CHARACTERIZATION OF NK CELLS IN THE DOG WITH A FOCUS ON THE ACTIVATING RECEPTOR NCR1

Philosophiae Doctor (PhD) Thesis

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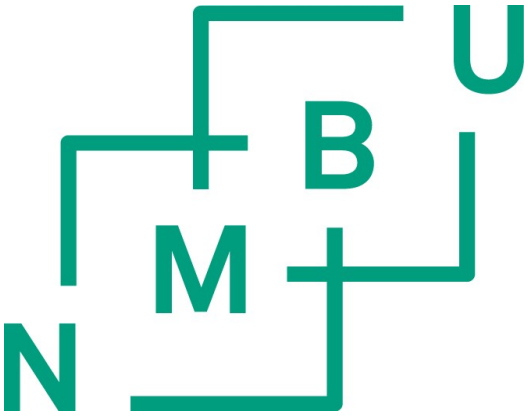

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Christine Grøndahl-Rosado

ABBREVIATIONS

ADCC	antibody dependent cell mediated cytotoxicity
αGalCer	α-galactocylceramide
BCR	B cell receptor
CBC	complete blood count
CLPD-NK	chronic lymphoproliferative disorders of natural killer cells
CTAC	canine thyroid adenocarcinoma cell line
CTL	cytotoxic T cell
CD	cluster of differentiation
DC	dendritic cell
EBV	Epstein Barr Virus
HLA	human leukocyte antigen
IFN	interferon
IL	interleukin
ILC	innate lymphoid cell
iNKT	invariant natural killer T
ITAM	immunotyrosine activation motif
ITIM	immunotyrosine inhibitory motif
KIR	killer immunoglobulin like receptor
LAK	lymphokine-activated killer
LGL	large granular lymphocyte
mAb	monoclonal antibody
MHC	major histocompatibility complex
NCR	natural cytotoxicity receptor
NK	natural killer
NKT	natural killer T
PARR	PCR for Antigen Receptor Rearrangement
PBL	peripheral blood lymphocytes
PBMC	peripheral blood mononuclear cell
PCR	polymerase chain reaction
S1P	sphingosine 1-phosphate
TCR	T cell receptor
TLR	toll like receptor
TNF	tumour necrosis factor
Treg	T regulatory cell
qPCR	quantitative polymerase chain reaction

LIST OF PAPERS

Paper I

NCR1^{+} cells in dogs show phenotypic characteristics of natural killer cells

Grøndahl-Rosado C, Bønsdorff TB, Brun-Hansen HC, Storset AK.

Vet Res Commun. 2015 Mar;39(1):19-30.

Paper II

NCR1 is an activating receptor expressed on a subgroup of canine NK cells

Grøndahl-Rosado C, Boysen P, Johansen GM, Brun-Hansen HC, Storset AK.

Submitted to Veterinary Immunology and Immunopathology

Paper III

Lymphoproliferative Large Granular Lymphocyte (LGL) disorders in three dogs

Grøndahl-Rosado C, Johansen GM, Moe L, Storset AK, Brun-Hansen HC.

Manuscript

SUMMARY

NK (natural killer) cells are part of the innate immune system and are important for protection against infectious diseases and destruction of potential malignant cells that can develop into cancer. NK cells represent a link between the innate and adaptive immune system. As the name implies, NK cells are cells with the ability to kill other cells but they are also important producers of cytokines that modulates the immune response. NK cell features show variation between species and are therefore difficult to generalize. NK cells are precisely defined in species like humans, rodents, cattle, sheep and swine, but canine NK cells are poorly characterized.

NCR1 is a receptor consistently expressed by NK cells in all species examined except for the pig where $NCR1^-$ NK cells are present. NCR1 is an activating receptor and binding of this receptor alone can mediate killing of target cells.

The immune system is controlled in a highly refined way. Dysregulation can result in a number of different diseases, for example haematopoietic cancers. This is a heterogeneous group of conditions affecting all ages and is a growing cancer type in the western world. Cancer originating from mature NK cells is called large granular lymphocyte (LGL) leukaemia/lymphoma because of their morphological appearance. This term also encompasses certain cancers originating from T cells.

The goal of this study was to phenotypically and functionally characterize canine NK cells by investigating NCR1 as a potential marker. Furthermore, LGL disorders in the dog was going to be studied.

In paper I validation of a cross reactive antibody binding to the canine NCR1 was presented. A $CD3^+$ and a $CD3^-$ $NCR1^+$ cell population was demonstrated indicating that NCR1 was not expressed exclusively by NK cells. $CD3^-NCR1^+$ cells were presumed to represent canine NK cells but the cell population identified was much smaller than expected compared to other species. $CD3^-NCR1^+$ cells further showed a $CD8^-CD56^-GranzymeB^+$ phenotype. $CD3^-NCR1^+$ cells were detected in blood, liver, spleen and lymph nodes. A dog with chronic T LGL leukaemia expressing NCR1 on neoplastic cells was also presented.

In paper II the definition of NK cells was expanded to encompass $CD3^-GranzymeB^+$ cells. It was shown that dogs both have $NCR1^+$ and $NCR1^-$ cells within the $CD3^-GranzymeB^+$ cell population. A relative increase was observed in $CD3^-GranzymeB^+NCR1^-$ cells after routine

vaccination. Further, cell cultures dominated by $CD3^{-}GranzymeB^{+}$ cells were established of which a fraction expressed NCR1. After cytokine stimulation NCR1 was upregulated on $NCR1^{-}$ cells. NCR1 was demonstrated as an activating receptor and defined functionally different NK cell subset as only $NCR1^{+}$ cells produced IFNγ. Consequently, NCR1 can be regarded as a marker of NK cell activation in the dog.

In paper III 3 dogs with an abnormal level of LGLs detected in the circulation were presented. NCR1 expression was not observed in these cells. Two of the dogs were diagnosed with T LGL leukaemia and one dog was diagnosed with a reactive lymphocytosis and a carcinoma.

To conclude, canine NK cells can be divided into two subsets according to their NCR1 expression. As NCR1 was also identified on $CD3^{+}$ cells, NCR1 is not exclusively expressed by NK cells. NCR1 was detected on malignantly transformed cells of T cell origin. NCR1 is an activating receptor upregulated during cytokine stimulation and can therefore be considered a marker for activation on NK cells. NCR1 defines functionally different NK cells as only $NCR1^{+}$ cells produce IFNγ. Until a specific marker found on all NK cells in dogs is identified, canine NK cells should be referred to as $CD3^{-}GranzymeB^{+}$ cells.

SAMMENDRAG (SUMMARY IN NORWEGIAN)

NK celler (naturlige dreperceller) er en del av det medfødte immunforsvaret og er viktig i bekjempelsen av infeksjoner og i eliminering av potensielt maligne celler som kan gi opphav til kreftsykdom. Denne celletypen representerer en kobling mellom det medfødte og det ervervede immunforsvar. Som navnet tilsier er celledrap en viktig rolle for NK celler, men de er også viktige produsenter av cytokiner – signalstoffer som modulerer immunresponsen. NK cellens egenskaper viser artsvariasjoner og derfor er det vanskelig å trekke generelle konklusjoner som gjelder for alle pattedyr. NK celler er grundig karakterisert bla hos menneske, gnagere, storfe, sau og gris, men hos hund vet vi lite om denne celletypen.

NCR1 er en reseptor på NK cellens overflate som har vist seg å uttrykkes på alle NK celler hos alle arter undersøkt, med unntak av gris. Hos gris finnes det en undergruppe av NK celler som er $NCR1^-$. Dette er en aktiverende reseptor og binding av denne reseptoren alene kan resultere i drap av målceller.

Selv om immunforsvaret er nøye kontrollert, kan sykdom oppstå som følge av feilregulering. En slik feilregulering kan gi opphav til kreft i blod og lymfoide organer. Dette er en heterogen gruppe kreftsykdommer som kan ramme alle aldersgrupper. Totalt utgjør disse ca. 5 % av nye kreftdiagnoser hos menneske i Norge og er en stadig økende krefttype. Kreft utgått fra modne NK celler kalles «large granular lymphocyte (LGL)» leukemi/lymfom fordi NK celler er store og har granula i cytoplasma. LGL neoplasi omfatter også enkelte krefttyper med opphav i T celler.

Målet med det presenterte arbeidet var å beskrive NK celler hos hund både fenotypisk og funksjonelt ved å anvende NCR1 til å definere NK celler hos denne arten. Videre skulle hunder med øket mengde LGLs i blod studeres.

I Paper I ble det vist at antistoffet "AKS6" bandt seg til NCR1 hos hund. NCR1 ble påvist på både $CD3^+$ og $CD3^-$ lymfocyttære celler og følgelig var ikke NCR1 eksklusivt uttrykket på NK celler hos hund, men også på en undergruppe av T celler. $CD3^-NCR1^+$ celler representerte trolig NK celler, men utgjorde en mindre cellepopulasjon enn det man har observert i andre arter. Disse cellene var $CD8^-CD56^-GranzymeB^+$. $CD3^-NCR1^+$ celler ble påvist i blod, lymfeknuter, lever og milt. I paper I beskriver vi også en hund med kronisk leukemi av T celletype som uttrykker NCR1.

I Paper II utvidet vi definisjonen av NK celler til å omfatte $CD3^-GranzymeB^+$ celler. Vi fant at hunder både har en $NCR1^+$ og en $NCR1^-$ $CD3^-GranzymeB^+$ cellepopulasjon. Vaksinasjon førte til en økning av $CD3^-GranzymeB^+NCR1^-$. Vi lyktes i å etablere cellekulturer dominert av $CD3^-GranzymeB^+$ celler hvorav en fraksjon av disse uttrykket NCR1, men ved cytokinstimulering ble NCR1 oppregulert. Funksjonelle studier viste at NCR1 fungerte som en aktiverende reseptor slik som vist hos andre arter. NCR1 definerte funksjonelt forskjellige NK celler fordi IFNγ produksjon kun ble observert i $NCR1^+$ celler, og ikke i $NCR1^-$. NCR1 kan følgelig anses som en aktiveringsmarkør.

I Paper III beskrev vi tre hunder med unormalt høy forekomst av LGL celler i sirkulasjonen. Det ble ikke påvist økt uttrykkelse av NCR1 i blod hos noen av de tre kasusene. To av hundene hadde trolig en klonal og malign ekspensjon av LGL celler, mens den tredje antakeligvis hadde en reaktiv lymfocytær hyperplasi som følge av annen kreftsykdom.

For å konkludere kan NK celler hos hund deles inn i en $NCR1^+$ og en $NCR1^-$ subpopulasjon. NCR1 uttrykkes på både $CD3^-$ og $CD3^+$ celler, og dermed er ikke NCR1 uttrykk eksklusivt for NK celler hos hund. Uttrykkelse av NCR1 på maligne T celler ble påvist. NCR1 er en aktiverende reseptor og kan anses som en aktiveringsmarkør da den oppreguleres ved cytokinstimulering. NCR1 definerer også funksjonelt forskjellige NK celler. Inntil en mer spesifikk markør for NK celler hos hund er beskrevet, bør NK celler hos denne arten defineres som $CD3^-GranzymeB^+$.

INTRODUCTION

The immune system is a collective term of organs, cells and signalling molecules that protects an organism from both external and internal threats. It is divided in an innate and an adaptive entity that work and collaborate in a refined and highly regulated manner. The innate immune system consists of external barriers composed of specialized cells, the complement system and leukocytes initiating an immediate response by recognizing general structures on pathogens and abnormal cells. Foreign antigen having compromised the outer barriers is detected in the periphery by macrophages among others and is transported to the closest lymphoid organ were it is presented to the rest of the immune system. The adaptive system primarily comprises B and T cells. B cells produce antigen specific antibodies while T cells play a vital role in cell mediated immunity. Both B and T cells have a somatic rearrangement of receptor genes resulting in an immense repertoire of antigen receptors. The adaptive immune system is capable of generating memory cells accountable for a quicker and more efficient response the next time the antigen in question is encountered.

Natural Killer (NK) cells are defined as large granular lymphocytes (LGL) with a cytotoxic and cytokine producing function. NK cells have germline encoding receptors and belong to the innate immune system. NK cell deficiencies are rarely seen reflecting the importance of this cell type [1]. Natural cytotoxicity was first described in the dog in 1960 [2] and NK cells were discovered in the 70`s [3]. Since then researchers have been intrigued by NK cells multifunctional role in the immune system.

Although essential in eliminating real and potential dangers from the body, cells of the immune system themselves can give rise to a large number of different diseases. Dysregulation of lifesaving processes can cause autoimmune disorders and a wide array of cancers. Haematopoietic cancers are a diverse group of conditions ranging from benign disease to highly aggressive disorders with a poor prognosis. These diseases can affect all ages and account for about 5 % of new cancer diagnoses set according to the Cancer Registry of Norway. There is currently no common cancer register in veterinary medicine, although between 1990 and 2011 a Norwegian Cancer registry for dogs and cats existed. Nevertheless, haematopoietic tumours also account for a substantial number of canine oncology patients [4]. Cancers arising from mature NK cells are termed LGL leukaemia/lymphoma. LGL leukaemia/lymphoma can also originate from T cells.

NK cells – General aspects

General NK cell biology

NK cells were identified more than 30 years ago and were characterized as cells capable of killing tumour cells without prior sensitization [3,5]. It is now recognized that NK cells have multiple functions in the innate immune system and represent a link between the innate and adaptive immune system. Recent discoveries show that NK cells also have features originally thought exclusive to the adaptive immune system like memory and probably also some antigen specificity [6]. NK cells are important in defence against viral, bacterial and parasitic pathogens [7] as well as in anti-tumour response [8], graft vs host disease [9] and in transplantation medicine [10]. NK cells are also important producers of cytokines and chemokines [11]. Most importantly is the production of IFNγ contributing to the initiation of a Th1 response [12]. NK cells have also been shown to produce the immunosuppressive cytokine IL-10, illustrating NK cells as a downregulator of cell mediated immunity [13,14].

Development, anatomical distribution and trafficking

Distribution of NK cells at different stages of development, maturation and activation is most extensively described in humans and mice.

NK cells develop in the bone marrow from haematopoietic stem cells through a common lymphoid progenitor characterized by CD34 expression. This development takes place both during foetal life and after birth. Immature NK cells migrate from the bone marrow out to the periphery where they go through their final differentiation [15], although there is evidence that NK cells can develop from $CD34^+$ cells outside the bone marrow [16–20]. NK cells found in the periphery show a great heterogeneity, both between and within the same organ, and it is not always clear if the different populations of NK cells represent subsets or different stages of differentiation. Nor do we know the importance of tissue specific factors on NK cell differentiation in different body compartments [15]. $CD56^{bright}$ and $CD56^{dull}$ cells represent two well characterized functional subsets of human NK cells illustrate an example. $CD56^{bright}$ cells are known to be efficient cytokine producers while $CD56^{dull}$ cells are highly cytotoxic and predominates in human peripheral blood (>90 %). Several studies now show that $CD56^{bright}$ cells might be a progenitor of the $CD56^{dull}$ cells [21,22].

NK cell differentiation can be divided into three stages characterized by a specific phenotype and functional characteristics: NK cell precursors, immature NK cells and mature NK cells [16]. As the NK cell differentiate, the cell`s cytotoxic and cytokine producing abilities increase, and specific NK cell receptors are acquired [6]. An important step in the maturation of NK cells is called educating or licensing. This refers to the NK cells expression of inhibitory receptors recognizing MHCI molecules on self-cells and is crucial for NK cells self-tolerance [23].

Different organs contain different quantities of NK cells possessing certain properties important for that particular organ. An example of a highly specialized category of NK cells are NK cells that accumulate in the decidua during pregnancy. This subset regulate trophoblast invasion by secreting specific cytokines as well as inducing vascular growth in the decidua by secreting angiogenetic factors [24]. During the differentiation process, NK cells acquire homing receptors that regulate NK cell trafficking within the body [25]. Homing of NK cells and other cells of the immune system, both during development, steady state and during inflammation, is regulated by a chemokine (chemoattractant cytokine) system consisting of chemokines and chemokine receptors. Chemokine receptors are a family of seven-transmembrane G-protein coupled receptors and chemokines are soluble signalling proteins secreted by a wide array of cells [26]. Chemokines produced in the different organs guide the homing of NK cells according to the chemokine receptors they express on their surface. For example, under normal conditions a considerable amount of human NK cells are found in the lymph nodes as they express chemokine receptors like CCR7 making them migrate towards CCR7 ligands CCL19 and CCL21 found in lymph nodes [25]. Other response proteins not belonging to the chemokine superfamily are also able to attract NK cells. Sphingosine 1-phosphate (S1P) is important in recirculation of B and T cells, and is also involved in homing of NK cells in a steady state and in mobilizing NK cells to inflamed tissue [27]. Chimerin, a pro-inflammatory protein, has been shown to be important in recruiting NK cells from blood to pathological peripheral tissues [28].

NK cells in healthy humans and in mice are found in the circulation and in lymphoid organs like lymph nodes, spleen and liver [29,30]. In the spleen, NK cells are mainly found within the red pulp [30], and in the liver NK cells are situated in the hepatic sinusoids often adhering to the endothelial cells. NK cells represent a substantial proportion of lymphocytes found in the human liver comprising 20-30 % of the hepatic lymphocytes [31]. A dramatic increase in hepatic NK cells can be observed during infections [32]. NK cells are also found in non-

lymphoid organs like the lamina propria of the gut, the pancreas, the central nervous system and the lungs [29,30]. In the mice and rat it has been shown that the lung contains the largest number of NK cells among the non-lymphoid organs [33,34]. NK cells are also present in the human healthy dermis [35]. It is not fully understood to what degree NK cells re-circulate through tissues, i.e. how anatomically restricted NK cells really are. It has been shown in mice that NK cells recycles between tissues. When transferring murine spleen derived NK cells into a naive host, these NK cells were found in all organs where NK cells are localized [36].

Priming and activation

It was originally thought that NK cells were ready to carry out their function without needing any form of triggering beforehand. Now it is well known that NK cells need priming and subsequent activation to display cytotoxic and cytokine producing properties. Comparing NK cells in humans and pathogen free laboratory mice reveals a difference in distribution and triggering capacity [37–39]. When further comparing laboratory mice with free living mice, the latter display a distribution and activation status of NK cells similar to what is seen in humans indicating that environmental factors influence pre activation of NK cells [40]. Another study shows that injecting mice with a toll-like receptor ligand leads to NK cells migrating to their draining lymph node where trans presentation of IL-15 on dendritic cells occurs [41]. This chain of events is termed priming. Primed NK cells express CD69 and they accumulate intracellular granzyme B. However, they do not spontaneously produce IFNγ or show cytotoxic abilities [39]. Full activation of NK cells needs further signals.

NK cells can be activated in different ways, either by signalling through NK receptors or by cytokines produced by collaborating cells. Different cytokines and in diverse combinations have distinct effect on differentiation, function and survival of NK cells [42]. Cytokines can also control up and down regulation of NK cell receptors [43]. A selection of cytokines is addressed in more detail elsewhere in this thesis.

