NKp46 defines a subset of bovine leukocytes with natural killer cell characteristics

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Natural killer (NK) cells have not previously been precisely identified or characterized in cattle or any other ruminant species. We have generated a monoclonal antibody against bovine NKp46, which is expressed exclusively by NK cells in man. NKp46$^+$ cells comprised 1–10% of blood mononuclear cells in cattle, and did not stain with antibodies against CD3, CD4, TCR1, B cell or granulocyte markers. The majority of the NKp46$^+$ cells expressed CD2, and a variable fraction also expressed CD8. The tissue distribution of NKp46$^+$ cells in cattle was compatible with the tissue distribution of NK cells in other species. Bovine NKp46$^+$ cells had typical, large granular lymphocyte morphology, and proliferated vigorously in response to bovine IL-2 for a limited number of cell divisions. IL-2-activated NKp46$^+$ cells killed the bovine kidney cell line MDBK. This cytotoxicity was inhibited by preincubation with antibody against NKp46. In a redirected lysis assay, IL-2-activated NKp46$^+$ cells killed the FcγR$^+$ target cell line P815 after preincubation with antibody against NKp46. Together, these data indicate that bovine NKp46 is an activating receptor and demonstrate the existence of a subset of leukocytes in cattle that, in terms of surface markers, morphology and function, represent NK cells.

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1 Introduction

Natural killer (NK) cells are large granular lymphocytes with the ability to spontaneously lyse certain tumor cells, virally infected cells or normal, MHC disparate cells [1–3]. NK cells express both activating and inhibitory receptors. Many of these receptors recognize MHC class I or class I-like ligands, and the balance between activating and inhibitory receptors determines the final outcome of the interaction of NK cells with possible target cells [4]. NK cells also provide a link between innate and acquired immunity through production of cytokines and interaction with antigen-presenting cells [1, 5–7]. Although bovine NK-like cells responding to mycobacteria-infected dendritic cells have been described [8], and cells with natural cytotoxicity have been found in cattle and sheep [9–11], the lack of mAb specifically recognizing NK cells has hampered the characterization of these cells in ruminants. Recently, genes encoding several bovine NK receptors have been identified [12, 13]. NKp46, previously characterized in primates and rodents, is a type I transmembrane glycoprotein with two extracellular C2-type Ig-like domains [14–17]. In the human, NKp46 is an activating receptor expressed exclusively by NK cells [18], and is involved in NK cell-mediated lysis of several targets including antigen-presenting cells [7]. The transmembrane region contains an arginine residue thought to interact with the FcRγ chain [14, 18], which contains immunoreceptor tyrosine-based activation motifs (ITAM). This region is conserved between the bovine, rodent and primate NKp46.

We here report the generation of an mAb against bovine NKp46. This mAb is used to characterize a subset of bovine leukocytes that, in terms of surface markers, morphology and functional characteristics, represent NK cells. We describe a method for isolation and culture of bovine NK cells, and demonstrate that bovine NKp46 activates cytotoxicity.

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2 Results

2.1 Generation of an mAb against bovine NKp46

A soluble fusion protein consisting of the extracellular region of NKp46 and the hinge and Fc regions of mouse IgG2b was generated and used to immunize mice. Hybridomas were screened for reactivity against 293T cells transfected with an NKp46-FLAG construct. One clone was isolated and termed AKS1. This clone produced an mAb of the IgG1 isotype that specifically recognized bovine NKp46 (Fig. 1A). In Western blot analysis of 293T cells transfected with NKp46-FLAG, AKS1 weakly stained a band of ~37 kDa and also a faint band of ~47 kDa. These bands were also stained with an anti-FLAG antibody (Fig. 1B). Immunoprecipitation with AKS1 of surface-biotinylated IL-2-activated NKp46$^+$ cells (see below) revealed a band of ~47 kDa both under reducing and non-reducing conditions (Fig. 1C), suggesting that bovine NKp46 is expressed as a monomer. The observed molecular mass is comparable with human NKp46. The theoretical mass of the unglycosylated NKp46 polypeptide is 32.2 kDa [13]. The difference is likely due to glycosylation.

