Published in final edited form as:
Placenta. 2012 May ; 33(5): 379–386. doi:10.1016/j.placenta.2012.01.018.

Molecular Evidence for Natural Killer-Like Cells in Equine Endometrial Cups

L. E. Noronha[a], **K. E. Huggler**[a], **A. M. de Mestre**[b], **D. C. Miller**[a], and **D. F. Antczak**[a,*]

[a]Baker Institute for Animal Health, College of Veterinary Medicine, Cornell University, Hungerford Hill Road, Ithaca, New York, 14853, USA

[b]The Royal Veterinary College, Royal College Street, London NW1 OTU, UK

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.

Keywords

Horse; NKp46; CD16; CD94; CD56; NK cell

[*]**Corresponding author**: Prof. Doug Antczak, Baker Institute for Animal Health, College of Veterinary Medicine, Cornell University, Hungerford Hill Road, Ithaca, New York 14853, USA, Telephone: 607-256-5633, Fax: 607-256-5608, doug.antczak@cornell.edu.

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 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 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].

2.2. 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.

2.3 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, 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.

Equine *CD94* was located in a region of ECA6 syntenic with the natural killer gene complex (NKC) of HSA12 [18]; and a splice variant conserved between human, horse, and cow [19] was identified (data not shown). The translated dominant *CD94* sequence shares 51–67% identity with other species (Fig. 1C, Table S2). The C-type lectin domain, lack of a cytoplasmic signaling motif, and structure-stabilizing cysteine residues of the human protein are conserved in the equine homolog.

Equine *CD56* was found on ECA7 and demonstrated very high homology with other species at the amino acid level (96–98%). It shared the highest homology of all selected species to the human protein (Table S2). Due to its low abundance in PBMC, the full 6kb mRNA was difficult to clone in its entirety. A region spanning predicted exons 2–4 was cloned, sequenced, and used for the studies below.

3.2 Expression of equine NK cell receptor genes in peripheral lymphocytes

In order to determine expression levels of NK marker genes in the circulating immune cells of the horse, quantitative PCR was performed on cDNA generated from equine PBL. Lymphocyte expression levels were compared to those from a non-immune tissue: chorionic girdle (CG) trophoblast microdissected from conceptuses recovered at days 33–34 of pregnancy. These invasive cells are the immediate precursors of the endometrial cup trophoblast. In addition to NK marker genes, expression levels for the T-cell marker CD3 gamma (*CD3G*) and the trophoblast marker glial cells missing homologue 1 (*GCM1*) [17] were used as controls for the two cell types.

Transcripts for the NK markers *CD16*, *CD94*, and *NKP46* were present in PBL, with *CD16* and *CD94* demonstrating robust expression and *NKP46* exhibiting modest expression (Fig. 3A, 3B, 3D). Trophoblast expressed at most trace amounts of these three genes. *CD56* exhibited barely detectable expression levels in PBL, consistent with the low expression levels of antigen observed on the predominantly $CD56^{dim}$ peripheral NK cells of other species (Fig 3C). Somewhat surprisingly, *CD56* expression was detected in the CG trophoblast. This expression pattern is also observed in human and macaque invasive trophoblast [20, 21]. Predictably, *CD3G* demonstrated high expression in lymphocytes, but

none in trophoblast (Fig 3E). *GCM1* was highly expressed in trophoblast but absent in PBL (Fig 3E).

We next determined the expression of these NK cell markers in CD3+ and CD3− blood lymphocyte populations, in order to compare the patterns to those seen in other species. cDNA was generated from peripheral lymphocytes magnetically sorted according to expression of cell-surface CD3 antigen. As a control, *CD3G* expression was measured and found to be 24-fold higher in the CD3-enriched (CD3+) population (Fig. 4A). *NKP46* was expressed at 4-fold higher levels in the CD3-depleted (CD3−) population (Fig. 4B). This is consistent with the canonical CD3− NKp46+ NK cell phenotype exhibited by most species. However *CD16*, which is usually associated with peripheral CD3− NK cells, was 5-fold more highly expressed in the CD3+ population (Fig. 4C). This higher *CD16* expression among the CD3+ cells may therefore represent NKT cells, a CD16+ T lymphocyte subset [22], or a species-related difference in NK cell phenotype. *CD94* expression did not differ between the two-lymphocyte groups (Fig. 4D). This is not surprising as, in addition to CD3− NK cells, conventional CD3+ lymphocytes and NKT cells can express *CD94*. Changes in *CD56* levels were difficult to detect due to its low expression levels in the periphery (Fig. 3C). After increasing the input cDNA by 6-fold, transcript levels were still low, but we observed a trend toward increased expression in CD3− lymphocytes (Fig. 4E).

3.3 NK cell receptor expression is higher in endometrial cup lymphocytes compared to PBMC

Expression of NK cell marker genes was next investigated in the equine uterus during early pregnancy. Lymphocytes were isolated from the endometrial cups of five mares pregnant between days 43 and 46 of gestation. Expression levels of NK markers in these endometrial cup lymphocyte (ECL) samples were compared to PBMC isolated from the same mares just prior to euthanasia.

