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# Characterization of natural killer cells in healthy goats (*capra aegagrus hircus*)

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Title: Characterization of natural killer cells in healthy goats (*capra aegagrus hircus*)

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**Abbreviations**

| | |
|---|---|
| ADCC | Antibody – dependent cellular cytotoxicity |
| BSA | Bovine serum albumin |
| CD | Cluster of differentiation |
| Cr | Chrome |
| DMSO | Dimethyl sulfoxide |
| DPBS | Dulbecco's phosphate buffer saline |
| EMEM | Eagle's minimum essential medium |
| FCS | Fetal calf serum |
| GSM | Goat synovial membrane |
| IFN-γ | Interferon gamma |
| IL | Interleukin (rhIL/rbIL = recombinant human/bovine IL) |
| Ig | Immunoglobin |
| mAb | Monoclonal antibody |
| MHC1 | Major histocompatibility complex 1 |
| NK | Natural killer |
| NCR1 | Natural cytotoxicity triggering receptor 1 |
| LAK | Lymphokine activated killer |
| LAMP-1 | Lysosomal-associated membrane protein-1 (CD107a) |
| LGL | Large granular lymphocytes |
| PBMC | Peripheral blood mononuclear cell |
| PCR | Polymerase chain reaction |
| PPRV | Peste de petits ruminants' virus |
| RPMI | Roswell Park Memorial Institute |
| TCR | T – cell receptor |
| TWEAK | Tumor necrosis factor like weak inducer of apoptosis |

**Summary in Norwegian**

Tittel: *Karakterisering av naturlige dreperceller hos friske geiter* (*capra aegagrus hircus*)

Forfatter: Cathinka Celine Jørgensen

Veileder: Preben Boysen

Medveileder: Hege Lund

**Summary in English**

Title: *Characterization of natural killer cells in healthy goats* (*capra aegagrus hircus*)

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**Abstract**

NK cells are essential cells of the innate immune system characterized by their ability to spontaneously eliminate virus-infected cells, tumor cells and all cells that are missing evidence of self. In addition to their immunoprotective properties, they have immunoregulatory functions, such as contributing to a tolerant immune environment during the establishment of pregnancy. NK cells have been thoroughly characterized in humans, mice, pigs, and cattle as well as to some extend in sheep. However, NK cells in goats have not been characterized before. In a world of virus- induced chronic infections, tumor manifestations and complex immune interactions, it is necessary to establish basal knowledge of these important immune cells, to meet the growing demand for immunological model organisms. The main objective of this project was to establish essential methods for describing the phenotype and function of goat NK cells. Several cell selection approaches were performed. One finding was that the anti-ovine NKp46 EC1.1 antibody seemed to result in a higher cell outcome than the anti-bovine NKp46 AKS6 antibody, when performing cell selection with magnetic beads. A functional effector cell assay, including cytotoxicity based on CD107a expression as well as IFN-γ production, was established in goats. Using this method, NKp46+CD3- cells showed typical NK-cell like properties like cytotoxic activity and cytokine production. An observed circulating population of NKp46+CD3+ cells were suspected to be γδT cells. Furthermore, a population of cells that were frequently contaminating NKp46+ cell cultures were CD3+ and were also hypothesized to be γδT cells. To resolve these hypotheses, cell sorting strategies were attempted with the aim to subsequently confirm genes specific for certain cell types by PCR. Time constraints of the research track project did not allow for the fulfillment of the cell sorting tasks and PCR; nevertheless, these data will provide a useful basis for the optimalization of these methods, and the verification of these heterogenous cell populations in goats.

## 1. Introduction

In the 1970s, NK cells were considered an insignificant "background population" of cells, before being recognized as key cells of the innate immune system with importance roles within virus and tumor elimination. The knowledge about NK cells and their applications have expanded since then (Hokland and Kuppen, 2005), both in human and veterinary medicine.

NK cells are defined as cytotoxic and cytokine-producing large granular lymphocytes (LGL) of the innate immune system, contributing to the first line of defense against infectious intruders (ABUL K. ABBAS, 2014, Chapter 2, p.34-36). In addition to killing virus- infected and tumor cells, NK cells non- specifically eliminate cells lacking self-markers, such as the major histocompatibility complex 1 (MHC1) molecule. This is referred to as the "missing self-hypothesis" (Ljunggren and Kärre, 1990, Kärre, 2002).

The innate and adaptive immune response have often been presented as separate systems. However, they interact, often through the production of cytokines. For instance, interleukin 12 (IL-12) produced by macrophages stimulates NK cell interferon-gamma production (IFN-γ), which activates the macrophages to kill their ingested microbes (ABUL K. ABBAS, 2014, Chapter 2, p. 34-36). Also, activated T cells produce interleukin 2 (IL-2), which influences NK cell function and cytokine production (Pillet et al., 2011, Fehniger et al., 2003).

The balance between multiple activating and inhibiting receptors determine whether the NK cells are activated or not (Chiesa et al., 2005). It is the combination of these signals and cytokines that regulate NK cell activation, resulting in cytotoxic activity and cytokine production (O'Connor et al., 2006). Activated NK cells can perform four types of cytotoxic behavior as reviewed in (Boysen and Storset, 2009). A dominance of activating signals, due to lack or absence of inhibition, promotes spontaneous lyse of target cells by the NK cell. This process is referred to as natural cytotoxicity. NK cells can also be activated *in vitro* by cytokines such as IL -2 and become lymphokine activated killers (LAK cells) (Grimm et al., 1982). They can also kill target cells coated with antibodies and perform antibody dependent cellular cytotoxicity (ADCC). The ADCC, or redirected lysis, occurs when NK cells are activated by antibodies against activating receptors on the NK cell (Boysen and Storset, 2009). A redirected lysis assay with bovine IL-2 activated NKp46+ cells have been attempted against the P815 target cell line (Storset et al.,

2004). In human NK cells, the surface expression of the lysosomal-associated membrane protein-1 (LAMP-1 or CD107a) have been suggested as a functional marker for detecting NK cell cytotoxicity, due to a proven correlation between CD107a expression and cytotoxic activity. The CD107a assay is suggested as an alternative to other assays measuring NK cell cytotoxic activity, allowing the study of effector cells in heterogenous cell populations (Alter et al., 2004, Aktas et al., 2009). CD107a is expressed intracellularly in most cells of the body (Andrejewski et al., 1999), but on the surface of cells only when they show cytotoxic activity (Aktas et al., 2009). In addition to cytotoxic activity, NK cells produce cytokines like IFN-γ and TNF-α. NK cells are also affected by cytokines. For instance, IL-12 contributes to the NK cell's production of IFN- γ and increased cytotoxic activity, and interleukin 15 (IL-15) promotes NK cell development and maturation (ABUL K. ABBAS, 2014, Chapter 2, p. 34-36).

Prior to the invention of monoclonal antibodies (mAbs), NK cells were characterized as non-B, non-T cells or "null cells" with the ability to kill without previous stimulation (Grossi et al., 1982). Today, after the invention of monoclonal antibodies and the development of several immunological methods, the phenotypical and functional properties of NK cells have been further characterized in humans, mice and domestic species.

Central immunological methods used for the characterization of NK cells are based on the ability of antibodies to identify cell specific structures (antigens) on the surface of cells and intracellularly. Antibodies, or immunoglobins (Ig), are glycoproteins naturally produced by plasma cells in host organisms as part of the adaptive immune response. They have a characteristic Y- structure which represents a heavy and light chain of polypeptides that are held together by disulfide and nonconvalent bonds. There are different classes of immunoglobins and in mammals there are five classes: IgG, IgM, IgA, IgD and IgE. The IgG and IgA classes are divided into subclasses (isotypes) in some mammals, based on the presence of polymorphisms in the conserved region of the antibody's heavy chain. These antibody characteristics determine the difference in immune response(Lipman et al., 2005) The antibodies bind a part of the antigen (epitope) with a specific affinity and specificity (Lipman et al., 2005), which makes them ideal for the identification of cell populations based on their expression of cell markers (antigens). Usually, different antigens are recognized by a variety of several lymphocytes, leading to their activation and differentiation into plasma cells that produce different antibodies. This can be

referred to as a polyclonal antibody response, with several lymphocyte clones producing different antibodies. However, when only one B- lymphocyte clone produces antibodies, the response is referred to as a monoclonal antibody response (Lipman et al., 2005). A technique to generate monoclonal antibodies with a chosen specificity was developed based on this response (KÖHler and Milstein, 1975). Thus, species-specific monoclonal antibodies targeting specific antigens could be made and used to define specific cell populations based on their characteristic expression of cell markers, or phenotype. Different immunological methods utilize the qualities of monoclonal antibodies for the purpose of selecting specific cell populations, for instance during immunomagnetic cell selection, immunophenotyping and flowcytometric analysis and cell sorting (Arnold and Lannigan, 2010, Safarík and Safaríková, 1999, Cossarizza et al., 2019). During flowcytometric analysis, monoclonal antibodies conjugated with fluorochromes are used to detect cell markers (McKinnon, 2018). Flowcytometry has many applications. For instance, it can be used for phenotypic characterization of blood cells and intracellular cytokine detection among other uses (Adan et al., 2017). Prior to phenotypic characterizations, cells are selected from different material, for instance blood. This can be done with a gradient centrifugation extraction and magnetic beads (Böyum, 1968, Safarík and Safaríková, 1999). The selected cells can then be used to establish cell cultures or immunophenotyped by staining the cells with different antibodies prior to flowcytometric analysis (McKinnon, 2018). Flowcytometric analysis does not enable the physical sorting of single cells from a heterogenous cell population into wells or tubes that can be used to establish cell cultures or in further down-stream assays. However, this is possible with flowcytometric cell sorting (Arnold and Lannigan, 2010, Adan et al., 2017).

NK cells can thus either be selected from blood with magnetic beads, or by flowcytometric cell sorting. The functional assays in this study tested cells for both cytotoxic activity and cytokine production. CD107a (LAMP-1) has been used as a marker for cytotoxic activity in humans (Alter et al., 2004), and could also be used in goats if there is a cross-reaction between human CD107a and goat cells. Cytokine production can be measured by IFN-γ expression, which can be detected by immunophenotyping and flowcytometric analysis (Adan et al., 2017).

There are fewer immunological reagents specifically developed for veterinary species compared to humans and mice. The need for species-specific tools and reagents have been recognized for a while (Evans and Jaso-Friedmann, 1993). There is however, evidence of a cross-reactivity

between immunological reagents specifically developed for ruminant species, even though it cannot be guaranteed. It is likely that the cross-reactivity of monoclonal antibodies is greater when the monoclonal antibodies have been generated in phylogenetically closely related species such as ruminants (Hope et al., 2012, Chen et al., 2019). Goat specific tools and reagents would be ideal. However, since the bovine NK cells have been thoroughly characterized with the development of bovine specific reagents and monoclonal antibodies (Storset et al., 2004), these immunological tools can be tested on goat cells based on the likely cross-reactivity between ruminant species. A study on lymphocyte subpopulations in goat peripheral blood showed that monoclonal antibodies against the following surface markers; MHC1, MHC2, CD2, CD4, CD8, γδT cells, and the IL-2 receptor of bovine cells, worked to recognize the same lymphocyte subpopulations in goats (Navarro et al., 1996), strengthening the hypothesis that bovine tools and reagents can be used with goat cells. Monoclonal antibodies against bovine and ovine cells have been tested for reactivity with goat cells, yielding an overview of different molecules that cross-react, including the CD8 molecule, that can be used to separate different lymphocyte subpopulations (Davis and Ellis, 1991).

The phenotype of NK cells is usually defined by the presence or absence of species-specific surface cell markers. For instance, the human NK cell is defined by the expression of CD56 and CD16 (Michel et al., 2016, Fehniger et al., 2003), and the absence of CD3 expression, which is a cell marker for the T cell receptor (αβγδ) (Hercend and Schmidt, 1988). The CD45b marker, recognized by the DX5 monoclonal antibody, is used to define NK cells in mice, and can be used in all mice strains (Arase et al., 2001). NCR1 (natural cytotoxicity triggering receptor 1, CD335, NKp46) has been proposed as a characteristic NK cell marker in many mammals (Walzer et al., 2007).The activating NK cell receptor NKp46 has been used as a cell marker to define NK cells in both cattle and sheep (Storset et al., 2004, Connelley et al., 2011) among other species, for instance in pigs (Mair et al., 2012). However, the NKp46 marker does not define the entire NK cell population in pigs, but could be used to differentiate between different porcine NK cell populations with different functions (Mair et al., 2012). NK cells in domestic species were early reviewed in porcine, bovine, chicken, canine, feline and equine NK cells (Evans and Jaso-Friedmann, 1993) with a description of their phenotype, target cell specificity and cytokine regulation. However, a lot of phenotypical and functional descriptions were not complete at the time. Later, bovine NK cells have been thoroughly characterized (Boysen and Storset, 2009), and

defined as NKp46+CD3- cells (Storset et al., 2004). Canine and equine NK cells have been described, but the characterization is not complete (Grøndahl-Rosado et al., 2015, Viveiros and Antczak, 1999). Ovine NK cells have also been further characterized (Elhmouzi-Younes et al., 2010, Olsen et al., 2013, Connelley et al., 2011). The number of NK cells in peripheral blood also varies between species. In humans, NK cells constitute 10% of the total amount of lymphocytes in blood and peripheral lymphoid organs (ABUL K. ABBAS, 2014, Chapter 2, p.34-36), while in cattle, the percentage of NK cells in peripheral blood varies between 0.5 to 10%, with a lower relative number of NK cells in cattle compared to humans (Kulberg et al., 2004, Storset et al., 2004). In terms of ruminant NK cell biology, the bovine and ovine NK cells have been characterized, but there is little to no descriptions of NK cells in goats.