2.2 NKp46 is expressed on a cell population with an NK cell phenotype

Two-color flow cytometric analysis was used to investigate surface expression of NKp46 on leukocytes in blood and selected tissues from 6–24-month-old animals. NKp46 was expressed on 1–10% of blood mononuclear cells. The NKp46$^+$ population was CD4$^-$, TCR1$^-$, and negative for the γδ T cell marker WC1, suggesting they were not a subset of T cells (Fig. 2A). The NKp46$^+$ population was further negative with mAb against B cells and granulocytes, respectively. Although a small fraction of the NKp46$^+$ cells expressed MHC class II, this population was CD14$^-$, suggesting that it did not represent monocytes. The NKp46$^+$ population was largely CD2$^+$, and between 4% and 15% of the NKp46$^+$ cells expressed CD8. NKp46$^+$ bovine leukocytes thus share key surface marker characteristics with NK cells in other species [19, 20]. NKp46$^+$ cells were found in liver, lung and spleen. Significant numbers of NKp46$^+$ cells were not present in the thymus or retropharyngeal lymph nodes, but between 1.3% and 3.9% of mesenterial lymph node cells expressed NKp46. While the majority of NK cells in liver, lung and blood expressed CD2, the majority of NKp46$^+$ cells in mesenterial lymph nodes and to a lesser degree in spleen were CD2$^-$ (Fig. 2B). NKp46$^+$ cells from all organs tested were negative for CD4, TCR1, WC1 and MHC class II, and did not stain with mAb against B cells or granulocytes (data not shown).

2.3 Isolation and culture of NKp46$^+$ cells

NKp46$^+$ cells were purified from PBMC by incubation with AKS1 mAb and positive selection with immunomagnetic beads. Culture in the presence of recombinant bovine IL-2 led to the up-regulation of CD25 expression within 1–3 days, and to cell proliferation. Cultures could typically be maintained between 7–14 days, yielding 10- to 20-fold increases in cell numbers. Human recombinant IL-2 did not induce up-regulation of CD25 or proliferation. Proliferating NKp46$^+$ cells grew as nonadherent cells with prominent lamellipodia (Fig. 3A). However, shortly after isolation, cells from some animals were adherent for several hours. In Giemsa-stained cytospin preparations, IL-2-activated NKp46$^+$ cells as well as

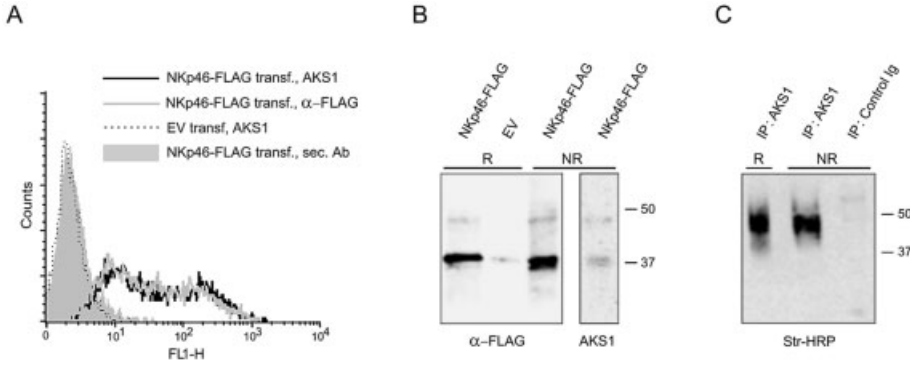

Fig. 1. (A) Flow cytometric analysis of 293T cells transfected with an NKp46-FLAG construct or empty vector (EV), and stained with supernatant from the AKS1 hybridoma or an anti-FLAG mAb. (B) Western blot of 293T cells transfected with an NKp46-FLAG construct or empty vector (EV) incubated with a rabbit anti-FLAG polyclonal Ab or the AKS1 mAb. (C) Immunoprecipitation of surface-biotinylated IL-2-activated NKp46$^+$ cells with AKS1 or an irrelevant isotype-matched control antibody. R: reducing conditions, NR: non-reducing conditions. Relative molecular masses in kDa are indicated.