Expression levels of all NK markers were dramatically increased in ECL samples compared to the paired PBMC (Fig. 5). *NKP46*, which was modestly expressed in the periphery, was expressed at 7–43 fold (mean=14) higher levels in the endometrial cups (Fig. 5A). *CD16*, which was highly expressed in PBL, shows a dramatic 20-fold higher expression in ECL (Fig. 5B). Based upon the slight decrease in *CD3G* expression in the endometrial cups (Fig. 5E), the increase in *CD16* is not likely due to cells like the CD3+CD16+ population seen in the periphery (Fig. 4C). *CD94*, also highly expressed in the periphery, is 8-fold higher in the ECL (Fig. 5D); again this is unlikely attributable to CD3+ cells. Finally, *CD56*, a marker that is nearly undetectable in the periphery, exhibits a 44-fold increase in the endometrial cup lymphocyte samples (Fig. 5C). Because *CD56* expression was also observed in chorionic girdle trophoblast, we measured *GCM1* in the ECL samples in order to detect contaminating trophoblast cDNA. *GCM1* expression was detected (Fig. 5F), but at levels 1/5 of those in the trophoblast samples (Fig. 3F) and the difference between ECL and PBMC expression was not statistically significant. Therefore, contaminating trophoblast cannot account for the dramatic increase in *CD56* expression seen in the ECL samples. While all markers were dramatically higher in the ECL population, the copy numbers of *NKP46* and *CD56* were much lower than the more abundant *CD94*, *CD16*, and *CD3G*, suggesting these markers my be expressed by a smaller number of cells.

Because $CD56^{bright}$ uNK cells described in other species produce IFNγ ?[23], we examined the relationship between *CD56* and IFNγ expression in the ECL. We have previously observed an increase in the number of IFNγ + lymphocytes among the same ECL samples studied here using flow cytometric analysis [16]. We compared these data to the *CD56* transcript numbers in the corresponding samples and found no statistically significant correlation (Fig. S1).

4. Discussion

Using a highly specific and sensitive quantitative molecular assay, we have demonstrated that the four primary markers used to determine NK cell phenotype in other species are expressed by horse lymphocytes. These genes exhibit conservation of critical structural and functional elements despite the approximately 100 million years of divergence between horses and humans [24]. This suggests that NK cell markers were conserved against evolutionary pressure to maintain their functional motifs, and are therefore likely to have biological activity. The conservation of alternative splicing patterns of human and horse NK marker transcripts also seen here is not a broadly observed phenomenon across genomes [25]. This suggests an evolutionary pressure at the mRNA processing level, further supporting preservation of gene functionality.

The activating receptor NKp46 is considered to be a specific marker for NK cells in multiple species, and is present on all NK subsets in primates and rodents at stable expression levels [26]. Therefore, the dramatic increase of *NKP46* in ECL suggests the presence of lymphocytes with an NK phenotype in the mare endometrium. *CD16*, while not associated with the NK cells found in the decidua during human pregnancy, is expressed in the pregnant uteri of the mouse [27], rhesus monkey [28], and pig [29].

The expression patterns of equine *CD94* and *CD56* genes seen here are particularly interesting because to date, expression of these molecules at the maternal-fetal interface has only been observed in primates. The CD94 proteins, a co-receptor for non-classical MHC class I molecules, is more highly expressed in the human decidua compared to the periphery [30], as seen here with the equine gene. CD56, perhaps the most important marker of primate decidual NK cells, is minimally expressed in the periphery but highly expressed in the decidua [23], similar to what we observed in the ECL. Neither marker is expressed by mouse uterine NK cells.

The $CD56^{bright}$ NK cells of the primate decidua are characterized by a poor cytolytic capacity and the secretion of high levels of IFNγ [23]. Interestingly, we have previously observed an increase in the number of IFNγ+ lymphocytes among the ECL [16]. When we investigated the relationship between *CD56* expression and IFNγ in these ECL, we found no significant correlation. However, of the four data points tested, three demonstrated a relationship suggesting a strong correlation (r=0.99); the fourth outlying data point was from ECL collected from a twin pregnancy. This sample size is insufficient to determine if a statistically significant relationship exists, but the pattern clearly suggests further investigation is warranted to determine if these two molecules are expressed by the same cells. Better characterization of such relationships will be possible with the development of antibody reagents to equine NK cell markers.

The stage of pregnancy studied here, days 43–46, is roughly one week following trophoblast invasion. The presence of NK cells in the equine uterus during this period could help explain the poorly understood phenomenon of paternal class I MHC expression by the chorionic girdle trophoblast. Interaction between uNK activating receptors and allogeneic paternal MHC I has recently been shown to be important for trophoblast invasion, vascular development, and fetal growth in both humans and mice [31, 32]. The horse uterus does not show the same spiral artery alteration seen in species with hemochorial placentae, so this uNK-cell function may operate irrespective of placentation in order to facilitate trophoblast invasion and endometrial remodeling. It will be interesting to further explore what role this population of cells plays in equine pregnancy, and what new insights it will lend to our knowledge of evolutionarily-conserved maternal-fetal interactions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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

Acknowledgments

The authors thank Emily Silvela for horse breeding and management, and Rebecca Harman for technical assistance. This work was supported by grants from the Zweig Memorial Fund and the US National Institutes of Health (R01 HD049545). DFA is an investigator of the Dorothy Russell Havemeyer Foundation, Inc. LEN is supported by NIH F32 HD 055794.