Previous unpublished data in goats that were not included in this study, but which this study is based on, showed that a fraction of PBMCs from goats was positive for the anti-bovine NKp46 antibodies AKS6, AKS4, AKS2 and the anti-ovine antibody EC1.1 (Appendix 1). The NKp46+ cells showed no overlap with any non-NK cell markers, except for a subset that was also positive for the GB21a antibody, which is specific for the TCR1 delta chain on bovine γδT cells (Davis et al., 1996). Furthermore, previous results showed that NKp46+ cells could be selected with a positive selection method using magnetic beads and showed expansion in cell cultures containing IL-2. These cells showed cytotoxic activity against the P815 target cell line and produced IFN-γ. Efficient killing was found with the use of an anti-NKp46 antibody targeting the NKp46 marker. The overlap between NKp46 and TCR1 could indicate that a proportion of the NKp46+ cells were not true NK cells, but whether they were most closely associated to NK cells or γδT cells, or a totally different entity was not known. Assessing the expression of CD3 (present on all T cells including γδT cells) on these cells was therefore of interest. In the goat, surface antibodies recognizing CD3 was not available, but an antibody against the intracellular domain of CD3 was available. However, this would require a permeabilization of the cells, resulting in the killing of these cells. This would not enable the use of these cells in further functional assays, which require live cells to work. Following permeabilization of PBMCs, a proportion of NKp46+ cells were shown to be positive for intracellular CD3. The CD8b surface marker was found to be a useful surface substitute marker for the intracellular CD3 marker. The use of this marker would allow for the separation between CD3+ and CD3- cells within the NKp46+ population without killing the cells, enabling the use of these cells in further functional assays. However, the

combined study of NKp46, CD3, TCR1 and CD8b had not been achieved, due to incompatible isotypes of available antibodies, when using unconjugated antibodies in an indirect staining method.

An overlap between NK cells and T cells have also been reported in other species. For instance, the NKp46+CD3+ population have been defined as a nonconventional T cell subset in cattle, showing both NK cell and T cell features (Connelley et al., 2014). A study on bovine γδT cells have shown that γδT cells sometimes express NKp46 and produce IFN-γ (Johnson et al., 2008), which raises the question of whether the NKp46+TCR+ cells are in fact γδT cells expressing NKp46 or NK cells expressing a γδT cell marker. γδT cells are considered to be a class of lymphocytes within the innate immune system, but with some T cell characteristics (ABUL K. ABBAS, Chapter 2, p. 36). γδT cells are expected to be positive for γδT cell markers and the CD3 T cell marker, but negative for the NK cell marker NKp46. The functions of γδT cells are still poorly understood, and their biology is under investigation, for instance in the bovine model, as γδT cells are especially rich in the circulation of cattle and sheep (Telfer and Baldwin, 2015). However, much remains when it comes to the characterization of γδT cells in ruminants, which was not the main focus of the research track project.

Small ruminants all over the world face several chronic infections, caused by different viruses, for instance the *PPRV virus* (Qi et al., 2020) and the *Caprine arthritis encephalitis virus* (CAEV), and bacteria, for instance *Mycobacterium avium subsp. Paratuberculosis* (Lybeck et al., 2011) and *Corynebacterium pseudotuberculosis* (Williamson, 2001*)*. It is important to characterize the cells that make up the first line of defense against these infectious agents. Also, goat uterine NK cells, regulated by proteins such as TWEAK, are proposed to play an important part in the estrous cycle and establishment of pregnancy in the goat (Lei et al., 2015), which further substantiates the important role of these cells, not only in the defense against infection, but as mediators of pregnancy as well. An immunological analysis of goat kids lacking the prion protein could also have included NK cells, if goat NK cells had been characterized prior to the study (Reiten et al., 2015). This further underlines the importance of characterizing these cells in healthy individuals, to acquire basic knowledge that can serve as a starting point and reference when conducting immunological analysis.

## Knowledge gaps

A short summary of essential knowledge gaps is presented below. The selection method for goat NKp46+ cells is based on the selection method for cow NK cells (Storset et al., 2004). However, this selection method has resulted in a low amount of goat cells after selection, which complicates the establishment of cell cultures and further downstream assays. Several anti-bovine NKp46 antibodies have been found to cross-react with goat lymphocytes (Appendix 1), but the NKp46 marker is not sufficient to define these cells alone. A negative marker is necessary as well, preferably a surface marker targeting other non-NK cells, such as T-cells. However, only one antibody against an intracellular domain of the T cell receptor CD3 is available. This antibody requires a permabilization of the cell membrane, which kills the cell and is not consistent with the performance of further functional assays. Previous results have shown that the surface CD8b marker coincide with the intracellular CD3 marker (Appendix 1). This marker can thus be used as a substitute for the intracellular CD3 marker for the selection of CD3+ cells without killing cells. A cytotoxic assay (Chrome (Cr) release assay) used on bovine NK cells have been tested on goat cells in previous studies (Appendix 1), but the CD107a assay have not been established in goat cells before. The CD107a assay is based on the detection of CD107a expression as an indicator of cytotoxic activity in the effector cell, and CD107a can be identified in the same cell that is immunophenotyped without a prior selection of cells. The intracellular CD3 antibody can thus be used because immunophenotyping occurs after the completion of the assay, and cells can be permeabilized and killed. In contrast, the Cr release assay measures the death of target cells, and therefore requires a selection of live cells prior to the assay, in order to assign the killing to specific effectors. Previous results (Appendix 1) have showed that goat NKp46+ cells were positive for a γδT cell marker (TCR1). Since γδT cells are expected to be CD3+, these cells were investigated with an intracellular CD3 marker, showing a population of NKp46+CD3+ cells in goats. These cell populations could be sorted with flowcytometric cell sorting and investigated further with PCR to detect typical NK cell, γδT cell or other cell specific genes.

## Aims and Objectives

The main objective of this study was to establish essential methods for describing the goat NK cell phenotype and function. A selection of cells from goat peripheral blood with a NKp46

antibody and magnetic beads could be performed, which is a method successfully used to select cow NK cells. Only a low number of goat cells have been selected based on this approach, and an optimalization of the selection method is required, to achieve a higher number of cells after selection. If enough cells could be gained in cell culture after selection, functional tests could be performed to test the NKp46+ cell's capacity for cytotoxic activity and cytokine production. A CD107a assay could be established in goat cells, to test the cytotoxic activity of goat NKp46+ cells, and cytokine production could be tested by detecting the expression of the intracellular IFN-γ marker on these cells. The NKp46+CD3- and NKp46+CD3+ cell populations could be investigated with flowcytometric cell sorting followed by establishment of cell cultures and PCR. After cell sorting of NKp46+CD3- and NKp46+CD3+ populations, PCR could be performed to identify these cell populations based on the detection of NK cell, γδT cell or other cell-specific markers.

**Hypotheses**

Our predictions were that:

1) An optimized selection method with the NKp46 antibody AKS6 would result in a high enough number of cells for the establishment of cell cultures and further downstream assays.

2) The human CD107a antibody would cross-react with goat cells, and the CD107a assay could be established with goat cells and used to measure cytotoxic activity.

3) Goat NKp46+CD3- cells would show NK typical functional properties like cytotoxic activity and cytokine production, and these cells would have similar phenotypical and functional characteristics to cows and sheep.

4) The flowcytometric cell sorting method, using CD8b as a CD3 substitute, would enable the sorting of true NKp46+CD3- and NKp46+CD3+ cell populations, suitable for establishment of cell cultures and further investigations in downstream assays. After the flowcytometric cell sorting, the different cell populations could be identified by PCR, for specific NK cell, γδT cell and αβT cell markers.

5) The NKp46+CD3+ population could be confirmed to be γδT cells, using another γδT cell antibody (GD3.5) in addition to TCR1.

## 2. Materials and Methods

### *2.1 Animals and blood sampling*

Blood sampling was approved by the Norwegian Animal Research Authority (FOTS ID 8194). Bovine and goat cells were isolated from a total of twenty healthy individuals, consisting of sixteen Norwegian Dairy Goats and four Norwegian Red cows, as listed in table 1. Limited availability of animal material did not allow us to systematically stratify the material for age, sex or breed. The blood was extracted by jugular venipuncture (venoject) and collected in 9 mL tubes with EDTA. The pelt was shaved off in a small area around the jugular vein to ensure easy access to the vein. The blood from goat ID1 to ID8 was collected in sealed glass bottles with citrate from goats from Fatland, Oslo, while the rest of the goats sampled, were either from PRODMED at NMBU, SHF at Ås, or from a research facility connected to the Norwegian Veterinary Institute.

**Table 1: Individuals (ID) in study**

| ID number | Animal | ID (ear mark) | Journal number | Volume | Location |
|---|---|---|---|---|---|
| BovineID1 | Adult cow | 2105 | 2016-705 | 95 mL | |
| BovineID2 | Calf 3 months | 1236 | 2016-712 | 90 mL | |
| BovineID3 | Calf | - | - | 70 mL | |
| BovineID4 | Adult cow | 0891 | 777 | 160 mL | |
| ID1 | Goat | 6002 | - | - | Fatland, Oslo |
| ID2 | Goat | 504 | - | - | Fatland, Oslo |
| ID4 | Goat | 503 | - | - | Fatland, Oslo |
| ID5 | Kid goat | 6010 | - | - | Fatland, Oslo |
| ID6 | Kid goat | 6009 | - | - | Fatland, Oslo |

| | | | | | |
|---|---|---|---|---|---|
| ID7 | Kid goat | 6005 | - | - | Fatland, Oslo |
| ID8 | Goat | 6001 | - | - | Fatland, Oslo |
| ID11 | Goat | 11529 | 2014-578 | 1)160 mL<br>2) 165 mL | PRODMED, NMBU |
| ID12 | Goat | - | 2016 – 65 | 1)120 mL<br>2) 75 mL | PRODMED, NMBU |
| ID13 | Goat | 40431 | 2014 | 100 mL | |
| ID14 | Goat | 22137 | 2016 | 100 mL | |
| ID15 | Goat | | 2002 | 35 mL | |
| ID16 | Goat | | 2004 | 35 mL | |
| ID17 | Goat | 4105 | | 50 mL | |
| ID18 | Goat | 4048 | | 50 mL | |
| ID19 | Goat | 2088 | | 50 mL | |

*Table 1: An overview of the individuals in our study with ID number, specification of animal, ID number from earmark, journal number, volume of blood sampled and location.*

### *2.2 Phenotypical investigations of goat cells*

#### *2.2.1 Monoclonal antibodies*

Antibodies from phylogenetically closely related species were used, relying on cross-reactivity between species. "In house" produced primary monoclonal antibodies (mAbs), and antibodies from external vendors were used, as listed in table 2.

**Table 2: Overview of monoclonal antibodies**

| **Cell type / use** | **Molecular target** | **Raised against** | **Clone** | **Isotype** | **Conjugate** | **Supplier / reference** |
|---|---|---|---|---|---|---|
| NK cells | NKp46 (NCR1/ | Bovine | AKS4 | Mouse(m) IgG1 | Unconjugated (U) | Crozat JEM 2010 |

| | | | | | | |
|---|---|---|---|---|---|---|
| | CD335) | | | | | (Crozat et al., 2010) |
| | | Bovine | AKS6 | mIgG2b | U | Lund VetImm 2012 (Lund et al., 2012) |
| | | Ovine | EC1.1 | mIgG1 | U | Kind gift from Timothy Connelley, The University of Edinburgh (Connelley et al., 2011) |
| Cytotoxic activity in NK cells | CD107a | Bovine | 9f6e2 | mIgG1 | U | Kind gift from Timothy Connelley, The University of Edinburgh |
| | | Human | eBioH4A3 | mIgG1 | PE | eBioscience / Thermo Fisher |
| | IFN – gamma | Bovine | 6,19 | mIgG2b | U | Kind gift from Gregers Jungersen |

| | | | | | | |
|---|---|---|---|---|---|---|
| | | | | | | (Olsen et al., 2005) |
| γδT cells | TCR1 | Bovine | GB21a | mIgG2b | U | VMRD/ Kingfisher Biotech |
| | GD3.5 | Bovine | G3.5 | mIgG1 | U | Kind gift from Mark Jutila (Jones et al., 2007) |
| T cells and subsets | CD3 | Human | CD3-12 | Rat (r) IgG1 | Pacific Blue | Bio-Rad |
| | CD8a | Bovine | BAQ111A | mIgM | U | VMRD/ Kingfisher Biotech |
| | CD8b | Bovine | BAT82A | mIgG1 | U | VMRD/ Kingfisher Biotech |
| | CD4 | Bovine | CACT138 A | mIgG1 | U | VMRD/ Kingfisher Biotech |
| NK cells, neutrophils, monocytes and macrophages | CD16 | Human | KD1 | mIgG2a | U / FITC | Bio-Rad |
| T cells and NK cells | CD2 | Bovine | MUC2a | mIgG2a | U | VMRD/ Kingfisher Biotech |
| | | Bovine | B26A4 | mIgM | U | |
| B-cells | CD21 | Bovine | GB25A | mIgG1 | U | VMRD/ Kingfisher Biotech |

| | | | | | | |
|---|---|---|---|---|---|---|
| Monocytes | CD14 | Bovine | CAM36A | mIgG1 | U | VMRD/ Kingfisher Biotech |
| Controls | E.g., Mouse IgG1 isotype control | | | | Various | Various |

*Table 2: An overview of monoclonal antibodies used in this study.*

*2.2.2 Conjugation and validation of antibodies*

Several protocols and kits were available for the conjugation of antibodies. The protocols of the conjugation kits were performed according to the manufacturer's instructions.