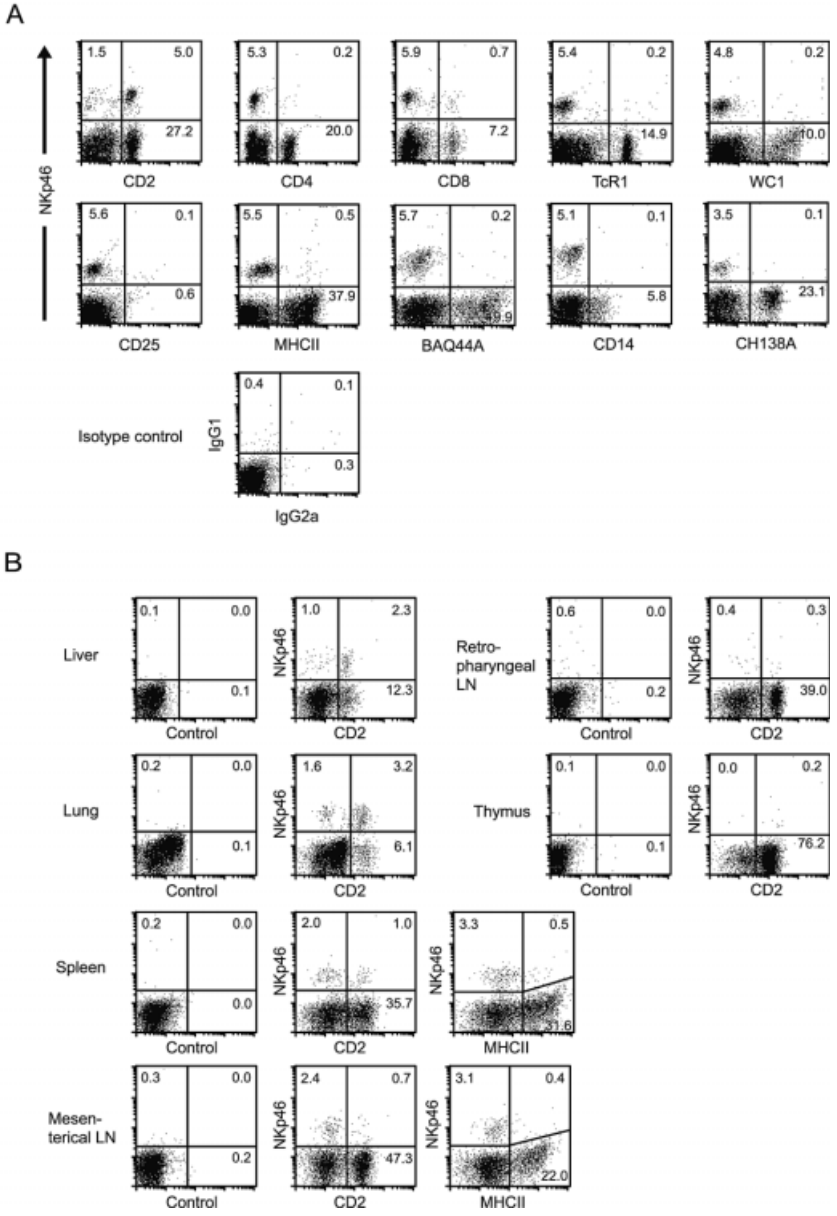

Fig. 2. Two-color flow cytometric analysis of NKp46 expression. (A) Blood cells stained with the AKS1 mAb and mAb against selected leukocyte markers. WC1 is a marker for a subset of γδ T cells; BAQ44A stains a major subset of B cells (B2); CH138A stains granulocytes. A forward/side scatter gate was set to include viable mononuclear cells, except for the granulocyte marker staining, where a larger gate also included viable granulocytes. (B) Cells from the indicated tissues stained with AKS1 and mAb against CD2 or MHC class II. The results shown are representative for experiments with eight (A) and five (B) different animals, respectively. LN: Lymph node.

freshly isolated NKp46$^+$ cells from blood were large granular lymphocytes, containing acidophilic granules polarized to one side of the cell (Fig. 3B). From all donors, 99.5% of IL-2-activated NK cells displayed a uniform NKp46^{bright}, CD3$^-$ phenotype (Fig. 4A, B). While the majority of NK cells in blood expressed CD2, the proportion was lower following IL-2 culture. In contrast, the level of CD8 expression was increased, and showed a continuous expression level from negative to brightly positive. Double labeling with CD8α and CD8β was not feasible due to steric interference between the two mAb. However, CD8α was more frequently expressed than CD8β, suggesting that some cells expressed a CD8α/α homodimer, while the majority expressed theCD8α/β heterodimer. CD8 was expressed on both CD2$^+$ and CD2$^-$ cells (Fig. 4B).

