Published in final edited form as:

Vet Immunol Immunopathol. 2012 June 15; 147(1-2): 60–68. doi:10.1016/j.vetimm.2012.04.003.

Generation and characterization of monoclonal antibodies to equine NKp46

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Abstract

The immunoreceptor NKp46 is considered to be the most consistent marker of NK cells across mammalian species. Here, we use a recombinant NKp46 protein to generate a panel of monoclonal antibodies that recognize equine NKp46. The extracellular region of equine NKp46 was expressed with equine IL-4 as a recombinant fusion protein (*rIL-4/NKp46*) and used as an immunogen to generate mouse monoclonal antibodies (mAbs). MAb were first screened by ELISA for an ability to recognize NKp46, but not IL-4, or the structurally related immunoreceptor CD16. Nine mAbs were selected and were shown to recognize full-length NKp46 expressed on the surface of transfected CHO cells as a GFP fusion protein. The mAbs recognized a population of lymphocytes by flow cytometric analysis that was morphologically similar to NKp46+ cells in humans and cattle. In a study using nine horses, representative mAb 4F2 labeled 0.8-2.1% PBL with a mean fluorescence intensity consistent with gene expression data. MAb 4F2+ PBL were enriched by magnetic cell sorting and were found to express higher levels of *NKP46* mRNA than 4F2- cells by quantitative RT-PCR. CD3-depleted PBL from five horses contained a higher percentage of 4F2+ cells than unsorted PBL. Using ELISA, we determined that the nine mAbs recognize three different epitopes. These mAbs will be useful tools in better understanding the largely uncharacterized equine NK cell population.

Keywords

NCR1; Natural killer cell; Horse; Cell surface receptor; Leukocyte

1. Introduction

Natural killer (NK) cells serve a vital role in the innate immune response due to their ability to destroy foreign cells, such as virus-infected cells and tumor cells, during a primary encounter. Their cytotoxic activity is determined by a complex interaction of activating and

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Conflict of Interests

The authors have no conflict of interests.

inhibitory cell-surface receptors (Joncker et al., 2009). While many of these receptors can be expressed on multiple cell types, the activating receptor NKp46 (NCR1, CD335) appears to be specific to NK and NK-like cells (Sivori et al., 1997). Thus, NKp46 is currently considered the most reliable identifying marker for NK cells across species (Walzer et al., 2007).

NKp46 is a type-I glycoprotein belonging to the immunoglobulin (Ig) superfamily. NKp46, with NKp30 (NCR3), and NKp44 (NCR2) comprise the natural cytotoxicity receptors (NCRs): activating receptors capable of inducing NK cell mediated cytotoxicity. Although the NCRs have similar cellular functions, NKp46 is structurally distinct from the other two molecules and is located in a different region of the genome (Biassoni et al., 2002). It also appears to be more stably expressed, and is generally present on all resting and activated human NK cells (Sivori et al., 1997).

The structure of NKp46 consists of two extracellular C2-type Ig-like domains, a transmembrane region, and a short cytoplasmic tail (Ponassi et al., 2003). The receptor alone cannot transmit an activating signal; it acts by complexing with intracellular signaling molecules, such as CD3 ζ and Fc ϵ RI γ , which contain immunoreceptor tyrosine-based activation motifs that initiate signal-transduction cascades resulting in NK cell activation (Biassoni et al., 2001). Known ligands that engage NKp46 include viral hemagglutinins and cellular heparan sulfate proteoglycans (Bloushtain et al., 2004; Mandelboim et al., 2001). Based upon the NKp46-mediated cytotoxicity of tumor cells, additional unidentified cellular ligands are presumed to exist (Halfteck et al., 2009). Indeed, mice that lack NCR1, the murine NKp46 ortholog, are more susceptible to influenza and the growth of some types of tumors (Gazit et al., 2006; Halfteck et al., 2009).

