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Research paper

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 (*r*IL-4/NKp46) and used as an immunogen to generate mouse monoclonal antibodies (mAbs). MAbs 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.

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 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).

Abbreviations: FACS, fluorescence-activated cell sorter; CDS, coding sequence; MFI, mean fluorescence intensity.

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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 CD3ζ and FcεRIγ, 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 cytolysis 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., 2012b). 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., 2012a), 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., 2012b). 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., 2012a; Wagner et al., 2012). CHO K-1cells 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., 2012). *r*IL-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., 2012). One microgram 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 *r*IL-4/NKP46 and *r*IL-4/IgG using ELISA, and for cell surface labeling of equine PBMC using flow cytometry. Antibodies that labeled PBMC and detected *r*IL-4/NKP46 but not *r*IL-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 *r*IL-4/NKP46 by ELISA as described previously (Wagner et al., 2012) and against *r*IL-4/IgG1 (Wagner et al., 2005) and *r*IL-4/CD16 (Noronha et al., 2012a) to confirm their specificity to NKP46. For epitope ELISA, plates were coated with 1 μg purified mAbs, washed, then followed with *r*IL-4/NKP46 fusion protein in transfected CHO cell supernatant. Following washing, 1 μg 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., 2012a). CHO-K1 cells were transfected with the vectors using the Geneporter2 system and assayed for protein expression 48 h post-transfection. Successful expression of GFP was confirmed by fluorescence microscopy and indicated correct reading frame cloning of