Antibodies used during immunophenotyping can be either unconjugated or directly conjugated. Using directly conjugated antibodies allows for only one step during staining, which is the addition of a specific primary antibody bound to a specific fluorochrome. It is then important that the combinations of colors in the fluorescence emission spectrum does not overlap. However, most of the antibodies used in immunological assays with ruminant cells are unconjugated. Unconjugated antibodies can either be conjugated or used with an indirect staining method. A prerequisite for indirect staining is that the primary antibodies have different isotypes, so that they are a fit for secondary antibodies with different fluorochromes on top of them. If these antibodies have similar isotypes and cannot be combined, the antibodies can be directly conjugated to allow for more combinations of antibodies in one flowcytometric analysis.

Both unconjugated and conjugated antibodies were used in our assays. Newly conjugated antibodies (CD8b and NKp46biotin-streptavidin) were tested on goat cells by flowcytometry to validate their function. The following conjugation strategies were performed or attempted; The CD107a antibody was conjugated with the Alexa Fluor Ab Labeling Kit (Thermofisher). The CD8 antibody (BAT82A) was conjugated with the Lightning-Link PE-Cy7 Tandem Conjugation Kit (Innova biosciences). The AKS6/DSB-X antibody was conjugated with the DSB – X Biotin Protein Labeling Kit (D – 20655) with DYNABEADS Flowcomp flexi part A for the positive selection of NKp46+ cells directly from PBMC. The principle behind this kit is that DSB-X biotinylated antibodies bound to the intended cell population (in our case NKp46+ cells), are

captured by modified streptavidin-coupled Dynabeads. Then, a release buffer gently releases this modified biotin-streptavidin connection, resulting in bead-free cells that are ready for use in other downstream assays such as flowcytometric cell sorting. The conjugated antibodies were validated either with immunophenotyping and flowcytometric analysis, or with the "Conjugate check and go!" kit (Innova Biosciences).

*2.2.3 Immunophenotyping and flow cytometric staining of cells*

A standard protocol for immunophenotypic staining was used followed by flowcytometric analysis with the Gallios flowcytometer. A description of the different steps of immunophenotyping and flowcytometric analysis is presented below.

The following buffers and reagents were used: flowbuffer (DPBS, 0,5% BSA and 0,005% azide), Protein-free DPBS buffer with 2mM EDTA (DPBS/EDTA), LIVE/DEAD yellow Fixable Dead Cell Stains (Life Technologies), primary mononuclear antibodies conjugated with fluorochrome, 40% goat serum, the cell fixation/permeabilization kit (BD Biosciences) and FACS Lysing Solution (BD Biosciences).

Cells (PBMC or NK cells/target cells) were dissolved in protein – free DBPS with 2mM EDTA (DBPS/EDTA) before being aliquoted in wells. Live/dead staining, surface staining and/or intracellular staining was performed after this, depending on which markers that were tested for. Non – sterile flow 96 - well microplates were used. Unless otherwise stated, the different markers or plasma were added in small volumes to each well according to a pre-made scheme, followed by an incubation on ice in the dark. The pre-made scheme showed an overview of markers and associated flowcytometric channels. After each incubation, the plate was washed by adding flowbuffer (DPBS with 0,5% BSA and 0,005% azide) and spun down.

A LIVE/DEAD Fixable Dead Cell Stains (Life Technologies) marker, e.g., L/D yellow marker was used to separate dead cells from live cells. Primary monoclonal antibodies towards extracellular antigens were added according to scheme, followed by adding 40 % normal goat serum (FcR block). Then secondary antibodies were added according to scheme.

To facilitate intracellular staining, Cytofix/Cytoperm buffer (Cell fixation/Permabilization kit) was added to each well, followed by an incubation on ice in the dark. 1x Perm/Wash was then added, followed by centrifugation. The step was repeated with1x Perm/Wash, before adding

intracellular antibodies according to scheme. The plate was washed twice with fixation buffer, before resuspending the cells in flowbuffer. In some cases, secondary antibodies were used for the intracellular staining as well, which added a few steps to the staining process. After resuspension in flowbuffer, the samples were analyzed on the Gallios Flowcytometer (Beckman Coulter).

Prior to flowcytometric analysis, UltraComp eBeads™ Compensation Beads from Thermofisher were used according to the manufacturer's instructions. These beads have several applications, such as calibrating relevant channels in the flowcytometer, compensating for signaling spillage across fluorescent channels, and saving goat cells for the flowcytometric analysis, since beads were used instead of goat cells during calibration. The following antibodies were used: (FL9 Anti-human CD3 *Pacific blue* (Table 2), FL10 Anti-mouse/human B220 *BV570* (BioLegend), FL1 Anti-mouse CD16 *FITC* (Table 2), FL2 Anti-mouse Ly49A *PE* (eBioscience) and FL6 Anti-mouse *CD62L APC* (eBioscience)).

### *2.3 Selection of goat NKp46+ cells*

Due to the more well-known NK cell biology of bovine NK cells (Boysen and Storset, 2009), as well as the availability of material, bovine NK cells were first used to test the methods that were later tested on goat cells.

#### *2.3.1 Preparation of goat peripheral blood mononuclear cells (PBMC)*

The blood was extracted with venoject collection tubes with EDTA to avoid blood clotting (section 2.1). Unless otherwise stated, polypropylene tubes were used. The blood was distributed in tubes and diluted 1:2 with DPBS buffer to exploit resources, before the diluted blood was carefully applied onto Lymphoprep (Fresenius Kabi Norge/Axis-Shield) prior to centrifugation. After the density gradient centrifugation on Lymphoprep, the cell gradient layer (PBMC) was collected and transferred to tubes. The cells were washed twice with Dulbecco's phosphate – buffer with saline and EDTA (DPBS/EDTA), and then dissolved in DPBS/EDTA with BSA (bovine serum albumin) prior to counting the cells in Countess (Thermo Fisher).

#### *2.3.2 Cryopreservation*

The surplus of cells was frozen by distributing suspensions of cells into NUNC cryotubes. The freezing medium (80% FCS+20%DMSO) was added in drops until the 1 mL mark. The frozen

cells were put in a -80 ℃ freezing box, followed by transfer to liquid nitrogen the following day or within a week.

*2.3.3 Positive selection of goat NKp46+ cells and in vitro expansion in cell culture*

After counting the cells in Countess (Thermo Fisher) (last step of section 2.3.1), the volume of the sample was reduced by centrifugation. The cells were dissolved in a tube in 1xDPBS/EDTA buffer with 0.5% BSA. The surplus of cells could then be frozen by cryopreservation (section 2.3.2), or the positive selection could ensue by adding the NKp46 antibody, prior to incubation on ice, or on a rotating device in a cooling room at 4℃. The IgG pan mouse DYNABEADS were prepared. The samples were washed twice, first with DPBS/EDTA buffer with BSA and then with DPBS/EDTA buffer. The sample and buffer were transferred to polystyrene tubes, and the cells were dissolved in 1xDPBS/EDTA with 0.5% BSA. The washed and resuspended DYNABEADS Pan Mouse IgG were added in a specific ratio of volume per mL cells, and the tubes were put on ice in a polystyrene box on a shaking device. After positive selection with the IgG pan mouse DYNABEADS, the cells were incubated in an $CO_2$ incubation chamber at 37℃ for three days. The cells were investigated in the microscope to check whether the right cell type had been selected, and to check if the cells had sufficient conditions for growth and proliferation. After three days, the magnetic beads were removed by placing the tubes in a magnet and retaining the liquid content containing the cells. The cells were then left to expand in NK cell medium. The NK cell medium consisted of RPMI (Roswell Park Memorial Institute) 1640 medium supplemented with P/S, 10% FCS (fetal calf serum), sodium pyruvate, non-essential amino acids and 2 – mercaptoetanol. To optimize proliferation and growth, the cells were transferred into a 96 – well microplate with NK cell medium and cytokines (ovIL – 2 (Connelley et al., 2011), 200 IU/mL, and hIL – 15, 10mg/mL, eBioscience). Standard protocols for the maintenance and sustainment of cell cultures were followed. Cells that were in their exponential growth phase was used in the assays, to ensure an accurate representation of the cells phenotypical and functional properties.

To determine the optimal cytokine profile for cell proliferation in cell cultures, cells receiving only ovIL -2 were compared to cells receiving a combination of ovIL – 2 and hIL – 15. Instead of 6- well microplates, 96-well microplates were used in the initial phase of cell culturing to

maximize cell-to-cell contact, before the cells were transferred to 12 and 6-well plates when the number of cells increased.

### *2.3.4 Modification of cell selection methods*

To improve the cell output after selection, different selection methods were attempted. In addition to *the positive selection with DYNABEADS* (section 2.3.3), a *negative depletion of cells with a MACS LD column* (section 2.3.5) resulting in an NKp46+ enriched population, and a *positive selection with the DYNABEADS Flowcomp Flexi kit and the AKS6/DSB-X antibody* (section 3.1.5) was attempted.

### *2.3.5 Flow cytometric cell sorting*

The flowcytometric cell sorting enabled the physical separation of cell populations into tubes or wells, followed by establishment of cell cultures or use in further downstream assays.

The proceedings prior to cell sorting were performed in the same manner as described in section 2.3.1. Instead of a positive selection with a NKp46 antibody and DYNABEADS (section 2.3.3), a negative selection of cells with a MACS LD column was attempted (section 2.3.4). The purpose of the negative selection method was to remove non – NKp46+ cells surrounding the NKp46+ cells, resulting in an NKp46+ enriched population. A negative selection cocktail of surface markers against non-NKp46+ cells was prepared, as listed in table 3. After counting the cells in Countess (Thermo Fisher), the volume of the sample was reduced by centrifugation. The cells were dissolved in 1xDPBS/EDTA with 0.5% BSA. Antibodies were added to the tube and left to rotate on a rotator (cooling temperature). The content was then washed twice with DBPS/BSA buffer without EDTA. The pellet was resuspended before adding anti – mouse IgG microbeads/million cells. The blend was incubated on a rotator in a cooling room. After incubation, the cells were washed by adding buffer followed by centrifugation. The cells were then resuspended to a certain number of cells per mL buffer. The magnetic separation and negative selection of cells with an LD column was executed according to the manufacturer's instructions (Miltenyi Biotec MACS). The NKp46+ enriched cell population was counted in Countess (Thermo Fisher), to check the number of cells after selection. The cells were then diluted in 50 mL tubes, and a certain amount of the solution was put aside in NUNC tubes for later calibration.

**Table 3: The negative selection cocktail**

| Cell type | Surface marker | Clone number | Isotype | Stock concentration |
|---|---|---|---|---|
| B cells | CD21 | GB25A | Mouse IgG1 | 1 mg/mL |
| γδ- T cells | TCR1 | GB21A | Mouse IgG2b | 1 mg/mL |
| CD4 T cells | CD4 | CACT138A | Mouse IgG1 | 1 mg/mL |
| Monocytes | CD14 | CAM36A | Mouse IgG1 | 1 mg/mL |

*Table 3. The negative selection cocktail consisting of surface markers known to define different non – NK cell populations.*

Prior to sorting, the NKp46+ enriched population was labelled with antibodies following the standard protocol for *Immunophenotyping and flowcytometric staining of cells* described in section 2.2.3.