NKp46 appears to be well conserved among species and its expression has been identified in primates, mice, rats, cattle, sheep, and pigs (Biassoni et al., 1999; Connelley et al., 2011; De Maria et al., 2001; Jozaki et al., 2010; Sivori et al., 1997; Storset et al., 2003). Monoclonal antibodies (mAbs) have been developed to recognize NKp46 in most of these species, and thus far, its expression is limited to NK or NK-like cytotoxic lymphocyte populations.

Our group has recently described the identification of the equine ortholog of the *NKP46* gene (Noronha et al., *in press*). It is expressed by lymphocytes, and its sequence contains conserved domains required for protein function. The predicted equine protein shares 65% identity with the human and bovine proteins. This degree of similarity is apparently insufficient to permit cross-reactivity with anti-human and -bovine NKp46 mAbs, as our attempts to label horse lymphocytes with several have shown a lack of recognition (data not shown). Therefore, using a system we recently employed to develop mAbs to equine CD16 (Noronha et al., accepted pending minor revisions, resubmission submitted), we generated a panel of novel mAbs that recognize equine NKp46.

2. Materials and Methods

2.1. Recombinant IL-4/NKP46 (rIL-4/NKP46)

Sequence IDs and PCR primers are listed in Table 1. The full-length coding sequence (CDS) of equine *NKP46* was previously cloned and sequenced (Noronha et al., *in press*). The extracellular domain (bases 62-668 of the CDS) was predicted by performing Clustal W alignments with validated *NKP46* sequences of other species, and was directionally cloned into a pcDNA3.1 vector (Invitrogen, Carlsbad, CA) downstream from the CDS of equine *IL-4* as previously described (Noronha et al., accepted pending minor revisions, resubmission submitted; Wagner et al., accepted pending minor revisions, resubmission submitted). CHO K-1 cells were transfected with linearized IL-4/NKP46 plasmid using the

Geneporter2 system (Genlantis, San Diego, CA). Stable transfectants were selectively cultured in G418 (Invitrogen), cloned by limiting dilution, and screened for IL-4 production by flow cytometry and ELISA as previously described (Wagner et al., accepted pending minor revisions, resubmission submitted). *hIL-4/NKP46* was purified from serum-free supernatant by fast protein liquid chromatography using an anti-IL-4 affinity column as previously described (Wagner et al., accepted pending minor revisions, resubmission submitted). One μ g of the purified fusion protein was resolved by SDS-PAGE on a 10% non-reducing polyacrylamide gel to determine molecular weight.

2.2. Immunization and splenic fusion

Mice were maintained at the Baker Institute for Animal Health rodent facility at Cornell University. Animal care was performed in accordance with the guidelines set forth by the Institutional Animal Care and Use Committee (IACUC) of Cornell University. Immunization was performed as previously described (Wagner et al., 2003). Animal response was measured by monitoring serum titers to equine IL-4 using ELISA. Spleen cells were fused to SP2/0 myeloma cells as previously described (Appleton et al., 1989). Nascent hybridomas were plated into 96 well tissue culture plates and supernatants from all wells were screened for reactivity to *hIL-4/NKP46* and *hIL-4/IgG* using ELISA, and for cell surface labeling of equine PBMC using flow cytometry. Antibodies that labeled PBMC and detected *hIL-4/NKP46* but not *hIL-4/IgG* were selected for further study. All hybridoma cultures except mAb 8F9 were cloned by performing three rounds of limiting dilution, measuring sensitivity and specificity of secreted immunoglobulin by ELISA and flow cytometry as above after each round. Mouse immunoglobulin isotypes of secreted antibodies were determined by ELISA (Sigma, St. Louis, MO). Antibodies were purified by fast protein liquid chromatography using a protein G affinity column (GE Healthcare, Piscataway, NJ). Proteins were quantified using a Bradford assay (Bio-rad, Hercules, CA). Selected antibodies were biotinylated using Sulfo-NHS-Biotin (Thermo Fisher Scientific, Waltham, MA).