References

1. Bacon SJ, Ellis SA, Antczak DF. Control of expression of major histocompatibility complex genes in horse trophoblast. Biol Reprod. 2002; 66(6):1612–1620. [PubMed: 12021038]
2. Donaldson WL, Oriol JG, Pelkaus CL, Antczak DF. Paternal and maternal major histocompatibility complex class I antigens are expressed co-dominantly by equine trophoblast. Placenta. 1994; 15(2): 123–135. [PubMed: 8008728]
3. Allen WR, Hamilton DW, Moor RM. The origin of equine endometrial cups. II. Invasion of the endometrium by trophoblast. Anat Rec. 1973; 177(4):485–501. [PubMed: 4762726]
4. Grunig G, Triplett L, Canady LK, Allen WR, Antczak DF. The maternal leucocyte response to the endometrial cups in horses is correlated with the developmental stages of the invasive trophoblast cells. Placenta. 1995; 16(6):539–559. [PubMed: 8570575]
5. Antczak DF, Miller JM, Remick LH. Lymphocyte alloantigens of the horse. II. Antibodies to ELA antigens produced during equine pregnancy. J Reprod Immunol. 1984; 6(5):283–297. [PubMed: 6481697]
6. Croy BA, van den Heuvel MJ, Borzychowski AM, Tayade C. Uterine natural killer cells: a specialized differentiation regulated by ovarian hormones. Immunol Rev. 2006; 214:161–185. [PubMed: 17100884]
7. Tabiasco J, Rabot M, Aguerre-Girr M, El Costa H, Berrebi A, Parant O, Laskarin G, Juretic K, Bensussan A, Rukavina D, Le Bouteiller P. Human decidual NK cells: unique phenotype and functional properties -- a review. Placenta. 2006; 27 Suppl A:S34–S39. [PubMed: 16516963]
8. Harris LK. IFPA Gabor Than Award lecture: Transformation of the spiral arteries in human pregnancy: key events in the remodelling timeline. Placenta. 2011; 32 Suppl 2:S154–S158. [PubMed: 21167598]
9. Lash GE, Bulmer JN. Do uterine natural killer (uNK) cells contribute to female reproductive disorders? J Reprod Immunol. 2011; 88(2):156–164. [PubMed: 21334072]
10. Croy BA, He H, Esadeg S, Wei Q, McCartney D, Zhang J, Borzychowski A, Ashkar AA, Black GP, Evans SS, Chantakru S, van den Heuvel M, Paffaro VA Jr, Yamada AT. Uterine natural killer cells: insights into their cellular and molecular biology from mouse modelling. Reproduction. 2003; 126(2):149–160. [PubMed: 12887272]