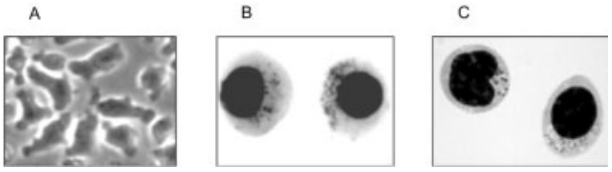

Fig. 3. (A) Phase-contrast microscopy image of NKp46$^+$ cells cultured in IL-2 for 6 days. (B) Giemsa-stained cytospin preparation of NKp46$^+$ cells cultured in IL-2 for 8 days and (C) Giemsa-stained cytospin preparation of NKp46$^+$ cells positively selected from blood.

2.4 Bovine NKp46$^+$cells spontaneously lyse tumor cells

In 4-h cytotoxicity assays, IL-2-activated NKp46$^+$ cells from one donor efficiently lysed the murine tumor targets YAC-1 and P815, as well as the human leukemic cell line K562 (Fig. 5A). A second donor showed lower levels of cytotoxicity, whereas six other animals did not show significant cytotoxicity against these cell lines. However, after preincubation with mAb against NKp46, effector cells from all eight donors induced efficient lysis of the FcγR-expressing cell line P815 even at low effector to target ratios. Preincubation with mAb against CD2 or CD8 did not induce lysis (Fig. 5B). Lysis of FcγR$^-$ target cells (YAC-1) was not induced by antibody against NKp46 (data not shown). Moreover, NKp46$^+$ cells from all animals tested killed the bovine kidney cell line MDBK

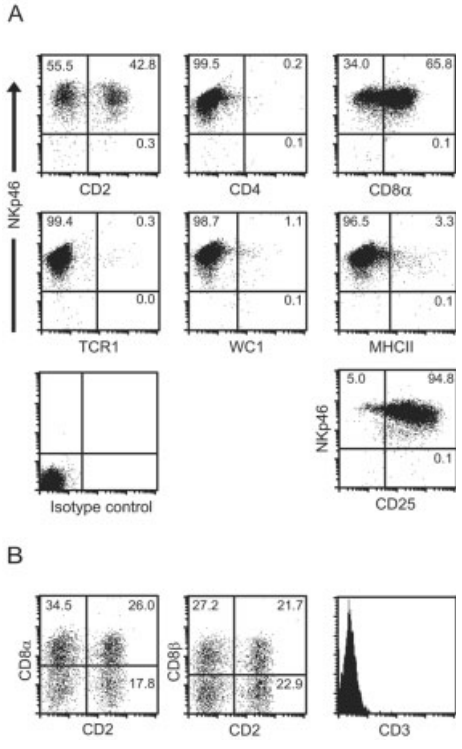

Fig. 4. Flow cytometric analysis of bovine IL-2-activated NKp46$^+$ cells. (A) Two-color staining with the AKS1 mAb and mAb against selected leukocyte markers. WC1 is a marker for a subset of γδ T cells. (B) Double staining with anti-CD2 mAb and mAb against CD8α or CD8β; and (far right) histogram displaying surface expression of CD3. The results shown are representative for experiments with five different animals.

