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Molecular evidence for natural killer-like cells in equine endometrial cups

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ABSTRACT

Objectives: To identify equine orthologs of major NK cell marker genes and utilize them to determine whether NK cells are present among the dense infiltration of lymphocytes that surround the endometrial cup structures of the horse placenta during early pregnancy.
Study design: PCR primers were developed to detect the equine orthologs of *NKP46*, *CD16*, *CD56*, and *CD94*; gene expression was detected in RNA isolated from lymphocytes using standard 2-step reverse transcriptase (RT) PCR and products were cloned and sequenced. Absolute real-time RT-PCR was used to quantitate gene expression in total, CD3+, and CD3- peripheral lymphocytes, and invasive trophoblast. Lymphocytes surrounding the endometrial cups (ECL) of five mares in early pregnancy were isolated and NK marker gene expression levels were assayed by quantitative RT-PCR.
Main outcome measures: Absolute mRNA transcript numbers were determined by performing quantitative RT-PCR and comparing values to plasmid standards of known quantities.
Results: NKP46 gene expression in peripheral CD3- lymphocytes was higher than in CD3+ lymphocytes, *CD16* levels were higher in the CD3+ population, and no significant differences were detected for *CD56* and *CD94* between the two groups. Expression of all four NK cell markers was significantly higher in lymphocytes isolated from the endometrial cups of pregnant mares compared to PBMC isolated from the same animal on the same day (*NKP46*, 14-fold higher; *CD94*, 8-fold higher; *CD16*, 20-fold higher; *CD56*, 44-fold higher).
Conclusions: These data provide the first evidence for the expression of major NK cell markers by horse cells and an enrichment of NK-like cells in the equine endometrium during pregnancy.

1. Introduction

Placentation in the mare is diffuse, epitheliochorial, and primarily non-invasive. However, the horse placenta contains a population of invasive placental cells called the chorionic girdle trophoblast. These cells of fetal origin express both maternal and paternal polymorphic MHC class I antigen at very high levels [1,2]. During a period in early pregnancy, the chorionic girdle trophoblasts invade the uterus of the pregnant mare, differentiate, and organize to form discrete tissue structures in the superficial endometrium known as endometrial cups [3]. Maternal mononuclear leukocytes are recruited into the endometrial stroma around the cups, forming a dramatic cellular infiltrate at the cup periphery. Our lab has previously identified these leukocytes as primarily CD4+ and CD8+ lymphocytes, most of which are also CD3+ [4]. Despite the seemingly hostile environment in which the trophoblast cells of the cups exists, they persist in situ until their eventual death approximately two months later. During this time, the paternal MHC class I antigen expressed on the surface of these trophoblasts is recognized by the maternal immune system and induces a robust humoral immune response in nearly all pregnant mares [5]. It is not clear how the highly antigenic trophoblast cells are able to evade the maternal immune response for such an extended period.

In humans and mice, NK cells are the primary leukocyte population in the decidua during early pregnancy. Despite the implications of their name, they are weakly lytic and promote the establishment and maintenance of pregnancy. In most species, NK cells of the uterus and decidua are distinct from peripheral populations in terms of phenotype and function [6]. Their role in pregnancy is not completely understood, but they have been implicated in vascular remodeling and facilitation of trophoblast invasion [7,8]. Changes in NK cell numbers and phenotype have been associated with multiple reproductive disorders in women [9]; and NK cell-depleted mice demonstrate aberrant spiral artery

Abbreviations: PBMC, peripheral blood mononuclear cells; ECL, endometrial cup lymphocytes; PBL, peripheral blood lymphocytes; uNK cell, uterine natural killer cell; CDS, coding sequence; WGS, whole genome sequence.

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modification [10]. Based upon the important role that NK cells play in species with invasive placentae, we wanted to determine whether they are present among the leukocyte infiltration that surround the invasive trophoblast of the equine endometrial cups.

There is some evidence for an NK cell presence at the maternal-fetal interface of the horse. Electron microscopy studies of the equine endometrium during early pregnancy reveal large granular lymphocytes consistent with uterine NK cells (uNK) [11]. But progress in the investigation of equine NK cells has been inhibited by a lack of reagents. Our lab has previously described a population of peripheral lymphocytes that exhibit NK cell-like characteristics by using a cross-reactive monoclonal antibody to a catfish vimentin-like protein shown to identify human NK cells [12]. Also, expression of *LY49* family genes has been detected by screening a horse spleen cDNA library [1]. However, to date, none of the primary markers used to phenotype NK cells, or methods to detect them, have been described in the horse. In order to explore whether NK cells might play a role at the equine fetal–maternal interface, we identified the equine orthologs of four NK cell marker genes and using molecular methods, investigated their expression in the endometrium during early pregnancy.

2. Materials and methods

2.1. Animals

Horses used in this study were maintained at the Cornell Equine Genetics Center; all procedures were performed in accordance with guidelines established by the Institutional Animal Care and Use Committee of Cornell University. Adult horses of mixed genetic backgrounds, sexes, and ages were used (Table S1). Pregnancies were established as previously described [13]. Major Histocompatibility Complex haplotypes were assigned to horses using serological and genomic methods [14,15].