NK cell receptors

NK cells display both inhibitory and activating receptors on their surface and the balance in the stimulation of these receptors decides the final outcome of cell-to-cell contact. Unlike B and T cells expressing a specific antigen receptor, each NK cells express a number of different combinations of receptors. These receptors are germ-line encoded transmembrane

receptors and do not go through genetic rearrangement as TCRs and BCRs do. NK cell receptors can grossly be divided into two groups: C-lectin receptors and immunoglobulin-like receptors. In addition, NK cells express a number of different receptors also found on other immune cells.

"The missing-self hypothesis" and inhibitory receptors

Inhibitory receptors recognize self-cells and ensure self-tolerance. Most somatic cells express MHCI (in humans: HLA), presenting peptides from within the cell to $CD8^+$ T cells. Inhibitory receptors on NK cells recognize MHCI, but also other non-MHCI ligands expressed by self-cells.

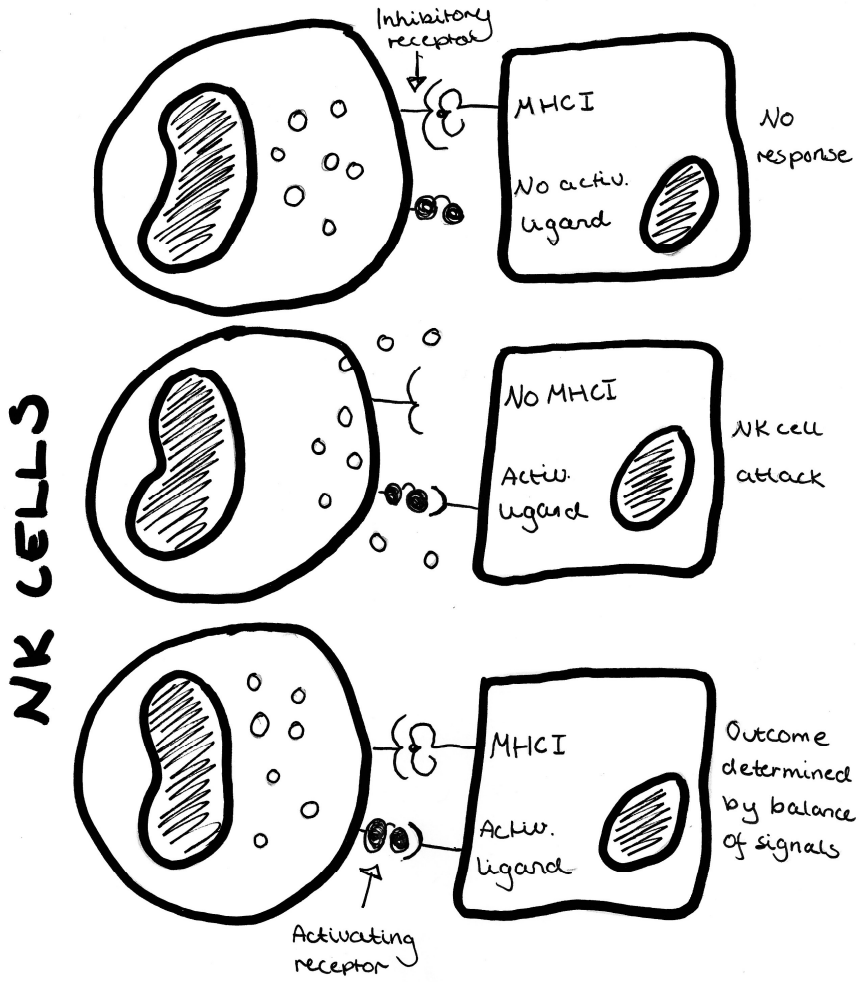

Fig 1. Schematic illustration of NK cells meeting target cells expressing different combination of activating and inhibitory ligands. NK cells to the left and target cells to the right.

Under normal conditions, self-cells are spared by sending strong and dominating inhibitory signal to the NK cell. This principle is called "The missing-self hypothesis" [44]. Under certain

conditions like during viral infections and malignant transformation, self-cells downregulate their MHCI in an attempt to hide away from the cellular immune system [45–47]. When an important suppressing signal is absent, the balance shifts towards stimulation and NK cells are activated. MHCI is not expressed on red blood cells and in nervous tissue and other NK protective mechanisms must be involved here [48].

Two important groups of inhibitory receptors are killer cell immunoglobulin (Ig) like receptors (KIR) in humans [49] and Ly49 receptors in rodents [50] both recognizing MHCI/HLA. KIR molecules are variably expressed on individual NK cells as well as being highly polymorphic resulting in a high degree of variation from person to person [51]. Both KIRs and Ly49 have been demonstrated in other species and traditionally it has been thought that co-expression of KIRs and Ly49 has not been possible. However, this is observed in cattle and horses. Cattle have polymorphic and polygenic KIRs as well as a single polymorphic Ly49 gene [52–56]. Several Ly49 genes are demonstrated in the horse, and horses also have at least one KIR [55,57]. A single Ly49 gene has also been identified in domestic cat, pig and dog [55].

NKG2A/CD94 is another inhibitory receptor recognizing non-classical MHCI molecules, thus functionally complementing the KIRs [58,59]

The inhibitory receptors have different extracellular domains but they have a common intracellular structure. KIRs are a type I glycoprotein of the immunoglobulin superfamily while Ly49 and NKG2A/CD94 are type II glycoproteins with a C-type lectin-like scaffold. All the inhibitory receptors have a long intracytoplasmic tail with two immunoreceptor tyrosine-based inhibition motifs (ITIM) [60].

Activating NK cell receptors

The lack of MHCI is not singlehandedly sufficient to activate NK cells and additionally stimulatory signals through activating receptors are necessary [61].

NKG2D/CD94 is an activating receptor on NK cells binding to MHCI related chain A and B (MICA/MICB). MICA and MICB are not expressed in normal tissue, but are induced on stressed cells like virus infected cells or cells going through malignant transformation [62].

Another example of an activating receptor is CD16. CD16 is a low affinity Fc receptor (FcγRIII). When binding to the Fc portion of antibodies covering a target cell it can mediate antibody dependent cell mediated cytotoxicity (ADCC), and its binding alone is enough to

initiate activation and degranulation of NK cells [63]. CD16 is also expressed on monocytes and granulocytes.

Toll like receptors (TLR) are pattern recognition receptors found on many innate immune cells and binding of TLRs leads to triggering of an innate immune response. There are a total of ten different TLRs in humans and a majority of them can be expressed on NK cells [64].

Also activating KIRs have been identified. Activating KIRs probably recognize MHCI molecules with a lower affinity than the inhibitory counterpart. During viral infection when viral particles are expressed on MHCI the affinity seems to increase. The function of activating KIRs is therefore probably influenced by the nature of the peptide displayed on the MHCI molecule. This is in contrast to inhibitory KIRs recognizing the MHCI protein regardless of the protein presented [64].

NCRs are an important group of activating receptors and consist of NCR1 (NKp46, CD335), NCR2 (NKp44, CD336) and NCR3 (NKp30, CD337), all of them discovered in the late 1990`s [65–67]. NCRs are glycoproteins and belong to the immunoglobulin superfamily.

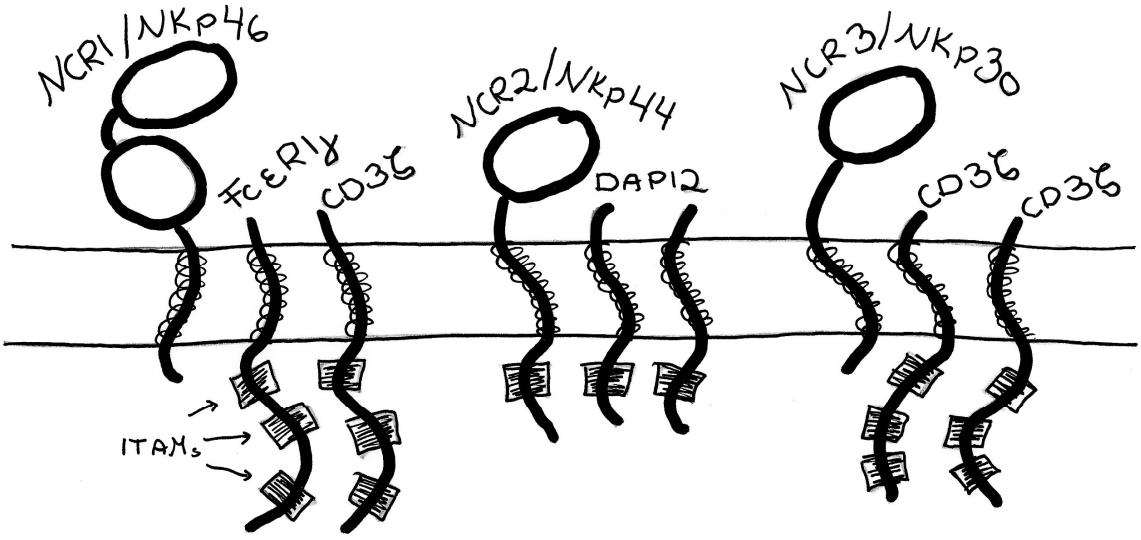

Figure 2. Schematic illustration of the structure and associated molecules of NCR1, NCR2 and NCR3.

In humans, NCR1 and NCR3 are expressed on both resting and activated NK cells while NCR2 is upregulated upon activation. It was originally thought that NCRs are exclusively

expressed by NK cells, but NCRs are also expressed on other lymphocytes. All NCRs signal through immune receptor tyrosine-based activation motif (ITAM)-bearing adaptor proteins [68].

NCR1

NCR1 consists of two extracellular C2-type Ig-like domains arranged in a 85 ° angle, a transmembrane region and a short cytoplasmic tail [69,70]. The cytoplasmic tail is lacking a signalling motif and activation of this receptor is therefore dependent on association with CD3ζ and FcεRIγ which together bear multiple intracellular ITAM molecules [71]. Engagement of NCR1 leads to tyrosine-phosphorylation and a subsequent activation of cytotoxicity and Ca^{2+} influx leading to cytokine production [65,72]. Even if triggering of NK cells is generally dependent on multiple signals, it is shown that NK cytotoxicity can be mediated by NCR1 alone [73].

In both human and mice the cell surface expression of NCR1 is initiated on immature NK cells in the bone marrow [74,75], hence NCR1 is expressed on both resting and activated NK cells in the periphery [65]. However, NCR1 expression can vary greatly within the peripheral NK cell population. Most human NK cells have a high expression of NCR1 (^{bright}), although in some donors NK cells with a lower expression (^{dull}) of NCR1 can be demonstrated. NCR1 phenotype of the NK cell correlates with their natural cytotoxicity [65]. An investigation of NK cells in human secondary lymphoid organs found NK cells lacking NCR1 in uninflamed tonsils. [76]. Also a very small population of $CD3^{-}CD56^{+}$ cells in peripheral blood mononuclear cells (PBMC) has been identified expressing a very low density of NCR1 or may even lack NCR1 proposing that $NCR1^{-}$ NK cells might be present in humans [77]. Human NK cells can downregulate NCR1 under certain conditions such as in patients with HIV viremia [78] or neoplastic conditions [79,80].

The cellular ligand of NCR1 is still unknown although certain pathogens are recognized by the receptor, for example the haemagglutinin (HA) of the influenza virus and the haemagglutinin-neuraminidase of the parainfluenza virus [81]. NCR1 is also involved in immunity against the hepatitis C virus [82], *Mycobacterium tuberculosis* [83] *Streptococcus pneumonia* [84] and the malaria parasite *Plasmodium falciparum* [68,85]. NCR1 is additionally involved in various non-infectious disease processes. An upregulation of an NCR1 ligand on β cells in the pancreas have been shown to result in the presence of large numbers of $NCR1^{+}$ cells in the pancreatic islands contributing to autoimmunity and type I diabetes [86]. Treatment

strategies using blocking NCR1 might prevent development of type I diabetes in early stages of the disease [87]. NCR1 is also shown to prevent tumour growth and metastasis [88–90]. The receptor was originally thought to only be expressed by NK cells, but it is now clear that also certain groups of T cells can express NCR1 [68,91] or upregulate NCR1 under certain conditions, like in patients with celiac disease [92].

Mechanisms of NK cell cytotoxicity

NK cells exert their cytotoxic function through calcium dependent degranulation resulting in the release of perforin and granzymes. It has also been shown that NK cells can kill target cells through the Fas-FasLigand pathway [93]. Cytotoxic T cells use the same two mechanisms [94]. Activation through the Fas-FasLigand pathway results in cells showing features of apoptotic cell death, mediated by caspase activation [95]. However this mechanism of killing is not as efficient as granzyme/perforin mediated cytotoxicity [96]

NK cells release granules containing perforin and granzyme after activation. Perforin creates a pore in the target cell`s membrane through which granzyme can pass and enter the cell in a controlled manner. Granzymes are serine proteases which induce apoptosis through activation of caspase-driven cell death pathways [97]. Also cells like NKT cells and γδ T cells use perforin and granzyme to exert their function. Granzyme B is the most extensively studied of the granzymes and is considered the most powerful pro-apoptotic granzyme [97].

Four types of cytotoxic phenomena is reported in NK cell research [98]:

1. Killing in the absence of stimulation called “natural cytotoxicity”.
2. Killing after cytokine stimulation of NK cells. *In vivo* these cells are termed “activated” while *in vitro* IL-2 stimulated NK cell cultures are termed LAK-cultures (lymphokine-activated killer cells)
3. Killing following antibody opsonisation of target cells, called antibody dependent cell mediated cytotoxicity (ADCC)
4. Redirect lysis (reverse ADCC) where antibodies attached to proteins on the NK cell surface are bound by Fc receptors on a target cell.

Cytokines important for NK cell function

Cytokines are small, soluble proteins important in cell signalling. A broad range of cytokines are produced by cells in the immune system. Cytokines bind to their target cells expressing

appropriate receptors and this leads to a relevant response. Studies of knockout mice lacking specific cytokines or cytokine receptors have allowed us to get a unique insight into the role of each cytokine individually [99,100]. Different cytokines, mainly IL-2, IL-12, IL-15, IL-18, IL-21 and the type I interferons are crucial for NK cell homeostasis [101].

IL-2 and IL-15 are important for development, survival, proliferation and effector functions of NK cells. They share a medium-affinity receptor formed by two chains, and association with a third chain, IL-2Rα (CD25) or IL-15α, forms a high affinity receptor for IL-2 and IL-15 respectively. On resting human NK cells IL-15α is expressed in low numbers, while IL-2α is barely detectable. During activation, IL-15α and IL-2α are expressed in a sequential way[102]. Expression on IL-15α peaks after 16-20 hours followed by a gradual decline, while IL-2α expression reaches a maximum at around 48 hours. Consequently, NK cells only responds to free IL-15 early in the immune response, while later T cell derived IL-2 and trans-presented IL-15 on dendritic cells are important for NK cell function [102]. Even if the receptors for IL-2 and IL-15 are very similar, they induce different responses [103].

IL-2 and IL-15 are individually poor inducers of cytokine production, but together and in combination with IL-12, production of cytokines from NK cells is initiated. IL-12 is produced by monocytes, macrophages and dendritic cells as a response to microbial antigens. IL-12 is essential in polarizing the immune response towards a Th1 response partially because of IL-12`s effect on NK cells to initiate IFNγ production. IL-12 can trigger cytotoxicity and influence NK cell proliferation [101]. It has also been shown that IL-12 is involved in upregulation of NCR1 [104].

Memory-like NK cells

Immunological memory is traditionally associated with T and B cells in the adaptive immune system. Following activation and antigen specific response, long-lived memory cells are generated. These can mount a quicker and more potent immune response when re-challenged by the appropriate antigen. Recent findings indicate that also NK cells can show adaptive features and memory-like properties. A study done on mice showed that infection with cytomegalovirus resulted in an extensive proliferation of NK cells that resided in the body for many months. When re-challenged these NK cells quickly produced cytokines and de-granulated [105]. A second study, also done in mice, described how *in vitro* stimulated NK cells transferred into a naive host had a prolonged survival and produced more IFNγ when re-stimulated [106].

Vaccination, adjuvants and NK cells

Vaccines contain an attenuated or killed/inactivated pathogen often together with an adjuvant to induce an immune response and immunological memory, but without resulting in disease. Vaccines are primarily aimed to give a humoral immunity and cellular immunity has in comparison received little attention. NK cells have therefore been neglected in vaccinology. Since the development of the first vaccines, it has been known that additional factors than the antigen itself are important for a successful outcome. Adjuvants are a heterogeneous group of molecules, and using adjuvants in a vaccine can increase and modify the immune response to an antigen [107–109]. Antigen uptake, transport to the local lymph node and presentation by antigen-presenting cells can be facilitated by adjuvants. Also, adjuvants can provide a depot effect that gives prolonged antigen release and antigen presentation and/or results in an inflammation at the injection site, amplifying the desired immune response. Aluminum-hydroxide based adjuvants (Alum) are the most commonly used adjuvants in human vaccines, due to their well-documented safety record. Alum is known to induce a strong antibody response [110–112].

A few studies describe how immunization influences NK cells. Injection of mature dendritic cells (DCs) together with different adjuvants resulted in rapid recruitment of NK cells to the lymph node where they served as an early source of IFNγ [12]. Peripheral TLR stimulation in mice lead to NK cells travelling to the lymph node followed by interaction with DCs resulting in emerging of effector NK cells in the periphery [41]. Another study characterized *in vitro* cellular response after vaccination with an inactivated rabies vaccine in healthy humans [113]. PBMC was collected before and 21 days after the first vaccine and then re-challenged with the inactivated rabies virus *in vitro*. Interesting observations were done in PBMC taken after vaccination. Shortly after virus re-challenging both CD69 and IFNγ was upregulated in both T and NK cells. However, NK cells were the dominating IFNγ producers early in the immune response. Also degranulation and release of perforin from NK cells were seen. Co culturing post vaccinated PBMC and inactivated rabies virus gave an extensive proliferation of both T and NK cells, but a higher proportion of NK cells compared to T cells were proliferating. This demonstrates that NK cells contribute notably in the immune response following re-challenge of the antigen and especially in the early phases. Changes in NK function when reintroducing rabies antigen in PBMC after vaccination also indicated memory features of NK cells.

ILCs, NKT and NKT-like cells

Traditionally the division of cells belonging to the innate and to the adaptive immune system has been regarded absolute. This perception has recently been challenged as innate like properties of B and T cells are described and adaptive features have been identified in NK cells. Consequently, the innate and adaptive immune system overlap to a larger extent than previously thought.

A wide array of innate lymphoid cells is characterized and new ones are constantly emerging. These cells represent a bridge between the innate and adaptive immune system and have been found important in many disease processes [114,115]. A common classification system for innate lymphoid cells (ILCs) has been lacking until recently when it was proposed to group innate lymphoid into three different groups according to their cytokine expression profile. Lack of rearranged receptors, lack of markers for myeloid or dendritic cells and a lymphocyte morphology are common features for ILCs. NK cells are placed in group I where it they considered a prototype [116].