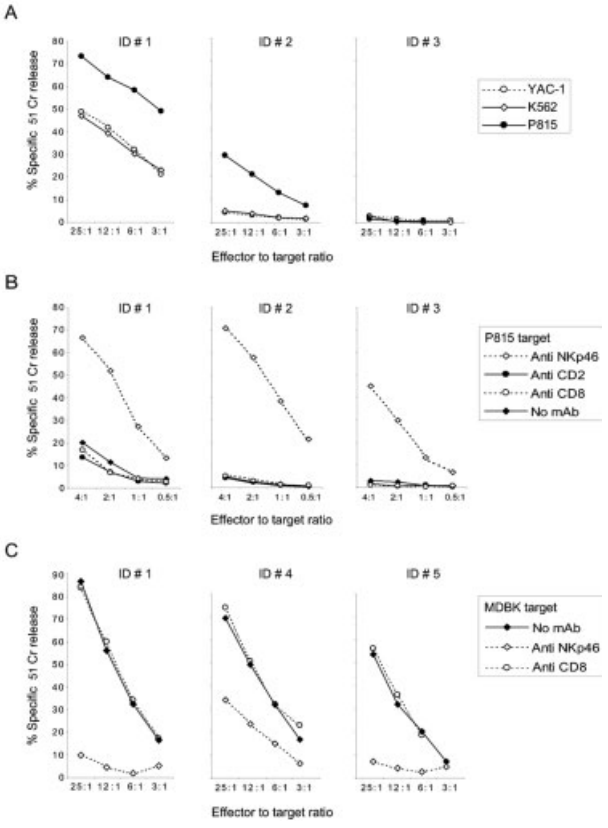

Fig. 5. (A) IL-2-activated NKp46$^+$ cells from eight different animals were tested for spontaneous cytotoxic activity against murine and human tumor cell lines in a 4-h ^{51}Cr-release assay. Effector cells from one of the animals (ID #1) showed cytotoxic activity against all three cell lines tested, while cells from a second animal (ID #2) only lysed P815 targets. Six other animals tested did not show spontaneous cytotoxic activity as exemplified by ID #3. Results shown are representative for at least two independent IL-2 cultures from each animal. (B) Redirected lysis assay. IL-2-activated NKp46$^+$ cells from the indicated animals were preincubated with the anti-NKp46 mAb AKS1 or mAb against CD2 or CD8 (all of IgG_1 isotype) and used as effector cells against the FcγR$^+$ tumor target P815 in a 4-h ^{51}Cr-release assay. The results shown are representative for all eight animals tested. (C) IL-2-activated NKp46$^+$ cells from all donors tested efficiently lysed the bovine kidney cell line MDBK, and preincubation with mAb AKS1 reduced this activity. Results shown are representative for at least two analyses from seven individual donors.

(Fig. 5C). Thus, NKp46$^+$ cells from all donors had the capacity for natural cytotoxicity. Together with the morphology and flow cytometry data, these functional data indicate that bovine NKp46$^+$ leukocytes represent NK cells.

Lysis of MDBK cells was reduced following preincubation of effector cells with mAb against NKp46, although to varying degrees in cultures from different animals (Fig. 5C), suggesting that MDBK cells express a ligand for NKp46. Together with the redirected lysis data, this also indicates that the NKp46 receptor activates cytotoxicity in bovine NK cells.

3 Discussion

In this study, we have generated an mAb against bovine NKp46 and used this mAb to define a subset of leukocytes in blood and other organs that functionally as well as phenotypically represent NK cells. This definition relies on several observations: (a) the majority of the NKp46-positive cells expressed CD2, and a variable fraction also expressed CD8, corresponding to the characteristics of NK cells in other species [19, 20]. Moreover, B cells, T cells, monocytes and granulocytes did not express NKp46. In concurrence with this, NKp46 has been shown to be specifically expressed by NK cells in the human [18]. (b) The tissue distribution of NKp46$^+$ cells in cattle is compatible with the tissue distribution of NK cells in mouse, rat and man [21, 22]. (c) Bovine NKp46$^+$ cells proliferate vigorously in response to IL-2 for a limited number of cell divisions, as is the case for NK cells in other species [19, 22]. (d) NKp46$^+$ cells had a typical NK cell morphology in culture as well as on Giemsa-stained cytospins. (e) IL-2-activated NKp46$^+$ cells had the capacity for spontaneous killing of bovine, murine and human target cell lines. We thus conclude that most or all bovine NK cells express NKp46. We have not detected NKp46 expression on other cell types than NK cells in cattle. Previously, cell surface markers such as CD16, CD56, CD94 and NKR-P1 have been used to define NK cells in man, mouse and rat. Antibodies against bovine orthologues of these markers are presently not available, and NK cells specifically express none of these markers. Based on our results, NKp46 should be regarded as a valid marker for bovine NK cells.

While there were almost no NKp46$^+$ cells present in the retropharyngeal lymph nodes, a small population of NKp46$^+$ cells could be detected in the mediastinal lymph nodes. Because the majority of these cells were CD2$^-$, they most likely did not represent contamination from blood NK cells present in the prepared tissue. Although significant numbers of NK cells are not believed to reside in lymph nodes under normal conditions, they have been reported to accumulate at these sites after regional challenges [23]. This may be an enduring state in the intestine of healthy farmed cattle, as used in this study. Subpopulations of human NK cells have been described that express CCR7 and have the ability to home into secondary lymphoid organs through high endothelial venules [24].

