterally pregnant ewes at day 140 of pregnancy and nine nonpregnant ewes were tested for lysis of BHV-1 infected D17 cells using the ^{51}Cr release assay at effector:target cell ratios of 25:1, 50:1, and 100:1.

Statistical Analysis. Data were analyzed by least square analysis of variance using the General Linear Models Procedure of SAS (29). Analyses included effects of ewe (lymphocyte donor), treatments, effector:target ratio, and all possible interactions. Ewe was considered as a random effect and other main effects were considered fixed. Error terms were determined based on calculation of expected mean squares. In some analyses, variance for multiple-degree-of-freedom effects was partitioned using orthogonal contrasts to determine individual comparisons. In addition, the pdiff mean separation test of SAS was performed.

Results

Immunoneutralization of NK-Like Activity. As shown in Figure 1, lysis of BHV-1 infected D-17 cells by PBL was reduced when incubation was conducted in the

presence of 100 μ g anti-FAM ($P < 0.05$). In contrast, there was no significant reduction of lysis caused by incubation with anti-CD8, anti-CD4, anti- $\gamma\delta\text{T}$ or isotype controls (Fig. 1). In a second experiment using PBL from a nonpregnant ewe, 50 μ g anti-FAM also neutralized activity, whereas 50 μ g IgM control had no effect (results not shown).

Lysis of BHV-1 infected D-17 cells by EEC was also reduced by coincubation with anti-FAM ($P < 0.01$), whereas other antibodies and isotype controls tested did not exert any effect on EEC lytic activity (Fig. 2).

Magnetic bead depletion and complement-mediated lysis using antibodies against CD4, CD8, or $\gamma\delta\text{T}$ cells did not affect NK-like lysis of BHV-1 infected D-17 cells by PBL (results not shown). The effectiveness of depletion by magnetic bead separation was confirmed by flow cytometry. The percentage of positive cells with depletion using control mouse ascites fluid or specific antibody were as follows: CD8 $^+$, 25.8% \pm 0.26% vs 3.65% \pm 0.20%; $\gamma\delta\text{T}^+$, 3.06% \pm 0.26% vs 0.27% \pm 0.20%.

Flow Cytometry and Immunohistochemistry Using Anti-FAM. No FAM $^+$ cells were identified in PBL using flow cytometry (results not shown). The percentage of PBL staining with FAM by flow cytometry (~3%-4%) was similar to the proportion of cells nonspecifically stained with the mouse IgM isotype control. Similarly, there was no detection of FAM $^+$ cells in PBL or endometrium using immunohistochemistry. This was the case using either ethanol or acetone as a fixative. As a positive control, immunohistochemistry of endometrium and PBL was also performed using anti-CD45R as an antibody; antigen was detected in cells in both tissues (results not shown).

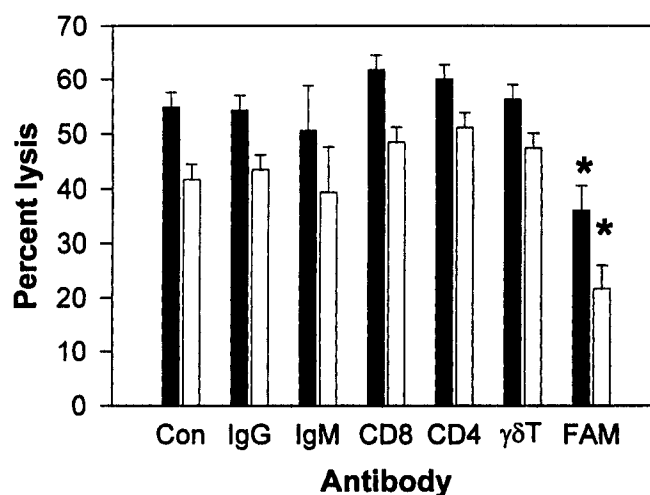


Figure 1. Immunoneutralization of NK-like activity in preparations of PBL. Lysis of BHV-1 infected D17 cells by PBL was conducted in the presence of various antibodies (anti-CD8, anti-CD4, anti- $\gamma\delta\text{T}$ and anti-FAM), isotype controls (mouse IgG and mouse IgM), or a DPBS control (Con). Lysis was performed at 100:1 (black bars) and 50:1 (open bars). Shown are least square means \pm SEM for results from seven nonpregnant ewes. Anti-FAM neutralized lysis of BHV-1 infected D17 cells by PBL ($P < 0.05$). *Significantly different from control ($P < 0.05$ or less).