The flowcytometric cell sorting was performed at Oslo University Hospital, Ullevaal with a BD FACS ARIA flowcytometric cell sorter (Beckton Dickinson). During cell preparation prior to flowcytometric cell sorting, the cells were filtrated in buffer with a nylon mesh to remove clumps and doublets. Polypropylene tubes were used to avoid adherence. The cells were kept on ice during sorting. The cell capture medium was supplemented with HEPES buffer (Sigma-Aldrich) to maintain adequate pH. Sterility was ensured by adding P/S (penicillin/streptavidin) to the NK cell medium. The cells were diluted in NK cell medium after cell sorting, supplemented with hIL -15 and ovIL – 2, and then transferred to 96-well microplates before incubation at 37 ℃ with $CO_2$. Standard protocols for the maintenance and sustainment of cell cultures were followed. The different subpopulations of sorted cells were phenotyped after eighteen days, to survey which populations of cells were present.

### *2.4 Functional investigations of goat NKp46+ cells*

#### *2.4.1 Validation of the CD107a antibody*

The CD107a antibody, which binds intracellularly, was validated for functionality and specificity on bovine NK cells and goat NKp46+ cells, using intracellular staining and flowcytometry, as described above (section 2.2.3). To determine which antibody to use, previously collected bovine NK cells were stained with different CD107a molecule markers (huCD107a

(eBio/Thermofisher), bovCD107a unconjugated, kind gift from Timothy Connelley, table 2). Antibodies against bovine CD8a (VMRD/Kingfisher Biotech) and humane CD16 (Bio-rad) were included as negative controls (table 2).

*2.4.2 Cytotoxic activity of goat NKp46+ cells stimulated in monoclonal antibody-coated wells*

To promote cytotoxic activity in the NKp46+ cells, these cells were stimulated in wells coated with monoclonal antibodies against activating receptors like NKp46. The antibodies were diluted in carbonate buffer and incubated in wells overnight at 4℃. The coated plated were washed with buffer. PBS with 10% FCS was added to achieve the blocking of other irrelevant binding sites and increase the probability of correct specific binding. The cells were then incubated for two hours at 37℃. The plates were washed with PBS, spun down and dissolved in NK cell medium after counting. The cell concentration was adjusted, and a certain number of NKp46+ cells was distributed per well. Carbonate buffer was added to negative control wells. The cells were centrifuged and the CD107a antibody (stock concentration: 1,6 mg/mL) diluted in NK cell medium was added. The plates were centrifuged to pellet cells, and to ensure contact between cells and the monoclonal antibody coat. The cells were then incubated for three hours at 37℃ at 5% $CO_2$, one hour without Golgistop$^{TM}$ (BD biosciences), a protein transport inhibitor, and then two hours with Golgistop. The cells were then transferred to a U – bottomed 96 – well plate and centrifuged, before being washed twice with PBS and analyzed in the flowcytometer.

*2.4.3 In vitro combined CD107a and IFN-γ assay with P815 and GSM target cells*

The measurement of cytotoxic activity (CD107a expression) and cytokine production (IFNγ expression) was combined in this in vitro assay, as described below.

Target cells were mixed with cultured ovIL – 2 stimulated NKp46+ cells (effector cells) at different concentrations to elucidate the NKp46+ cell's CD107a expression (cytotoxic activity indicator) and IFN-γ production at different effector target ratios (Effector:Target ratio, E:T ratio).

Two different cell lines were used; P815 and the goat synovial membrane (GSM) adherent cell line (Rolland et al., 2004). Both cell lines were cultured in RPMI 1640 medium or EMEM (Eagle's minimum essential medium), respectively, supplemented with P/E and 10% FCS, and maintained at 37℃, 5% $CO_2$ in an incubation chamber. The GSM cell line medium was supplemented with 0.5% fungizone in addition. Prior to the assay, cell lines were retrieved from

the liquid nitrogen container and thawed in warm water, before being washed in 10 mL medium at 1250 rpm for 5 minutes. Cells were diluted 1:10 in a heated medium in a cell culture bottle and stored at 37℃ with 5% $CO_2$. The cells were regularly observed in a microscope to determine when to replace the medium, or trypsinate the adherent GSM cells to release them from the cell culture bottle. Every three to four days the GSM cell cultures were diluted, while the P815 cells (which expanded very quickly in culture), were diluted every to every second day. Standard protocols for the maintenance and sustainment of cell cultures were followed.

Both effector (NKp46+) cells and target cells were sustained in a medium consisting of ovIL-2 and hIL-15 in addition to HEPES buffer. The following antibodies were used; Anti – NKp46 unconjugated AKS6 and CD107 – Alexa647. Mouse IgG-Alexa 647 was used as an isotype control. The cell suspension was prepared in a medium consisting of RPMI 1640 medium, FCS and cytokines (ovIL – 2/hIL-15). Different amounts of target cells were seeded in a 96-well microplate, followed by a constant amount of NKp46+ cells (effector cells), resulting in a range of different E:T ratios across wells (16:1 to 0.5:1). There were two wells for controls, one well for effector cells only, and one for target cells only. The CD107a antibody or isotype control antibodies, and AKS6 or medium, were added to the wells according to a pre-made scheme. The mixture was spun down before the cells were incubated at 37℃ for one hour using an incubator with motion properties. Brefeldin A (protein release inhibitor) was then added, before incubating at 37℃ for two hours. After a three-hour incubation in total, the standard protocol for flowcytometry was followed (section 2.2.3) with the markers CD3 (FL-9), L/D (FL – 10), IFN-γ (FL-1), NKp46 (FL-2) and CD107a (FL-6) in each channel, and four rows with different target cell mixtures. The capacity for NK cell cytokine production (IFNγ) was determined by immunophenotyping and intracellular flowcytometry in the presence of protein release inhibitor (Brefeldin A).

# 3. Results

## *3.1 Results*

The results from this study is presented below. All raw data is accessible.

### *3.1.1 Immunophenotyping and flowcytometric analysis of goat PBMC*

PBMC isolated from goats were phenotyped with different antibodies directed towards known NK and non-NK cell surface and intracellular markers prior to flowcytometric analysis (results from stainings with the CD3 and CD2 markers are shown). Unless otherwise stated, the AKS6 antibody was used for NKp46 labeling.

To investigate whether the population of NKp46+ cells overlapped with the intracellular CD3 marker, the cells were stained intracellularly for CD3. A variable proportion of the NKp46+ population was CD3- (27-89%), as illustrated in figure 1A and 1B. A proportion of the NKp46+ population was CD3+ (11-73%), as illustrated in figure 1A and 1B. The CD2 marker was also tested, which has been shown to divide bovine NK cells into distinct subsets (Boysen et al., 2006). Results showed a population of NKp46+CD2+ cells (33%) among the NKp46+ cells, as illustrated in figure 1C. The results with the CD2 marker were in accordance with previous results with the CD2 marker in goats (Appendix 1).

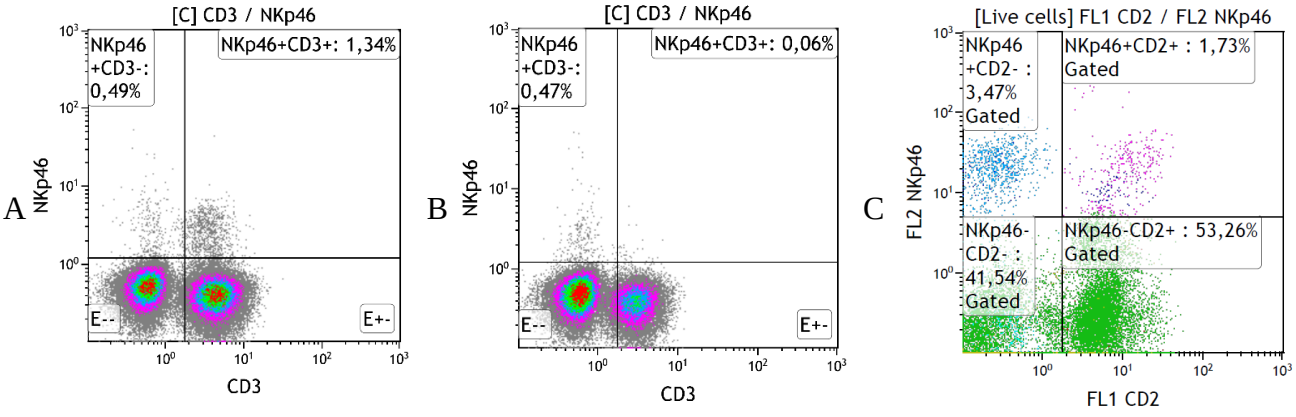



*Figure 1. Flow cytometric analysis of goat PBMC. Figure 1A and 1B are representative of seven goats. (A) ID4 is shown. There is a majority of NKp46+CD3+ cells. (B) ID6 is shown. There is a majority of NKp46+CD3- cells. (C) ID11 is shown. Figure 1C is representative of two goats. Approximately a third of the NKp46+ cells were CD2+.*

### *3.1.2 Cell selection and culture*

NKp46+ cells were selected using a positive selection with magnetic beads, based on binding of the AKS6 antibody. The NKp46+ cells showed expansion in cell culture in the presence of ovIL – 2 or the combination of ovIL-2 and hIL – 15. The latter combination of cytokines resulted in better proliferation (results not shown). Goat NKp46+ cells mostly reached maximum numbers around 14 days. The use of 96 well plates rather than 6 well plates in the initial phase of culturing goat cells, resulted in an increase in total cell count after selection (results not shown). Non-NK like cells also appeared in several cultures that were specifically selected for NKp46+ cells with a NKp46 antibody. Since previous results have shown an overlap between AKS6 (NKp46+) and a TCR1 recognizing antibody, γδT cells can also be expanded with IL-2, and prior experience with cell cultures describe the presence of these cells in culture (P.Boysen, personal communication), these cells might be γδT cells.

### *3.1.3 Distinction of goat NKp46+ cells from other cell types*

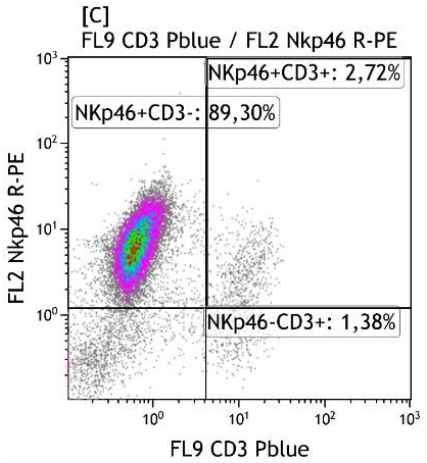
To investigate the overlap between NKp46+ and TCR1+ cells observed in previous studies, the CD8b, NKp46 and γδT cells markers (TCR1 and GD3.5) were tested together by immunophenotyping and flowcytometric analysis. However, the CD8b and NKp46 antibodies shared the same isotype (mouse IgG). Since indirect staining requires a combination of antibodies with different isotypes, the Lightning Link PECy7 Tandem Conjugation Kit was used to directly conjugate the CD8b antibody, to enable the combination of antibodies against NKp46 and CD8b in the same analysis. However, this option resulted in inefficient staining, leading us to lay this direct conjugation strategy aside and prioritize other aims in this study (results not shown).

NKp46+ cells grown in culture was phenotyped with NKp46, CD3 and TCR1 antibodies after 14 days, and tested in flowcytometry. The majority of the NKp46+ cell population was CD3- (97%), as illustrated in figure 2A. Even though the goat cells had been positively selected for NKp46+ cells, the cell culture contained cells that were not NKp46+, as illustrated in figure 2A. A minor proportion of the cells were CD3+ (4%), and some were double positive for NKp46 and CD3 (3%), as illustrated in figure 2A.

Previous results have shown an overlap in PBMC between NKp46+ and TCR1+ cells (Appendix 1). Thus, investigations were done to find out whether the NKp46+ cells in culture were in fact

TCR1+. Indeed, a proportion of the cells in culture were positive for the γδT cell marker TCR1 (5-18%), as illustrated in figure 2B. This could be indicative of γδT cells in the culture, or of an unspecific binding of the TCR1+ antibody. If these cells were positive for another γδT cell marker (GD3.5) as well, these cells were likely γδT cells.

A

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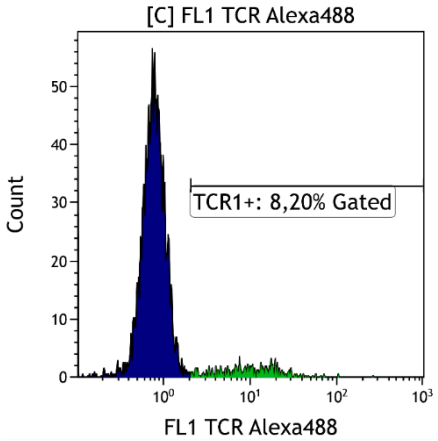



*Figure 2. Flowcytometric analysis of selected and cultured goat NKp46+ cells. Figure is representative of eight goats. ID2 is shown. (A) The NKp46+ cell culture contained cells that were CD3+. (B) The NKp46+ cell culture also consisted of a TCR1+ population.*

In NKp46+ cell cultures (figure 3A and 4A), the NKp46+CD3- population was negative for the GD3.5 marker and the TCR marker, as illustrated in figure 3B and 4B, respectively. This is consistent with the phenotype of NK cells as they are known in cows and sheep. The NKp46+CD3+ cells were GD3.5+ (3%), as illustrated in figure 3C, and TCR1+ (87%), as illustrated in figure 4C. If a higher amount of the NKp46+CD3+ cells had been GD3.5+, these cells would have been positive for two γδT cell markers. This would have been evidence enough to assume that the CD3+ cells in goat NKp46+ cell cultures were in fact γδT cells. However, since only a small proportion of the NKp46+CD3+ cells were GD3.5+, there is not enough evidence to claim that these cells are γδT cells.