2.3. Antibody screening and ELISA

Cell culture supernatants were screened for mAbs to *hIL-4/NKP46* by ELISA as described previously (Wagner et al., accepted pending minor revisions, resubmission submitted) and against *hIL-4/IgG1* (Wagner et al., 2005) and *hIL-4/CD16* (Noronha et al., accepted pending minor revisions, resubmission submitted) to confirm their specificity to NKP46. For epitope ELISA, plates were coated with 1 μ g purified mAbs, washed, then followed with *hIL-4/NKP46* fusion protein in transfected CHO cell supernatant. Following washing, 1 μ g of mAb in biotinylated form was then added, followed by streptavidin-HRP. Reactions were developed and analyzed as previously described (Wagner et al., 2006). For mAb 8F9, purified and biotinylated antibodies were not available; capture antibody was in the form of hybridoma supernatant.

2.4. GFP fusion protein expression and flow cytometric analysis

Full-length sequence (minus termination codon) for the equine *NKP46*, *CD16*, and *IL-4* genes were PCR-amplified and cloned into the pEGFPN1 vector as previously described (Noronha et al., accepted pending minor revisions, resubmission submitted). CHO-K1 cells were transfected with the vectors using the Geneporter2 system and assayed for protein expression 48 hours post-transfection. Successful expression of GFP was confirmed by fluorescence microscopy and indicated correct reading frame cloning of the fusion protein, as GFP sequence was downstream of the protein of interest. Cells were detached with trypsin and used either fresh or fixed with 2% PFA for 20 minutes. Cells were labeled and analyzed by FACS as previously described (Noronha et al., accepted pending minor revisions, resubmission submitted).

2.5 Lymphocyte isolation, flow cytometry, and immunohistochemistry

Heparinized blood samples were collected from horses maintained at the Equine Genetics Center, Baker Institute for Animal Health, Cornell University (animal details in Table S1). Animal care was performed in accordance with the guidelines set forth by the Cornell University IACUC. Lymphocytes were isolated by incubation with carbonyl-iron followed by density gradient centrifugation as previously described (de Mestre et al., 2010). PBMC were similarly isolated but without use of carbonyl-iron. Cells were assayed for viability using trypan blue exclusion and phase contrast microscopy. For flow cytometry experiments, one million fresh cells were labeled with mAb 4F2 or a monoclonal antibody recognizing anti-canine parvovirus (CPV) as an isotype control. Dead cells were excluded following staining for viability with propidium iodide. For immunohistochemistry specimens, five hundred thousand leukocytes were adhered to a glass slide with a Cytospin centrifuge, fixed in acetone, and labeled with mAbs as previously described (de Mestre et al., 2010).

2.5 Magnetic cell sorting and qPCR

CD3 cell sorting was performed using an AutoMACS cell sorter (Miltenyi Biotec, Auburn, CA) following incubation of 10^8 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 populations were a mean 8% CD3+ as verified by FACS. 4F2 sorting was similarly performed using 5×10^8 PBL and mAb 4F2. Total RNA isolation and cDNA synthesis were performed as previously described (de Mestre et al., 2010). SYBR Green (Applied Biosystems, Carlsbad, CA) real time PCR reactions for amplification of *NKP46* or the housekeeper gene equine ubiquitin-conjugating enzyme E2D 2 (*UBE2D2*), were performed using an ABI 7500 Fast sequence detector (Applied Biosystems) as previously described (de Mestre et al., 2010). Primers were designed with Primer3 software (MIT, Cambridge, MA) to cross intron/exon boundaries to prevent amplification of genomic DNA. 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. Differences between groups were determined using paired two-tailed Student's t tests. Values were considered significantly different at P values <0.05.