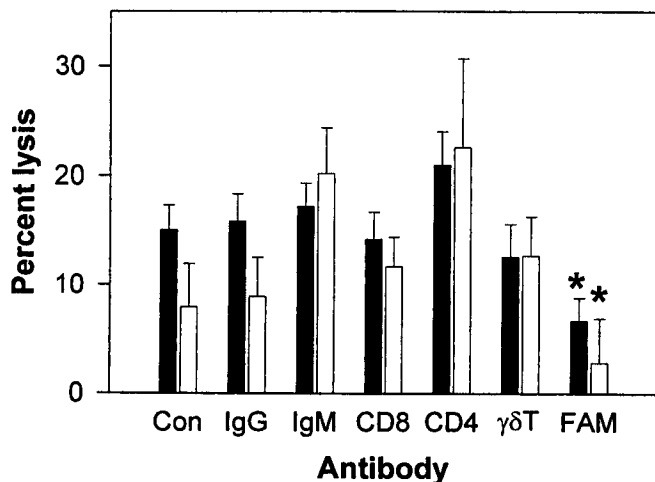


Figure 2. Immunoneutralization of NK-like cell activity in preparations of EEC. Lysis of BHV-1 infected D17 cells by EEC was conducted in the presence of various antibodies (anti-CD8, anti-CD4, anti-γδT, anti-FAM, control mouse ascites fluid, mouse IgM, or a DPBS control). Lysis was performed at 100:1 (black bars) and 50:1 (open bars). Shown are least square means \pm SEM for results from 9 cyclic ewes and 1 pregnant (day 91) ewe. Anti-FAM neutralized lysis of BHV-1 infected D17 cells by EEC ($P < 0.01$). *Significantly different from control ($P < 0.05$ or less).

Effect of Perforin Processing Inhibitors on Lysis of D17 Cells by PBL. As compared to controls, both concanamycin-A and leupeptin significantly reduced NK-like lysis of BHV-1 infected D17 cells at an effector:target cell ratio of 50:1. Pepstatin-A and ammonium chloride significantly reduced NK-like lysis of BHV-1 infected D17 cells at both ratios tested (Fig. 3).

Perforin Expression in PBL. A representative result of flow cytometry for perforin expression in PBL is shown in Figure 4. The percentage of perforin positive cells in PBL ($n = 4$) averaged $8.83 \pm 0.68\%$.

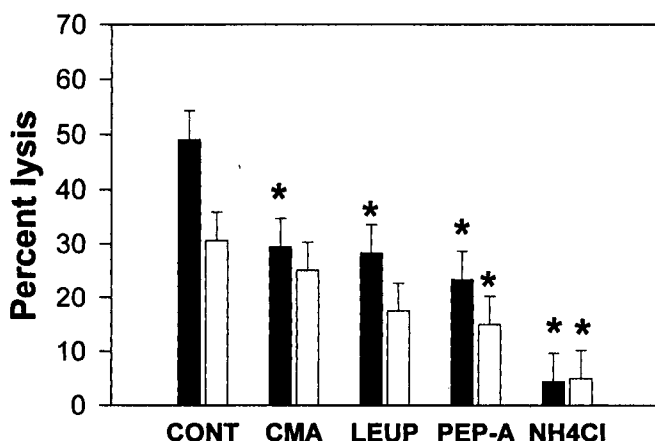


Figure 3. The effect of inhibitors of perforin processing on lysis of BHV-1 infected D17 cells by PBL. The assay was performed with a DPBS control (CONT), concanamycin-A (CMA), leupeptin (LEUP), pepstatin-A (PEP-A) and ammonium chloride. Lysis was at 50:1 (black bars) and 25:1 (open bars). Data are least square means \pm SEM for results from 6 nonpregnant ewes. Overall, lysis was affected by treatment ($P < 0.01$) and ratio ($P < 0.05$). As compared with controls, lysis was reduced by all inhibitors at 50:1 and by leupeptin and ammonium chloride at 25:1 ($P < 0.01$). *Significantly different from control ($P < 0.05$ or less).