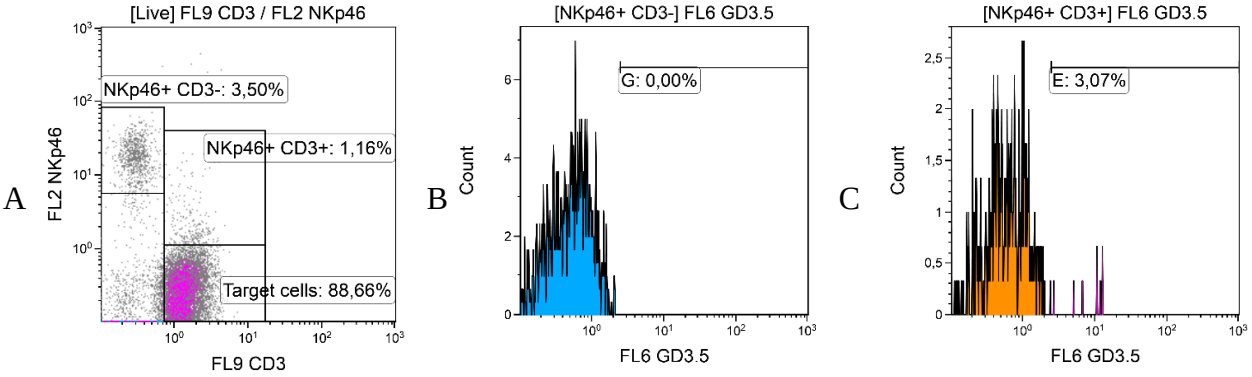



*Figure 3. Flowcytometric analysis of NKp46+ cells from cell cultures with target cells. Figure is representative of two goats. ID11 is shown. (A) The majority of the NKp46+ cells were CD3-. (B) The NKp46+CD3- population was GD3.5-. (C) A small percentage of the NKp46+CD3+ population was GD3.5+. Note that the results shown are from cultures where target cells were present as detailed in 2.4.3, but the target cells were omitted by flow cytometric gating in Kaluza as shown.*

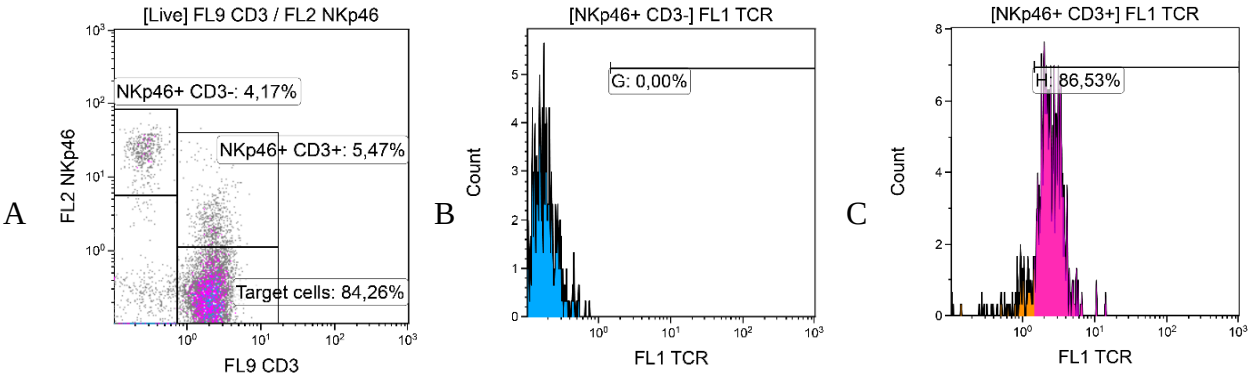



*Figure 4. Flowcytometric analysis of NKp46+ cells from cell cultures with target cells. Figure is representative of two goats. ID11 is shown. (A) The majority of the NKp46+ cells were CD3+. (B) The NKp46+CD3- population was TCR1-. (C) A high percentage of the NKp46+CD3+ population was TCR1+. Note that the results shown are from cultures where target cells were present as detailed in 2.4.3, but the target cells were omitted by flow cytometric gating in Kaluza as shown.*

### *3.1.4 Flowcytometric cell sorting*

To further investigate the NKp46+CD3- and NKp46+CD3+ cell populations without having to kill the cells by permeabilization, a physical separation of cells with flowcytometric cell sorting and the NKp46 and CD8 markers was attempted.

The flowcytometric cell sorting was attempted twice. After each cell sorting, the results were verified in our lab to investigate whether the cells were in accordance with cell markers that were sorted for. Then the cell populations were cultured. The different populations were then phenotyped after a certain number of days in culture, to investigate which markers that were present on these cells.

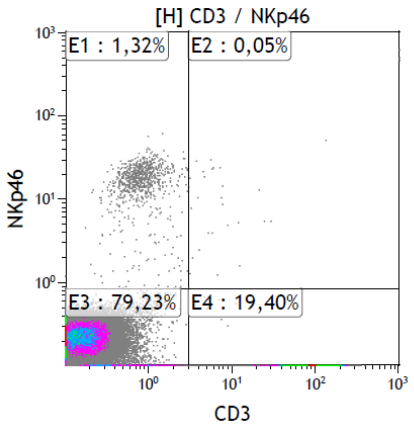
As noted above, results have shown that TCR1+ cells follow into the culture when positive selection with magnetic beads and the NKp46 antibody have been attempted, as illustrated in figure 2B and 4. The flowcytometric cell sorting would allow for a more precise selection, because of its ability to physically separate cells without magnetic beads with several markers. Also, since the number of cells after selection was low to begin with, a pre-enrichment of cells prior to cell sorting was recommended, since this would ensure the inclusion of all NKp46+ cells (our intended cell population), resulting in a better separation after cell sorting.

To achieve a pre-enriched cell population prior to the first cell sorting attempt, Nkp46+ cells were selected using a negative selection of cells with a MACS column that removed surrounding non NKp46+ cells. The NKp46+ enriched cell population was sorted into NKp46+CD8b+ and Nkp46+CD8b- populations. However, the separation between them was not convincing, as illustrated in figure 6A. The majority of the cells in culture were TCR1+ (results not shown), despite the negative selection with non – NK cell markers including TCR1 (table 3). This lead us to believe that the negative selection method did not work entirely as expected.

Because the negative selection method did not work entirely as expected, a positive selection method with modifications (The Dynabeads Flowcomp Flexi kit with a DSB-X conjugated antibody, 2.3.4) was attempted prior to the second cell sorting attempt. A positive selection with magnetic beads should give a more effective selection because of its specific attachment to the cells. However, since the magnetic beads are normally attached to the cells for a while, rendering them unavailable, and the cells were going straight to cell sorting, a different method was required. The Dynabeads Flowcomp Flexi kit presented us with a solution, since this method

yields positively selected bead-free cells that can be analyzed directly in the flowcytometric cell sorter. The Dynabeads Flowcomp Flexi kit required a DSB-X conjugated antibody, so the AKS6 antibody was conjugated with DSB-X. The DSB-X/AKS6 conjugated antibody was validated in both bovine and goat NKp46+ cells by selecting these cells from PBMC with this antibody prior to immunophenotyping and flowcytometric analysis. In bovine cells, the majority of the selected NKp46+ cells were CD3- (96%) and a very small percentage was CD3+ (4%), as illustrated in figure 5A. In goat cells, the majority of the NKp46+ cells were CD8b+ (80%) and a small percentage was CD8b- (20%), as illustrated in figure 5B. However, the majority of the total population was NKp46- in both bovine (99%) and goat NKp46+ cells (97%), as illustrated in figure 5A and 5B, even though the cells were positively selected for NKp46. This might suggest that the cells that presented as NKp46- at this stage were in fact NKp46+ cells that for unknown reasons had ceased to express surface NKp46.

A

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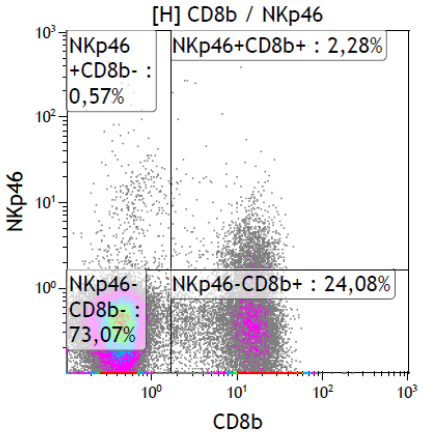
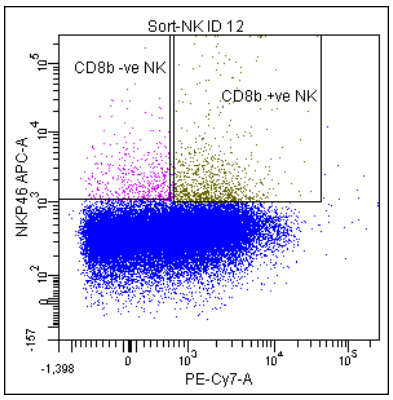



*Figure 5. Flowcytometric analysis of bovine and goat NKp46+ cells after positive selection with the DSB-X conjugated AKS6 antibody. (A) The majority of the bovine NKp46+ cells were NKp46+CD3- (E1). (B) The majority of the goat NKp46+ cells were NKp46+CD8b+.*

The positive selection of cells with the DSB-X conjugated antibody and Flowcomp Flexi kit did not increase the number of NKp46+ cells as much as expected. Despite of this, we tested whether this method had contributed to the exclusion of the majority of other non-NKp46+ cells by using this method before the second cell sorting attempt. During the second cell sorting attempt, cells were sorted into four subpopulations per individual: NKp46+CD8b-, NKp46-CD8b-, NKp46-CD8b+ and NKp46+CD8b+, as illustrated in figure 6B. The separation was improved from last

time, but the majority of cells were negative for both markers (NKp46-CD8b- cells), as illustrated in figure 6B, despite the positive selection for NKp46+ cells. The NKp46- sorted populations were immunophenotyped and analyzed in the flowcytometer, and a substantial proportion of the NKp46- cells were actually NKp46+ (results not shown).

A




B

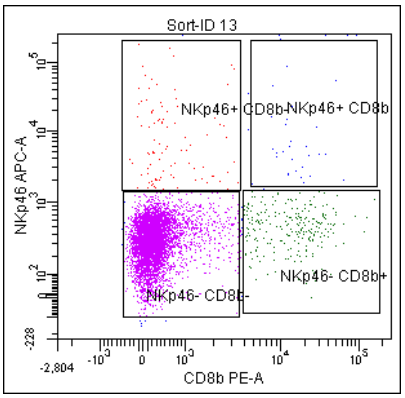



*Figure 6. Flowcytometric cell sorting of NKp46+ cells at Oslo University Hospital, Ullevaal. Both figures are representative of two goats. (A)ID12 is shown. The separation between Nkp46+CD8b- cells and NKp46+CD8b+ cells was not convincing. (B)ID13 is shown. The separation between NKp46+CD8-, NKp46-CD8-, NKp46-CD8+ and NKp46+CD8+ cell populations are shown. The majority of the total cell population was negative for both markers (NKp46-CD8b-).*

### *3.1.5 Functional assays*

The functional properties of the goat NKp46+ cells were tested (cytotoxic activity and cytokine production) by detection of CD107a and IFN-γ expression.

A prerequisite for the establishment of a CD107a assay in goat was a cross-reaction between the human CD107a antibody and goat cells. To investigate whether the antibody against human CD107a cross-reacted with goat cells, PBMC from goat was permeabilized and incubated with anti-CD107a among other antibodies. The results showed that human CD107a is present on all goat cells, as illustrated in figure 7. This is compatible with the expected expression of CD107a on the surface of cells only when they show cytotoxic activity. The cross-reaction between human CD107a and bovine PBMC was also tested, showing the same results as with goat PBMC (results not shown). The CD107a antibody was tested both in bovine and goat NKp46+ cells

stimulated in monoclonal antibody-coated wells, confirming that the CD107a antibody worked with both bovine and goat cells (results not shown).