3. Results

3.1. Expression of rIL-4/NKp46 and selection of mAbs to equine NKp46

To generate monoclonal antibodies to equine NKp46, we employed a system that we recently used to generate mAbs to equine CD16 (Noronha et al., accepted pending minor revisions, resubmission submitted). This method utilizes a recombinant protein made by tagging equine IL-4 to a target antigen (Wagner et al., accepted pending minor revisions, resubmission submitted). To create this fusion protein, the extracellular domain of NKp46 was predicted by comparing the CDS of *NKP46* to annotated sequences from other species and identifying homologous regions. The extracellular region was PCR-amplified from equine lymphocyte cDNA and inserted into a mammalian expression vector downstream of equine IL-4 (Fig. 1A); CHO-K1 cells transfected with this construct secreted the soluble protein. Secreted protein was purified from the culture medium by fast protein liquid chromatography using an affinity column designed to capture equine IL-4 (Wagner et al., accepted pending minor revisions, resubmission submitted). SDS-PAGE of the purified protein showed a molecular weight consistent with 39.9 kDa, the predicted molecular weight of rIL-4/NKp46 (Fig. 1B). The three bands observed on SDS-PAGE likely represent

differentially glycosylated species of the fusion protein, as it contains three predicted N-linked glycosylation sites with an N-X-S/T-X motif (Fig. 1B, arrowheads). The purified protein was used in combination with an adjuvant to immunize Balb/c mice from which splenocytes were recovered and used to generate hybridomas. Media samples from hybridoma cell clones were screened for the ability to recognize *rIL-4/NKp46* in ELISA (Fig. 1C). Specificity of this recognition was determined by also testing the samples against *rIL-4/IgG1* and *rIL-4/CD16* fusion proteins (Fig. 1C). CD16 was used as a specificity control because like NKp46, it has two extracellular C2-type Ig-like domains, and is therefore more structurally similar than the other NCR family members. Antibodies that recognized only the NKp46 fusion protein were considered to be specific for NKp46. Nine hybridoma clones produced mAbs that were selected for further characterization based upon fulfillment of these initial screening criteria.

3.2. MAbs recognize full-length equine NKp46

We next wanted to determine if the selected mAbs could recognize NKp46 in its native conformation of the surface of a cell. To test this, full-length CDS (minus the stop codon) of *NKP46* was inserted into the pEGFPN-1 expression vector upstream of the *EGFP* gene (Fig. 2A). This fusion protein was designed to express NKp46 on the cell surface, with EGFP confined to the cytosolic compartment, thereby mimicking the conformation of NKp46 on NK cells. CHO-K1 cells were transfected with the expression vector, and successful expression was verified by fluorescence microscopy. EGFP fluorescence also verified in-frame cloning of *NKP46*. Transfected cells were then labeled with the nine mAbs and analyzed by FACS. In order to demonstrate specificity of recognition, additional cells were transfected with IL-4- and CD16-EGFP constructs. Labeling was performed with both fresh cells and fixed, permeabilized cells, as permeabilization was required to detect intracellular IL-4-GFP. All nine mAbs demonstrated recognition of the NKp46-expressing cells, but not IL-4 (Fig. 2B). Eight of the nine clones failed to label CD16-transfected cells. One clone, 3B7, showed faint recognition of CD16 in a pattern similar to what was seen with the polyclonal post-immune sera (Fig. 2B).

3.3. MAbs recognize NKp46 on equine lymphocytes

We next tested the ability of the antibody panel to recognize NKp46 on intact horse cells. The mAbs were first tested on isolated PBMC. Labeling was only observed in the lymphocyte population (Fig. S1); therefore PBL were used for all subsequent analyses. All nine mAbs labeled lymphocytes with a similar pattern (Fig. 3A). The percent of labeled PBL varied between 1.0 and 1.5 (median=1.25%) among the nine mAbs on cells from the same horse. PBL were then isolated from eight additional horses, and representative mAb 4F2 was used for labeling. The percent of labeled lymphocytes varied from 0.8%-2.1% (mean=1.3%) among animals (Fig. 3B).

In order to determine if the cells labeled with mAb 4F2 expressed NKp46, we labeled PBL with mAb 4F2 and separated the cells into positive and negative fractions by magnetic cell sorting. The 4F2-enriched fraction increased from an initial 1.1% to 49.1% 4F2+ cells (Fig. 3C). cDNA was prepared from these cells as well as the 4F2-depleted (4F2-) fraction, and expression of *NKP46* transcripts was measured by real-time quantitative PCR (Fig. 3D). *NKP46* expression in the enriched population was 23-fold higher than in the depleted population.