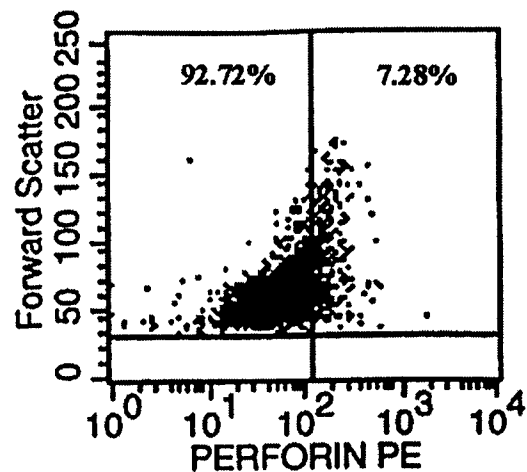


Figure 4. The expression of perforin on PBL stained sequentially with mouse anti-human perforin and goat anti-mouse IgG-PE. Shown is a representative result of experiments from four nonpregnant ewes.

Regulation of NK-Like Lysis of PBL and EEC by OvUS. Both of two separate purification batches of OvUS tested inhibited NK-like lysis of BHV-1 infected D-17 cells by PBL ($P < 0.001$) (Fig. 5A). Lysis of BHV-1 infected D-17 cells by EEC was also inhibited by OvUS ($P < 0.05$) but not by the control protein, OVAL (1 mg/ml) (Fig. 5B).

Regulation of NK-Like Lysis of PBL by IFN- τ . At a final concentration of 100 ng/ml, ovine and bovine IFN- τ each significantly increased lytic function of PBL against BHV-1 infected D-17 cells ($P < 0.01$) (Fig. 6) as compared

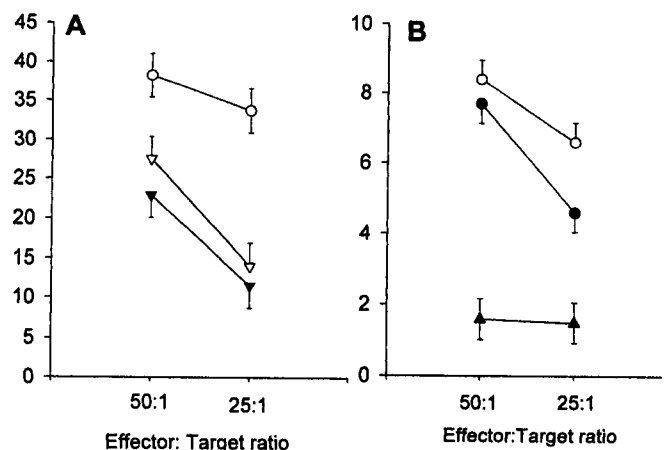


Figure 5. Inhibitory activity of OvUS on lysis of BHV-1 infected D17 cells by PBL (A) and EEC (B). PBL were preincubated with two separate OvUS batches (open and filled triangle) or an equal volume of DPBS (open circle) for 1 hr before the ^{51}Cr release assay. Data are represented as least square means \pm SEM for results by PBL from six nonpregnant ewes. Lysis was affected by ratio ($P < 0.01$) and treatment ($P < 0.01$). Using orthogonal contrasts, OvUS differed from controls ($P < 0.001$) but there was no difference between the two batches of OvUS. The EEC were cultured with OvUS (filled triangle), OVAL (filled circle), or an equal volume of DPBS (open circle) for 1 hr before the ^{51}Cr release assay. Data are least square means \pm SEM for results by EEC from three nonpregnant ewes. Lysis was affected by ratio ($P < 0.01$) and was greater for DPBS control and OVAL as compared with OvUS ($P < 0.05$). There was no significant difference between DPBS and OVAL.