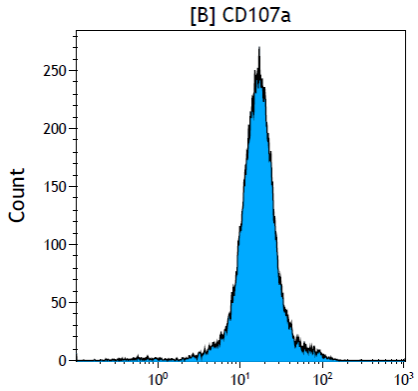
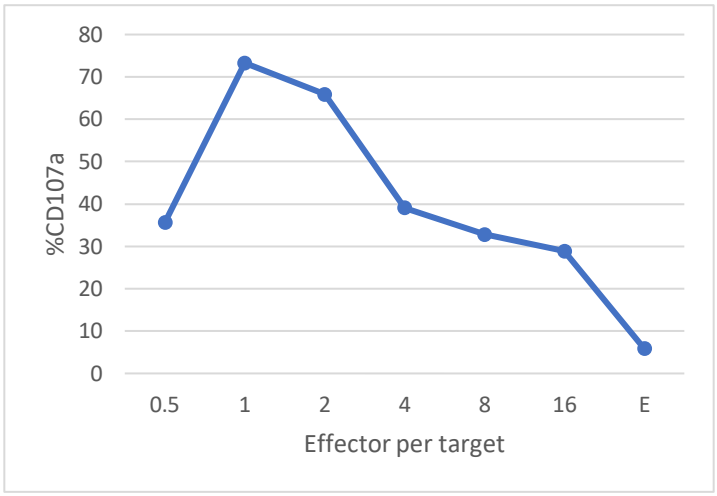
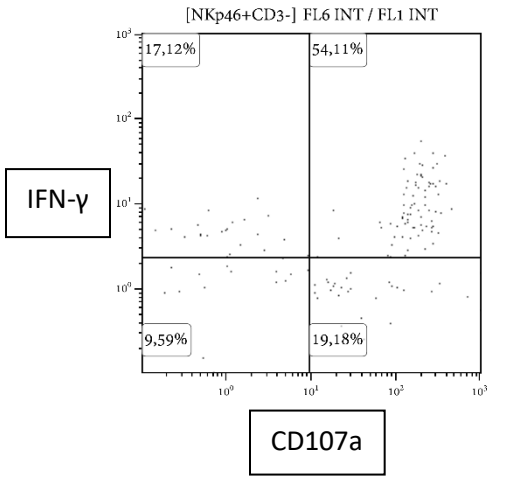



*Figure 7. Flowcytometric analysis of goat PBMC. Figure is representative of eight goats. ID1 is shown. Goat cells cross-reacted with the human CD107a marker following cell permeabilization.*

To investigate the cytotoxic capacity of goat NKp46+ cells in relation to target cells, target cells were mixed with cultured ovIL – 2 stimulated NKp46+ cells (effector cells) at different concentrations in an in vitro combined CD107a and IFN-γ assay. The effector cells killed both the goat synovial membrane cell line (GSM), which is a natural target for the goat effector cells (natural cytotoxicity), and the murine P815 cell line, which is killed through redirected lysis. The CD107a expression was detected by immunophenotyping and flowcytometric analysis, and the capacity for IFN-γ production was detected by intracellular staining and flowcytometry, as previously performed on bovine NK cell subsets (Boysen et al., 2006). The goat NKp46+ cells expressed CD107a, as illustrated in figure 8A and 9A, and they produced IFN-γ, as illustrated in figure 8B and 9B. The highest expression of CD107a was reached at an E:T ratio of 1:1 with both P815 and GSM target cells, as illustrated in figure 8A and 9A (plot to the right), and the highest production of IFN-γ was reached at an E:T ratio of 2:1 with both P815 and GSM target cells, as illustrated in figure 8B and 9B (plot to the right).

A

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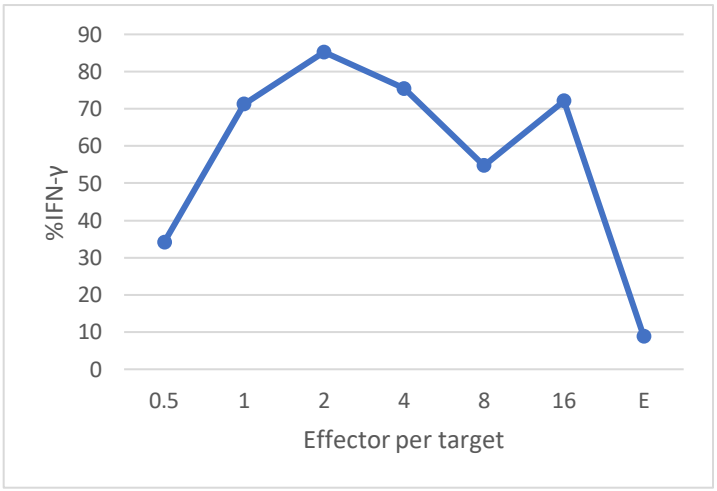


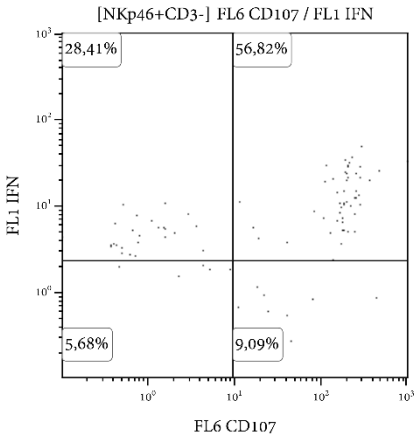
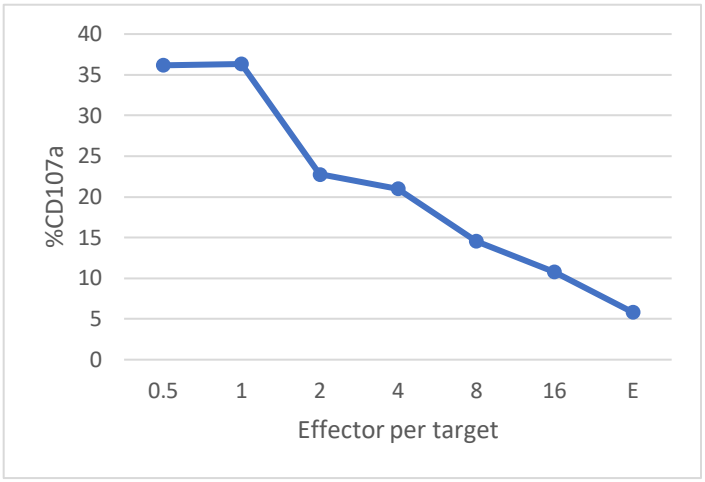
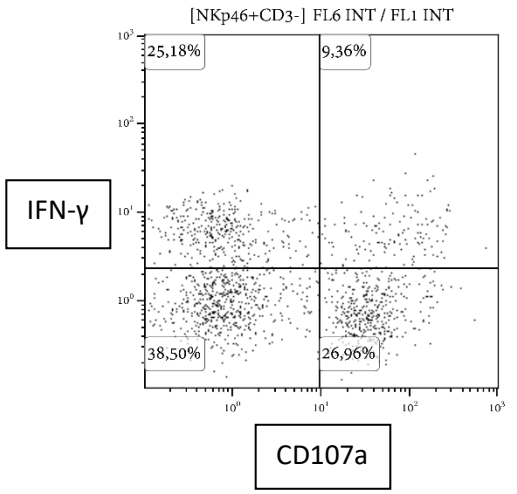




*Figure 8. Flowcytometric analysis of NKp46+ cells (effector cells) and mab stimulated P815 cells (target cells). Figure is representative of two goats. ID11 is shown. (A) To the left: %CD107a at different concentrations of effector to target cells. E represents only effector cells. To the right: The plot of the highest expression of CD107a at an E:T ratio of 1:1. (B) To the left: %IFNγ at different concentration of effector to target cells. To the right: The plot of the highest expression of IFNγ at an E:T ratio of 2:1.*

A

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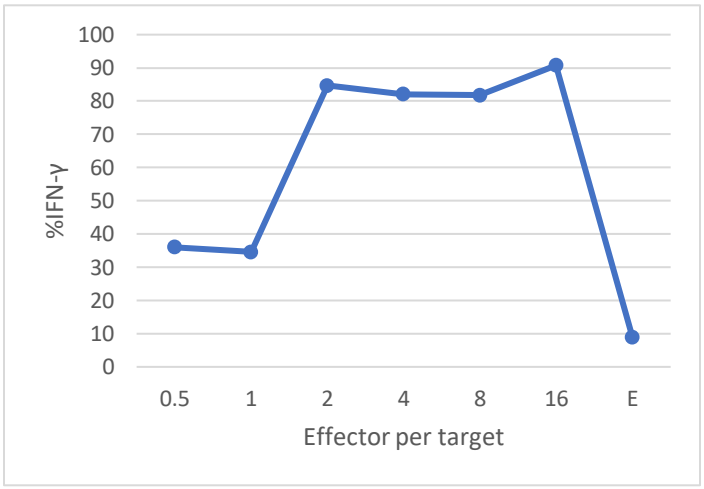


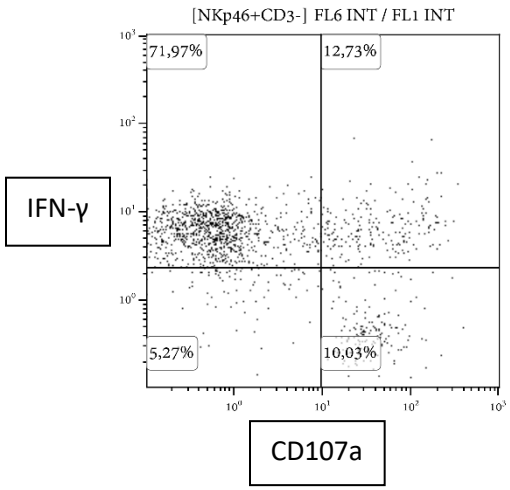


*Figure 9. Flowcytometric analysis of NKp46+ cells (effector cells) and mab stimulated GSM cells (target cells). Figure is representative of two goats. ID11 is shown. (A) To the left: %CD107a at different concentration of effector to target cells. E represents only effector cells. To the right: The plot of the highest expression of CD107a at an E:T ratio of 1:1. (B) To the left: %IFNγ at different concentration of effector to target cells. To the right: The plot of the expression of IFNγ at an E:T ratio of 2:1.*

### *3.1.5 The EC1.1 antibody for the positive selection of goat NKp46+ cells*

The low amount of NKp46+ cells after positive selection could be explained by a low antibody affinity of the AKS6 antibody. The selection ability of two NKp46 antibodies, AKS6 (anti-bovine) and EC1.1 (anti-ovine), was compared to investigate whether the EC1.1 antibody could have a higher antibody affinity than the AKS6 antibody. In a CD107a assay with target cells, both the AKS6 and the EC1.1 antibody were used for the positive selection of goat NKp46+ cells with magnetic beads. Cells selected with either of the antibodies were grown in different cultures. Cell cultures selected with the EC1.1 antibody had more visible cells, better growth and showed many proliferation isles compared to the cell cultures selected with the AKS6 antibody, which showed few cells and less signs of proliferation. This could indicate that the EC1.1 antibody has a better antibody affinity and that selection with this antibody could result in a higher number of cells after selection. Since the EC1.1 and the CD8b antibody share the same isotype (IgG1), a direct conjugation of CD8b or biotinylation of EC1.1 would make the combination of these markers in flowcytometric analysis possible. This was however not pursued due to the time constraints of the research track project.

# 4. Methodological considerations and general discussion

## *4.1 Methodological considerations*

Method establishment was a central part of the research track project, and thus the methodological considerations section is a central part of the discussion. Goat specific tools and reagents would have been optimal, but due to the time constraints of the research track project, the development of goat specific reagents and antibodies was not our main focus.

Blood sampling from live animals was quite efficient, even though the protocol enabled us to only work with a few individuals at a time. Blood sampling from newly euthanized goats at the slaughterhouse ensured larger amounts of blood, but there was a greater contamination risk with the use of a more unpredictable collection method, and the experience was overall less pleasant than working with live goats. Extraction of blood from live animals was more practical to conduct, and the results were better with EDTA tubes compared to glass bottles with citrate.

Since this was a descriptive study of NKp46+ cells in healthy goats, few individuals from different locations were used per assay. It would have been optimal to sample blood from a standardized group of goats, but sampling from a few individuals from different locations was satisfactory for this descriptive study.

Selecting a large amount of goat NKp46+ cells from PBMC proved to be challenging due to several factors: a low amount of NKp46+ cells after selection with the AKS6 NKp46 antibody, contamination of non-NKp46+ cells in culture, and the lack of goat specific tools, reagents, and antibodies. It is possible to perform the assays with a low number of cells. However, a higher total cell count would be optimal, and enable the use of more controls in our flowcytometric analysis to verify our results. The purpose of controls is to make it possible to distinguish results from background variation and non-specific effects, and there are many different controls that can be included in a flowcytometric assay (Maecker and Trotter, 2006). Due to the low number of cells after selection, only essential controls could be included, depending on the number of cells per assay. To improve cell output, the antibody and NKp46+ cells were left on a rotating device in a cooling room (at 4°C) rather than on ice, to ensure better interaction between the antibody and NKp46+ cells. To ensure optimal bead-cell interaction, the time the cells spend with the beads on was increased from 2 days (48 hours) to 3 days.