We next wanted to determine if the 4F2+ population demonstrated the typical CD3- phenotype seen in NKp46+ cells of most species. To do this, we examined whether the depletion of CD3+ affected the number of 4F2+ cells. PBL from five horses were labeled with a CD3 mAb and CD3+ cells were depleted by magnetic cell sorting (Fig. 3E). Unsorted

cells and CD3-depleted (CD3⁻) cells were labeled with mAb 4F2 and the paired samples were compared (Fig. 3F). The number of 4F2⁺ cells increased by 2.3-fold following CD3-depletion. The fold-change for the five individual animals ranged from 1.1-3.4.

In order to visualize the morphology of the cell population recognized by these mAbs, PBMC were adhered to slides and labeled with the mAbs by immunohistochemistry (Fig. 3G). A small population of dimly stained lymphocytes was observed (arrow). The frequency of positive cells was approximately 10-fold lower than that observed by flow cytometry.

3.3. MAbs recognize three different epitopes

Lastly, the epitope-recognition of the nine mAbs were analyzed by ELISA. Each of the mAbs was first used to capture rIL-4/NKp46. Remaining free sites on the recombinant protein were then labeled with a biotinylated version of each mAb in a checkerboard fashion (Table 2). Failure to bind by the second antibody indicated a blocked site, and therefore recognition of the same epitope as the “capture” mAb. Using this assay, we determined that the nine mAbs could be divided into three groups of epitope recognition.

4. Discussion

Using a combination of ELISA, flow cytometry, and molecular methods, we have demonstrated that the mAbs described here demonstrate specific reactivity with equine NKp46. These mAbs show recognition of recombinant NKp46, as well as native protein on the surface of equine lymphocytes.

The size and granularity of equine leukocytes labeled by these mAbs, as determined by FACS forward and side scatter (Fig. S1), was similar to what is seen with peripheral NKp46⁺ cells of humans and cattle (Almeida-Oliveira et al., 2011; Kulberg et al., 2004). However, the intensity of labeling, i.e. the mean fluorescence intensity (MFI) measured by FACS (Fig. 3A), appears to be lower than in other species. It is unlikely that this relatively dim labeling is due to poor antibody affinity for NKp46 because all of the mAbs have a similar pattern, which cannot be overcome by increasing the antibody concentration. Also, CHO cells transfected with NKp46-EGFP demonstrated a high MFI following labeling. Therefore, it is more likely that the low signal is due to a low antigen density on the cell surface. This is consistent with quantitative RT-PCR studies we have previously reported showing that lymphocytes express a modest level of *NKP46* mRNA (Noronha et al., *in press*). This low level of antigen expression may explain why the number of cells detected by immunohistochemistry (IHC) was less than what was seen with FACS, as colorimetric IHC is a less sensitive assay. While these mAbs appear to be capable of labeling cells in IHC assays, under the conditions here we are only able to visualize the cells expressing the most antigen. More sensitive immunofluorescence microscopy assays may be required to visualize the entire population.

The 1.3% of lymphocytes recognized here in adult horses is less than what is seen in adult humans (7%), but is similar to what is reported for adult cows (1.8-2.6%) (Almeida-Oliveira et al., 2011; Kulberg et al., 2004). It is also lower than what is observed in juvenile sheep (3-16%). However, this may reflect an age-related effect as a progressive decline in the percentage of NKp46⁺ cells from birth to adulthood has been observed in some species (Almeida-Oliveira et al., 2011; Kulberg et al., 2004).