Classical NKT cells comprise a group of innate like T cells that recognize lipid antigens presented by a monomorphic MHCI molecule termed CD1d. They are grouped according to their TCR usage and antigen specificities [117]. Type I NKT (invariant NKT) cells express an invariant TCR α chain that is paired with a limited number of TCRβ chain. Type II NKT cells lack the invariant TCR α chain and express a more diverse TCR repertoire than iNKT cells. TCR on type I NKT recognizes the α-galactocylceramide (αGalCer), a derivative from a sponge, while type II NKT cells do not [114,118]. NKT cells are potent cytokine producers, most important are IFNγ and IL-4, and they display granzyme and perforin meditated cytotoxicity [117]. NKT can both enhance and suppress cellular immune response. [114].

Also subsets of conventional T cells expressing rearranged TCR αβ and NK cell receptors are described in the literature, but they have not been intensively studied like the classical NKT cells. These have been termed "non-classical NKT", "CD1d independent NKT" or "NKT-like" cells [119]. Cells displaying both CD3 and NCR1 are described in humans, mice [120], cattle [91] and swine (Mair et al., unpublished results). Murine $CD3^+NCR1^+$ cells were found to be $CD4^-$ non CD1d restricted NKT-like cells producing more IFNγ than $NCR1^-$ NKT cells [120]. In cattle, $CD3^+NCR1^+$ cells have been shown to be split into a TCRαβ and a TCRγδ subset, and $TCR\alpha\beta^+$ cells expressed a diverse TCR repertoire. The bovine $CD3^+NCR1^+$ cells showed cytotoxic activity but required crosslinking of CD3 to produce IFNγ [91].

Phenotypic and functional definition of NK cells – a comparative view

The definition of NK cells is in constant change and differ between species. NK cells were first described as cytolytic cells that could spontaneously kill malignant cells [3,5] and these cells were morphologically described as large granular lymphocytes [121,122]. Nowadays phenotypic characteristics are used to define and subgroup NK cells. Common for all definitions used is the lack of CD3, co-receptor for TCR [123]. Human NK cells are $CD3^-$ granular lymphocytes also expressing perforin and granzyme [124]. Human NK cells are further classified according to the level of CD56 (bright/dim) and CD16 [125]. CD56, also known as N-CAM, is expressed on neurons and muscle cells as well as NK cells. The function of CD56 in NK cells is unknown [126]. NK cells in cats have also been characterized as $CD3^-CD56^+$ cells [127]. Rodent NK cells do not express CD56 and are defined differently. NK cells in mice are divided into functional subgroups according to CD11b and CD27 [128]. NK cells in rats are defined as $CD3^-$ cells expressing CD49b or NKR-P1A [129].

The activating receptor NCR1 has been suggested as a pan species NK cell marker [130]. NK cells have been defined as $CD3^-NCR1^+$ cells in humans [65,131], monkeys [132,133], rats [134], mice [75,131], cattle [135], sheep [136] and horses [137]. However, as only about half of the NK cells in the pig express NCR1 [138], this receptor does not mark all NK cells in all species.

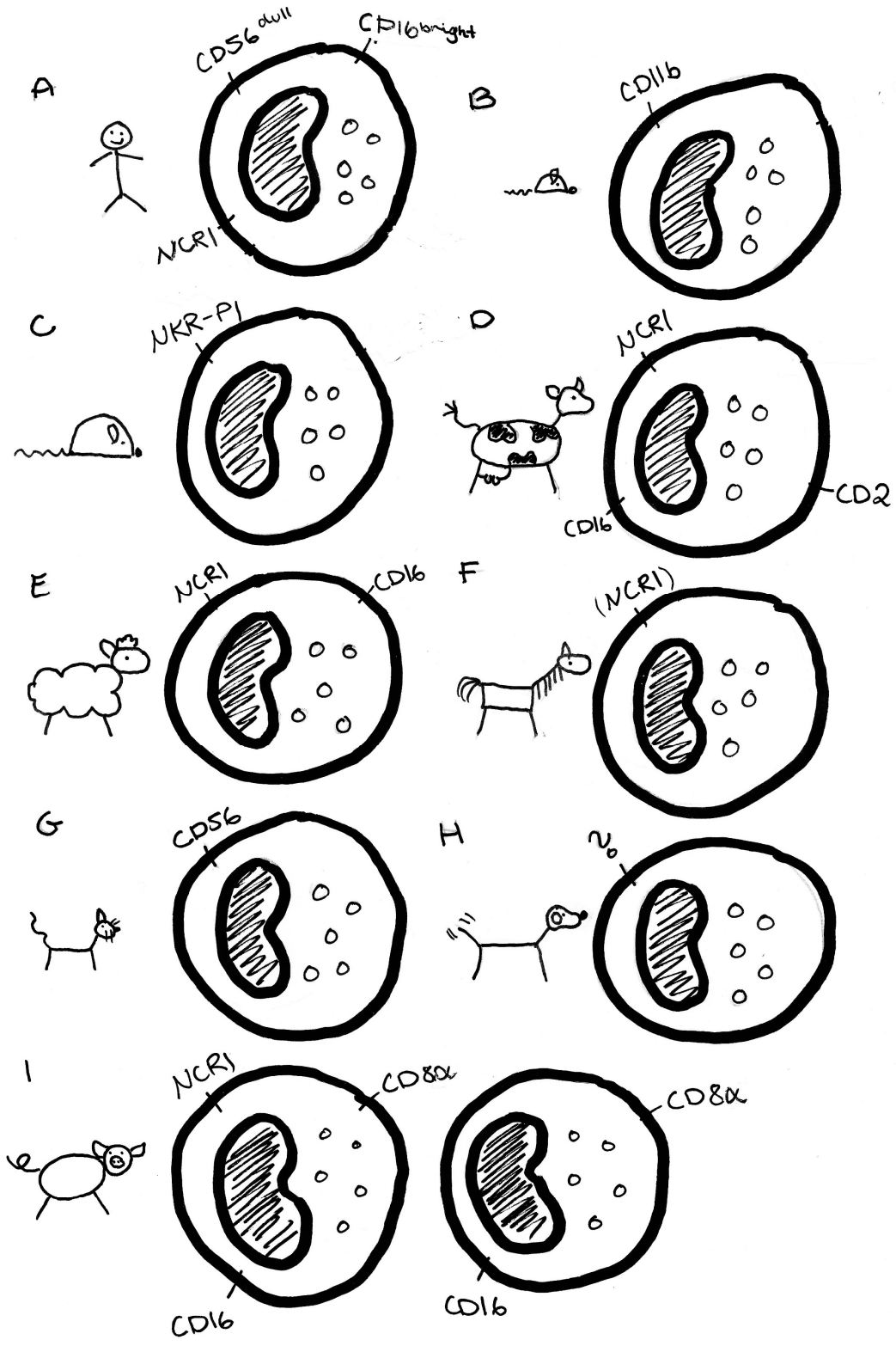

Fig 3. Schematic illustration of the dominating NK cell subset in the circulation of assorted species A Man [77] B Mouse [129] C Rat [129,139] D Cow [135,140] E Sheep [136,141] F Horse [137] G Cat [127] H Dog I Pig [138]

NK cells in the dog

Cytotoxic activity in PBMC was first described in the dog in 1960 [2]. Canine lymphocytes with NK cell activity have been characterized using different approaches without coming to general definition of NK cells in this species.

CTAC, a canine thyroid adenocarcinoma cell line established in 1964 [142], has been a valuable tool for studying NK cell activity in the dog as this is a susceptible target for NK cell cytotoxicity [143–145], probably because of lack of MHCI. Canine cells making conjugates with CTAC have been described morphologically as cells with a kidney bean-shaped nucleus and electron-dense cytoplasmic granules [146], the typical morphological features of NK cells. In 1985 canine cells with natural killer cell activity against CTAC were characterized as Ig^- and Thy-1(CD90)$^+$ cells [147]. Cells with natural cytotoxic activity against CTAC and morphological features consistent with NK cells have been described as $CD5^{low}$ [148] and $CD8^+$ [149]. In both these studies, quantitative levels of mRNA encoding NK cell receptors were determined by qPCR. This included NKp30 (NCR3), NKp44 (NCR2), 2B4, NKG2D and CD16 and both studies showed that the cell population characterized expressed a higher level of NK-related genes than control cells. However, in both these studies the cells described also expressed CD3. An *ex vivo* expansion of canine cytotoxic large granular lymphocytes exhibiting natural killer cell characteristics against CTAC, has been described, displaying a $CD3^+CD5^{dim}TCR\alpha\beta^-TCR\gamma\delta^-$ phenotype and not expressing the invariant TCRα chain (iTCRα) [150]. As it is highly unlikely that canine NK cells express CD3, the cells described here most likely represent NKT-like cells and not NK cells. An invariant TCRα chain has been shown to be expressed by canine $CD3^+$ cells probably representing NKT cells [151]. LAK cells established using recombinant human IL-2 displaying natural cytotoxicity have been described in several studies [148,149]. LAK cultures are established by isolating PBMC followed by IL-2 stimulation resulting in a mixture of T and NK cells [152]. Therefore, LAK cultures cannot be considered NK cell cultures.

A recent study presented a new way of culturing canine non-B non-T cells, probably representing NK cells [153]. PBMC was isolated followed by a CD5 depletion eliminating T cells. CD5 was chosen instead of CD3 based on the authors experience with the performance of these antibodies. Recombinant human IL-2 and IL-15 were tried separately and together and showed the best results in cultures stimulated with both cytokines. The cells had a $CD3^-$

$CD4^-CD22^-MHCII^+CD11/18^+CD11b^+$ phenotype. The cultured cells were negative for CD56, NKp46 (NCR1) and CD94 but as the antibodies used were anti-human, a poor cross reactivity could be the reason for the negative result. The cultured cells showed cytotoxic activity towards CTAC but did not possess the typical morphological appearance of NK cells as granules were not present when staining with Diff Quick and/or Wrights stain.
In these studies, human recombinant interleukins have been used. A canine recombinant IL-15 has recently been presented indicating that rcIL-15 stimulation enhances proliferation and antitumor effect [154]. Quantitative measurements of mRNA for NK cell related molecules comparing rhIL-15 and rcIL-15 were also presented and showed divergent results. The same research group also produced a rcIL-21 showing a synergistic effect with rhIL-2 and rhIL-15 [155].

As human NK cells are defined according to their CD56 expression, CD56 has been the centre of attention in a handful of publications. Three isoforms of CD56 are found in the dog, CD56-120, CD56-140 and CD56-220, which arise from alternative splicing of one single gene located on chromosome 5 [156,157]. mRNA encoding canine CD56 is found in high levels in nervous and endocrine tissue but in low levels in lymph node, spleen, intestine and lung and undetectable levels in liver and leukocytes [156]. Another study using qPCR to determine organ specific expression of CD56 detected substantial expression of CD56 in the canine brain and smaller amounts in lung, liver and kidney [158]. Furthermore, CD56 expression has also been shown in odontogenic tissue during odontogenesis in dogs [159]. Flow cytometry using a cross reactive anti human CD56 antibody (Leu-19) showed that CD56 was expressed on canine lymphocytes but all of the lymphocytes expressing CD56 were also $CD3^+$ indicating that CD56 is only found on T cells. Leu-19 used in immunohistological stainings showed expected patterns in nervous tissue from the dog [160]. Several studies have measured mRNA using qPCR on cell cultures displaying NK cell activity and have not showed increased levels of CD56 encoding mRNA compared to control cells [149,150,154,155]. One study however, found an increase in CD56 encoding mRNA in LAK cells compared to PBMC [161]. To conclude, it is unlikely that canine NK cells express CD56.

A small number of studies have assessed canine NK cell activity in dogs with malignant disease processes. Comparison of LAK cultures from dogs with different neoplasias with healthy dogs showed that cytotoxic activity is reduced in dogs with malignant disease [162]. The same group also evaluated cytotoxic activity in specific cancer types, showing that dogs with mammary carcinomas had a significantly depressed cytotoxic activity [163].

Taken together there are many papers describing NK cell activity in the dog, but in several of these publications the cell population characterized express CD3. A method has been established to successfully culture non-B non-T cells probably representing NK cells[153]. However, as long as no marker specific to canine NK cells has been identified, the search for canine NK cells continues.

Lymphoproliferative disorders of Large Granular Lymphocytes (LGLs)

Cancer and haematological malignancies – fitting the dog into the big picture

Haematopoietic malignancies encompass both leukaemia and lymphoma and is a broad and heterogeneous group of disorders both in human and veterinary medicine. Different classification systems of haematological malignancies have been used interchangeably, though none of them have combined morphological features with clinical behavior and survival in a feasible fashion. In 2001 The World Health Organization finally succeeded joining the current systems together making "The WHO classification of neoplasms of the hematopoietic and lymphoid tissues" widely used today. An update was published in 2008 [164]. Though based on human disease, the WHO system is relevant also in veterinary medicine. Dogs develop many of the same types of cancers as humans do, and neoplastic disease in the dog often behaves in a similar way as observed in people [165]. Similarities between haematological malignancies in the dog and humans have been described both on a morphological [166] and cytogenetic level [167].

Diagnosing lymphoproliferative malignancies – a short overview

Diagnosing lymphoproliferative disorders can be difficult both in human and veterinary medicine. Separating haematological malignancies from reactive proliferation represent a challenge, especially in the early stages when the disease can be slowly progressive or stable. Morphological evaluation of atypical cells is a key element in the diagnosis, although additional diagnostic tests are essential to set a final diagnosis [168,169]. Differentiating between neoplastic and reactive disease is done by assessing clonality. Polymerase chain reaction (PCR) is a long regarded golden standard to separate polyclonal from monoclonal T cell populations. This method is termed the PARR test (PCR for Antigen Receptor Rearrangement). A PARR test has been developed for diagnosing canine lymphocytic diseases [168,170].

Flow cytometry and other antibody based methods can be used to detect proteins expressed by the expanded lymphocyte population. By using panels of monoclonal antibodies directed against specific markers, it is possible to get a unique immunophenotypic characterization of

the lymphocyte population. Presence of cells with an abnormal immunophenotype and/or expansion of a lymphocyte population with a homogenous phenotype are important features of malignant disease [171]. No single antigen is specific for any neoplasm, but combinations of markers are used diagnostically, prognostically and therapeutically in human medicine. Flow cytometry and immunohistochemistry are important in diagnosing haematopoietic neoplasia in dogs, although the small number of canine antibodies available represents a limitation [168,172]. In human medicine new and innovative methods for diagnosing haematopoietic neoplasia are continuously being established. Cytogenetic analysis and mutational studies use genetic sequencing to get new insight into tumour biology [173].

Classification of lymphoproliferative disorders of LGLs

NK cells and cytotoxic T cells present morphologically as large granular lymphocytes (LGLs) because of cytoplasmic azurophilic granules giving them a characteristic appearance. It is not possible to separate the two cell types without using specific markers [174]. Lymphoproliferative disorders of LGLs have been reported in cats [175–177], Fischer 344 rats [178,179] and horses [180–183] as well as being well known in humans and dogs.

In the WHO classification system, tumours of mature NK cells and T LGLs are currently split into four groups, although constantly revised:

1) T cell large granular lymphocytic leukaemia
2) Chronic lymphoproliferative disorders of NK cells - CLPD-NK (still provisional)
3) Aggressive NK-cell leukaemia
4) Extranodal NK/T cell lymphoma, nasal type

Lymphoproliferative disorders of LGLs was first described in humans in the mid-70s [184–186] and has since then been subject for discussion. Disorders of LGLs range from benign LGL lymphomatosis to malignant aggressive NK LGL leukaemia [187], but it is not clear where normal state ends and pathology begins. Clonality is considered an important feature of malignant disease, however it is shown that healthy individuals can exhibit clonal populations of LGLs [188–190]. Detecting clonality in a cell population of NK cell origin represents a challenge, as NK cells do not express a uniquely rearranged receptor. Concurrent disease is often present, complicating the clinical picture. Diagnosing LGL disorders is therefore considered difficult [191].

T-LGL leukaemia and chronic lymphoproliferative disorders of NK cells (CLPD-NK)

T-LGL leukaemia and NK-chronic lymphoproliferative disorders (CLPD-NK) are considered two separate entities according to the WHO classification. It has been proposed to merge these two diseases into one group comprising all chronic LGL disorders as the pathological mechanisms, clinical features and treatment are very similar [192]. The strong biological difference between T and NK cells however, supports keeping these as two different groups of diseases.

T cell LGL leukaemia is the most common of the LGL leukaemias and constitutes about 85 % of all LGL disorders while CLPD-NK are rare. They typically affect older individuals [193]. Many patients are asymptomatic by the time of diagnosis while others have symptoms connected to cytopenias often observed [194,195]. Splenomegaly, hepatomegaly and skin lesions are sometimes seen. The expanded cell population typically display a $CD3^+TCR\alpha\beta^+CD8^+CD57^+CD16^+$ or $CD3^-CD56^+CD16^+$ phenotype in T and NK cell disorders respectively [196]. Often other forms of pathology exist simultaneously. Rheumatoid arthritis has been reported to be present in as much as 30 % of the patients with T LGL leukaemia. Other autoimmune diseases such as systemic lupus erythematosus, celiac disease and immune mediated cytopenia, certain viral infections, other haematological malignancies as well as non-haematological malignancies have all been observed concurrent to T LGL leukaemia [197–199]. CLPD-NK are associated with rheumatoid arthritis, immune mediated haemolytic anemia, hepatitis, pure red cell aplasia and other malignancies among others [193,199]. The prognosis is considered good and an aggressive course is rare [193,196]

Aggressive NK cell leukemia

The aggressive NK LGL leukaemia is one of the most fatal diseases seen in medical oncology with a short survival time of days to weeks [200]. This form of leukaemia affects younger people and has an ethnic predisposition for oriental countries, although cases in the western population have been reported [201,202]. The disease has a disseminated character and neoplastic infiltrations showing a destructive and diffuse pattern of cells slightly larger than normal large granular lymphocytes. Disseminated intravascular coagulation and haemophagocytic syndrome are complications seen [200] and several cytogenetic abnormalities have been detected [203] . Epstein Barr Virus (EBV) is considered an etiological agent [204]. The prognosis is poor with virtually 100 % mortality and response to therapy is poor [205].

Extranodal NK/T lymphoma

Extranodal NK/T lymphoma, nasal-type is most often of NK cell origin and strongly connected to EBV. This disease has an ethnic predisposition and it is most commonly seen in Latin America and East Asia [200]. As the name implies, this is a tumour occurring in the nasal cavity and the adjacent tissues and the tumour is locally invasive. Clinical symptoms are connected to the local lesions, though if the disease disseminates systemic symptoms occur. The clinical outcome varies and is connected to the stage of disease when diagnosed [204]. Extranodal (extranasal) NK/T lymphoma has been described. This disease can affect any part of the body but the histological findings are similar to the nasal type and typically has a poor prognosis [200].

How to detect clonality in NK cells

Disorders originating from NK cells are a source of discussion as markers of malignancy and strategies for detection of clonality is still under investigation. Therefore, it has still not been determined if CLPD-NK mainly consist of monoclonal or polyclonal conditions. As a large number of cases are needed to conclude on diagnostic criteria, the rarity of the disease makes this work challenging.