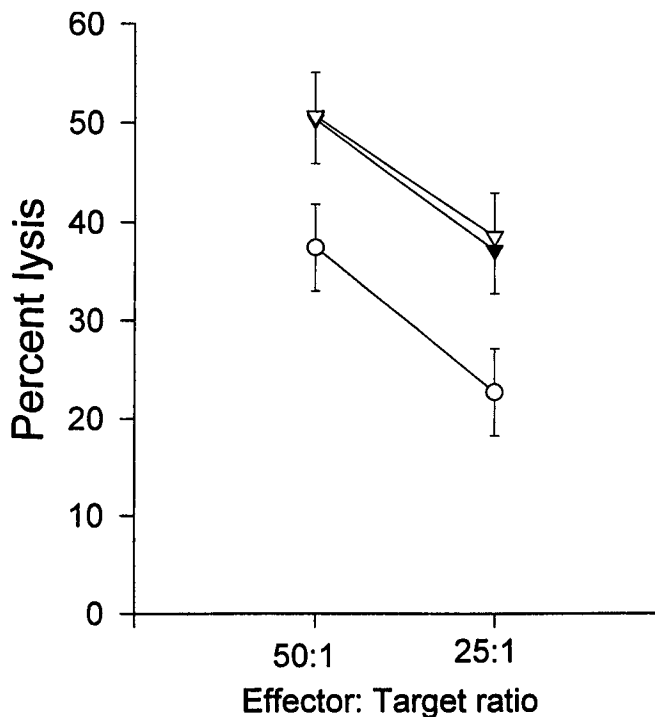


Figure 6. Effect of IFN- γ on lysis of BHV-1 infected D17 cells by PBL. Before the ^{51}Cr release assay, PBL were preincubated with ovine (filled triangle) and bovine IFN- γ (open triangle) or an equivalent volume of DPBS (open circle) for 1 hr. Shown are least squares means \pm SEM for using six separate nonpregnant ewes. The lytic activity was affected by ratio ($P = 0.05$) and treatment ($P < 0.01$). Using orthogonal contrasts, the two interferons were different from controls ($P < 0.01$) but there was no difference between bovine and ovine IFN- γ .

with DPBS controls. There was no difference between ovine and bovine IFN- γ in inhibitory activity.

Effect of Pregnancy Status on Lysis of BHV-1 Infected D17 Cells by PBL. Peripheral blood lymphocytes from pregnant (day 140) and nonpregnant ewes were tested for lysis of BHV-1 infected D17 cells. PBL lysed BHV-1 infected D17 cells and lysis increased as ratio of effector:target cells increased ($P < 0.05$). Though there was a tendency for PBL from nonpregnant ewes to cause more lysis, the effect of pregnancy status was not statistically significant (Fig. 7).

Discussion

The present study confirms an earlier one that sheep PBL can lyse BHV-1 infected D17 cells. In that study (6), lysis of D17 cells was increased by presence of virus and IL-2. Exposure to BHV-1 increases target sensitivity because it downregulates expression of MHC class I molecules (7,8), whereas IL-2 activates NK cells and transforms them into LAK cells (11,30). Present experiments provide additional evidence that lysis of D17 cells is mediated by cells with properties characteristic of NK cells. Experiments using complement-mediated lysis and magnetic bead depletion indicated that lytic activity of PBL was not caused by cells expressing CD4, CD8, or the $\gamma\delta\text{T}$ cell receptor. Ad-