Our data clearly support that the mAbs described here recognize the equine NKp46 ortholog on horse cells. However, it is not yet clear if these cells are functional NK cells. As discussed above, NKp46 is generally considered the most reliable identifying marker of NK cells across species. Here, we see that the percentage of mAb 4F2⁺ PBL increases following

depletion of CD3+ cells, which is consistent with the canonical CD3-NKp46+ phenotype used to describe NK cells in most species. However the 2.3-fold increase was not proportional to the 8.4-fold reduction in CD3+ cells, and one animal increased only marginally (1.1-fold). These data suggest that some NKp46+ cells could also be CD3+. Such cells could be NKp46+ NKT cells similar to those recently described in the human and mouse (Yu et al., 2011). In cattle, CD3+ γ/δ T cells have also been shown to express NKp46 under some conditions (Johnson et al., 2008).

Alternatively, these cells could represent a species-related difference in the horse. CD3+ natural killer-like cells have previously been observed in lymphoid tissues recovered from severe combined immunodeficiency (SCID) foals that were devoid of conventional lymphocytes (Lunn et al., 1995).

Our group has previously identified equine lymphocytes with an NK cell phenotype using a cross-reactive monoclonal antibody to a catfish vimentin-like protein ("FAM," function-associated molecule) (Viveiros and Antczak, 1999). Those cells constitute a larger percentage of the lymphocyte population (4.5 to 23.3%) than what is seen here, and are all CD3-. Interestingly, in those studies, when CD3+ cells were depleted then labeled with anti-FAM mAb, the increase in presumptive NK cells was roughly 2.6 fold, very similar to the 2.3 fold increase seen here (Fig. 3F). It is not yet clear what the relationship is between FAM+ cytotoxic cells and the NKp46+ cells we have identified. Multiple overlapping phenotypic subsets are seen among the NK cells of other species (Lanier, 2005), and it is reasonable to think that equine NK cells would be equally complex.

5. Conclusions

The mAbs described here demonstrate the ability to recognize native and recombinant NKp46 in multiple assays (summarized in Table 3). These new reagents, in conjunction with our recently described antibodies recognizing equine CD16, will allow immunologists to better describe and understand the largely uncharacterized population of equine NK cells. They will also facilitate new study in areas with clinical relevance such as viral immune responses and tumor immunology.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors thank Ms. Julie Hillegas, Ms. Susanna Babasayan, and Ms. Esther Kabithe for expert technical assistance, and Dr. Colin Parrish for anti-CPV mAbs. DFA is an investigator of the Dorothy Russell Havemeyer Foundation, Inc. This research was funded by the Harry M. Zweig Memorial Fund for Equine Research, and NIH grants R01 HD049545 and F32 HD 055794. The development of the IL-4 expression system was supported by the USDA Grant #2005-01812 (The US Veterinary Immune Reagent Network) to BW.

Abbreviations

FACS	fluorescence-activated cell sorter
CDS	coding sequence
MFI	mean fluorescence intensity

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Figure 1.

Construction and expression of *hIL-4/NKP46*, and utilization to identify anti-NKp46 hybridomas. (A) The predicted extracellular domain of equine *NKP46* was directionally inserted 3' to equine *IL-4* in a pcDNA3.1 expression vector (*CMV*, Human cytomegalovirus immediate-early promoter; *pA*, Bovine Growth Hormone polyadenylation signal). (B) Soluble *hIL-4/NKP46* was expressed in CHO cells, affinity purified, and resolved by SDS-PAGE on a 10% gel under non-reducing conditions (lane 2). Arrowheads denote multiple glycosylation forms. (C) Culture media from hybridomas were tested by ELISA for the ability to recognize *hIL-4/NKP46*, *hIL-4/IgG1*, and *hIL-4/CD16*.

Figure 2.

Generation of full-length NKP46-, CD16-, and IL4-GFP expression constructs and flow cytometric analysis showing recognition by anti-equine-NKP46 mAbs. (A) Full length *NKP46*, *CD16*, and *IL-4* coding sequences (minus stop codons) were directionally inserted into the pEGFP-N1 mammalian expression vector 5' to *EGFP* (CMV, Human cytomegalovirus immediate-early promoter; *pA*, SV40 polyadenylation signal). (B) CHO cells transfected with fusion constructs were labeled with anti-equine-NKP46 mAbs and a fluorescently-labeled anti-mouse immunoglobulin secondary antibody (goat-anti-mouse-IgG (H+L)-647) and analyzed by flow cytometry. MAb 4F2 is shown as representative mAb. Anti-equine-IL-4 mAb (13G7) and sera taken from mouse spleen donor prior to immunization (*pre-immunization*) and at spleen collection (*post-immunization*) were used as controls.