Considerable variation was observed between donors with regard to the level of spontaneous cytotoxicity towards the murine and human target tumor cell lines tested. The level of NK cytotoxicity against these targets for each donor remained unchanged between independent IL-2 cultures. Moreover, the cytotoxic potential was intact and similar between NK cells from all donors, as demonstrated by preincubating with anti-NKp46 mAb in a redirected lysis assay. This suggests that the difference in cytotoxicity is not due to individual differences in cytotoxic potential, but rather to different receptor repertoires between different donors. IL-2-activated cultures from all bovine donors expressed NKp46 at a similar level, in contrast to observations with human NK cells [25]. Thus, the difference in cytotoxicity was not due to different levels of NKp46 expression.

In contrast to the murine and human targets, the bovine target MDBK was efficiently lysed by NK cells from all donors. Due to interspecies sequence differences, mouse and human target cells might express few ligands efficiently recognized by bovine activating NK receptors.

In the redirected lysis assay, mimicking a ligand for NKp46 induced vigorous cytotoxicity even at low effector to target cell ratios. Thus, in animal 1, $\sim$70% specific ^{51}Cr release from P815 targets was obtained at a 25:1 ratio without antibody, whereas the same level of cytotoxicity was observed at a 4:1 ratio following incubation with AKS1. The lower cytotoxicity in the spontaneous lysis situation probably indicates that only a subset of NK cells express the appropriate receptors that allow killing, whereas all cells expressed NKp46 and could be activated via the AKS1 mAb. Lysis of the FcR$^-$ target MDBK was inhibited by incubation with AKS1, suggesting that the mAb blocked interaction with a ligand on the target cells that was important for induction of cytotoxicity. Our data do not exclude that cross-linking of NKp46 alone may be sufficient to activate the cytolytic machinery, but further studies investigating a larger array of NK receptors on single clones will be required to address this question.

The bovine IL-2-activated NK cells expressed CD8 mainly in the heterodimeric (α/β) form. This contrasts to the situation in man and rat where NK cells predominantly express the CD8α homodimer [26, 27], whereas

murine NK cells do not express CD8 [22]. The functional role of CD8 on NK cells is not clear. In this study, preincubation with anti-CD8 mAb had no effect on the level of cytotoxicity towards FcR^- or FcR^+ targets (Fig. 5B, C).

We have described a method whereby bovine NK cells can be purified and cultured. In contrast to bovine T cells that proliferate in response to human recombinant IL-2 [28], bovine IL-2 was required for the culture of bovine NK cells. Bovine $NKp46^+$ cells could also to some extent be expanded in supernatant from Con A-stimulated bovine $CD4^+$ T cells (data not shown).

This initial characterization of NK cells in cattle and the availability of an mAb specifically reacting with bovine NK cells will hopefully facilitate future studies of innate immunity in cattle.

4 Materials and methods

4.1 Generation of an anti-NKp46 mAb

An expression construct containing the extracellular region of bovine NKp46 [13] at the N-terminal side of the Fc region of mouse IgG2b was constructed. Briefly, the NKp46 signal sequence and extracellular region (nucleotides 4–774) was amplified by PCR using *PfuTurbo* DNA polymerase (Stratagene, La Jolla, CA) and gene-specific primers including HindIII and BamHI restriction sites. The PCR product was purified by agarose gel electrophoresis, incubated with Taq DNA polymerase (Promega, Madison, WI), cloned into pCR2.1 TOPO vector (Invitrogen, San Diego, CA), released by HindIII and BamHI digestion (New England Biolabs, Beverly, MA) and ligated into a mammalian expression vector containing the hinge, C_H2 and C_H3 regions of the mouse IgG2b gene (obtained from H. C. Aasheim, The Norwegian Radium Hospital, Oslo, Norway). Of this construct, 20 μg was mixed with 120 μl Lipofectamine (Invitrogen) in 2.8 ml Opti-MEM (Invitrogen) and incubated for 20–30 min at room temperature; 11.2 ml Opti-MEM were then added and the mixture transferred to a 160-cm^2 flask containing an ~70% confluent layer of 293T cells. After 6 h, 14 ml of complete RPMI 1640 medium/20% FCS (Invitrogen) was added. The cells were then incubated for 18 h, washed three times in Opti-MEM or PBS and finally cultured in serum-free AIM-V medium (Invitrogen) for 4 days. bNKp46-mFcγ2b fusion protein was purified from the culture supernatant on a protein G column (Amersham Biosciences, Little Chalfont, GB) according to the manufacturer's instructions. Female young adult BALB/c mice were immunized by four intraperitoneal injections of 100 μg fusion protein in Freund's complete (first injection) or incomplete adjuvant (following two injections, Invitrogen) or in PBS (final injection), and spleen cells were fused with NS-0 cells by conventional techniques. Single-clone hybridoma supernatants were screened by flow cytometry (see below) for reactivity against 293T cells transiently transfected with an expression construct containing the entire coding region of NKp46 and a FLAG tag in the N-terminal end (generated by amplifying the coding region of NKp46 without the signal sequence (nucleotide 88 to 951) by PCR and inserting it into pFLAG-CMV-1 (Sigma-Aldrich, St. Louis, MO). One specifically reacting hybridoma clone, with a staining pattern similar to that of anti-FLAG antibody M2 (Sigma-Aldrich), was subcloned twice and named AKS1.