11. Enders AC, Liu IK. Trophoblast-uterine interactions during equine chorionic girdle cell maturation, migration, and transformation. Am J Anat. 1991; 192(4):366–381. [PubMed: 1781447]
12. Viveiros MM, Antczak DF. Characterization of equine natural killer and IL-2 stimulated lymphokine activated killer cell populations. Dev Comp Immunol. 1999; 23(6):521–532. [PubMed: 10512462]
13. Adams AP, Antczak DF. Ectopic transplantation of equine invasive trophoblast. Biol Reprod. 2001; 64(3):753–763. [PubMed: 11207188]
14. Lazary S, Antczak DF, Bailey E, Bell TK, Bernoco D, Byrns G, McClure JJ. Joint Report of the Fifth International Workshop on Lymphocyte Alloantigens of the Horse, Baton Rouge, Louisiana: 31 October-1 November 1987. Anim Genet. 1988; 19(4):447–456. [PubMed: 2466424]
15. Tseng CT, Miller D, Cassano J, Bailey E, Antczak DF. Identification of equine major histocompatibility complex haplotypes using polymorphic microsatellites. Anim Genet. 2010; 41 Suppl 2:150–153. [PubMed: 21070289]
16. de Mestre A, Noronha L, Wagner B, Antczak DF. Split immunological tolerance to trophoblast. Int J Dev Biol. 2010; 54(2–3):445–455. [PubMed: 19876828]
17. de Mestre AM, Miller D, Roberson MS, Liford J, Chizmar LC, McLaughlin KE, Antczak DF. Glial cells missing homologue 1 is induced in differentiating equine chorionic girdle trophoblast cells. Biol Reprod. 2009; 80(2):227–234. [PubMed: 18971425]
18. Colucci F, Boulenouar S, Kieckbusch J, Moffett A. How does variability of immune system genes affect placentation? Placenta. 2011; 32(8):539–545. [PubMed: 21665273]
19. Storset AK, Slettedal IO, Williams JL, Law A, Dissen E. Natural killer cell receptors in cattle: a bovine killer cell immunoglobulin-like receptor multigene family contains members with divergent signaling motifs. Eur J Immunol. 2003; 33(4):980–990. [PubMed: 12672064]
20. Golos TG, Bondarenko GI, Dambaeva SV, Breburda EE, Durning M. On the role of placental Major Histocompatibility Complex and decidual leukocytes in implantation and pregnancy success using non-human primate models. Int J Dev Biol. 2010; 54(2–3):431–443. [PubMed: 19876826]
21. Proll J, Blaschitz A, Hartmann M, Thalhamer J, Dohr G. Human first-trimester placenta intra-arterial trophoblast cells express the neural cell adhesion molecule. Early Pregnancy. 1996; 2(4): 271–275. [PubMed: 9363226]
22. Lanier LL, Kipps TJ, Phillips JH. Functional properties of a unique subset of cytotoxic CD3+ T lymphocytes that express Fc receptors for IgG (CD16/Leu-11 antigen). J Exp Med. 1985; 162(6): 2089–2106. [PubMed: 2415663]
23. Kalkunte S, Chichester CO, Gotsch F, Sentman CL, Romero R, Sharma S. Evolution of non-cytotoxic uterine natural killer cells. Am J Reprod Immunol. 2008; 59(5):425–432. [PubMed: 18405313]
24. Cao Y, Fujiwara M, Nikaido M, Okada N, Hasegawa M. Interordinal relationships and timescale of eutherian evolution as inferred from mitochondrial genome data. Gene. 2000; 259(1–2):149–158. [PubMed: 11163972]
25. Yeo GW, Van Nostrand E, Holste D, Poggio T, Burge CB. Identification and analysis of alternative splicing events conserved in human and mouse. Proc Natl Acad Sci U S A. 2005; 102(8):2850–2855. [PubMed: 15708978]
26. Narni-Mancinelli E, Chaix J, Fenis A, Kerdiles YM, Yessaad N, Reynders A, Gregoire C, Luche H, Ugolini S, Tomasello E, Walzer T, Vivier E. Fate mapping analysis of lymphoid cells expressing the NKp46 cell surface receptor. Proc Natl Acad Sci U S A. 2011
27. Yadi H, Burke S, Madeja Z, Hemberger M, Moffett A, Colucci F. Unique receptor repertoire in mouse uterine NK cells. J Immunol. 2008; 181(9):6140–6147. [PubMed: 18941204]
28. Dambaeva SV, Durning M, Rozner AE, Golos TG. Immunophenotype and Cytokine Profiles of Rhesus Monkey CD56bright and CD56dim Decidual Natural Killer Cells. Biol Reprod. 2011
29. Engelhardt H, Croy BA, King GJ. Evaluation of natural killer cell recruitment to embryonic attachment sites during early porcine pregnancy. Biol Reprod. 2002; 66(4):1185–1192. [PubMed: 11906940]

30. King A, Allan DS, Bowen M, Powis SJ, Joseph S, Verma S, Hiby SE, McMichael AJ, Loke YW, Braud VM. HLA-E is expressed on trophoblast and interacts with CD94/NKG2 receptors on decidual NK cells. Eur J Immunol. 2000; 30(6):1623–1631. [PubMed: 10898498]

31. Hiby SE, Apps R, Sharkey AM, Farrell LE, Gardner L, Mulder A, Claas FH, Walker JJ, Redman CW, Morgan L, Tower C, Regan L, Moore GE, Carrington M, Moffett A. Maternal activating KIRs protect against human reproductive failure mediated by fetal HLA-C2. J Clin Invest. 2010; 120(11):4102–4110. [PubMed: 20972337]

32. Madeja Z, Yadi H, Apps R, Boulenouar S, Roper SJ, Gardner L, Moffett A, Colucci F, Hemberger M. Paternal MHC expression on mouse trophoblast affects uterine vascularization and fetal growth. Proc Natl Acad Sci U S A. 2011; 108(10):4012–4017. [PubMed: 21300875]