Figure 3.

Recognition of horse peripheral blood lymphocytes by anti-NKP46 mAb 4F2. (A) Isolated lymphocytes were labeled with IgG1 control mAb (anti-CPV, left) or mAb 4F2 (right) and analyzed by flow cytometry. (B) Graph depicting percentages of 4F2-labeled lymphocytes for nine horses. (C) Histogram showing mean fluorescence intensity (MFI) of 4F2-labeled lymphocytes prior to (shaded curve) and following (open curve) enrichment of 4F2+ cells using magnetic cell sorting. Bars reflect the percentage of labeled lymphocytes (lower, pre-enrichment; upper, post-enrichment). (D) Real-time quantitative PCR analysis of *NKP46* transcripts in 4F2-depleted (4F2-) and enriched (4F2+) lymphocyte populations. (E) Histogram showing MFI of CD3-labeled lymphocytes prior to (shaded curve) and following (open curve) depletion of CD3+ cells using magnetic cell sorting. Bars reflect the percentage of labeled lymphocytes (upper, pre-depletion; lower, post-depletion). (F) Graph comparing percentages of 4F2-labeled lymphocytes of five horses prior to (total) and following (CD3-) CD3 depletion. (G) Immunohistochemical labeling of PBMC with mAb 4F2 (1000X) showing a representative dimly-stained lymphocyte (arrow).

Table 1
Sequences of genes and primers used

Gene	Accession #	Primers (5'-3')
<i>NKP46</i>	JN808451	<i>IL-4 fusion construct</i>
		F-GGCGGATCCCCAGAAGCGTACTCCCTCTAAAC R-GGCAAGCTTTCATGAATCAGGAGAAGAGATGTGC
		<i>GFP fusion construct</i>
		F-GGCGCTAGCATGCCTTCTATACTCACTGTCCTGCTC R-GGCGGTACCTCTTTGTCCAGGGATCTTTGTGTTCTGAAC
		<i>qPCR</i>
		F-CACCTGGAATGATGAACAAAG R-CCTGGGATGAACTGAGAGG
<i>IL-4</i>	GU139701	<i>GFP fusion construct</i>
		F-GGCGCTAGCATGGGTCTCACCTACCAACTGATTCCAG R-GGCGGTACCTCACACTGGAGTATTCTCTTTCATGATCGTCTTTAGC
<i>CD16</i>	JN795139	<i>GFP fusion construct</i>
		F-GGCGCTAGCATGTGGCAGATGCTATCACCAACGG R-GGCGGTACCTCAGAGCCCCGGCTCCATGTG

Table 2

ELISA and Epitope Recognition Group Assignments

		2. Biotinylated Antibody								Group Assignment
		3B7	4D10	4F2	7C2	8A3	8E8	8G3	9C1	
1. Capture Antibody	3B7									A
	4D10									A
	4F2									A
	7C2									A
	8A3									B
	8E8									A
	8F9*									C
	8G3									A
	9C1									A

Black: Reduced binding by biotinylated antibody; White: No reduction in binding

* 8F9 was only used as a capture antibody

Table 3**Summary of αNKp46 mAbs**

Epitope Group	mAb	Isotype	Application	
			ELISA	FACS ¹
A	3B7	IgG1	+ ²	++ ²
	4D10	IgG1	+	+
	4F2	IgG1	+	++
	7C2	IgG1	+	++
	8E8	IgG1	+	+
	8G3	IgG1	+	+
	9C1	IgG1	+	+
B	8A3	IgG1	+	+
C	8F9	IgG1	+	++

¹Labels both fresh and PFA-fixed cells

²Slight crossreactivity with CD16 observed in ELISA and FACS with transfected cells