4.2 Western blotting and immunoprecipitation

293T cells transfected with the FLAG-NKp46 construct or empty vector were harvested 48 h post transfection and lysed with 1% Igepal CA-630 (Sigma-Aldrich) in lysis buffer [25 mM Tris-HCl pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.02% NaN_3, 1 mM PMSF, 1 mM Na_3VO_4, 10 mM NaF and protease inhibitor mix (Sigma-Aldrich)] at 4°C. For immunoprecipitations, IL-2-activated NK cells were surface-biotinylated as follows: cells were washed three times in PBS pH 8.0, incubated for 30 min at room temperature with 0.5 mg/ml NHS-biotin (Sigma-Aldrich) in PBS pH 8.0 at 25×10^6 cells/ml, then washed three times in PBS pH 8.0. Biotinylated cells were lysed in 1% Triton X-114 (Sigma-Aldrich) as described [29]. The membrane-enriched lysate fraction from 7×10^6 cells (~20 μl) was diluted in 400 μl lysis buffer (as above) containing 0.5% digitonin (Calbiochem, San Diego, CA), and precleared with 30 μl protein A/G beads (Santa Cruz Biotechnology, Santa Cruz, CA) precoated with irrelevant antibody (5 μg mAb W6/32 [30] per 100 μl beads) three times at 4°C; twice for 1 h, and the third step overnight. Precleared lysate was then immunoprecipitated for 2 h at 4°C with 30 μl beads precoated with 5 μg AKS1 mAb, and the beads were washed three times by centrifugation for 4 min at 2,300×g and resuspension in lysis buffer/0.5% digitonin.

Protein samples were diluted in SDS sample buffer, boiled for 2 min, separated by SDS-PAGE, and transferred to PVDF membrane by semi-dry transfer. Membranes were blocked with blocking buffer (TBS/0.05% Tween 20/3% BSA) and incubated with rabbit anti-FLAG antibody diluted 1:1,000 (Sigma-Aldrich) or AKS1 at 4 μg/ml in blocking buffer for 2 h at room temperature. Membranes were washed six times in TBS/0.05% Tween 20 and incubated with peroxidase conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, PA) in blocking buffer. Detection of secondary Ab was with SuperSignal West Pico substrate (Pierce Biotechnology, Rockford, IL) and a high-performance CCD camera (Kodak ImageStation 2000, Eastman Kodak, Rochester, NY).

4.3 Culture of bovine NK cells

PMBC were separated from blood collected in EDTA tubes by Lymphoprep (Axis-Shield, Oslo, Norway) gradient centrifugation (1,150×g, 20 min) and washed twice in PBS with 2 mM EDTA. Subsequently, 3×10^8 cells in 6 ml PBS with

2 mM EDTA and 0.5% BSA were added to 18 μg AKS1 mAb and incubated at 4°C for 30 min. After washing twice, 24×10^6 immunomagnetic beads coated with an anti-mouse pan IgG mAb (Dynal, Oslo, Norway) were added and incubated at 4°C for 30 min with shaking, fixed in a magnet and washed three times in PBS with 0.5% BSA. Positively selected cells were cultured in a six-well tray in 3 ml of RPMI 1640 supplemented with 60 μg/ml penicillin, 100 μg/ml streptomycin, 1 mM sodium pyruvate, nonessential amino acids, 50 μM 2-mercaptoethanol, 10% FCS (all Invitrogen) and recombinant bovine IL-2 (10 biological U/ml final concentration) obtained by expression in COS cells. After 24–48 h, magnetic beads were collected and the cells were transferred to new wells. Additional medium was added when necessary. The IL-2 concentration in added medium was reduced to 4 or 2 biological U/ml after 5–6 days when cultures were proliferating.