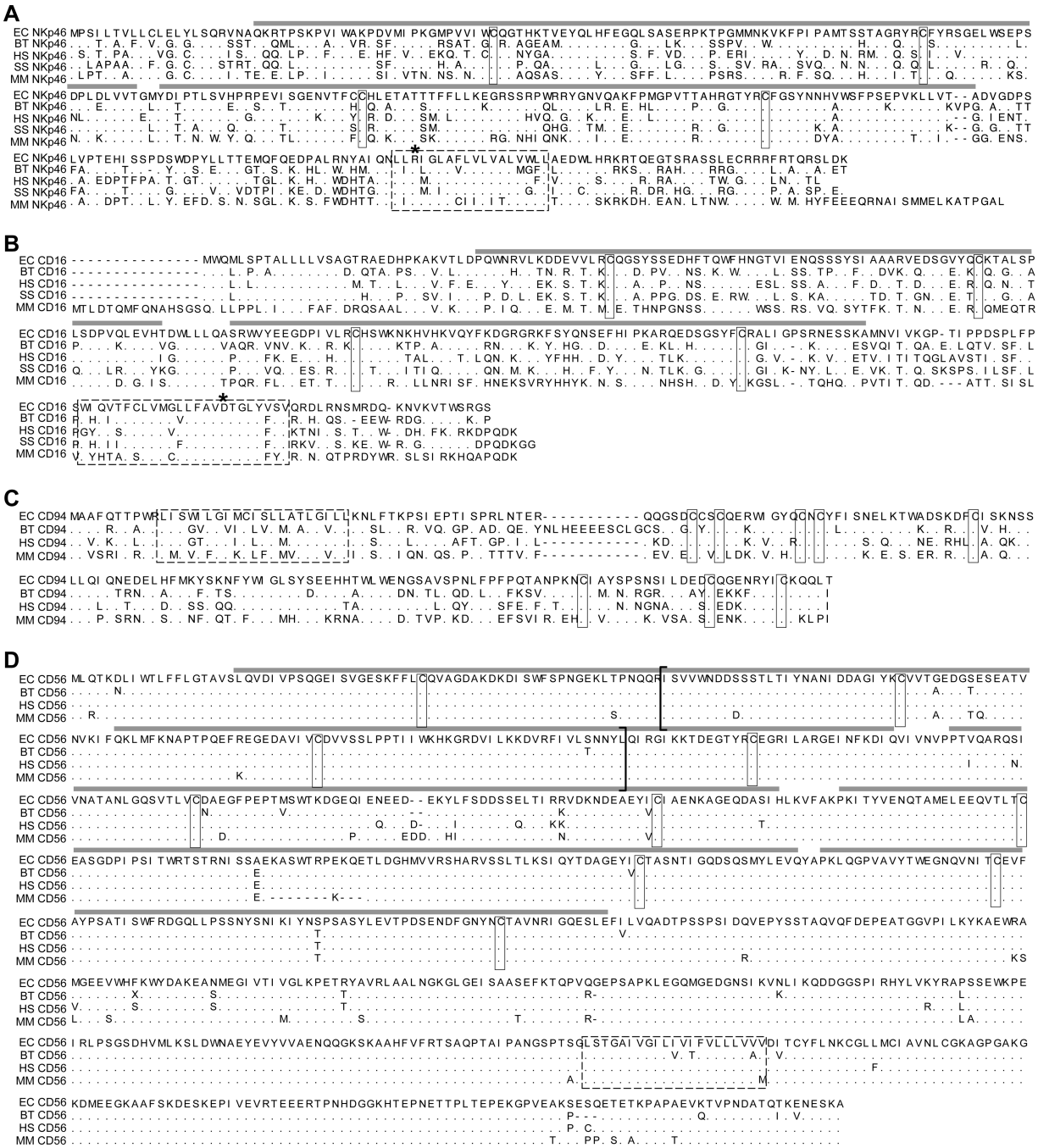

Figure 1.

Multi-species alignments of NK cell marker protein sequences. Amino acid sequences of equine NKp46 (A), CD16 (B), CD94 (C), and CD56 (D) orthologs were determined by translation of cDNA sequences determined from a combination of bioinformatic analysis of the equine WGS and amplification from horse PBMC mRNA by RT-PCR. *Equus caballus* (EC) sequences were aligned with sequences of *Homo sapiens* (HS), *Bos taurus* (BT), *Sus scrofa* (SS) and *Mus musculus* (MM) using Clustal W. Full- length clones were obtained for *NKP46*, *CD16*, and *CD94* genes; a 247 bp partial clone was obtained from *CD56*, as indicated by brackets. Dots represent identities, dashes represent gaps. Dashed boxes represent transmembrane domains and grey bars represent conserved immunoglobulin-like

domains. Asterisks highlight highly conserved transmembrane domain charged residues (arginine and aspartic acid) required for association with adaptor molecules responsible for intracellular signaling. Closed boxes represent cysteine residues known to stabilize intramolecular secondary structures. Genbank IDs for sequences used: NP_004820 (HS), NP_899209 (BT), NP_001116615.1 (SS), NP_034876 (MM), NKp46; AAH17865.1 (HS), AAI12757.1 (BT), NP_999556 (SS), NP_034318.2 (MM), CD16; NP_001107868 (HS), NP_001002890 (BT), EDK99936 (MM), CD94; NP_851996.2 (HS), NP_776824.1 (BT), NP_001074914.1 (MM), CD56.