An NK cell culture consisting of mainly live cells and visible signs of proliferation (proliferation isles) would be ideal, as illustrated in figure 11. The low amount of NKp46+ cells after selection could affect the establishment of cell cultures by affecting the growth of cells in culture. This, because there would be less "cell to cell" interaction and exchange of paracrine signals, which are necessary for proliferation (Domenech et al., 2009). At first, the cultures consisted of a lot of dead cells when using 6 well plates and ovIL – 2 as the only cytokine. This could have been due to the size of the wells, which resulted in a lack of contact between live cells, and the combination of cytokines used in the NK cell medium. A study on different cell culture devices have suggested that macro cell culture devices, such as open wells, could minimize the concentration of important paracrine signaling components in the cell cultures, thus affecting cell growth. This because the volume among other factors lead to dilution and reduced cell intimacy needed for optimal cell cultures, as described above. Measures were taken to increase "cell to cell" contact and the exchange of paracrine signals, by using 96 well plates instead of 6 well plates. This would reduce the volume in the cell culture device and possibly increase the interaction between paracrine signals and cells. The cells were then transferred to a 12 and 6 well plate later when the number of cells increased. hIL – 15 was also added to the NK medium to see whether the combination of IL-2 and IL-15 cytokines would increase cell culture conditions, which it did. Since IL-2 and IL-15 seem to exert different effects on the NK cells (Pillet et al., 2011), it only seems natural to assume that the combination of these cytokines would be better for NK cell culture conditions than only one or the other. The proliferation of cells in culture was documented by subjective observation of proliferation isles in the culture. The NK cell proliferation ability could also have been checked with a carboxyfluorescein succinimidylester (CFSE) assay (Quah and Parish, 2010), instead of subjectively evaluating the visible proliferation isles in culture.

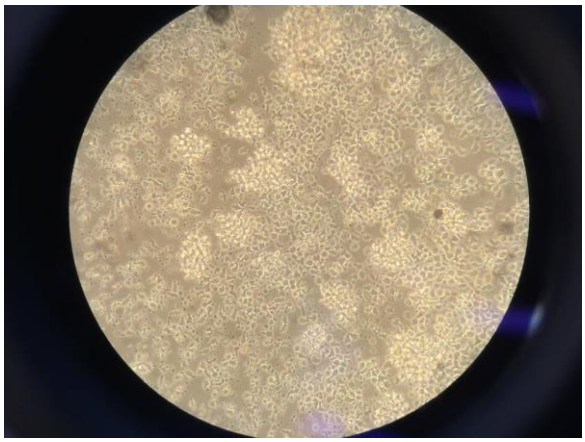

*Figure 11. Microscopic image of NKp46+ cells in culture. An NKp46+ cell culture showing mainly live cells and visible signs of proliferation (proliferation isles). The active NKp46+ cell has a characteristic "slipper" shaped morphology, and the light clusters of cells form characteristic proliferation isles.*

A lot of the method establishment throughout the research track project focused on the selection of NKp46+ cells from PBMC, which was an essential method to establish to enable the establishment of homogenous NK cell cultures, and the performance of later assays. Cells were selected with the NKp46+ antibody, which resulted in a low amount of cell after selection. This could mean that the antibody affinity to antigen was insufficient. The anti-ovine EC1.1 antibody seemed to have a higher antibody affinity than the anti-bovine AKS6 antibody, which might be because the goat is phylogenetically closer to the sheep than the cow (Chen et al., 2019). A study on the isolation of T cells suggested that the low affinity between MHC and the T cell receptor complicated the capture of cells by MHC-coated beads (Luxembourg et al., 1998). Antibodies usually bind a small part of an antigen, and this enables a cross-reaction with epitopes on other antigens. However, the cross-reaction binding usually has a lower affinity (Lipman et al., 2005). This might indicate that antibodies based on cross-reactivity between species bind with less affinity, which might result in a less efficient selection with magnetic beads. A high antibody antigen affinity seems to be a prerequisite for the selection of cells with magnetic beads. Another prerequisite for magnetic selection is that the cell population can be identified with only one cell marker. However, if the cell population is identified by several markers, the flowcytometric cell sorting might be a better solution, due to its ability to sort several markers at the same time.

Another possibility is that the NKp46 marker is not sufficient to select the entire population of goat NK cells, and that this marker only selects a part of the goat NK cell population. Ideally, several cell population phenotypes should be tested before concluding which cells can be referred to as NK cells based on their phenotype and function. Since NK cells in other species, such as humans, are not selected by the NKp46 marker, and this marker doesn't define all NK cells in pigs (Mair et al., 2012), the NKp46 marker might not define all NK cells in goats or be suitable to define goat NK cells at all. However, this speculation was outside of the scope of the study, as the study assumed that the NKp46 marker was sufficient to define goat NK cells, based on results in phylogenetically related species such as cows and sheep. Since goats are phylogenetically closer to other ruminants such as cows and sheep, it is likely that the NK cell phenotype is more similar to other ruminants than pigs, humans and mice (Chen et al., 2019). In addition, the search for another likely cell marker would have been too extensive for the research track project as well, and these were the antibodies that were available for this project. Also, previous results (Appendix 1) have showed a selection of these cells with the NKp46 antibody. If sorting of cells into distinct populations had resulted in a clearer separation of cell populations, PCR could have been used to investigate whether the NKp46- cell population expressed typical NK cell genes. This would have uncovered whether the NKp46+ marker actually defines all NK cells in goats or not.

The flowcytometric cell sorting assay required transportation of cells to the cell sorter at the Oslo University Hospital, Ullevaal. This transportation could have increased the risk of cell abnormalities and cell death, which could have been avoided if the machine was in our lab. The negative selection with the LD MACS column during the first cell sorting attempt did not work as well as expected. The cells were not kept cool during the MACS column flowthrough, which is lengthy and could have impacted the cells by exposing them to room temperature. A solution would be to execute the column flowthrough in a cooling room. Even though this method removed many of the surrounding non-NKp46+ cells, a lot of NKp46- cells remained. This could be explained by the antibodies ability to select the non-NK cell populations (table 3), the antibody antigen affinity (proposed also as an explanation for the selection with the AKS6 antibody versus EC1.1 antibody), or that there was an occurrence in the eluation process in the columns, which might have led to cells following into the eluate. After the second cell sorting attempt, the majority of cells were NKp46-, and some of the NKp46- cell populations were

found to be NKp46+. This could indicate that some of the cells that were NKp46- after the pre-enrichment might in fact be NKp46+ cells with only a temporarily absent NKp46 expression. This could be explained by competing or secondary antibodies blocking the NKp46 antibodies (epitope competition), resulting in a blocked NKp46 molecule, or an internalization of the NKp46 receptor. Both explanations would result in false negatives.

Several selection methods were attempted prior to flowcytometric cell sorting. A positive selection of cells would be the best option, because it specifically selects the NKp46+ cells. However, a prerequisite for this method is that the NKp46 selecting antibody has a good enough antibody antigen affinity. If the antibody does not work as expected, it would be better with a negative selection, ending up with an NKp46+ enriched population of cells, which would also ensure a sufficient number of cells for flowcytometric cell sorting. The disadvantage with the positive selection with magnetic beads is that the cells are activated by stimulation of the NKp46 activating receptor, and the receptors can thus be internalized, resulting in false negatives. Still, a positive selection of cells before sorting might result in a higher amount of NKp46+ cells because of its specific selection for the NKp46 marker. Also, it could have been possible to first perform a positive selection to specifically select for NKp46 followed by a negative selection removing surrounding cells, but this would be very time consuming and impractical. A time-consuming assay could also increase the risk of cell abnormalities and death. An efficient NKp46 antibody is a prerequisite for this double selection method, and with an efficient NKp46 antibody, the positive selection with magnetic beads might be sufficient. One individual was excluded after cell sorting because of an aggregation of cells, potentially caused by an interaction between buffer and NK cell medium (Hans Christian Aas, personal communication). The cells from another individual were washed two times before adding buffer, but the separation was still poor, which might indicate that the buffer/NK cell medium was not the main cause of the aggregation of cells. Cell preparation and pre/post conditions should be considered, as there are some practical aspects to consider when using high speed cell sorting (Arnold and Lannigan, 2010). The flowcytometric cell sorting method was new to us, and within the research track project there was limited time to fully optimize this protocol, which was not the main focus of this project either.

Few cells were available when performing the CD107a assay with target cells. Due to the shortage of cells, fewer controls could be included. A higher number of cells would have enabled us to include more controls and better isotype controls, in addition to more E:T ratios for an even more nuanced reflection of cytotoxic capacity. Both the GSM and P815 cells worked as target cells. However, the P815 cells were easier to grow in culture, due to the high proliferation rate as well as the non-adherent properties demanding less maintenance. In addition to the CD107a assays on coated plates and with target cells, other cytotoxic assays could also have been attempted. For instance the 51Cr target release assay, which has been used to measure the cytotoxic activity of bovine NK cells (Boysen et al., 2006). It would also have been interesting to investigate the difference between cell lines in a 51Cr release assay and a CD107a assay, to see if there is a difference in cytotoxic capacity and IFN-γ production of the NKp46+ cells after exposure to different target cell lines.

### *4.2 General discussion*

Our results were mostly in accordance with our predictions (hypotheses). However, due to the time constraints of the research track project, there was not enough time to pursue all our hypotheses or investigate all the questions that were raised throughout method establishment.

Even though the selection method was modified from the original method, there was still a low amount of NKp46+ cells present after selection. This was not entirely in accordance with our predictions. The low amount of NKp46+ cells after selection made it challenging to grow enough cells in culture, and the cells in culture were not a homogenous population either. Even with the changes in cytokine combinations in the NK cell medium, and in the use of different wells, there were few cell cultures consisting of mainly live cells and visible signs of proliferation (proliferation isles). The negative selection method was designed to remove surroundings cells, but the cell population remained heterogenous. Towards the end of the research track project, a potential reason for this outcome was discovered when investigating the NKp46 antibody affinity. The anti-ovine EC1.1 antibody showed promising potential when used in magnetic beads selection compared to the anti-bovine AKS6 antibody.

The human CD107a antibody cross-reacted with goat cells and could be used to establish a CD107a assay that was used to measure cytotoxic activity. This was in accordance with our predictions. The goat NKp46+CD3- cells showed NK typical functional properties like cytotoxic

activity and cytokine production, which was also in accordance with our predictions. The cultured goat NKp46+CD3- cells showed cytotoxic activity against P815 cells in the presence of an anti-NKp46 antibody, coinciding with previous results using the Cr51 release assay (Appendix 1). Similarly, they were cytotoxic against a species-specific cell line (GSM). These cultured cells also produced IFN-γ.

The phenotypical and functional properties of goat NKp46+CD3- cells were mostly consistent with NK cell phenotypical and functional properties in cattle and sheep as expected. Goat cells were positive for the AKS6 anti-bovine NKp46 antibody, as previously documented in Appendix 1. The combination of markers in the goat NKp46+CD3- cell population was similar to the bovine NK cell phenotype, and the percentage of goat NKp46+ cells in goat PBMC (0,5-2%) was overlapping with the range of NKp46+ cells in bovine PBMC (1-10%). A third of the NKp46+ cells were CD2+ in goat cells, which is a bit less than in bovines. A high percentage of the goat NKp46+ cells were CD8b+ (80%), which is a bit higher than the bovine CD8b expression which was variable. The bovine NK cells were negative for the CD3, CD4, TCR1, B cell markers, which was similar for the goat NKp46+CD3- cell population. Bovine NK cells have been characterized as large granular lymphocytes. These cells grew in culture after IL-2 stimulation, were cytotoxic against a bovine kidney cell line and the P815 cell line (Storset et al., 2004), and produced IFN-γ after stimulation with IL-2 and IL-12 (Lund et al., 2013), and in response to mycobacterial proteins (Olsen et al., 2005). Like the bovine NK cells, goat NKp46+ cells grew in culture after stimulation with IL-2, were cytotoxic against a species-specific cell line (GSM) and the P815 cell line, and produced IFN-γ after stimulation with IL-15 and IL-12. Previous results showed that goat cells were positive for the anti-ovine EC1.1 NKp46 antibody (Appendix 1). Similar to ovine NK cells, goat NKp46+ cells in PBMC have been showed to be CD14- (Appendix 1). Also like ovine NK cells, goat NKp46+ cells were cytotoxic against a species-specific cell line (GSM) and the P815 cell line, produced IFN-γ after stimulation with cytokines (Elhmouzi-Younes et al., 2010), and were positive for the NKp46 marker (Connelley et al., 2011).