4.4 Cytospin preparations

IL-2-activated NKp46$^+$ cells (5×10^4 in 50 μl) were centrifuged onto poly-L-lysine-coated glass slides at 80×g for 6 min, immediately fixed in methanol for 5 min, air dried, and stained with a 1:20 dilution of Giemsa stain (Sigma-Aldrich) in water for 20–30 min, and washed with water buffered to pH 7.0 (3 mM sodium phosphate). Blood NK cells were positively selected by AKS1 antibody and CELLection pan mouse IgG magnetic beads (Dynal), released by DNase treatment, washed twice in RPMI 1640/10% FCS prior to cytospin preparation and stained as described above.

4.5 Flow cytometry

Two-color flow cytometric analysis of cell surface receptors was used to determine the expression of leukocyte receptors on NKp46$^+$ cells in peripheral blood. Blood from eight 6–12-month-old cattle of the Norwegian Dairy Cattle breed was lysed hypotonically, and 10^6 leukocytes were incubated with AKS1 at 1 μg/ml in PBS with 1% BSA and 10 mM NaN_3 and one of the following antibodies: CD4 (IL-A11, 10 μg/ml), CD8α (BAQ111A, 5 μg/ml), CD2 (MUC2A, 10 μg/ml), CD25 (LCTB2A, 5 μg/ml), TCR1 (GB21A, 5 μg/ml), WC1 (B7A1, 10 μg/ml), MHC class II (H42A, 0.5 μg/ml), B cells (BAQ44A, 10 μg/ml), granulocytes [CH138A, 5 μg/ml, all from Veterinary Medical Research Diagnostics (VMRD), Pullman, WA] and CD14 (TÜK4, 0.6 μg/ml, DAKO, Glostrup, Denmark). Subtype-specific secondary antibodies conjugated with allophycocyanin (anti-IgG1 mAb, BD Biosciences, San Jose, CA) or FITC (goat anti-mouse Ab, against all other isotypes, Southern Biotech, Birmingham, AL) were used. Control stainings with secondary antibodies only and isotype control antibodies were performed. Cells were fixed by adding FACS lysing solution (BD Biosciences) prior to analysis. Gates were set to include mononuclear cells or the total leukocyte population in the granulocyte marker stainings. A total of 10,000 gated cells were analyzed.

Tissue samples from thymus, spleen, mesenterial and retropharyngeal lymph nodes, liver and lungs were collected from five animals of the Norwegian Dairy Cattle breed at 6–24 months of age at the abattoir, kept on ice and homogenized as previously described [31]. The surface expression of the leukocyte markers described above, including CD8β (BAT82A, 10 μg/ml) and CD3ε chain (MM1A, 10 μg/ml, both from VMRD) was also analyzed on IL-2-activated NK cells from five different animals.

4.6 Cytotoxic activity of bovine NK cells

The cytotoxic activity of IL-2-activated bovine cells against the bovine kidney cell line MDBK [32], the murine tumor cell lines YAC-1 [33] and P815 [34] and the human leukemic cell line K562 [35] was tested in a standard ^{51}Cr-release assay: target cells were maintained in complete RPMI 1640/10% FCS. Target cells (5×10^6 cells) were incubated with 100 μCi $Na_2{}^{51}CrO_4$ (Amersham Biosciences) in 1 ml medium at 37°C for 1 h and were washed three times in PBS/2% FCS. Twofold dilutions of effector cells (6–14 day NK cell cultures) were added to 1×10^4 ^{51}Cr-labeled target cells in 96-well round-bottom microtiter plates and incubated at 37°C for 4 h. The supernatant was harvested and radioactivity counted on a gamma-counter. Specific ^{51}Cr release was calculated on the basis of the ratio [(sample release – spontaneous release)/(total release – spontaneous release)]. The results were expressed as the median of three parallel samples. In blocking experiments with MDBK targets and in redirected lysis assays of the FcγR$^+$ target cell line P815 the effector cells were preincubated for 20-30 min with 1 μg/ml mAb (all IgG1) against NKp46 (AKS1), CD8 (CACT80C) or CD2 (BAQ95A, both from VMRD). MDBK grows as a monolayer, and was released by non-enzymatic treatment (Cell dissociation solution, Sigma) prior to the ^{51}Cr labeling.

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