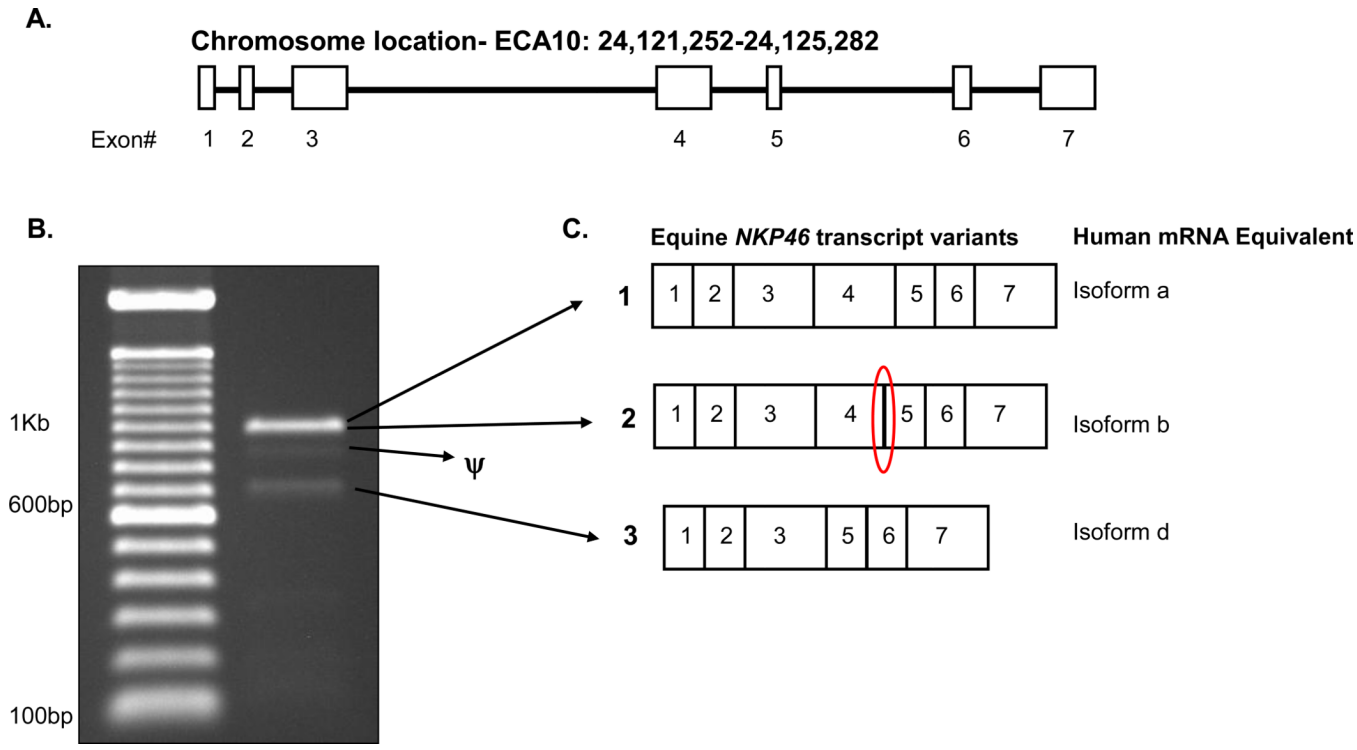

Figure 2.

Relation of equine and human *NKP46* splice isoforms. (A) Schematic diagram of *NKP46* genomic structure. (B) Agarose gel of PCR products generated from PBMC cDNA using primers designed to amplify the full-length CDS and partial 5' and 3' untranslated regions. Bands were extracted, cloned, and sequenced, yielding 3 transcripts with open reading frames (C). Equine transcript variant 1 is similar to full length human *NKP46* isoform a (NM_004829.5). Variant 2 uses an alternate in-frame splice site at the exon 4–5 boundary, similar to human isoform b (NM_001145457.1). Variant 3 has a deletion of exon 4, corresponding to the loss of one Ig-like domain, similar to the human exon 3 deletion isoform d (NM_001242356.1). The band observed at 900bp is an intron-retention mutant of variant 3 with a premature stop codon (ψ, pseudogene).