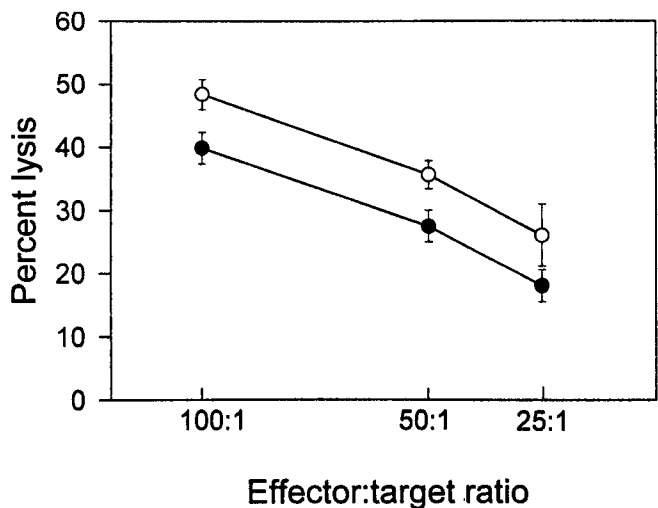


Figure 7. Effect of pregnancy status on lysis of BHV-1 infected D17 cells by PBL. Shown are least squares means \pm SEM of results from nine unilaterally pregnant (day 140, filled circle) ewes and nine nonpregnant ewes (open circle). Lysis was affected by ratio ($P < 0.05$) but not by reproductive status.

ditionally, lysis of BHV-1 infected D17 cells by PBL was blocked by the presence of antibody against FAM, a molecule previously shown to react with NK cells in different species including sheep and cattle (14). NK-like cells were also found in the endometrial epithelium because preparations of EEC contained cells that lysed BHV-1 infected D17 cells and lysis was inhibited by anti-FAM.

Both cytotoxic T cells and NK cells lyse targets through secretion of the pore-forming protein perforin (31). Perforin-mediated lysis by NK cells can be inhibited by concanomycin-A, a proton pump inhibitor, ammonium chloride, which increases the pH of endosomal compartments, and the proteinase inhibitor leupeptin (32–34). Treating sheep PBL with all these molecules reduced lysis of BHV-1 infected D17 cells by PBL. Therefore, lytic function of sheep NK-like cells in PBL is mediated by proteins, probably perforin, that reside in acidic compartments and require proteolytic processing. The presence of perforin-containing cells in PBL was also indicated by flow cytometry where about 9% of sheep PBL were positive for perforin.

Given the ability of anti-FAM to block NK-like activity in PBL and EEC, it was surprising that FAM $^{+}$ cells were not detected in PBL or endometrium by flow cytometry or immunohistochemistry. Other reports using flow cytometry, in contrast, showed anti-FAM labeled a small portion of PBL in several species including sheep (14,35,36). One plausible explanation for the failure to find FAM $^{+}$ cells in the present study is that the intensity of reaction of FAM $^{+}$ cells with antibody was too low to allow discrimination from the relatively high amount of nonspecific binding occurring with IgM.

Endometrial NK cells are also found in mice, humans, and pigs (16–18). Given that ovine trophoblast does not express classical MHC class I or II antigens (15), it is susceptible to lysis by NK cells during pregnancy. Indeed,

sheep NK-like and LAK cells were cytotoxic toward preattachment ovine conceptuses in early pregnancy (11). Sheep PBL did not kill primary trophoblasts from pregnant ewes at days 60–91 of pregnancy, but killing could be induced in some cases with IL-2 stimulation (6). Similarly, trophoblast from both mouse and human are resistant to NK lysis but susceptible to LAK-mediated killing (22,30,37). Accordingly, the survival of the conceptus may depend on regulation of cytotoxic effector cells such as NK cells.

One possibility is that NK-like lytic activity of maternal effector cells is regulated through immunoregulatory pregnancy-associated molecules released by the uterus and conceptus. One such molecule is OvUS, which can inhibit a variety of immune functions including inhibition of NK cytotoxicity and NK-mediated abortion in mice (10). Consistent with the idea that OvUS inhibits NK activity, it was found that OvUS inhibited lysis of BHV-1 infected D17 cells by both PBL and EEC. This is the first study that demonstrates that OvUS inhibits NK activity of not only PBL but also lytic cells from the uterine endometrium. In humans, HLA-G has been identified as a trophoblast-derived molecule that inhibits maternal NK cells (38). However, the sheep trophoblast is not known to produce a molecule similar to HLA-G; perhaps OvUS may play the same role in sheep as HLA-G does in the human.