Aberrant expression of NK cell receptors has been connected to malignant diseases of NK cells and deviating ratios between NK cell receptors might therefore be used as a diagnostic tool. Aberrant expression of KIRs has been associated with chronic and acute NK LGL leukaemia. Pathological NK cell populations can express only one single KIR, be negative for KIRs altogether or only express KIRs on a small fraction of NK cells. NK cells from healthy donors express a wide array of different KIRs. Patients with NK LGL disorder have a normal KIR genotype indicating a KIR dysregulation [206], although certain KIR gene repertoires characterized by a high level of activating genes have been connected to this disease [207]. Taken together, aberrant KIR expression represents promising markers for detecting abnormal populations of NK cells.

The C-lectin receptors have shown a divergent expression in patients with NK LGL disorders. Bright expression of NKG2A and NKG2D as well as bright expression of CD94 has been demonstrated [206]. CD94/NKG2A expression has also been associated with less aggressive behavior [208].

NCR1 expression on malignant cells

Not many studies have investigated NCR1 expression on malignant cells and no study has explored the connection between NCR1 expression and clinical outcome. One study looked into expression of NCRs measured by qPCR in 13 patients with NK cell LGL leukaemia, finding a downregulation or loss of NCR1 and NCR3 on malignant cells in some patients [206]. Another large study evaluated NCR1 expression in 292 cases of lymphoproliferative disorders [209]. NCR1 was detected on abnormal cells from six out of seven patients with CLPD-NK and in all patients with T LGL leukaemia (n = 3). In extranodal NK/T lymphoma NCR1 was expressed in 59/66 patients. Assorted T cell neoplasia were also tested for NCR1 expression. In cases of atypical T cell proliferations, anaplastic large cell lymphoma, peripheral T cell lymphoma, mycosis fungoides and mycosis fungoides with a large cell transformation, NCR1 expression was detected on the neoplastic cells.
A study of 34 patients diagnosed with T-LGL leukaemia showed a significant upregulation of NCR1 expression compared to PBMC from healthy controls measured by qPCR. In this study it was demonstrated that IL-15 might be involved in leukaemic transformation of NCR1 expressing T cells and IL-15 could represent a useful target for treatment in these patients [120]. Taken together, NCR1 seems to be commonly expressed in LGL leukaemia and extranodal T/NK neoplasia of both T and NK cell origin. Non-LGL T cell neoplasia can also express NCR1. NK neoplasia negative for NCR1 have been described. NCR1 therefore seems to be dysregulated in many categories of malignant cells of lymphoid origin.

Lymphoproliferative LGL disorders reported in the dog

Lymphoproliferative LGL disorders in the dog are rare conditions similar to what is reported in humans. Around 40 cases of canine lymphoproliferative LGL disorder have been described in the literature.

One study reported three cases with LGL lymphocytosis [210]. All three dogs were middle aged to old and presented with clinical disease, cytopenias and bone marrow involvement. A mediastinal mass was detected in one of the dogs. One dog had an aggressive disease, while the two others displayed a more chronic disease responsive to therapy.

One of the most extensive clinical studies of dogs with LGL lymphocytosis described 25 dogs having more than 10 % LGLs in peripheral blood lymphocytes (PBL) [211] Three of the dogs had an aggressive disease resulting in euthanasia within two weeks and the remaining

22 dogs had a chronic clinical course lasting for more than four months. Affected dogs where middle aged ranging from five to 14 years of age at the onset of disease. Most of the dogs were of large breed and females were affected more often than males (female: male ratio 1.8). An assorted number of breeds were represented, although Golden Retrievers (n = 5) and German Shepherd Dogs (n = 4) accounted for almost one third of the cases described. Twenty-one out of 25 cases were clinically ill at the time of diagnosis. Fifteen dogs were anaemic and 17 dogs had a neutrophilia. Only one dog had a neutropenia and this dog also had anaemia and thrombocytopenia. Splenomegaly was detected in 60 % of the dogs. Out of eight cases where bone marrow was examined, five cases had bone marrow involvement. Symptomatic dogs were successfully treated with immunosuppressive drugs and survival was reported to range between six months and three years. Three of the dogs were thought to have a reactive lymphocytosis rather than a monoclonal expansion. Of these three, one dog had a chronic bronchitis, one tested positive for *Ehrlichia canis* antibodies and the last had a thrombocytopenia. PBMC from all 25 dogs was examined using flow cytometry. All dogs were positive for CD8α, Thy 1, MHCII, VLA-4, CD18 and CD11a and negative for CD21 and CD11b. 92 % were $CD3^+$, 60 % were TCRαβ+ and 32 % were TCR $\gamma\delta^+$. Atypical cells from two dogs were $CD3^-$, postulated to be of NK cell origin and both of these were $CD8\alpha^+$ and $CD8\beta^-$. Of these two, one had an aggressive clinical course and the other had a chronic disease with an anaemia.

A 10 year old neutered female mixed breed dog was reported to have a cutaneous lymphoma with a concurrent LGL leukaemia of possible NK cell origin [212]. The dog had a generalized pruritic skin disorder and generalized lesions with diffuse erythema, patchy alopecia, scale, crusts and focal ulcerations. The dog had thrombocytosis as well as lymphocytosis, but otherwise CBC was normal. Neoplastic cells were found in the bone marrow and in lymph nodes and the dog had moderately enlarged lymph nodes and hepatomegaly. Both the leukaemic cells found in the circulation and the malignant cells detected in skin biopsies were $CD3^-$. The leukaemic cells were further phenotyped as $CD4^-CD8^-$ and positive for IL-2 receptor. The IL-2 receptor was functional and the neoplastic cells themselves produced IL-2 indicating that IL-2 might be important in the disease process.

Other examples of canine extranodal LGL lymphoma exist. A dog with LGL intravascular lymphoma [213] and a dog with LGL intestinal lymphoma [214] have been described. Both cases had a concurrent LGL leukaemia and neoplastic cells were negative for CD3 indicating a

possible NK cell origin. Recently, a report of a γδ T LGL lymphoma has been published described a two year old Labrador Retriever with a mediastinal mass [215].

One study has reported a possible connection between LGL lymphocytosis and positive *Ehrlichia* serology in a dog [216]. Also retrovirus corresponding to an oncovirus has been identified in a dog with LGL leukaemia [217] representing a possible causal factor for this disease in the dog. Canine LGL disorders have been observed together with other malignant diseases like histiocytic sarcoma [218] and thymoma [219].

Canine LGL lymphoproliferative disorders are a heterogeneous group of conditions and the clinical course can vary from a benign disorder to highly aggressive malignant disease with a poor response to treatment and a largely unfavourable prognosis like also seen in humans. The dog can be asymptomatic at the time of presentation or it can have symptoms reflecting the tumour burden itself and/or symptoms associated with other abnormalities in the blood, most often an anaemia. Neutrophilia seems to be more common in dogs than neutropenia often observed in people. Dogs with LGL disorders can display symptoms connected to coexisting conditions. In dogs, LGL disorders have been observed concurrent to other malignant diseases, positive *Ehrlicha* titer and infection. There is no reported connection between autoimmune disease and canine LGL disorders. Concurrent disease makes evaluation of prognosis difficult as most reports do not specify if euthanasia/death is caused by the LGL disorder itself or if it is caused by the coexisting condition. Many different breeds are represented in the case reports, although some breeds seem to be overrepresented like the Golden Retriever and the German shepherd. In human medicine, some LGL disorders are strongly connected to certain ethnical groups. Both in humans and in dogs $CD3^+$ T LGL disorders are much more common than LGL disorders lacking CD3 expression and chronic disease is more common than acute. An aggressive course is seen in both $CD3^+$ and $CD3^-$ LGL disorders in the dog while in humans, LGL disorders with a poor prognosis is most often of NK cell origin. Extranodal LGL lymphoma is observed in several locations in the dog, but no reports exist of a nasal type.

To conclude, it seems that canine lymphoproliferative disorders of LGLs resemble the human equivalent in many respects.

AIMS OF THE STUDY

Main objective

The main aim of the current work was to characterize canine NK cells phenotypically and functionally and to investigate NCR1 expression on canine NK cells and its functional role. Furthermore, the obtained knowledge was going to be applied in studying canine lymphoproliferative LGL disorders.

Sub goals

1. Obtain a monoclonal antibody directed towards canine NCR1 and further investigating NCR1 as a marker for canine NK cells (Paper I + Unpublished material).
2. Investigate NK cells in different body compartments in both healthy dogs and in dogs with medical conditions (Paper I).
3. Establish canine NK cell cultures by stimulation of recombinant canine IL-2 and examine the cultured cells for functional properties focusing on the importance of NCR1 (Paper II + unpublished material).
4. Characterize canine lymphoproliferative LGL disorders with regards to NCR1 (Paper I and III).

SUMMARY OF PAPERS

Paper I

NCR1+ cells in dogs show phenotypic characteristics of natural killer cells
Grøndahl-Rosado C, Bønsdorff TB, Brun-Hansen HC, Storset AK.
Vet Res Commun. 2015 Mar;39(1):19-30.

Peripheral blood mononuclear cells (PBMC) from 14 healthy dogs, 37 dogs with a clinical diagnosis, including a dog diagnosed with LGL leukaemia, and tissue samples from eight dogs were evaluated for $NCR1^+$ expression by a cross reacting anti-bovine NCR1 antibody. $CD3^-NCR1^+$ cells were found in the blood of 93 % of healthy dogs and comprised up to 2.5 % of lymphocytes in PBMC. In a selection of healthy dogs, sampling and immunophenotyping were repeated throughout a period of one year revealing a substantial variation in the percentage of $CD3^-NCR1^+$ cells over time. Dogs allocated to eight disease groups had comparable amounts of $CD3^-NCR1^+$ cells in PBMC to the healthy individuals. All organs examined including liver, spleen and lymph nodes contained $CD3^-NCR1^+$ cells. Circulating $CD3^-NCR1^+$ cells were further characterized as $CD56^-GranzymeB^+CD8^-$. $CD56^+$ lymphocytes were demonstrated but all $CD56^+$ cells were $CD3^+$ and $NCR1^-$. A $CD3^+NCR1^+$ population was observed in PBMC in 79 % of the healthy dogs examined representing at the most 4.8 % of the lymphocyte population. NCR1 was also demonstrated on neoplastic cells from a dog with a $CD3^+$ $GranzymeB^+CD8^+$ leukaemia. $CD3^-NCR1^+$ cells may represent canine NK cells.

Paper II

NCR1 is an activating receptor expressed on a subset of canine NK cells
Grøndahl-Rosado C, Boysen P, Johansen GM, Brun-Hansen HC, Storset AK.

In this study, canine NK cells are characterized as $CD3^-GranzymeB^+$ cells, further divided into $NCR1^+$ and $NCR1^-$ subsets. All dogs examined displayed both subsets in blood, although of quite variable magnitude. Following vaccination an increase was observed in the $CD3^-$ $NCR1^-$ cell population in blood, but not in the $CD3^-$ $NCR1^+$ population. Non-B non-T cell cultures stimulated with IL-2 and IL-15 were dominated by $CD3^-GranzymeB^+$ cells after

approximately two weeks and a large proportion of the $CD3^-GranzymeB^+$ cells expressed NCR1. IL-12 stimulation lead to a further upregulation resulting in an almost uniform expression of NCR1. The cultured cells expressed MHC class II, showed a variable expression of CD8 and were negative for CD4 and CD21. The cultures were able to kill known NK cell targets, and NCR1 was shown to be a major activating receptor. A proportion of the $NCR1^+$ cells, but none of the $NCR1^-$ cells, produced IFNγ in response to IL-12 stimulation. These results show that NCR1 defines two subsets of canine NK cells, likely to represent different activation stages, and that NCR1 acts as an activating receptor on canine NK cells.

Paper III

Lymphoproliferative Large Granular Lymphocyte (LGL) disorders in three dogs

Christine Grøndahl-Rosado, Lars Moe, Grethe M. Johansen, Anne K. Storset, Hege C. Brun-Hansen

Clinical, haematological and immunophenotypic findings in three dogs with lymphoproliferative LGL disorders are presented. Two dogs were diagnosed with T LGL leukaemia displaying a $CD8^+MHCII^+NCR1^-$ phenotype, of which one was additionally diagnosed with a non-B non-T cutaneous lymphoma. These two dogs had an acute clinical course and were euthanized shortly after initial presentation. The last dog presented most likely had a reactive lymphocytosis concurrent to a carcinoma.

UNPUBLISHED MATERIAL

Generation of a mouse anti-canine NCR1 monoclonal antibody

Background

In the presented thesis a mouse anti-bovine NCR1 antibody (AKS6) [220] cross reactive with canine NCR1 has been used. The AKS6 antibody however was applied in methods like western blot and in immunomagnetic cell isolation without success. In contrast, AKS6 can be used for cell separation of bovine NCR1$^+$ cells. A likely explanation for the poor performance is a low affinity between the canine NCR1 and the AKS6 antibody as this antibody is not specifically made for the canine NCR1.

Based on these observations we wanted to generate a mouse anti-canine NCR1 antibody.

Materials and Methods

An expression construct consisting of the extracellular region of the canine NCR1 at the N-terminal side of the Fc region of mouse IgG2b was created.

Canine NCR1 genomic sequences were identified by homology searches in GenBank with human and bovine NCR1 exon sequences. Primers were designed using Primer3 software [221]. Sequences were analysed and aligned by use of Vector NTI, Life Technologies (Thermo Fisher Scientific, Carlsbad, CA, USA) and Clustal W (EMBL-EBI, Cambridgeshire, UK)

PBMC from canine EDTA blood was isolated and stimulated with recombinant human IL-2 (25ng/ml final concentration, eBiosciences, San Diego, CA, USA) for 5 days before mRNA isolation with MACS mRNA isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany) followed by oligo dT primed cDNA synthesis using Superscript ® Master mix, Life Technologies (Thermo Fisher Scientific).

A part of the gene encoding NCR1 was then PCR amplified using primers designed to span the region encoding the extracellular fragment of *cf*NCR1:

Forward primer with HindIII restriction site underlined:
AAGCTTCTCCTCCATATGTGCCTGGT

Reverse primer with BamHI restriction site underlined:
GGATCCGCTTGCTGGAATTCTGCTTC

The PCR product of the appropriate size of 765 base pairs, was cloned into a pCR2.1TOPO vector, amplified in *E. coli*, purified (Qiagen miniprep, Hilden, Germany) and sequenced with vector specific primers (M13F and R). The gene segment encoding the extracellular domain of *cf* NCR1 was then released by BamH1 and HindIII digestion and ligated into the mammalian expression vector pMIg1 containing the hinge, C_H2 and C_H3 regions of the mouse IgG2b gene (obtained from H. C. Aasheim, The Norwegian Radium Hospital, Oslo, Norway) using T4 DNA Ligase, Invitrogen (Thermo Fisher Scientific). The *cf*NCR1-PMIg1 construct was then amplified in *E. coli*, purified (Nuckeoband ® Xtra Midi EF, Macherey-Nagel, Düren, Germany) and sequenced using vector specific primers (T7 and MIg1) before it was transfected into 293T cells using Lipofectamine, Invitrogen (Thermo Fisher Scientific) following the manufacturer`s instructions. Lipofectamine and DNA in Opti-MEM medium (Thermo Fisher Scientific) was incubated for 20-30 minutes and transferred to a 160 cm^2 flask containing a ~70 % confluent layer of 293T cells. After six hours RPMI 1640 supplemented with 10 % FCS was added. After 18 hours the medium was removed and replaced by 100 ml of AIM-V medium (Thermo Fisher Scientific). The supernatant was collected and after four days *cf*NCR1-mFcγ2b fusion protein was purified from the culture supernatant on a protein G column (Amersham Biosciences, Little Chalfont, GB) according to the manufacturer's instructions.

Three female young adult BALB/c mice were immunized by four intraperitoneal injections of 250 µg fusion protein in Freund`s complete (first injection) or incomplete adjuvant (following two injections, Invitrogen) or in PBS (final injection). Six weeks after the first immunization the mice were sacrificed and spleen cells were fused with NS-0 cells by conventional techniques.

Single clone hybridoma supernatant was screened using flow cytometry for reactivity towards 293T cells expressing the entire NCR1 protein with a FLAG tag at the N-terminal end. Creating the NCR1 FLAG construct was described in paper I. Mab ANTI FLAG M2 IgG1 (Sigma-Aldrich) was used to confirm complete expression of the product of the transfected gene and used as positive control during hybridoma screening

Results

Blood samples were taken from the three immunized mice before the mice were sacrificed. Serum was screened towards ConA stimulated PBMC from two dogs finding that serum from all three mice bound to a protein expressed on ConA stimulated canine cells (Fig. 4 A and B).

The serum was further tested towards *cf*NCR1FLAG expressing 293T cells showing that all three mice had produced antibodies binding to the screening cells (Fig. 4 C). Roughly 400 hybridoma clones were tested but no clones showed the same staining pattern as the ANTI FLAG M2 IgG1 mAb. A negative clone is presented in Fig. 4 D. Approximately five to six clones showed a moderate right shift but the same right shift was observed when screening the clones towards untransfected 293T cells. This could indicate that the mice had developed antibodies towards other proteins expressed by 293T cells in which the recombinant extracellular *cf*NCR1 protein used for immunization of the mice was made. The observed staining could also represent unspecific binding. Consequently, we did not succeed making a mouse anti-canine NCR1 antibody.

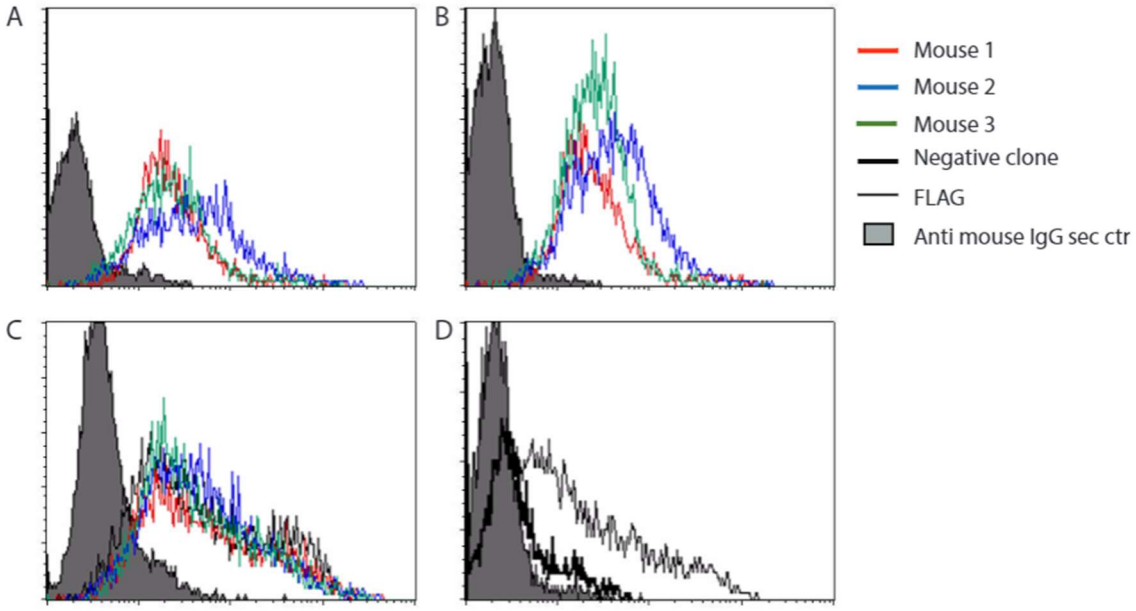

Fig 4. A + B Con A stimulated PBMC from two different dogs screened towards serum from three mice immunized with *cf*NCR1 recombinant protein. C *cf*NCR1 transfected 293T cells screened towards serum from three mice immunized with *cf*NCR1 recombinant protein. D A representative example of a negative clone.