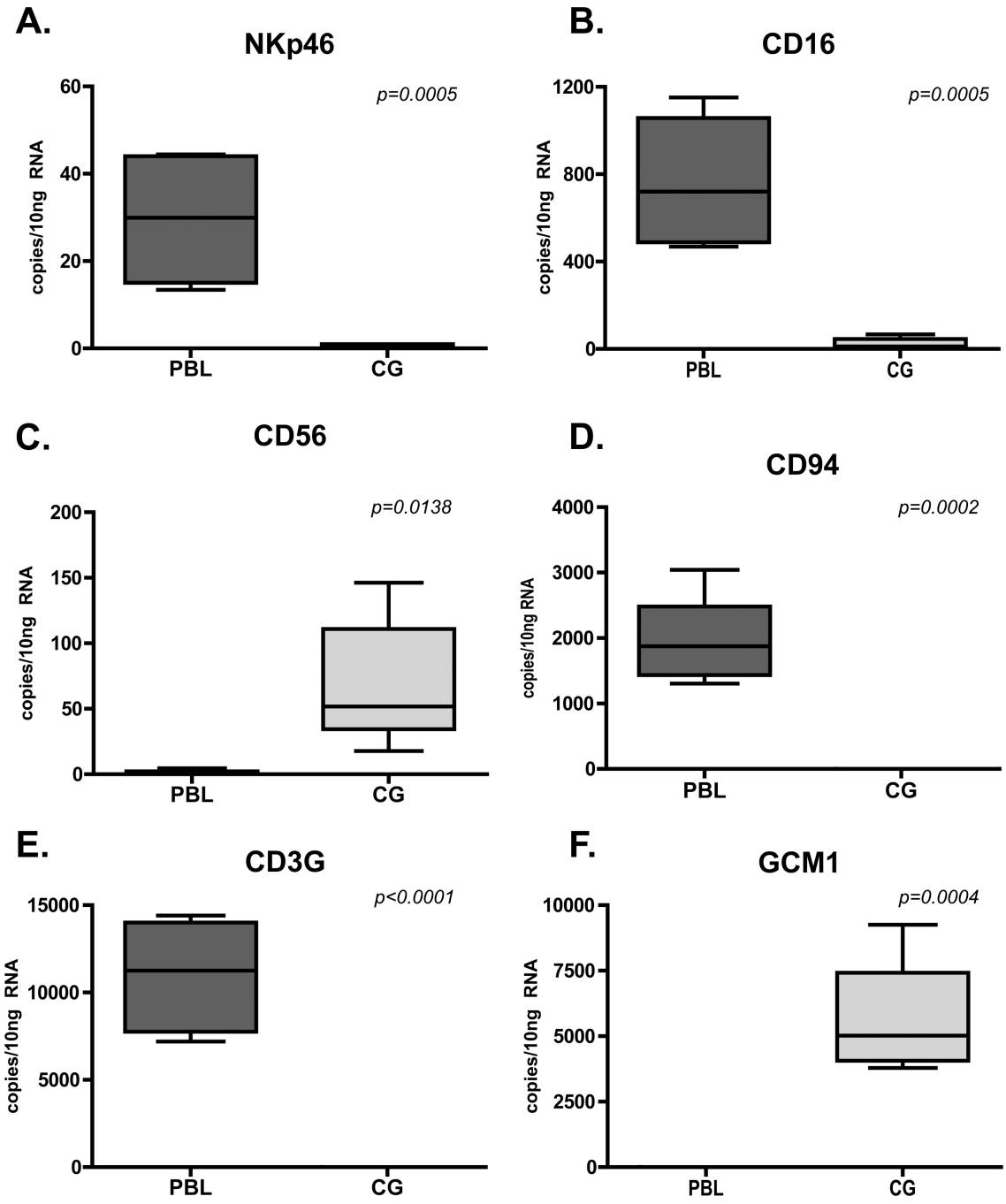

Figure 3.

Detection of NK cell marker expression in equine peripheral lymphocytes. Expression of equine *NKP46* (A), *CD16* (B), *CD56* (C), and *CD94* (D) was determined using quantitative RT-PCR performed on RNA isolated from PBL and chorionic girdle trophoblast (CG). *CD3G* (D) and *GCM1* (E) expression levels were also measured as controls; n=5.

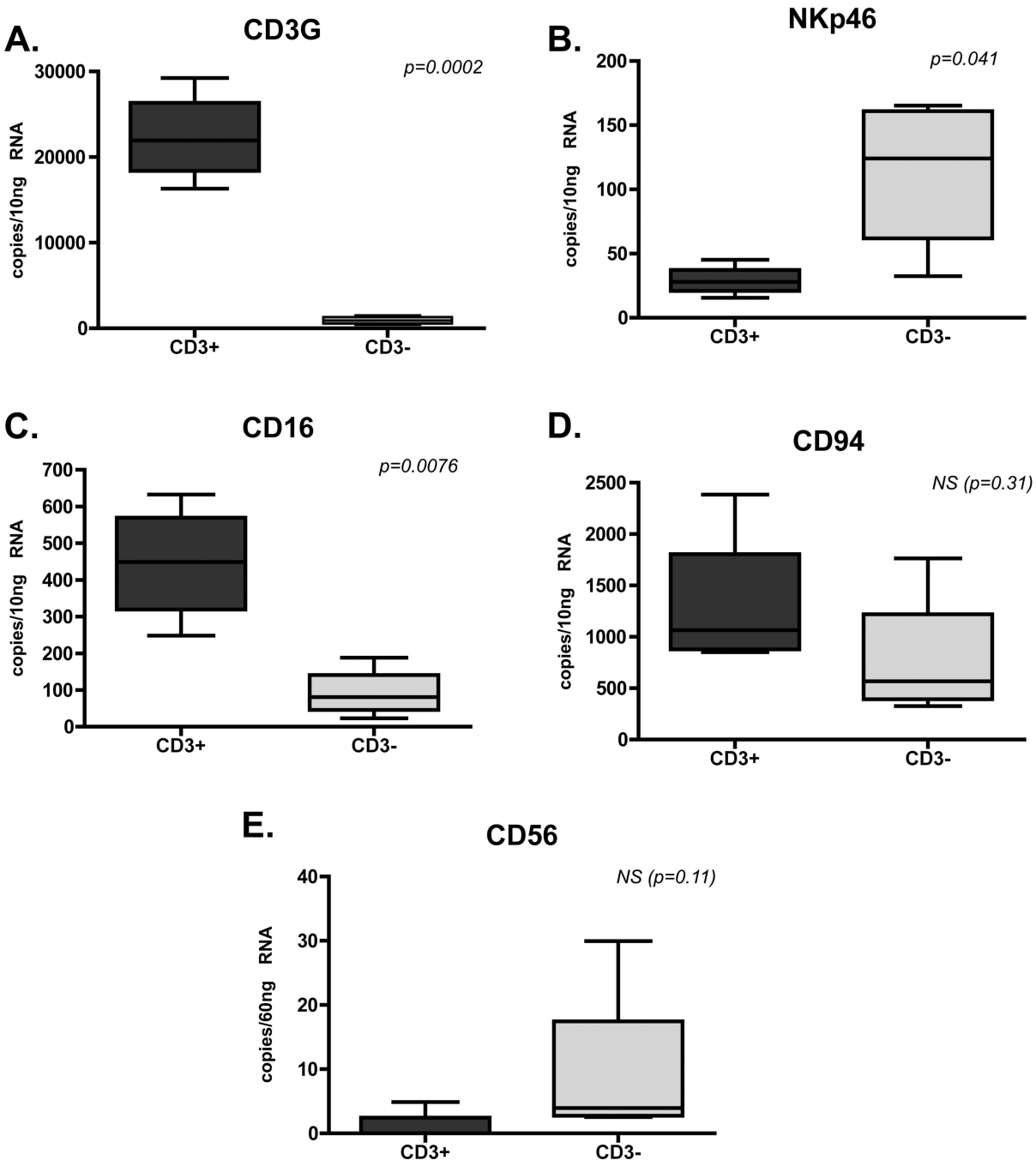

Figure 4.