Lytic activity of NK can be modulated through type I interferons such as IFN- α , β (39), and τ (9). Consistent with these reports is the present finding that both OvIFN- τ and BoIFN- τ enhanced NK-like lysis of BHV-1 infected D17 cells by sheep PBL. Perhaps secretion of IFN- τ by preimplantation conceptuses is one reason the preattachment conceptus is susceptible to NK and LAK cells (11). Although the preattachment conceptus is at risk to lysis from NK cells, it may be protected from such cells by other immunosuppressive molecules. One such molecule is a lactosaminoglycan-containing protein produced by the preattachment conceptus that can block lymphocyte proliferation (24). It may also be that some activation of NK cells may be beneficial for pregnancy. Knockout mice without NK cells had reduced litter size associated with perturbations of the vascular bed in the placenta (40). Additionally, interferon- γ released from NK cells was found beneficial for pregnancy in mice (41). Another possibility is that NK cells activated by IFN- τ facilitate placentation in sheep by causing limited lysis of luminal epithelial cells. Degradation of the luminal epithelium of uterine caruncles can first be seen around day 16 of pregnancy (42), when secretion of IFN- τ is maximal (43).

In conclusion, NK-like cells, which can lyse BHV-1 infected D17 cells and depend on FAM, exist in peripheral blood and endometrial epithelium of the sheep. Their lytic function likely depends on presence and enzymatic activation of perforin or other molecules that are processed and activated similar to perforin. The endogenous lytic activity of sheep NK-like cells is regulated negatively by OvUS and positively by OvIFN- τ and BoIFN- τ . Activation of NK may

be a consequence of IFN- τ secretion by the preattachment conceptus, whereas regulation of NK-like activity by OvUS is consistent with a role for OvUS in protecting the conceptus from maternal effector cell attack during pregnancy.

The authors are very grateful to Dr. G. Griffiths for donation of hybridoma cells producing anti-perforin, Dr D.L. Evans for anti-FAM mAb, and Dr. R.M. Roberts for OvIFN- τ 4 and BoIFN- τ . The authors acknowledge the assistance of the Flow Cytometry Core and the Hybridoma Core Laboratories of the University of Florida Interdisciplinary Center for Biotechnology Research.

Abbreviations: BHV-1, bovine herpes virus-1; BoIFN- τ , bovine interferon- τ ; BSA, bovine serum albumin; DPBS, Dulbecco's phosphate buffered saline; EEC, endometrial epithelial cells; FAM, function-associated molecule; HuIFN- α , human interferon- α ; mAb, monoclonal antibody; IL, interleukin; LAK, lymphokine activated killer cells; M-199, Tissue Culture Medium-199; MEM, Eagle's Minimal Essential Medium; MHC, major histocompatibility complex; NK, natural killer cells; OVAL, ovalbumin; OvUS, ovine uterine serpin; OvIFN- τ , ovine interferon- τ ; PBL, peripheral blood lymphocytes; PBS, phosphate-buffered saline; PE, phycoerythrin.