Generation of recombinant canine IL-2

Background

In most studies published characterizing canine LAK cultures the cultures are obtained using recombinant human IL-2. It is likely that the proliferation and expansion of NK cells in cultures are more pronounced using species specific cytokines [154,155]. As IL-2 is important for proliferation and survival of NK cells [101] we wanted to generate recombinant canine IL-2 to facilitate the establishment of canine NK cell cultures.

Materials and Methods

Cloning and expression of canine IL-2

cDNA was obtained using the same method as described for the generation of the mouse anti-canine NCR1 antibody. Primers were designed to span the entire coding region of canine IL-2:

Reverse primer with Xho1 restriction site underlined:
CTCGAGGTAGCAAAACGTACATCAAA

Forward primer with BamHI restriction site underlined:
GGATCCTCAACTCCTGCCACAATGTA

The PCR product gave a band around 580 base pairs corresponding to the theoretical mass of canine IL-2 estimated to be 574 base pairs (Fig. 5 A). The PCR product was cloned into a pCR2.1TOPO vector, amplified in *E. coli* and purified (Nuckeoband ® Xtra Midi EF, Macherey-Nagel). The gene segment encoding the entire coding region of IL-2 was then released by BamH1 and Xho1 digestion and ligated into the CIP treated expression vector pcDNA3.1. The vector containing the *cf*IL-2 gene was amplified in *E. coli*, purified (Nuckeoband ® Xtra Maxi EF, Macherey-Nagel) and sequenced with vector specific primers (T7 and BGH reverse) before it was transfected into 293T cells using Lipofectamine following the manufacturer`s instructions. The supernatant was removed after two to four days, centrifuged to remove cell debris and stored at –18 °C.

^{3}H (Tritium) proliferation assay

Proliferation of canine cells in response to recombinant canine IL-2 was evaluated using ^{3}H (Tritium) proliferation assay.

PBMC was isolated from canine EDTA blood and stimulated with ConA for four days to allow upregulation of the IL-2 receptor. The cells were then washed and counted, and 8 x 10^4 cells were placed in microtiter plates in 100 µl medium. Then the supernatant containing rcIL-2 was added in dilutions from 1:10 to 1:10^5 tested in triplicates and incubated at 37 °C overnight. The cells were then pulsed with ^{3}H and left for 16 hours before harvesting the supernatant and radioactivity was measured using a microplate scintillation counter.

Results

The recombinant canine IL-2 gave a proliferation of ConA stimulated canine PBMC in a dose dependent manner (Fig. 5 B). However, the concentration achieved from the supernatant of transfected cells was not sufficient to give a better result than commercially available rhIL-2. Two batches were produced yielding similar results.

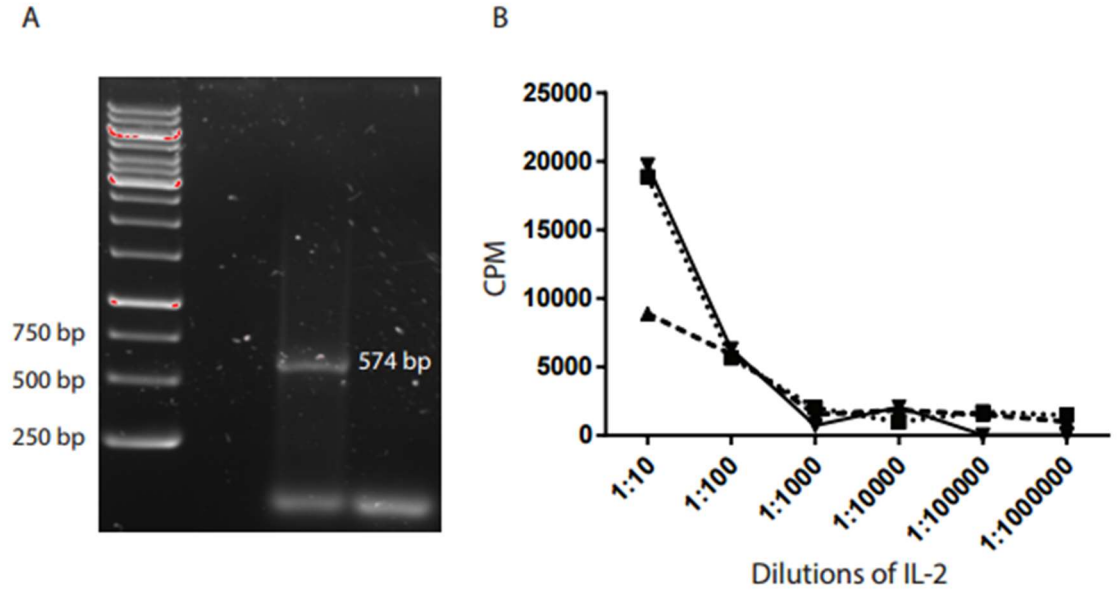

Fig. 5 A Gel showing a band approximately sized 574 bp. Ladder used was" O`GeneRuler^{TM} 1 kb DNA Ladder, ready to use" (Thermo Fisher Scientific) B Dilution series of IL-2 containing supernatant tested towards ConA stimulated PBMC from three dogs.

Generation of a *cf*CD16FLAG construct to screen CD16 antibodies

Background

CD16 is a low affinity FcγRIII receptor found on NK cells, neutrophils, monocytes and macrophages in humans. This receptor binds to the Fc region of antibodies and therefore initiate ADCC towards IgG coated cells. In humans NK cells are subdivided according to their CD16 and CD56 expression into two major subsets: $CD56^{bright}$ $CD16^{dim/-}$ and $CD56^{dim}$ $CD16^{+}$ [222]. These two subsets are functionally different. We wanted to investigate if CD16 could be used in characterizing canine NK cells.

The 3G8 clone is a mouse anti-human CD16 antibody promoted as cross-reactive with CD16 from assorted monkeys showing that it binds to an epitope well conserved among primates. The 3G8 antibody has earlier been described as cross reactive to canine CD16 [223]. In this study 3G8 stained canine neutrophils, but not eosinophils. The 3G8 antibody is probably also used in another study, but here the clonal designation is not specified [224]. The 3G8 antibody has not been used in any recent studies of canine leukocytes and when discussing CD16 expression on potential canine NK cells, quantification of CD16 encoding mRNA measured by qPCR is used and not flow cytometry [148–150,154,225]. The producer does not sell the 3G8 as an antibody cross reactive to canine CD16.

The KD1 antibody is an antibody binding to CD16 in humans, cattle, sheep, horses and pigs and therefore the KD1 antibody probably binds to a well conserved epitope between species. It does not however, bind to CD16 in rats.

Materials and Methods

A *cf*CD16FLAG construct was generated using the same method as described for the *cf*NCR1FLAG construct. Primers used to amplify the entire *cf*CD16 gene segment were:

Forward primer: CCTTCAGAGCTCGGCATATC

Reverse primer: TCCAGAGAGAGGTCCAGAGG

And primers containing HindIII and BamHI restriction sites:

Forward primer with HindIII restriction site underlined:

AAGCTTCAAGCTGATGTCCCAAAGGC

Reverse primer with BamHI restriction site underlined:
GGATCCCTAAGAGCCCTGGCTCCATA

Three different antibodies were screened towards 293T cells expressing *cf*CD16FLAG:

1) Mouse anti-human CD16 mAb, clone GRM1 from Southern Biotech
2) Mouse anti-human CD16 mAb, clone KD1 from AbDSerotec
3) Mouse anti-human CD16 mAb (IgG1), clone c127, a kind gift from Dr. Alessandro Moretta, Instituto Nazionale per la Ricerca sul Cancro, Genova, Italy

The antibodies were also tested both towards the lymphocyte and granulocyte gate in canine leukocytes using flow cytometry. Red blood cells were removed by cold water haemolysis. Using this method, the following antibody was also tested:

4) Mouse anti-human CD16 mAb, clone 3G8 from BioLegend

Results

The KD1 and c127 CD16 antibody did not bind to *cf*CD16FLAG. The GRM1 antibody showed a similar profile compared to the FLAG antibody binding to the FLAG tag indicative of cross reactivity (Fig. 6 A). However, when screening the GRM1 antibody towards canine leukocytes, no binding was observed (Fig 6 B). As CD16 is an Fc receptor it might be that GRM1 bound to the transfected *cf*CD16FLAG with the Fc region and not the antigen specific Fab region. We also tested the 3G8 antibody towards canine leukocytes not finding convincing staining of canine leukocytes (Fig. 6 B). The GRM1 antibody did not stain $CD5^-$ $CD3^-$ cell cultures (results not shown). Therefore, we did not proceed our investigation of CD16 expression in canine NK cells.

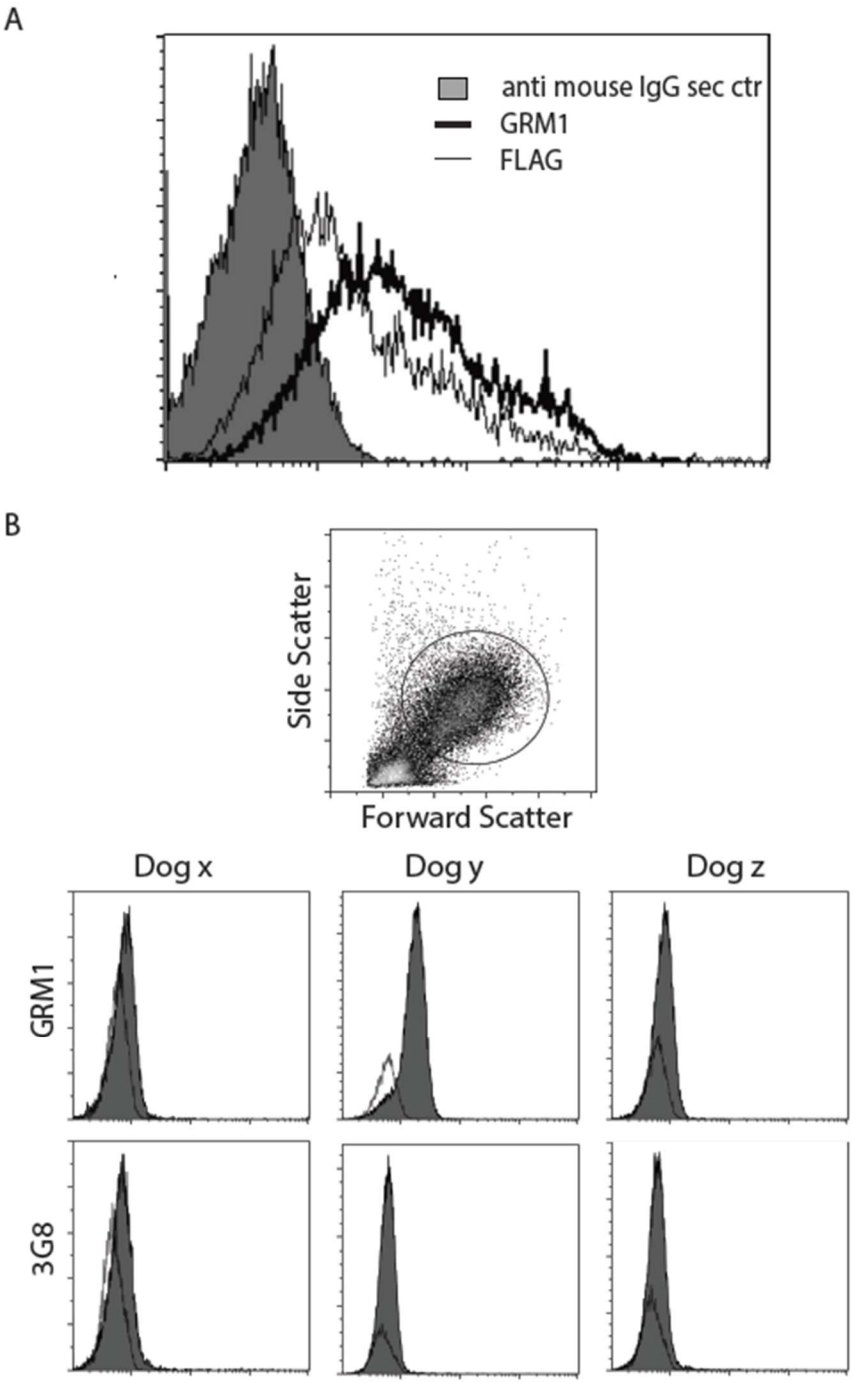

Figure 6 A Screening of the GRM1 clone towards *cf*CD16FLAG transfected 293T cells. B Testing of GRM1 and 3G8 towards canine leukocytes. Forward vs Side Scatter plot shows gating on granulocytes confirmed by CD14 expression (results not shown) and histograms showing GRM1 and 3G8 respectively towards leukocytes from three dogs.

METHODOLOGICAL CONSIDERATIONS AND GENERAL DISCUSSION

Collecting tissues from dogs

Collecting biological material from privately owned pets is time consuming. When working with farm animals, collection of large quantities of biological material from healthy individuals can be done in slaughter houses. Working with laboratory animals enables collection of tissues in an organized fashion within ethical guidelines. Collection of normal tissues from healthy individuals is, as in human medicine, not ethically acceptable in dogs. In order to investigate canine NK cells in different organs, biopsies were taken from a small number of dogs shortly after euthanasia with the consent of the owner and analysed within a couple of hours. For practical reasons analyses presented in the paper I and II are therefore predominantly done on circulating lymphocytes as blood can easily be taken without inflicting stress or pain. Also, repeated samples can be taken from the same dog over a period of time. Because a certain amount of blood was required at each sampling for many of the analysis performed, only medium and large breeds were selected to participate.

Isolating lymphocytes in dogs

Canine PBMC was isolated from EDTA blood using Lymphoprep ® from Axis Shield. Lymphoprep ® is an isosmotic medium with a density of 1.077 used to isolate PBMC in humans. Polymorph nucleated cells and red blood cells sediment through the medium while the mononuclear cells have a lower density and are therefore left in the interphase above the Lymphoprep medium after centrifugation. After isolation canine PBMC was analyzed using flow cytometry or put in cultures after cell depletion. However, on forward scatter vs side scatter plot a variably sized population with granulocytes appeared in some individuals. Cytospin preparation showed that these cells represented eosinophils. Consequently, eosinophils and mononuclear cells were found in the same interphase while neutrophils were sedimented together with red blood cells. Because of variable numbers of eosinophils in blood from normal dogs, this population also varied in size confirmed on CBC analyzed on ADVIA 2120 (Siemens, Munich, Germany). Problem shooting was performed both in preparation of blood before loading and conditions for centrifugation without successfully eliminating eosinophils. Therefore, one has to be aware that using this method does not result

in an elimination of all polymorph nucleated cells in blood. Consequently, canine cells isolated from the top layer of a 1.077 gradient can strictly speaking not be termed PBMC even if this has been done in this thesis and also elsewhere in the literature.

Validation of antibodies

General Aspects: Commercially made antibodies – a friend or a foe?

Monoclonal antibodies are an important tool in research and diagnostics to detect specific proteins. Antibody failure can have great consequences, both economically and for patients wrongly evaluated. Even so, there is no common agreement on standardization of antibody validation and all methods applied in antibody validation has their short comings. Validation implies "demonstrating that the antibody is specific, selective and reproducible in the context for which they are used" [226].

Commercially produced antibodies often have sparse documentation available. Detailed information about antibodies are considered trade secrets and therefore not made accessible for the consumers to evaluate. Critical evaluation of antibody specificity has started to emerge parallel to the growing realization that reproducibility in biomedical research is low. One group evaluated reproducibility of 53 landmark studies within cancer research finding that only six studies could be confirmed [227]. A study evaluating 5436 antibodies from 51 different antibody providers found that half of the antibodies did not perform as expected [228]. Two studies testing antibodies in knockout mouse lacking the target protein showed that staining patterns were similar to the wild type in a majority of the antibody tested [229,230]. Taken together, this demonstrates that many commercially available antibodies are poorly defined. A shortcoming of some of these studies was that a proportion of the antibodies were not validated for the methods used, but this also illustrates the need for validation for each method in which the antibody is going to be applied. Different methods provide different experimental conditions and this can influence how the target protein folds. If the binding site of the antibody is changed, the antibody may not bind to its target. Some antibodies might therefore recognize the native protein, but not the denatured one and vice versa. Different batches of the same antibodies have also been reported to not give comparable results [231,232]. Professor David Rimm, a pathologist from Yale University, developed a test based on commercially available antibodies. Particular staining patterns on biopsies from patients with melanomas would help to decide whether the patients needed aggressive therapy or not. However, when a new stock of antibodies was purchased, the new batch did not give the

same result even if the clone and producer was the same. It was therefore impossible to reproduce the original results and a potential lifesaving method had to be rejected [233]. It is not common for manufacturers to validate each batch of antibodies and therefore documentation presented for the validation process is most often from previous batches. Reproducibility is an important factor in all aspects of research. Antibodies must therefore perform as predicted on different time points and between batches.

Validation procedures – an overview

Western blot is a commonly used method to determine if a given antibody binds to the target protein. Specificity for an antibody can be tested using a synthetic, recombinant or purified target protein. Using a synthetic protein, the amino acid sequence is known, but it is hard to ensure that a synthetic protein assumes the same 3D structure as the native protein. A single band at the predicted molecular weight is a good indication that the antibody binds to the correct protein. However, post-translational modifications or different splice variants can cause multiple bands to show up [226]. Blocking peptides can be used evaluating antibody specificity. A blocking protein is added to a tissue known to express the target protein [234]. If staining is present, then this is indicative of unspecific binding. This method can therefore detect if an antibody is unspecific, but it cannot prove that the antibody binds to the target protein[226]. Defining the antibody down to the DNA level has been proposed to ensure reproducibility, but even sequential data does not ensure that the antibody actually works [233].

To prove absence of cross reactivity can be challenging. As mentioned earlier, there has been studies using knockout mice to test if antibodies only bind to the target protein. A less time consuming method is to test the antibody, not only towards the target protein but also towards lysates from a number of different cell lines expressing a big variety of proteins. Also important in validation of antibodies is correctly used controls. Every assay should have both a positive and a negative control. Knockout cells represent the most optimal negative control, while transfected cells provides the best positive control [226].