Expression of NK cell marker expression in CD3-depleted vs. CD3-enriched lymphocyte populations. PBL were magnetically sorted into CD3 depleted (CD3−) and enriched (CD3+) populations; isolated RNA was analyzed for expression of *CD3G* (A), *NKP46* (B), *CD16* (C), *CD94* (D), and *CD56*(E) using quantitative RT-PCR; n=4.

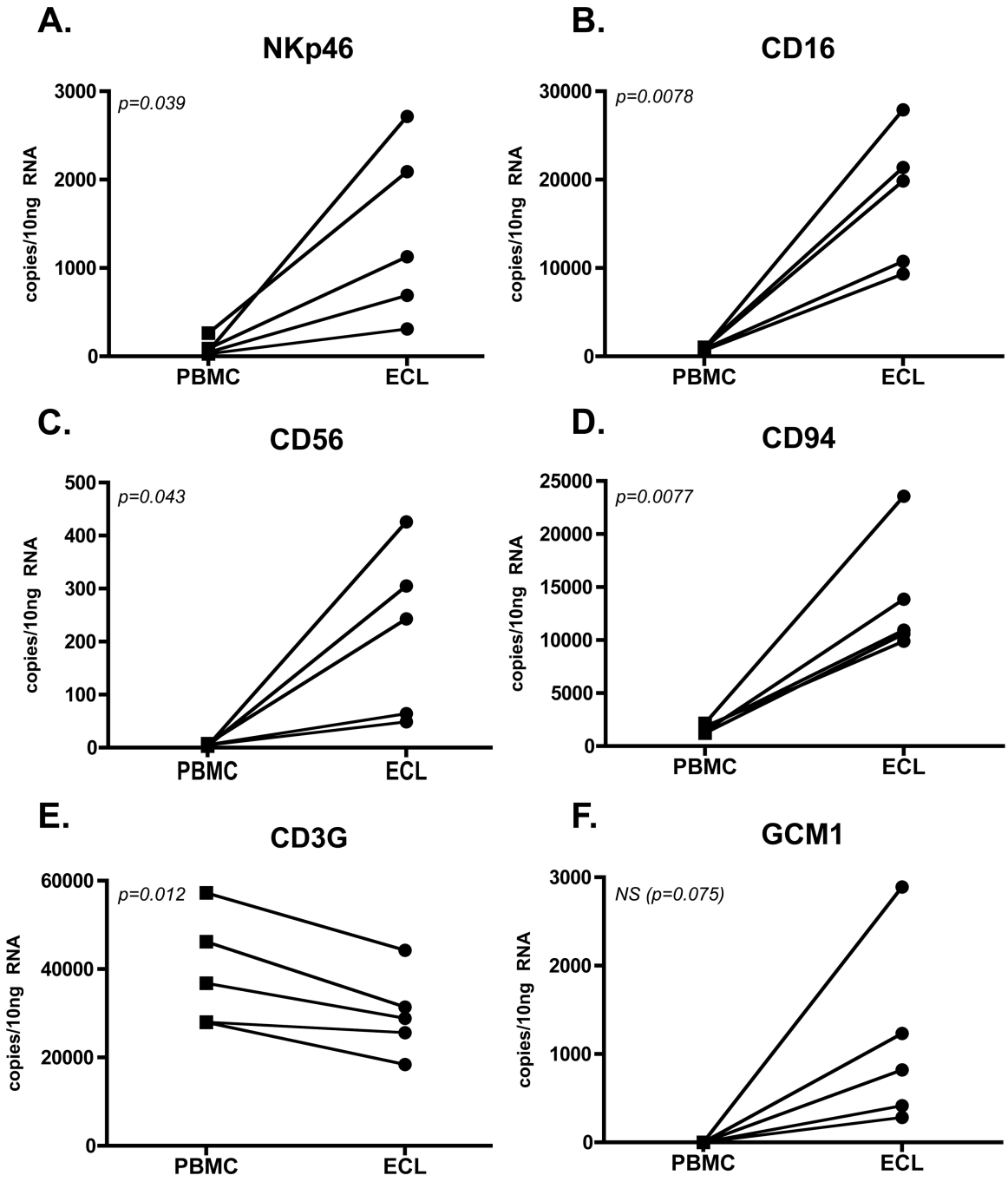

Figure 5.

Comparison of NK cell marker expression in peripheral and endometrial cup lymphocytes. Paired PBMC and ECL were isolated from five mares pregnant at days 43–46 of gestation. Expression of *NKP46* (A), *CD16* (B), *CD56* (C), *CD94* (D), *CD3G* (E), and *GCM1* (F) were measured using quantitative RT-PCR.

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