1. Lanier LL. NK cell receptors. *Annu Rev Immunol* **16**:359–393, 1998.
2. Karre K. Express yourself or die: peptides, MHC molecules, and NK cells. *Science* **267**:978–979, 1995.
3. Routes JM, Ryan JC, Ryan S, Nakamura M. MHC class I molecules on adenovirus E1A-expressing tumor cells inhibit NK cell killing but not NK cell-mediated tumor rejection. *Int Immunol* **13**:1301–1307, 2001.
4. Kambayashi T, Michaelsson J, Fahlen L, Chambers BJ, Sentman CL, Karre K, Ljunggren HG. Purified MHC class I molecules inhibit activated NK cells in a cell-free system in vitro. *Eur J Immunol* **31**:869–875, 2001.
5. Li W, Splitter GA. Bovine NK and LAK susceptibility is independent of class I expression on B lymphoblastoid variants. *Vet Immunol Immunopathol* **41**:189–200, 1994.
6. Tekin S, Hansen PJ. Lymphocyte-mediated lysis of sheep chorion: susceptibility of chorionic cells to specific and non-specific cytotoxic lymphocytes and presence of cells in the endometrium exhibiting cytotoxicity toward natural-killer cell targets. *Theriogenology*, in press.
7. Hariharan MJ, Nataraj C, Srikumaran S. Down regulation of murine MHC class I expression by bovine herpesvirus 1. *Viral Immunol* **6**:273–284, 1993.
8. Nataraj C, Eidman S, Hariharan MJ, Sur JH, Perry GA, Srikumaran S. Bovine herpesvirus 1 down regulates the expression of bovine MHC class I molecules. *Viral Immunol* **10**:21–34, 1997.
9. Tuo W, Ott TL, Bazer FW. Natural killer cell activity of lymphocytes exposed to ovine, type I, trophoblast interferon. *Am J Reprod Immunol* **29**:26–34, 1993.
10. Liu WJ, Hansen PJ. Effect of the progesterone-induced serpin-like proteins of the sheep endometrium on natural-killer cell activity in sheep and mice. *Biol Reprod* **49**:1008–1014, 1993.
11. Segerson EC, Gunsett FC. In vitro and in vivo effects of lymphokine-activated killer cells upon preattachment ovine conceptuses. *J Immunol* **152**:2938–2951, 1994.
12. Harris DT, Jaso-Friedmann L, Delvin RB, Koren HS, Evans DL. Identification of an evolutionary conserved function-associated molecule on human natural killer cells. *Proc Natl Acad Sci USA* **88**:3009–3013, 1991.
13. Kapur R, Evans DL, Harris DT. Evolutionary conservation of a human function-associated molecule on murine natural killer cells: expression and function. *Scand J Immunol* **40**:50–56, 1994.
14. Harris DT, Camenisch TD, Jaso-Friedmann L, Evans DL. Expression of an evolutionarily conserved function associated molecule on sheep,