There are plenty of good antibodies out there, but how can we know which ones to trust? And to what degree should suppliers be responsible for validation? Agreement of a common validation procedure would be useful so researchers could know what to expect from a commercially bought antibody independent of supplier. Algorithms for validation of antibodies have been suggested [226] but implication of such systems in the industry remains to be seen. For now, websites like antibodypedia.com and antibodyregistry.org are emerging

making it easier to find and share relevant information about both commercially available and home-grown antibodies. A website called pAbmAbs.com is an independent website for antibody reviews were researchers can share experiences and report results, both positive and negative. Very few antibodies within veterinary research are reviewed on these websites. It is however, very likely that we are facing the same challenges.

Validation of antibodies used in this study – the good, the bad and the ugly

In this thesis monoclonal antibodies are involved in a majority of the methods presented in all three papers. In veterinary research, and especially research in dogs, the limited selection of antibodies represents a restriction. Therefore, we are at the mercy of a handful of producers and there are seldom any alternatives if an antibody does not perform as expected. Most antibodies applied in the presented thesis are commercially produced antibodies. The majority of these are canine specific antibodies but some are made for other species and supposedly, cross-react with the canine equivalent.

Mouse anti-canine CD3 mAb (CA17.2A12)

The mouse anti-canine CD3 antibody CA17.2A12 supplied by AbDSerotec has been a source of frustration. Sparse with documentation of validation procedures of this antibody is available through AbDSerotec. On their website, the CA17.2A12 antibody is reported to work in flow cytometry among other methods, and the antibody is a valuable tool in diagnosing T cell lymphomas referring to a minor study [235]. A histogram from flow cytometric evaluation of canine lymphocytes is demonstrated showing a biphasic population. Eighteen references are sited referring to publications using the CA17.2A12 antibody in research settings, but neither of them present validation procedures. The CD3 CA17.2A12 antibody has in our hands shown great variability from batch to batch and individual variation between dogs (Fig. 7).

The CA17.2A12 antibody vial is not marked with an expiry date. However, after purchase the quality of the results given by this antibody gradually declines. The fridge used to store antibodies in our lab has a temperature control hence unstable temperature during storing can be ruled out. Also, combining the CD3 antibody with other antibodies in flow cytometry has been problematic because of unspecific binding. As a result, we did not obtain reliable data on the $CD3^{+}NCR1^{+}$ cell population from most dogs analysed in paper I. When using CD3 in multi-colour stainings we have incubated this antibody last to avoid interactions. Even when using this method we have observed differences in the number of positive cells and

differences in the profile of the histogram when compared to single colour staining. To conclude, results from assays using the mouse anti canine CA17.2A12 antibody need to be evaluated with care.

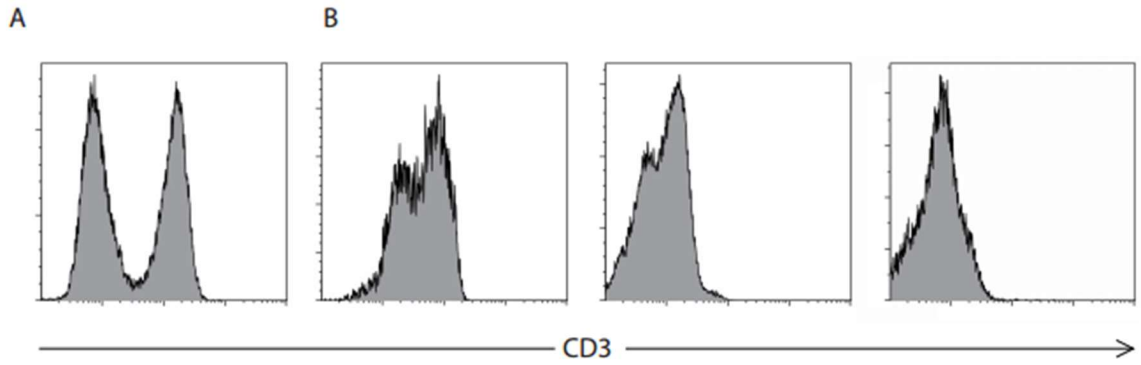

Fig. 7 Histograms showing different profiles for the CA17.2A12 CD3 antibody in healthy dogs. The cells are gated on lymphocytes and doublets are excluded. A An ideal histogram with an evident separation between the negative and positive population. B Histograms showing variable separation of negative and positive populations in three dogs demonstrating individual variations as the three presented graphs are from the same batch (0712).

Mouse anti-human CD25 mAb (ACT-1) and mouse anti-canine CD25 mAb (P4A10)

There are two antibodies on the marked binding to canine CD25. One cross-reactive mouse anti-human (ACT-1) supplied by Dako and one mouse anti-canine (P4A10) supplied by eBioscience. According to the latter, the P4A10 antibody has been reported to bind to the same epitope as ACT-1 but with greater affinity.

Validation for both of these antibodies are published in peer reviewed journals. The ACT-1 antibody is validated in two different studies. Both studies use cell lines overexpressing *cf*CD25 to confirm cross reactivity. One study uses 293T [236] cells and the other uses HeLa cells [237]. Both studies proceeded using the ACT-1 antibody in characterization of canine T regulatory cells (Tregs). In the former study, the ACT-1 antibody was tested towards unstimulated PBMC finding no binding of this antibody to non-stimulated lymphocytes. After ConA stimulation the antibody bound to more than 50 % of the lymphocytes. In the latter study CD25 expression was evaluated on Tregs in healthy dogs and dogs with osteosarcoma. This study showed one representative healthy dog where the ACT-1 antibody stained around 3 % of the lymphocytes. The P4A10 antibody was validated by using flow cytometry and western blot [238]. The antibody bound to a protein 55 kDa in size and in flow

cytometry it bound to 4.9 ± 2.1 % of the lymphocytes in PBMC. After PMA stimulation for 24 hours the proportion of P4A10 positive cells increased to 60 %.

Both ACT-1 and P4A10 were used in this study of canine NK cell. The ACT-1 antibody (batch: 00058284) was tested at several occasions towards rcIL-2, rhIL-2 and ConA stimulated PBMC yielding unexpected results. The ACT-1 antibody did not convincingly stain these cells.

Since the affinity of the P4A10 antibody to the canine CD25 was claimed to be better than for the ACT-1 antibody, the P4A10 antibody was tested. It was not possible to reproduce the reported results from the validation of the P4A10 antibody [238]. The P4A10 antibody bound between 0 and 25 % of lymphocytes in PBMC in healthy dogs (Fig. 8)

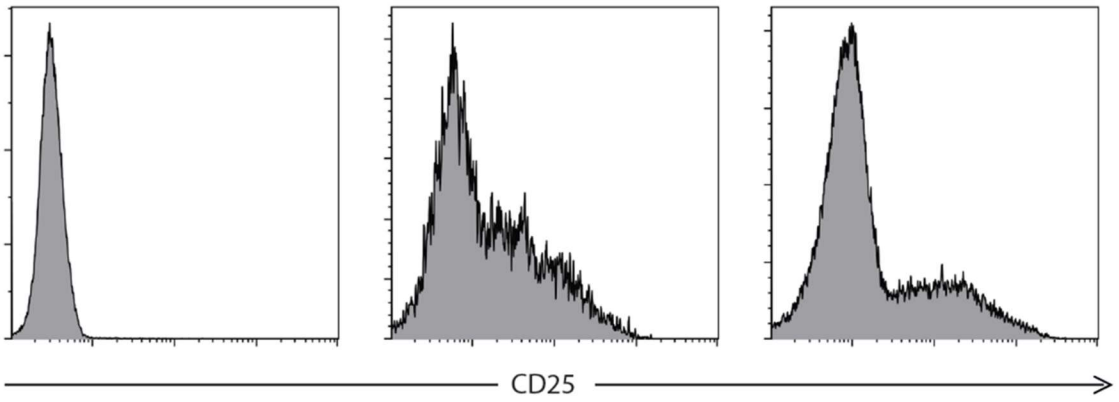

Fig. 8 Histograms showing different profiles for the P4A10 antibody of PBMC in three healthy dogs. The cells are gated on lymphocytes and doublets are excluded. These results are from batch E15239-101.

As it is unlikely that high affinity IL-2 receptor is expressed on 25 % of lymphocytes from PBMC in healthy dogs, this antibody probably binds unspecifically to non-CD25 proteins in some individuals.

After two weeks of IL-2/IL-15 stimulation of $CD5^-CD3^-$ cells a variable portion of the cultured cells were stained by P4A10. Some cultures where negative, many had between 10 and 30 % positive cells and one was completely positive for P4A10 (Paper II). It is expected that cell cultures obtained using the same method would express CD25 in a more consistent manner. 2 different batches of the P4A10 antibody (E15239-101 and E15239-102) were tried out giving similar deviating results.

Mouse anti-canine/human CD94 mAb (HP-3D9/ KLRD1/ KP43)

The CD94 antibody HP-3D9 is a mouse antihuman antibody sold as an anti-canine/human CD94 antibody. eBioscience refers to an article where 380 anti-human antibodies have been tested with flow cytometry towards canine blood to investigate if the antibodies stain canine cells [239]. In this paper the CD94 antibody is reported to stain a subpopulation of lymphocytes of unknown size and with no further characterization. In our hands this antibody did not stain cells in canine PBMC and all $CD5^{-}CD3^{-}$ cell cultures were negative except for one showing about 30 % positive cells.

Mouse anti-human granzyme B mAb (GB11)

In this study we chose Granzyme B as a marker for granulated lymphocytes using the mouse anti-human GB11 antibody. Granules also contain perforin and this marker was originally applied. The anti-human perforin antibody dG9 was tested, but did not stain canine lymphocytes. After personal communication with Dr. Dorothee Bienzle from The University of Guelph I learned that perforin is difficult to stain in canine lymphocytes as it is not expressed consistently. And, after personal communication with Dr Heiner Van Buttlar from The University of Leipzig I learned that the GB11 antibody against granzyme B had successfully been used in flow cytometry towards canine lymphocytes in his lab. The mouse anti-human granzyme B antibody GB11 was therefore used in this study. This antibody gave consistent results towards canine PBMC using flow cytometry and it did not show any variation between batches. To confirm that it bound intracellular to granulated cells we stained blood smears from a dog with $NCR1^{+}$ T LGL leukaemia (Paper I) using immunofluorescence technique. This demonstrated that the GB11 antibody bound in the intracellular department of large granular lymphocytes. Further characterization of canine $CD5^{-}CD3^{-}$ cultures where a majority of cells displayed an LGL appearance confirmed on cytospin preparation, showed that the percentage of $GB11^{+}$ cells corresponded well with percentage of LGLs observed by morphological observation (Paper II). However, neoplastic cells from dog 2 described in Paper III showed granzyme B expression on close to 100 % of cells using flow cytometry while morphological evaluation showed granules in approximately 20 % of the cells. As this dog had a $CD8^{+}MHCII^{+}$ T cell leukaemia, it is likely that all the neoplastic cells expressed granzyme B. Therefore, it could be that morphological evaluation sometimes underestimates the number of granulated cells and special stainings are required in some cases.

Michael et al. describing the culturing method applied in paper II did not detect granules in $CD5^-$ cultures established, even using the same staining techniques as we did [153]. This demonstrates that visualizing granules in canine LGLs is not straightforward as also demonstrated in other species [135].

Mouse anti-bovine NCR1 mAb (AKS6)

Making a mouse anti-canine NCR1 monoclonal antibody did unfortunately not end in success. Instead an in house cross reactive mouse anti-bovine NCR1 antibody, AKS6, [220] was used. AKS6 has been shown to cross react with NCR1 in sheep and it is therefore likely that the AKS6 recognizes an epitope well conserved across species. Cross reactivity for canine NCR1 was verified using flow cytometric evaluation of FLAG tagged *cf*NCR1 expressed by 293T cells showing that the AKS6 antibody gave a similar curve compared to an anti-FLAG antibody (Paper I). As the NCR1 showed an expected binding pattern in canine lymphocytes in flow cytometry we assumed that the recombinant protein made for screening was very similar to the native protein expressed by canine lymphocytes. AKS6 was tested in a western blot using *cf*-NCR1 FLAG and *bt*-NCR1-FLAG transfected 293T cells. This method did not give expected results for the bovine nor the canine NCR1 for unknown reasons (Paper I). However, using the anti-FLAG antibody gave a visualization of a 37 kDa protein in both dogs and cattle. A weak signal at around 47 kDa was also observed in both species. Human NCR1 is 46 kDa. The difference in weight observed here is probably due to differences in glycosylation between recombinant and native protein. This was consistent with what is previously demonstrated in cattle [135]. Consequently, the AKS6 antibody bound to a protein of correct size, but this was only showed indirectly using the anti-FLAG antibody.

Secondary antibodies and negative controls

When working with small cell populations and unreliable antibodies predictable secondary antibodies and negative controls are important. During this PhD period secondary antibodies and negative controls from different suppliers have been used finding differences in quality. Only secondary antibodies from Southern Biotech detected AKS6 on canine lymphocytes. Secondary antibodies from this producer has also shown to be the most reliable detecting other primary unconjugated antibodies in other species in our lab. Secondary antibodies from Invitrogen did not stain AKS6 convincingly. Negative controls have also shown variability between batches and producers. Commercially available negative controls are absorbed against human and murine cells and therefore, unspecific binding can be observed when used on non-human, non-murine cells. All antibodies tested showed a variable background and the

degree of background varied between individual dogs. No optimal negative control has been identified for canine flow cytometry. However, negative controls from AbDSerotec showed the most consistent results in our experiments.

Table 1 summarizes all antibodies applied in this study.

mAb	Clone	FC	WB	IMCS	IF
CD3	CA172A12	2	-	2	2
CD4	YKIX302.9	3	-	-	-
CD5	YKIX322.3	3	-	3	-
CD8	YCATE55.9	3	-	-	-
CD11c	CA11.6A1	3	-	-	-
CD14	Tük-4	3	-	-	-
CD16	GRM1	1	-	-	-
CD16	3G8	1	-	-	-
CD16	C127	1	-	-	-
CD16	KD1	1	-	-	-
CD21	CA2.1D6	3	-	-	-
CD25	P4A10	2	-	-	-
CD25	ACT-1	2	-	-	-
CD56	MOC-1	3	-	-	-
CD94	HP-3D9	1	-	-	-
Perforin	dG9	1	-	-	-
Granzyme	GB11	3	-	-	3
MHCII	YKIX334.2	3	-	-	-
NCR1	AKS6	3	1	1	2
IFNγ	CC302	3	-	-	-

Table 1. A review of all antibodies used in this study. FC = flow cytometry, WB = Western blot, IMCS = immunomagnetic cell separation, IF = immunofluorescence. 1 = does not work in hour hands, 2 = works, but critical evaluation of results is necessary, 3 = gives consistent results. (–) Not performed

Establishing cultures dominated by canine NK cells

As the AKS6 did not perform well in immunomagnetic separation, other approaches were tested in an attempt to isolate and culture canine NK cells. Mononuclear cells from both blood and lymphoid organs were stimulated with different combinations of human and canine

recombinant cytokines without giving reproducible results. Also CD3 depletion of PBMC was sought.

In paper II cultures dominated by canine NK cells were described. The method reported by Michael and colleagues was used [153] depleting $CD5^+$ cells and then the remaining cells were stimulated by recombinant human IL-2 and IL-15. The cultures obtained by Michael and colleagues were harvested and characterized after 14 or 21-25-days. At this time point, the cells were $CD3^-CD5^-CD4^-MHCI^+CD45^+$. Approximately 80 % expressed MHCII and around 10 % expressed CD8. Three ± seven % expressed CD94. In this article, there was no description of $CD3^+$ cells emerging, though an incomplete T cell depletion was mentioned for one culture eliminated from the study. However, in the present work a CD3 population reaching as much as 64 % was observed in many of the cultures (Fig. 9)

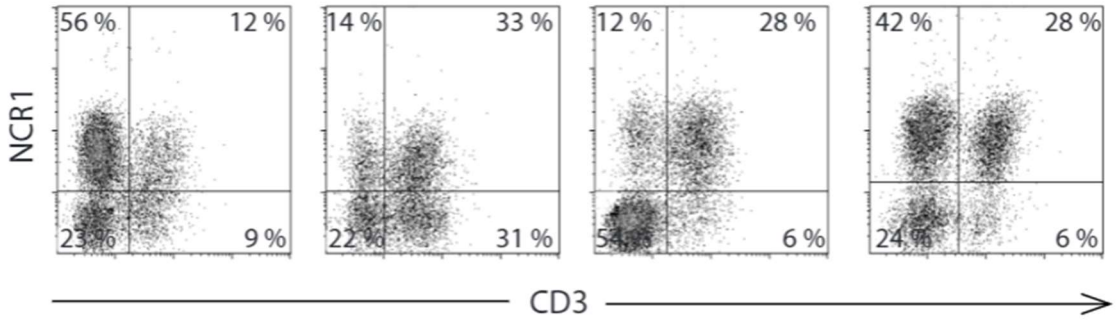

Fig. 9 CD3 vs NCR dot plots from two week $CD5^-$ cultures before CD3 depletion. Cells are gated on lymphocytes after exclusion of doublets and dead cells.

This could be explained by $CD3^+$ and $CD5^+$ cell populations not being completely overlapping. $CD3^+$ cells in dogs can be divided into a $CD5^{low}$ and a $CD5^{high}$ fraction [148]. It is possible that CD5 expression on $CD5^{low}$ cells is not dense enough for this cell population to be eliminated by immunomagnetic depletion and consequently $CD3^+CD5^{low}$ cells are included in the $CD5^-$ cultures. Therefore, after depletion of $CD5^+$ cells one is left with a heterogeneous population of NK cells, B cells, eosinophils (explained earlier), monocytes and probably $CD5^{low}CD3^+$ cells. Using an initial CD3 depletion would have been more optimal as CD3 is a better universal T cell marker than CD5 but problems with the CD3 antibody made this difficult. This issue is discussed by Michael and colleagues. However, in the present work the CD3 antibody worked satisfactory in the depletion of $CD3^+$ cells in established cultures, although this procedure did result in a loss of cells much greater than the $CD3^+$

population size estimated with flow cytometry. This made it hard to perform many functional assays on the same cultures.

Among the $CD3^+$ cells observed before CD3 depletion was performed, a large portion expressed NCR1 (Fig. 9). The study describing $CD5^{low}$ and $CD5^{high}$ cells in dogs reported an increased natural cytotoxicity and IFNγ production within the $CD5^{low}$ subset. Therefore, it is plausible that the $CD3^+NCR1^+$ cells observed in the cultures originate from a $CD5^{low}$ subset described in this study [148]. After CD3 depletion, the vast majority of the cultured cells expressed a $CD3^-GranzymeB^+$ phenotype and a subset of these expressed NCR1. They were also $MHCII^+$, had variable expression of CD8 and were negative for CD4 and CD21. The $NCR1^+$ and $NCR1^-$ cells roughly constituted the same percentage as observed in PBMC showing that $CD3^-GranzymeB^+NCR1^-$ cells seems equally responsive to IL-2 and IL-15 as $CD3^+GranzymeB^+NCR1^+$ cells.