2.2. Cells and tissues

Peripheral blood mononuclear cells (PBMC) and peripheral blood lymphocytes (PBL) were isolated using density gradient centrifugation with (PBL) or without (PBMC) pre-incubation with carbonyl iron followed by passage of cell suspension over a magnet to remove phagocytes. Endometrial cup lymphocytes (ECL) were similarly isolated following surgical dissection and collagenase digestion as previously described [16]. One ECL sample was obtained from the uterus of a mare carrying a twin pregnancy. Both conceptuses appeared viable based upon their tissue integrity and equal size. Chorionic girdle trophoblasts were microdissected from conceptuses collected at days 33–34 of pregnancy as previously described [17]. CD3 cell sorting was performed using an AutoMACS cell sorter (Miltenyi Biotec, Auburn, CA) following incubation of PBL with a mouse monoclonal antibody specific for equine CD3 (clone F6G, UC Davis, Davis, CA) and rat anti-mouse IgG1 MicroBeads (Miltenyi Biotec). CD3-depleted and enriched populations were verified by flow cytometry. Depleted populations were a mean 8% CD3+; enriched populations were 91% CD3+. RNA isolation and cDNA synthesis were performed as previously described [16].

2.3. Cloning

Equine *NKP46*, *CD16*, *CD56*, and *CD94* were amplified from horse PBMC cDNA using *Pfu* DNA polymerase (Stratagene, La Jolla, CA), gel purified/extracted, cloned into pCR4Blunt-TOPO vector (Invitrogen, Carlsbad, CA), and sequenced on an Applied Biosystems Automated 3730 DNA Analyzer at the Cornell Life Sciences Center. Sequences were analyzed using the DNAStar software suite.

2.4. qPCR

SYBR Green (Applied Biosystems, Carlsbad, CA) real-time PCR reactions for amplification of genes listed in Table 1, or the housekeeper gene equine ubiquitin-conjugating enzyme E2D 2 (UBE2D2), were performed using an ABI 7500 Fast sequence detector (Applied Biosystems). Primers were designed with Primer3 software (MIT, Cambridge, MA) to cross intron/exon boundaries to prevent amplification of genomic DNA (Table 1). A dissociation curve was performed after each experiment to confirm a single product was amplified. A standard curve was generated for all genes using known copy numbers of a plasmid that contained the DNA specific to the gene. Each sample was first normalized to 1.5×10^4 copies of UBE2D2. Data were analyzed using Graph Pad Prism Software. Data sets were checked for normality using the Kolmogorov–Smirnov test (for $n > 4$) or normal Q–Q plots (for $n \leq 4$). Differences between groups were determined using unpaired (Figs. 3 and 4) or paired (Fig. 5) two-tailed Student's t tests, or the Mann–Whitney test for non-parametric data (Fig. 4E). The relationship between previously reported flow cytometric analysis of intracellular IFNγ+ labeling (described in detail in Ref. [16]) and *CD56* transcript copy number in matched ECL samples was determined using the Spearman correlation coefficient (r).

3. Results

3.1. Comparative genomics of equine NK cell receptor gene orthologs

We chose the NK cell markers *NKp46*, *CD94*, *CD56*, and *CD16* to investigate equine NK cells based upon their expression patterns in peripheral and uterine NK cells of other species. PCR primers for these genes were designed by analyzing the equine whole genome sequence (WGS) for regions of homology with annotated genes of other species. Full-length (*NKP46*, *CD94*, and *CD16)* and partial (*CD56*) transcripts were amplified from cDNA generated from PBMC RNA derived from the WGS-donor animal maintained in our research herd. Coding sequences (CDS) were translated and aligned with the human, bovine, murine, and porcine protein sequences, as available (Fig. 1).

Equine *NKP46* was identified on chromosome 10 (ECA10) in a region syntenic with the leukocyte receptor gene complex (LRC) of human chromosome 19 (HSA19), where the *NKP46* gene is located [18]. Multiple splice variants were identified, most correlating closely to validated human transcript variants (Fig. 2). The translated sequence of the dominant *NKp46* isoform shares 54–67% identity with the protein sequences of other selected species (Fig. 1A, Table S2). Conservation of critical protein motifs such as two immunoglobulin (Ig)-like domains, a transmembrane domain containing an arginine required for activation, and cysteines that form stabilizing intramolecular disulfide bonds, suggest a functional capacity of the gene product.

Equine *CD16* was identified on ECA5, clustered with other predicted Fc receptor genes and syntenic to a homologous region on HSA1. The translated sequence shares 37–60% identity with other species (Fig. 1B, Table S2); the two Ig-like domains, stabilizing cysteine residues, and transmembrane domain with a required aspartic acid, are conserved in the equine ortholog.

Table 1
Gene sequences and quantitative PCR primers used.

Equine gene	Chromosome location	Genbank accession #	Quantitative PCR primers (5′-3′)
NCR1 (NKP46)	10:24121252–24125282	JN808451	F: CACCTGGAATGATGAACAAAG R: CCTGGGATGAACTGAGAGG
CD3G	7:26203732–26210259	JN808452	F: GGCCTCATCCTGGCTATCAC R: CCCAGATTCCGTGTAGTTTCTC
FCGR3 (CD16)	5:36222322–36228997	JN795139	F: AGACAGCCCTCTCACCACTC R: GTGCACATGCTTGTTCTTCC
NCAM1 (CD56)	7: 21413392–21712444	JN808450	F: CCGGCATTTACAAGTGTGTG R: GGGTTGGTGCATTCTTGAAC
KLRD1 (CD94)	6:37355730–37361350	JN795140	F: AGAATGGCTCTGCTGTCTCC R: CCCTTGGCAGTCTTCATCC
GCM1	20:50724492–50740140	XM_001503164.1	F: CAACTTCTGGAGGCACGAC R: CGCCTTCTTCATTGCTCTTC