- horse and cattle natural killer cells. *Vet Immunol Immunopathol* **38**:273–282, 1993.
15. Gogolin-Ewens KJ, Lee CS, Mercer WR, Brandon MR. Site directed differences in the immune response to the fetus. *Immunology* **66**:12–17, 1989.
 16. King A, Wooding P, Gardner L, Loke YW. Expression of perforin, granzyme A and TIA-1 by human uterine CD56+ NK cells implies they are activated and capable of effector functions. *Hum Reprod* **8**:2061–2067, 1993.
 17. Parr EL, Young LHY, Parr MB, Young JDE. Granulated metrial gland cells of pregnant mouse uterus are natural killer-like cells that contain perforin and serine esterases. *J Immunol* **145**:2365–2372, 1990.
 18. Croy BA, Yu ZM, King GJ. A review of the natural killer cell lineage in the uterus of the mouse and of the pig. *J Anim Sci* **72**(Suppl 3):9–15, 1994.
 19. Gendron RL, Farookhi R, Baines MG. Murine pregnancies predisposed to spontaneous resorption show alterations in the concentrations of leukotriene B₄ and prostaglandin E₂. *Biol Reprod* **47**:72–75, 1992.
 20. Duclos AJ, Pomerantz DK, Baines MG. Relationship between decidual leukocyte infiltration and spontaneous abortion in a murine model of early fetal resorption. *Cell Immunol* **159**:184–193, 1994.
 21. Kinsky R, Delage G, Rosin N, Thang MN, Hoffmann M, Chaouat G. A murine model of NK cell mediated resorption. *Am J Reprod Immunol* **23**:73–77, 1990.
 22. Zuckermann FA, Head JR. Murine trophoblast resists cell-mediated lysis. II. Resistance to natural cell-mediated cytotoxicity. *Cell Immunol* **116**:274–286, 1988.
 23. Skopets B, Hansen PJ. Identification of the predominant proteins in uterine fluids of unilaterally pregnant ewes that inhibit lymphocyte proliferation. *Biol. Reprod.* **49**:997–1007, 1993.
 24. Newton GR, Vallet JL, Hansen PJ, Bazer FW. Inhibition of lymphocyte proliferation by ovine trophoblast protein-1 and a high molecular weight glycoprotein produced by the peri-implantation sheep conceptus. *Am J Reprod Immunol* **19**:99–107, 1989.
 25. Tekin S, Ealy AD, Wang S, Hansen PJ. Differences in lymphocyte-regulatory activity among variants of ovine interferon- γ . *J Interferon Cytokine Res* **20**:1001–1005, 2000.
 26. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **7**:2248–2254, 1976.
 27. Bazer FW, Roberts RM, Basha SM, Zavy MT, Caton D, Barron DH. Method for obtaining ovine uterine secretions from unilaterally pregnant ewes. *J Anim Sci* **49**:1522–1527, 1979.
 28. Moffatt J, Bazer FW, Hansen PJ, Chun PW, Roberts RM. Purification, secretion and immunocytochemical localization of the uterine milk proteins, major progesterone-induced proteins in uterine secretions of the sheep. *Biol Reprod* **36**:419–430, 1987.
 29. SAS Institute . SAS/STAT User's Guide, Version 6, 4th Ed. Cary, NC: SAS Institute, 1998.
 30. Drake BL, Head JR. Murine trophoblast can be killed by lymphokine-activated killer cells. *J Immunol* **143**:9–14, 1989.
 31. Kagi D, Ledermann B, Burki K, Zinkernagel RM, Hengartner H. Molecular mechanisms of lymphocyte-mediated cytotoxicity and their role in immunological protection and pathogenesis *in vivo*. *Annu Rev Immunol* **14**:207–232, 1996.
 32. Hudig D, Redelman D, Minning LL. The requirement for proteinase activity for human lymphocyte-mediated natural cytotoxicity (NK): evidence that the proteinase is serine dependent and has aromatic amino acid specificity of cleavage. *J Immunol* **133**:2647–2654, 1984.
 33. Uellner R, Zvelebil MJ, Hopkins J, Jones J, MacDougall LK, Morgan BP, Podack E, Waterfield MD, Griffiths GM. Perforin is activated by a proteolytic cleavage during biosynthesis which reveals a phospholipid-binding C2 domain. *EMBO J* **16**:7287–7296, 1997.
 34. Savary CA, Phillips JH, Lotzova E. Inhibition of murine natural killer cell-mediated cytotoxicity by pretreatment with ammonium chloride. *J Immunol Meth* **25**:189–192, 1979.
 35. Camenisch TD, Jaso-Friedmann L, Evans DL, Harris DT. Expression of novel function associated molecule on cells mediating spontaneous cytotoxicity in swine. *Dev Comp Immunol* **17**:277–282, 1993.
 36. Viveiros MM, Antzack DF. Characterization of equine natural killer and IL-2 stimulated lymphokine activated killer cell populations. *Dev Comp Immunol* **23**:521–532, 1999.
 37. King A, Loke YW. Human trophoblast and JEG choriocarcinoma cells are sensitive to lysis by IL-2 stimulated decidual NK cells. *Cell Immunol* **129**:435–438, 1990.
 38. Rouas-Freiss N, Marchal-Bras G, Menier C, Dausset J, Carosella ED. Direct evidence to support the role of HLA-G in protecting the fetus from maternal uterine natural killer cytotoxicity. *Proc Natl Acad Sci USA* **94**:11520–11525, 1997.
 39. Biron CA, Nguyen KB, Pien GC, Cousens LP, Salazar-Mather TP. Natural killer cells in antiviral defense: function and regulation by innate cytokines. *Annu Rev Immunol* **17**:189–220, 1999.
 40. Guimond MJ, Luross JA, Wang B, Terhorst C, Danial S, Croy BA. Absence of natural killer cells during murine pregnancy is associated with reproductive compromise in TgE26 mice. *Biol Reprod* **56**:169–179, 1997.
 41. Ashkar AA, Croy BA. Functions of uterine natural killer cells are mediated by interferon- γ production during murine pregnancy. *Sem Immunol* **13**:235–241, 2001.
 42. Boshier DP. A histological and histochemical examination of implantation and early placentome formation in sheep. *J Reprod Fertil* **19**:51–61, 1969.
 43. Hansen PJ, Anthony RV, Bazer FW, Baumbach GA, Roberts RM. *In vitro* synthesis and secretion of ovine trophoblast protein-1 during the period of maternal recognition of pregnancy. *Endocrinology* **117**:1424–1430, 1985.