AKS6 was applied at several occasions in immunomagnetic selection of cultured $CD5^-CD3^-$ cells in order to establish $NCR1^+$ and $NCR1^-$ cell cultures. Both Dynabeads (Thermo Fisher Scientific) and MACS beads (Miltenyi Biotec) were tested without success.

The cultures presented in paper II consisted of cells with an LGL appearance in cytospin preparations stained by modified Wright`s stain. In phase-contrast microscopy adherent accessory cells were observed, probably representing monocytes in additional to cells with a typical NK cell appearance. It has been reported that presence of monocytes can enhance proliferation of human NK cells. This enhancement of proliferation is both caused by production of soluble factors and direct cell to cell contact [240,241]. If cultures were to be established by positive selection of NK cells in the future, additional feeder cells could be necessary.

$CD3^+$ cells expressing NK cell markers and displaying NK cell activity should be termed NKT-like lymphocytes.

Many descriptions of canine natural cytotoxicity in established cell cultures have been reported, but there seem to be some inconsistency of the basic definition of NK cells in canine immunology. NK cells are clearly defined as $CD3^-$ in the literature [123] and this definition should also be applied for dogs. Several publications however present $CD3^+$ cells as potential canine NK cells.

A study describing $CD5^{low}CD3^+$ cells with NK cell characteristics has already been discussed. These cells displayed LGL morphology and after IL-2 stimulation of PBL, they displayed cytotoxic activity towards CTAC and after IL-12 stimulation, they had the ability to produce IFNγ [148]. Also NK cytotoxic activity towards CTAC in IL-2 stimulated $CD3^+CD8^+$ cells have been described [149]. Both these studies demonstrated an increase in mRNA encoding assorted NK proteins measured by qPCR after cytokine stimulation. Another study described *ex vivo* expansion of canine LGLs exhibiting NK cell characteristics [150]. By quantifying mRNA by qPCR, a remarkable upregulation of NCR1 was observed compared to PBMC. The *ex vivo* expanded cells showed a $CD3^+CD8^+TCR\alpha\beta^-TCR\gamma\delta^-CD4^-CD21^-$ phenotype and was negative for invariant T cell receptor α chain. Cytotoxic activity towards CTAC was further demonstrated.

In these studies, no depletion of T cells was performed before cytokine stimulation and therefore it is likely that T cells dominate a majority of cultures established using this method. However, one of the studies also tested the expanded cells for TCR expression showing that the $CD3^+$ cells did not express TCRαβ or TCRγδ [150]. CD3 is a co receptor for TCR and together they form the TCR complex. CD3 expression without TCR is to the author's knowledge not described.

In the literature spontaneous killing of CTAC is referred to as a property specific for NK cells and several studies describe putative canine NK cells based on this observation (Ringler and Krakowka 1985; Knapp et al. 1993). Traditional T cells do not show spontaneous cytotoxic activity towards CTAC [148,150]. Several reports of canine $CD3^+$ cells spontaneously killing CTAC however exists. Taken together, the evidence for $CD3^+$ cells having NK cell characteristics in dogs are overwhelming. Describing CTAC as a strict NK cell target is consequently not accurate. $CD3^+$ cells expressing NK cell markers and/or displaying functional NK cell characteristics should be termed NKT like cells and not NK cells.

A similar $CD3^+NCR1^+$ cell population displaying many of the same features has also been described in bovines [91], swine (Mair et al,, unpublished results), humans and mice [120].

$NCR1^+$ lymphocytes in healthy dogs and in dogs with assorted diseases

NCR1 expressing lymphocytes were demonstrated in the circulation and in lymphoid organs like lymph nodes, spleen and liver (Paper I). NCR1 was detected on both $CD3^-$ and $CD3^+$ lymphocytes and therefore, NCR1 is not exclusively expressed by NK cells in dogs. The

$CD3^-NCR1^+$ cell population was presented as putative canine NK cells further phenotyped as $GranzymeB^+CD56^-CD8^-$. Great variations were seen in the relative number of circulating $CD3^-NCR1^+$ cells between individuals and within the same individuals in blood samples taken over a time period of 1 year. The $CD3^-NCR1^+$ cells accounted for a smaller population than NK cells in other species and in a few dogs no $CD3^-NCR1^+$ cells were observed. Percentages of NK cells in circulation and in lymphoid organs in cattle and humans compared to $CD3^-NCR1^+$ cells in dogs are presented in table 2.

Tissue	Dog	Human	Cattle
Circulation	0-2.5 %	7-31 % [242]	0.5-10 % [243]
LN	0.3-3.1%	5 % [76]	4.7-13 % [244]
Spleen	1.0-4.6 %		5.9-6.9 % [244]
Liver	0.9-1 %	20-30 % [31]	

Table 2 – Relative numbers of canine $CD3^-NCR1^+$ cells in the circulation and in lymphoid organs compared to values reported in NK cells in humans and cattle. The dogs evaluated had assorted diseases not connected to the organ presented.

In paper I blood and tissues from dogs with assorted diseases were evaluated. In the majority of cases, the $CD3^-NCR1^+$ measurements did not deviate from what was seen in healthy individuals. However, 2 dogs with gastrointestinal disease displayed 3.2 and 6.8 % $CD3^-NCR1^+$ cells in the circulation. Also in a liver from a dog with portosystemic shunt (PSS), 23.8 % $CD3^-NCR1^+$ cells were measured, although this is considered a normal number in the human liver. In dogs without liver pathology, only 1 % of lymphocytes were found to display a $CD3^-NCR1^+$ phenotype. Elevated numbers of NK cells in the liver have been observed during hepatic infections and NK cells are also shown to be involved in liver fibrosis [32]. Most of these dogs had disorders of non-infectious etiology. It could be that focusing more strongly on diseases caused by an infectious agent would have caused the conclusion to turn out differently.

$CD3^+NCR1^+$ cells ranged from 0 to 4.8 % of circulating lymphocytes. $CD3^+NCR1^+$ cells were $CD56^-$ and the vast majority expressed granzyme B^+ (Fig. 10). $CD3^+NCR1^+$ cells were unfortunately not evaluated in organs analyzed in paper I due to technical problems.

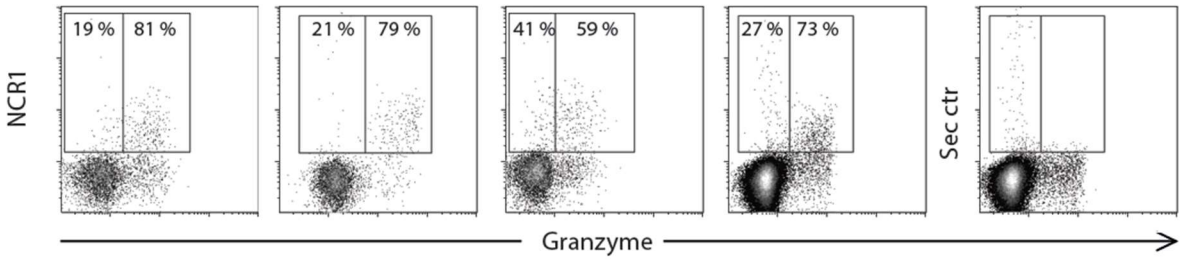

Fig. 10 Granzyme vs NCR1 dot plots from PBMC from four healthy dogs. Cells are gated on lymphocytes after exclusion of doublets.

In PBMC almost all $CD3^{-}NCR1^{+}$ cells were found to be $CD8^{-}$, while in the cytokine stimulated cell cultures $CD3^{-}GranzymeB^{+}$ cells showed a variably CD8 expression (Fig. 11)

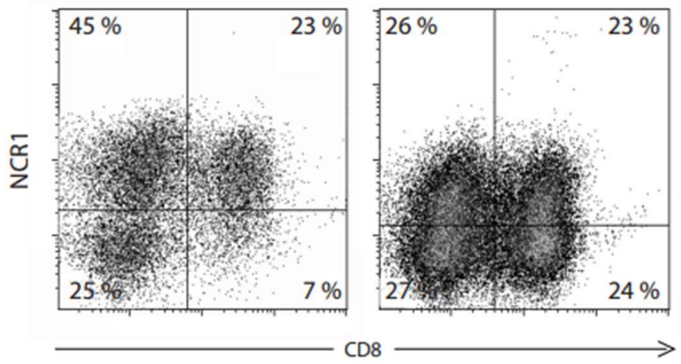

Fig. 11 CD8 vs NCR1 dog plots from two week old $CD5^{-}CD3^{-}$ cultures from two dogs. Cells are gated on lymphocytes after exclusion of doublets and dead cells.

Upregulation of CD8 on NK cells after in vitro cytokine stimulation has also been observed in cattle [135,140]. CD8 defines specific subsets of NK cells in different species [245,246]. CD8 is a co-receptor for MHCI restricted antigen recognition of T cells. CD8 is expressed as a heterodimer of CD8α and CD8β or as a CD8α homodimer [247]. CD8α homodimers are known to be expressed on subsets of NK cells, but the exact function of CD8 on NK cells is not known. The antibody used in this study does not differentiate between CD8α and CD8β.

NCR1 is probably not expressed on all canine NK cells and can therefore not be applied as a marker for NK cells in this species. $CD3^{-}GranzymeB^{+}$ has here been suggested as a more correct way of describing this cell population in dogs until a more specific marker is identified (Paper II). Expression of Granzyme B and perforin is known to be gradually

upregulated as the NK cell matures and goes through priming procedures [15,16,39]. Resting NK cells in humans express granzyme B [248] but in mice living under restrict hygiene conditions, granzyme is not found in resting NK cells. After microbial stimulation or TLR ligand priming granzyme B is however, strongly upregulated in mice [38,41,249]. Originally this observation was thought to be due to species differences but recent findings comparing laboratory and feral mice indicates that microbial exposure might be the reason for these discrepancies [40]. The degree of exposure to microbes are quite similar in humans and dogs, and according to this hypothesis it is reasonable to believe that granzyme B is expressed on all mature canine NK cells.

NCR1 and canine lymphoproliferative LGL disorders

From 2011 to 2015, five cases of lymphoproliferative LGL disorders were diagnosed at The Central Laboratory at NMBU, School of Veterinary Medicine and included in the study. One case of chronic $CD3^{+}NCR1^{+}$ LGL leukaemia is presented in paper I and three cases of $NCR1^{-}$ LGL disorders are presented in paper III. The first case included in the study was a seven-year-old female Golden Retriever with an aggressive T LGL leukaemia (Fig. 11) with disseminated disease involving spleen, liver, lymph nodes, lung tissue and bone marrow.

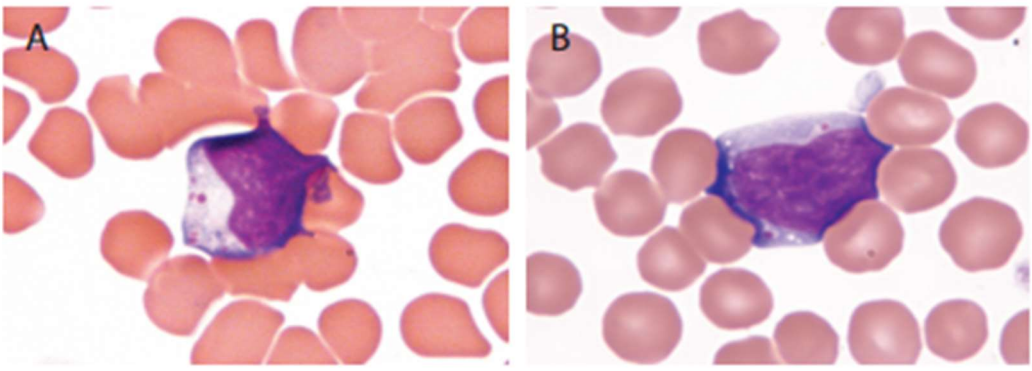

Fig. 12 Neoplastic cells in a blood smear from a dog with acute disseminated T $CD56^{+}$ LGL leukaemia.

This case was enrolled prior to the validation of the AKS6 antibody and was not characterized with respect to NCR1 expression and therefore not included in any publication. Flow cytometry showed a $CD3^{+}CD56^{+}CD8^{-}CD4^{-}MHCII^{+}$ phenotype of neoplastic cells.

A case of blastic $CD56^+$ leukaemia has been described in a Golden Retriever [250] but no LGL morphology was demonstrated. Neoplastic cells did not express CD3 and a conclusion of NK cell origin was made in this study. Although it is unlikely that CD56 is expressed on NK cells from healthy dogs, a dysregulation of CD56 in malignantly transformed NK cells cannot be ruled out in this particular case.

A handful reports of canine $CD3^-$ LGL lymphoproliferative disorders exist [168,211–214] and most conclude with a LGL leukaemia of NK cell origin without discussing the matter further. Downregulation of CD3 for example, needs to be considered. Malignant transformation from a chronic $CD3^+CD8^+$ LGL disorder to an acute $CD3^-CD8^-$ LGL leukaemia has been reported in a Golden Retriever [251]. Human lymphoproliferative LGL disorders are grossly split into two fractions: $CD3^+$ T cells and $CD3^-$ NK cells, but additional markers are used to confirm NK cell origin, like CD56 and CD16. No canine NK cell marker are currently available making it difficult to diagnose malignantly transformed NK cells. In the presented work NCR1 was initially proposed as a marker possible to apply in this setting in dogs, but as it became clear that NCR1 is also expressed on a distinctive $CD3^+$ lymphocyte population in healthy dogs, this hypothesis was rejected. For the time being, $CD3^-$ lymphoproliferative LGL disorders should therefore be termed “non-B non-T”.

Unfortunately, no cases of $CD3^-$ lymphoproliferative LGL disorders were diagnosed in the 5-year enrolment period and NCR1 expression was therefore not studied in non-B non-T LGL disorders. However, one of the cases included in the study was a chronic T cell leukaemia expressing NCR1 presented in paper I. Also in humans, it has been demonstrated that NCR1 is expressed in LGL leukaemia of both T and NK cell origin as well as other T cell malignancies. However, no study has investigated the clinical significance of NCR1 expression. Therefore, we wanted to study if NCR1 expression in LGL leukaemia was connected to certain clinical outcomes. Of the three last dogs enrolled in the study, two was diagnosed with acute T cell leukaemia not expressing NCR1. The enrolment period was too short and not enough dogs were included to conclude with a possible clinical significance of NCR1 expression.

Final remarks: Challenges within research in dogs

More than 500.000 dogs are registered in Norway according to the Norwegian Kennel Club. Consequently, dogs are important to a considerable number of Norwegians, and we are not unique. Since prehistoric times dogs and humans have lived together [252] and all over the world people keep dogs both for work and for company. Dog ownership has multiple beneficial effects on both physical and mental health [253] – an aspect included in the increasingly important "One Health concept" [254]. It has been shown that owning a dog is beneficial for patients with heart disease [255,256] and increased physical activities due to dog walking prevents obesity [257] . Pet ownership has been connected to reduced risk of diseases like cancer [258] and diabetes [259]. Also, interactions with dogs have been proven to reduce stress and depression [260–262] and increase quality of life [263,264]. Dogs are termed "man's best friend" and taking care of our dogs' health is therefore of great social and economic importance.

Even so, securing funding for research in dogs has been shown to be much more difficult than for other veterinary species, like for example fish, where commercial interests are substantial. Very few endowments and scholarships in Norway are designated for research in companion animals and establishing research groups within areas important to healthcare in dogs is difficult. Consequently, research projects within canine medicine are often discontinued because of lack of funding. Getting funding for the research presented here has been a challenge and the future perspectives in this area are uncertain due to lack of economic support.

In tune with the One Health Concept, dogs have been proposed as a model for human disorders. Dogs develop much of the same spontaneous diseases as humans, and many of them are connected to lifestyle and environmental factors. The recent release of the canine genome has led to the realization that dogs, not surprisingly, represent a better model for human cancer than laboratory animals commonly used today [165]. Dogs are selectively bred animals with a shorter life span than humans, making it possible to gain more data in a shorter period of time [265].

This PhD project originally included a clinical trial. A new cancer drug was tested in humans and dogs and we were going to look into changes in immunological response with a focus on NK cells in the dogs. After enrolling a couple of dogs in the study, the veterinary part of the project was unfortunately put to an end because it turned out to be too expensive. Also the knowledge gap between dogs and humans within NK cell immunology is currently big. A

deeper understanding of canine immunology is necessary before dogs can be used as a model for immunological responses and potential treatment strategies based on immunological mechanisms.

MAIN CONCLUSION

Canine NK cells were described using a mouse anti-bovine mAb (AKS6) cross reacting with canine NCR1 (Paper I).

Lack of CD3 and expression of granzyme B define mature NK cells in the dog. $CD3^-$ $GranzymeB^+$ cells can further be divided into $NCR1^+$ and NCR^- fractions. Consequently, NCR1 does not define all canine NK cells. NCR1 is a triggering receptor and defines functionally different NK cells as only $NCR1^+$ cells produce IFNγ. NCR1 can be upregulated on $NCR1^-$ cells following cytokine stimulation. Our results therefore indicates that NCR1 is a marker for activation in canine NK cells, and $NCR1^-$ NK cells probably represent a resting/ non-activated stage. Canine NK cells are detected in the blood, lymph nodes, spleen and liver. (Paper I and II).

A $CD3^+NCR1^+CD56^-GranzymeB^+$ lymphocyte population was demonstrated. This cell population probably displays some of the same features as NK cells and most likely represents NKT-like cells. (Paper I and II).

Cultures dominated by canine NK cell were obtained by CD5 depletion of PBMC followed by a CD3 depletion if necessary. After two weeks of IL-2 and IL-15 stimulation the vast majority of the cultured cells displayed a $CD3^-GranzymeB^+$ phenotype of which a subset express NCR1. The cultured cells showed LGL morphology.

Five dogs with lymphoproliferative LGL disorders were enrolled during a five-year period. Four dogs had a T LGL leukaemia and one dog had a probable reactive lymphocytosis. NCR1 expression was demonstrated in one case of chronic T LGL leukaemia. Canine lymphoproliferative LGL disorders coincide with what is known about the human equivalent (Paper I and III).

FUTURE PESPECTIVES

Still there is known no marker defining all canine NK cells. NKG2D and NCR3 (NKp30) are both receptors expressed on human NK cells with potential of defining canine NK cells, as high levels of mRNA encoding these two proteins have been detected in non-B non-T cell cultures [154,155]. Therefore, these two markers should be investigated next. The functional properties of $CD3^{-}GranzymeB^{+}NCR1^{-}$ cells need to be further explored to confirm the hypothesis of these cells representing resting NK cells. Also, $CD3^{+}NCR1^{+}$ cells need to be thoroughly characterized and functional features should be verified. A clear understanding and characterization of all cells involved in natural cytotoxicity is necessary in order to use canine NK and NKT-like cells in a clinical setting in the future.

In human medicine, NK cells are currently the centre of attention in cancer research. Dysfunction of NK cells has been shown to correlate with clinical outcome and therefore NK cells can be used as a prognostic biomarker. Reduced natural cytotoxicity has also been observed in dogs with cancer [162,163]. NK cells represent a valuable tool in cancer immunotherapy [266]. Promising approaches in preclinical studies include autocrine IL-2 and IL-15 stimulation, silencing of inhibitory NK receptors and redirect killing of neoplastic cells. Also genetic manipulation of NK cells have been suggested as a way of exploiting the full potential of natural killers in treatment of cancer [267].