The flowcytometric cell sorting method did not really enable us to establish or investigate the NKp46+CD3- or NKp46+CD3+ populations as intended. The CD8b marker seemed to work, but the cell populations were mostly NKp46- after both cell sorting attempts. Several pre-enrichment

strategies were tested, but without succeeding in obtaining homogenous NKp46+CD3- cultures. The Nkp46 antibody affinity could explain the vague separation of cell populations. Perhaps a selection with the anti-ovine EC1.1 antibody would result in a clearer separation.

In culture, the goat NKp46+ cells mostly reached maximum numbers around fourteen days, which coincided with previous results (Appendix 1). Cell cultures with goat NKp46+ cells were often contaminated by non-NK cells, mostly found to be CD3+. In assays with target cells, the NKp46+CD3- population expressed more CD107a than the NKp46+CD3+ population, and the NKp46+CD3- cells produced more IFN-γ than the NKp46+CD3+ population. This is consistent with these cells being NK cells. NK cells are known to produce IFN- γ, but γδT cells can also produce IFN-γ (McGill et al., 2014). This explains the IFN-γ production by the NKp46+CD3+ cells that could be γδT cells. The NKp46+CD3- cells were negative for both γδT cell markers as expected. The majority of the NKp46+CD3+ cells were positive for the TCR1 marker, but only a small proportion of cells were positive for the GD3.5 γδT cell marker (Jones et al., 2007). This was not entirely in accordance with our predictions, as the NKp46+CD3+ cells were expected to be positive for both γδT cell markers. This could indicate that these cells were γδT cells, but because they were only convincingly positive for one marker, there was not enough evidence to conclude that these cells were γδT cells. Despite using a negative selection with the TCR1 marker, TCR1+ cells were still present in the cultures. This could be due to an inefficient selection capacity, a particular capacity for the expansion of rare TCR1+ cells, or an upregulation of TCR1 in initially negative cells. This could be verified by detecting the gene of the delta chain of these cells by PCR, which would confirm that these cells were in fact γδT cells. Since the flowcytometric cell sorting did not work as expected, and also due to the time constraints of the research track project, PCR to identify these cell populations was not attempted.

At the beginning of the research track program, no studies about goat peripheral NK cells existed as to our knowledge. However, recently, a study was published about the regulation of peripheral NK cells by a tumor necrosis factor like weak inducer of apoptosis (TWEAK) during a Peste des petits ruminants virus (PPRV) infection (Qi et al., 2020). The NK cells in this study were selected by positive immunomagnetic selection with the CD16 and CD14 markers, as described in the study on ovine NK cells (Elhmouzi-Younes et al., 2010). They later labeled with different surface receptors against different molecules, including NKp46 and CD3. They did not specify

which clone of the NKp46 antibody they used. According to the information they provided, it seemed to be an NCR1 polyclonal antibody (IgG) with a species reactivity against humans. They also used a monoclonal CD3 antibody (IgG1) with a species reactivity against humans, which is the same CD3 antibody that was used in this project. The study showed that over 96% of the CD16+CD14- population, which defines ovine NK cells, was NKp46+CD3-. This coincides with previous results that have shown that goat NKp46+ cells in PBMC were CD14- (Appendix 1). This is also in accordance with the results attained throughout the research track project, which have shown that the NKp46+CD3- cells display phenotypical and functional characteristics consistent with NK cell properties.

Future research should focus on positively selecting NKp46+CD3- cells with the anti-ovine EC1.1 antibody, due to the vibrant cell cultures observed after selection with this antibody. This method should then be optimized until it results in a consistent amount of NK cells after selection. Another possibility is to try the approach described in the (Qi et al., 2020) article; first select CD16+CD14- cells and then label with surface receptors against NKp46 and CD3, or alternatively CD8b. After the selection method has been optimized, the method can be tested in more goats to enable more comparisons, both in relation to age and other parameters. If enough cells can be gained after selection, the flowcytometric cell sorting can be attempted again. Goat specific reagents and antibodies should be developed if cross-reaction with other species cannot be obtained. The development of a surface CD3 antibody, preferable anti-goat surface CD3, would make it possible to separate NKp46+CD3- cells from other cells without having to kill the cells in the process. If the positive selection with the ovine EC1.1 antibody proves to be as effective as anticipated, the cell sorting could be attempted again, followed by an indirect detection of NK cell associated markers and expression with PCR and RNA isolation. In addition to the CD107a assay, the Cr51 target release assay could also be attempted to further investigate the goat NKp46+CD3- cell cytotoxic capacity. A clearer definition of γδT cells would also be beneficial in terms of excluding them from the NKp46+ cell cultures. The NKp46+CD3+ population might be γδT cells. Goat γδT cells could express NKp46 as seen in bovine γδT cells (Johnson et al., 2008), or NKp46+ cells might express γδT cell markers. After flowcytometric cell sorting and PCR, this could be investigated further.

In conclusion, the selection method has been optimized and selection with the EC1.1 antibody seemed to result in more vibrant cell cultures after selection compared to the AKS6 antibody. A CD107a assay has been established in goats. Goat NKp46+CD3- cells showed typical NK cell like functional properties like cytotoxic activity and IFN-γ production. The NKp46+CD3+ cell population might be γδT cells, but more evidence is needed to confirm this. Refined strategies to achieve homogenous NK cell cultures have been suggested. Due to the time constraints of the research track project, PCR to confirm genes specific for certain cell types was not attempted.

**Ethical considerations**

The project was conducted in accordance with the R's (replacement, reduction, refinement). The use of animals was avoided and replaced by selecting cells from peripheral blood rather than from tissues which would have required euthanasia. The number of animals used per experiment was reduced by methods such as microsampling (small volumes) of blood from the same individuals (with time in between sampling) and freezing of excess material for later use. The refinement point underlines the importance of minimizing animal suffering and improving animal welfare, and we were conscious of this when handling the animals, for instance by reducing stress during blood sampling by gentle handling of the animals, limiting the amount of time spent on each individual and by reducing stressors in the environment throughout blood sampling.

**Statistics**

This was a descriptive study of the characteristics of goat NKp46+ cells, and only a few individuals were included in the different assays. Due to the low number of individuals in our test group/groups, the data could not be used to state anything about the general population of goats. Thus, statistical analysis was not conducted, as this would have required a higher number of individuals in our test group/groups to conduct.

## 5. Conclusion

In summary, goat NKp46+CD3- cells displayed typical phenotypical and functional characteristics of NK cells, in accordance with the criteria used to define NK cells in both cattle and sheep. These results contribute to the further characterization of ruminant NK cell biology. The relevance of this knowledge is extensive. If we know how to define these cells and how they work, we can also better understand how these cells work in vivo, and this knowledge will give valuable insight into host- pathogen interactions and immunoregulation in the healthy goat.

## Conflict of interest

The authors declare no competing interests.

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**Appendix 1 – An overview of previous unpublished results**

*Previous unpublished data*

The research track project builds on preliminary and unpublished data generated by the immunology unit at the Faculty of Veterinary Medicine at the Norwegian University of Life Sciences. Essential previous data are mentioned for the purpose of context, mostly omitting figures, as they were only observed in a few animals.

*Phenotypical investigations of caprine NK cells using bovine and ovine NKp46+ antibodies*

PBMC isolated from goats were tested in flow cytometry and were positive for the following anti-bovine NKp46 antibodies; AKS6, AKS4, AKS2 and anti-ovine antibody EC1.1, but negative for anti-bovine AKS1, AKS7 and AKS8. All AKS clones have been produced in the immunology lab. Unless otherwise stated, subsequent stainings were performed using AKS6. The amount of AKS6+ cells was found to be lower in young goats (1% of PBMCs in <1 year old goats) than in older (up to 3,5% in >3-year-old goats). Mostly, female goats were tested due to availability of animal material. To investigate if AKS6+ cells were overlapping with known non-NK cells in the PBMCs, cells were co-stained with either CD4 (T-cell subset), CD21 (B-cells), CD14 (monocytes) and WC1, TCR1–N6 and TCR1–N7 (γδT-cell subsets), and all were found to be negative. However, a TCRδ recognizing antibody (GB21A) showed marked overlap with AKS6+, as in young goats up to 20% of AKS6+ cells were TCRδ+, and in older goats the number was up to 40%. Furthermore, markers known to mark subsets of cattle NK cells were tested, CD16 (KD1) and CD8 (SBUT-8) being predominantly positive, CD2 variable (41-97 percent), while the activation marker CD25 was negative, as expected in the absence of stimulation (Boysen and Storset, 2009). Hence, with the exception of the puzzling GB21A/AKS6 overlap especially in older goats, these results were in agreement with data from cattle as well as from sheep where AKS6+ identifies NK cells.

*Cell selection, cell culture, and functional assays*

NKp46+ cells were isolated using positive magnetic selection and showed expansion in cell culture in the presence of ovine recombinant IL-2, typically reaching maximum numbers at around 9-16 days. Younger goats tended to proliferate more vigorously than older. In older goats, a proportion of cells stained positively for TCRδ (up to 15%). The capacity for NK cell IFN-γ production was measured by intracellular staining and flow cytometry in the presence of a protein release inhibitor (Brefeldin). PBMCs stimulated overnight with either human(h)IL-15 and hIL-12, or with ovIL-2 and hIL-12, responded by IFN-γ production, and around 30% of NKp46+ cells were IFN-γ+ in a young goat, whereas in an older goat this number was lower (8-12%). Cytotoxic capacity was tested in ovIL-2 stimulated cell cultures, as stated above, using Cr51 release assay against the target cell line P815. This FcR-bearing target cell line invokes NK cell cytotoxicity by adding a mouse monoclonal antibody against an activating receptor on the NK cell (redirected lysis effect). We found efficient killing with the use of anti-NKp46 antibody (an activating NK cell receptor) but not when adding antibodies against CD8 (a non-activating NK cell receptor) or γδT-cell receptor TCR1 (expected not to be present on NK cells).

*Distinction of NK cells from T cells*

The overlap of a γδT-cell marker and NKp46 in fresh as well as cultured NK-like cells, together with the reduced capacity for proliferation, cytokine production and cytotoxicity, lead us to hypothesize that a proportion of NKp46+ cells in older goats were not true NK cells. No antibody recognizing surface CD3 (present on all T-cells in most mammals) was available for goats, but an antibody reacting to an intracellular domain of human CD3 had previously been shown to cross react with several species (Jones et al., 1993). Following permeabilization of PBMCs, a proportion of NKp46+ cells were indeed positive for cytoplasmatic (cyt)CD3; up to 20% and up to 65% of NKp46+ cells were cytCD3+ in young and older goats, respectively.

The NKp46+CD3- and NKp46+CD3+ populations were crosschecked with antibodies against CD8a and CD8b markers, and the results showed that virtually all NKp46+CD3- cells were CD8b-, while all NKp46+CD3+ cells were CD8b+, as illustrated in figure 1. In contrast, both populations were positive for CD8a, although the NKp46+CD3- population stained less brightly with the CD8a marker. Hence, CD8b seemed to be a useful substitute surface marker to distinguish between CD3+ and CD3- cells within the NKp46 population, which would enable

further functional assays without having to permeabilize the cells like with the (cyt)CD3 antibody.

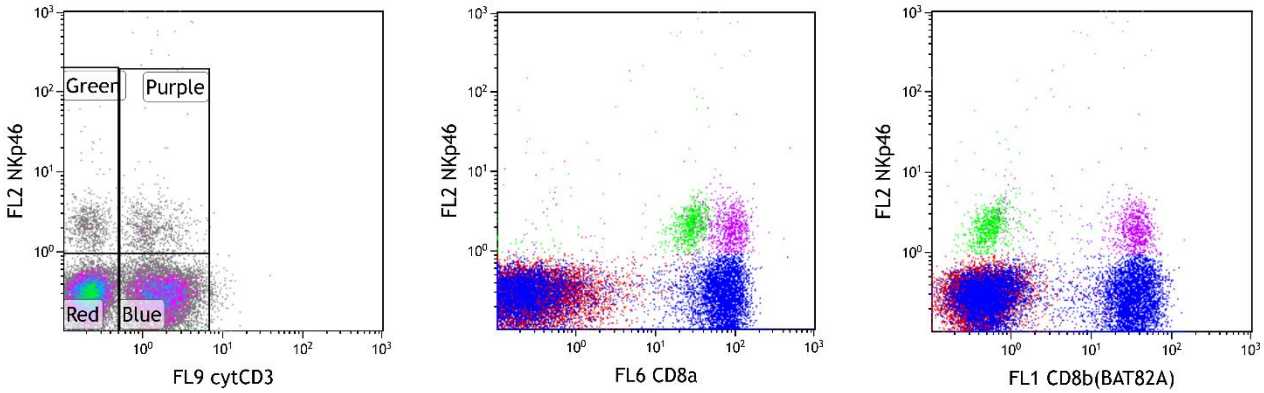


*Figure 1: Flow cytometric staining of NKp46 and cytoplasmatic (cyt)CD3 in PBMCs from one representative goat out of 8 tested. The two right hand panels were gated according to colouring indicated in the left panel.*

The above findings called for further investigation and founded the basis for the research track project.


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