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SCIENTIFIC PAPERS I-III

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ORIGINAL ARTICLE

NCR1$^+$ cells in dogs show phenotypic characteristics of natural killer cells

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Abstract No specific markers for natural killer (NK) cells in dogs have currently been described. NCR1 (NKp46, CD355) has been considered a pan species NK cell marker and is expressed on most or all NK cells in all species investigated except for the pig which has both a NCR1$^+$ and a NCR1$^-$ population. In this study peripheral blood mononuclear cells (PBMC) from 14 healthy dogs, 37 dogs with a clinical diagnosis, including a dog diagnosed with LGL leukemia, and tissue samples from 8 dogs were evaluated for NCR1$^+$ expression by a cross reacting anti bovine NCR1 antibody. CD3$^-$NCR1$^+$ cells were found in the blood of 93 % of healthy dogs and comprised up to 2.5 % of lymphocytes in PBMC. In a selection of healthy dogs, sampling and immunophenotyping were repeated throughout a period of 1 year revealing a substantial variation in the percentage of CD3$^-$NCR1$^+$ over time. Dogs allocated to 8 disease groups had comparable amounts of CD3$^-$NCR1$^+$ cells in PBMC to the healthy individuals. All organs examined including liver, spleen and lymph nodes contained CD3$^-$NCR1$^+$ cells. Circulating CD3$^-$NCR1$^+$ cells were further characterized as CD56$^-$GranzymeB$^+$CD8$^-$. A CD3$^+$NCR1$^+$ population was observed in PBMC in 79 % of the healthy dogs examined representing at the most 4.8 % of the lymphocyte population. In canine samples examined for CD56 expression, CD56$^+$ cells were all CD3$^+$ and NCR1$^-$. To our knowledge, this is the first examination of NCR1 expression in the dog. The study shows that this NK cell associated receptor is expressed both on populations of CD3$^+$ and CD3$^-$ blood lymphocytes in dogs and the receptor is found on a CD3$^+$ GranzymeB$^+$ CD8$^+$ leukemia. Our results support that CD56 is expressed only on CD3$^+$ cells in dogs and shows that NCR1 defines a different CD3$^+$ lymphocyte population than CD56$^+$CD3$^+$ cells in this species. CD3$^-$NCR1$^+$ cells may represent canine NK cells.

Keywords NCR1/NKp46 · Dog · Natural killer cell · LGL · LGL leukemia

Introduction

Natural killer (NK) cells have been defined as large granular lymphocytes (LGLs) with a cytotoxic and immune regulatory function. They recognize damaged, infected and cancerous cells by a repertoire of germ line encoded inhibitory and activating receptors. The balance in the stimulation of these receptors decides the final outcome of the cell-to-cell contact. NK cells kill target cells by granzyme and perforin release as well as through fas-fas ligand interactions. They also secrete cytokines, most important is the early production of IFNγ in immune response when no adaptive T cells are present yet. As NK cells do not express re-arranged T cell receptors (Triebel et al. 1987; Tutt et al. 1987), they belong to the innate part of the immune system. Human NK cells are CD3$^-$ granular lymphocytes that also express perforin and granzyme (Cella et al. 2014). Human NK cells are further classified according to the level of CD56 (bright/dim) (Lanier et al. 1989).

The activating receptor NCR1 (NKp46, CD355) has been suggested as a pan species NK cell marker. NK cells have

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been defined as $CD3^{-}NCR1^{+}$ cells in humans (Biassoni et al. 1999; Sivori et al. 1997), monkeys (De Maria et al. 2001; Kennett et al. 2010), rats (Westgaard et al. 2004), mice (Biassoni et al. 1999; Walzer et al. 2007), cattle (Storset et al. 2004) and sheep (Connelley et al. 2011). $NCR1^{+}$ cells have also been described in the horse (Noronha et al. 2012). However as only about half of the NK cells in the pig express NCR1 (Mair et al. 2012) this receptor does not mark all NK cells in all species. NCR1 is a natural cytotoxic receptor and belongs to the immunoglobulin superfamily. It consists of two extracellular C2-type Ig-like domains, a trans membrane region and a short cytoplasmic tail (Ponassi et al. 2003). NCR1 is connected to ITAM-bearing adaptor proteins and ligation of this receptor leads to activation of cytotoxicity and cytokine production (Sivori et al. 1997). NCR1 is found on both resting and activated NK cells. Although it binds to the haemagglutinins of influenza virus (Mandelboim et al. 2001) its cellular ligands are still unknown. NCR1 was traditionally thought to only be expressed by NK cells, but during the last couple of years it has been established that NCR1 and other natural cytotoxicity receptors can be expressed on certain subgroups of T cells (Connelley et al. 2014; Hudspeth et al. 2013).

Cytotoxic activity in PBMC was first described in the dog in 1960 (Govaerts 1960). Since then NK cell activity in canine PBMC has been described several times against CTAC, a canine thyroid adenocarcinoma cell line (Kasza 1964; Knapp et al. 1993; Krakowka 1983). Cells making conjugates with CTAC have been described morphologically as 5 to 6.5 μm in diameter with a kidney bean shape nucleus, and electron-dense cytoplasmic granules (Knapp et al. 1995). NK cells in the dog have been described as having a $CD3^{-}CD56^{-}$ phenotype (Bonkobara et al. 2005; Otani et al. 2002). Cells with natural cytotoxic activity have been described as $CD5^{low}$ (Huang et al. 2008) and $CD8^{+}$ (Lin et al. 2010). Recently ex vivo expansion of canine cytotoxic large granular lymphocytes exhibiting natural killer cell characteristics was described (Shin et al. 2013). The expanding population had a $CD3^{+}CD5^{dim}TCR\alpha\beta^{-}TCR\gamma\delta^{-}$ phenotype and did not express the invariant TCRα chain (iTCRα) expressed by NKT cells in dogs (Yasuda et al. 2009).

A handful of case reports describing non-T, non-B lymphoid neoplasias being indicative of a NK cell origin have also been published (Bonkobara et al. 2007; Lane et al. 2012) Neoplasias arising from NK cells are termed large granular lymphocyte (LGL) lymphomas/ leukemias. This designation also encompasses neoplasias arising from cytotoxic T lymphocytes (CTLs), making a diagnosis of NK vs T cell LGL lymphoma/leukemia based on morphological features alone impossible without NK cell markers. The clinical progression ranges from indolent to aggressive disorders and phenotypic features of the neoplastic cells are prognostic (Sokol and Loughran 2006).

To be able to describe canine NK cells and the neoplasias they give rise to, more knowledge of receptors expressed on canine NK cells and antibodies directed towards these receptors are needed. The object of this study was to identify and characterize canine NCR1 expression on lymphocytes in PBMC, liver, spleen and lymph nodes from both healthy dogs and dogs with different diseases.

Materials and methods

Identifiaction and cloning of canine NCR1 mRNAs

Canine NCR1 genomic sequences were identified by homology searches in GenBank with human and bovine NCR1 exon sequences. Primers were designed to span the entire putative coding region of canine NCR1 by Primer3 software (Rozen and Skaletsky 2000) (F: TGTGCCTGGTATAATCAGTGCT; R: CAAGGTCAATGCCAGTGTTC. PBMC from canine EDTA blood was isolated and stimulated with human recombinant IL-2 (25 ng/ml final concentration, eBiosciences, San Diego, CA, USA) for 5 days before mRNA isolation with MACS mRNA isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany). Oligo dT primed cDNA syntesis (SuperscriptIII, Invitrogen, Carlsbad, CA, USA) was performed prior to gene specific PCR amplification (DynaZyme, Invitrogen) with the canine primers. Amplification products of the expected length were cloned in TOPOpCR2.1, prepped (Qiagen mini-prep, Hilden, Germany) and sequenced with vector specific primers (M13F and R). Sequences were analysed and aligned by use of Vector NTI (Life Technologies, Carlsbad, CA, USA) and Clustal W (EMBL-EBI, Cambridgeshire, UK).

Blood and tissue

Whole blood from 14 clinically healthy dogs of various breeds ranging from 0, 4 to 10 years of age was collected from the cephalic vein into 3 ml vacutainer tubes containing EDTA. The dogs were all private pets of the staff at The Norwegian School of Veterinary Science. 8 of these dogs were sampled at several occasions over a period of 1 year to evaluate variations within the same individual over time. 3 ml EDTA anticoagulated blood from 36 dogs, including four dogs from which organs were also analyzed, of various breeds and ages undergoing diagnostic workup at the Teaching Hospital at The Norwegian School of Veterinary Science was collected to evaluate PBMC from individuals with a variety of different diagnoses. The dogs were divided in 8 categories: respiratory disease, gastrointestinal disease, neurological disease, endocrine disease, kidney/urinary tract disease, oncological disease, dermatological disease and intoxications. Owners consent was given for all blood sampling not included in a

diagnostic workup. According to Norwegian legislation, regular bloodsampling performed according to standard procedure does not require approval from an ethical committee.

Organs from 8 dogs of various breeds and ages were analyzed. The dogs were recruited from The Teaching Hospital at The Norwegian School of Veterinary Science and from PetVett Sandvika, Small Animal Clinic. These dogs were both client owned dogs euthanized for medical reasons and research dogs with various diagnoses taking part in other projects approved by appropriate ethical committees. Anesthesia was performed using Zoletil mix ® (zolazepam, tiletamin, xylazin, butorphanol) (Virbac, Carros Cedex, France) intramuscular and followed by euthanasia with an overdose of pentobarbital intravenously or by intracardiac injection Spleen, liver and/or lymph nodes were collected shortly after euthanasia and kept on ice until processing. Unfortunately not all organs were available from all dogs. All dogs included in the study are summarized in Supplementary table 1.

Analysis of cells from a dog with LGL leukemia

Dog #47 was a male Rottweiler 13 years of age presented to his regular veterinarian with a history of PU/PD. CBC showed a mild anemia (4,41 10^{12}/L, ref 5,1-8,5 10^{12}/L) and a marked lymphocytosis (69,24 10^{9}/L, ref 0,8-5,8 10^{9}/L). A morphological evaluation indicated a well-differentiated LGL lymphocytic leukemia. Pictures were taken using Nikon Digital Camera Dxm 1200 F and processed using softwear Nikon ACT-1. EDTA anticoagulated blood was further used for flowcytometric analysis. The dog was euthanized 4 months after diagnosis.

Antibodies

The commercially available antibodies used in this study were: Mouse anti-canine CD3 FITC (clone CA17.2A12, IgG1, AbDSerotec, Oxford, UK), mouse anti-human CD56 PE (clone MOC-1, IgG1, Dako Glostrup, Denmark), mouse anti-human Granzyme B PE (clone GB11, IgG1, eBioscience), mouse anti-canine CD21 (clone CA2.1D6, IgG1, Thermo Fisher Scientific Rockford, IL USA), rat anti-canine MHCII (clone YKIX334.2, IgG2a, eBioscience) and rat anti-canine CD4 (clone YKIX302.9, IgG2a, AbDSerotec). Production of a monoclonal antibody recognizing *bt* (*bos taurus*) NCR1 (AKS6, IgG2b) has been described earlier (Lund et al. 2012). Briefly a soluble fusion protein of *bt* NCR1 and the hinge and Fc region of the mouse IgG2b was generated and used to immunize mice. Hybridomas were screened for reactivity against 293 T cells transfected with *bt* NCR1FLAG. In flow cytometric analysis AKS6 was used in a final concentration of 2,5 μg/ml. Secondary antibodies used to detect *cf* (*canis familiaris*) NCR1 were goat anti-mouse IgG2b PE for staining of 293 T cells, IgG2b APC for characterization of dog cells and LGL neoplasia (Southern Biotech, Birmingham, AL, USA) and IgG2b Al488 (Invitrogen) for use in immunofluorescence. Isotype controls used were the clone MOPC-21 for IgG1 FITC and PE (BioLegend, San Diego, CA, USA). Anti-FLAG antibody M2 (Sigma-Aldrich, St Louis, MO, USA) was used to confirm a complete transcription after transfection with pFLAG-CMV-1 vector (Sigma-Aldrich). The antibodies were used in concentrations recommended from the producer.

Cloning and transfection of cf NCR1-FLAG and bt NCR1-FLAG constructs

A *cf* NCR1-FLAG construct was made by amplifying the cloned *cf* NCR1 sequence by primers AAG-CTT-CAG-AAG-CAG-ATT-CTT-TCA-AAA-C (*cf* NCR1-FLAGfHindIII) and GGA-TCC-TAA-CTG-TGG-CCA-GCA-CAT-CT (*cf* NCR1-FLAGrBamHI), restriction sites for HindIII and BamHI respectively underlined. The PCR product was ligated into TA cloning vector, amplified in *E. coli*, cut with appropriate restriction enzymes and ligated into cut and CIP treated pFLAG-CMV-1 vector (Sigma-Aldrich) and transfected into 293 T cells using PEI following the manufacturer's instructions. After 24 h the cells were harvested and used as screening cells. Mab ANTI FLAG M2 IgG1 (Sigma-Aldrich) was used to confirm complete expression of the product of the transfected gene. Cloning and transfection of *bt* NCR1-FLAG has been described earlier by (Storset et al. 2004).

Western blotting

293 T cells were transfected with *bt* NCR1-FLAG and *cf* NCR1-FLAG construct as described above and harvested 24 h post transfection using phosphate buffered saline (PBS) with 2 mM EDTA. The cells were washed once and counted. About 15 million cells were lysed with Pierce IP lysisbuffer (Thermo Fisher Scientific) with 1×Halt proteinase inhibitor (Thermo Fisher Scientific) at 4 °C for 30 min before centrifugation at 13,000 g for 5 min followed by collection of the supernatant from the pelleted debris. Protein samples were diluted in NUPAGE Sample Buffer × 4 (BioRad, Hercules, CA, USA), boiled for 5 min, separated by SDS-PAGE Criterion XT, 4–12 % (BioRad) and transferred to PVDF membrane by semi-dry transfer. The membrane was then blocked with 5 % dry milk in PBS-T (pH 7,4) +0,1 % Tween (blocking buffer) to prevent unspecific binding, followed by incubation with anti-FLAG antibody M2 (Sigma-Aldrich) diluted 1:1000 and with AKS6 4 μg/ml and 8 μg/ml in blocking buffer overnight at 4 °C. The membrane was washed 4 times in blocking buffer and then incubated with peroxidase conjugated anti-mouse secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) in blocking buffer.

Detection of secondary antibody was performed using SuperSignal West Pico substrate (Thermo Fisher Scientific) and visualization was done using Molecular Imager ® ChemiDox™ XRS+ (Bio Rad).

Preparation of EDTA anticoagulated blood and organs before flow cytometric analysis

Three millilitres EDTA blood was diluted 1:1 with Hanks Buffered Salt Solution (HBSS). Organs were homogenized by manual squeezing using a syringe plunger, diluted in 25 ml PBS containing 2 mM EDTA and filtrated through a 70 μm cell strainer (BD Bioscience, Franklin Lakes, NJ, USA). Cells were then loaded onto a Lymfoprep (Axis-Shield, Dundee, Scotland) and centrifuged at 1760 g for 30 min. The interphase was then carefully removed and washed 3 times with PBS with 1 % BSA and 10 mM NaN_3 (flowbuffer). The cells were counted and viability evaluated using trypan blue expression analyzed by the Countess ® Automated Cell Counter from Life Technologies.

Flow cytometry

Flow cytometric analysis was performed on transfected and non-transfected 293 T cells, on PBMC from healthy dogs and on PBMC and tissue samples from dogs with various diagnoses. 5×10^5 cells resuspended in 25 μl flow buffer were incubated with appropriate antibodies on ice. For all stainings involving AKS6 and the CD3 CA17.2A12 antibody the antibodies were incubated individually. AKS6 antibody was incubated first, then the appropriate secondary antibody followed by directly conjugated CD3 antibody. All cells marked with extracellular staining were analyzed without previous fixation. For intracellular staining for Granzyme B the BD Cytofix/Cytoperm ™ kit (BD Biosciences) was used according to the manufactures instructions. After the last washing the cells were diluted in 200 μl flow buffer and kept at 4 °C until analysis. Data was collected using FACSCalibur and (BD Bioscience) and Gallios (Beckman Coulter, Brea, CA, USA). Data was processed in CellQuestPro (BD Bioscience) or Kaluza (Beckman Coulter) respectively. Doublets were excluded by a FS INT vs. FS PEAK gate.

Staining of blood smears

Air dried blood smears from dog # 47 were kept on −80 °C until analysis and dried for 1 h in room temperature after thawing followed by rehydrating in PBS for 2 min. The BD Cytofix/Cytoperm ™ kit (BD Biosciences) was used for fixation of cells before staining. Briefly, the smears were covered with fix/perm solution for 20 min and then washed twice with perm/wash solution. PE conjugated Granzyme B antibody (GB11) and PE conjugated CD21 antibody used as a negative control were incubated for 30 min and then washed three times. The antibodies were both used in a 1:10 dilution. After the last wash the smears were rinsed with distilled water and mounted with Prolong Gold Antifade reagent with DAPI (Life Technologies). The smears were kept dark and evaluated within 2 h using Zeiss AxioImager M2 microscope and a×40 objective. Imaging software ZEN lite from Carl Zeiss Microscopy was used for processing of pictures.

Results

Identification of a canine NCR1 transcript

The canine NCR1 transcript identified in this study (JN790861) translates into a protein of 316 amino acids and includes both start and stop codons. The 316 amino acid canine NCR1 protein sequence shares 63 % identity to human NCR1, and 61 % identity to the bovine protein (Fig. 1). The JN790861 sequence has been used to generate a NCBI Reference sequence for canine NCR1, NM_001284448.1.

Cross reactivity of AKS6 towards the canine NCR1

The specificity of cross reactivity of anti-bovine NCR1 (AKS6) with the canine orthologue protein was tested by flow cytometric analysis of cells transfected with *cf* NCR1-FLAG constructs. MAb ANTI FLAG M2 IgG1 (Sigma-Aldrich) stained the transfected cells indicating a successful transfection and transcription for the *cf* NCR1-FLAG plasmid (Fig. 2a and b). The histogram for the AKS6 antibody showed a similar curve compared to mAb ANTI FLAG M2 IgG1 (Sigma-Aldrich). AKS6 did not bind to non-transfected 293 T cells (data not shown).

Western Blot analysis of protein lysates of both *cf* NCR1-FLAG and *bt* NCR1-FLAG transfected cells showed a band around 37 kDa with mAb ANTI FLAG M2 IgG1 (Sigma-Aldrich) (Fig. 2c). The AKS6 antibody did not show any specific band on neither *cf* NCR1-FLAG nor *bt* NCR1-FLAG transfected cells and it was concluded that AKS6 has a poor performance in the western blot application.

$NCR1^+$cells in blood lymphocytes sampled from healthy dogs

PBMC from 14 healthy dogs of various breeds and ages were analyzed using flow cytometry. Lymphocytes were gated in a forward scatter vs. side scatter plot as shown in Fig. 3a. In CD3 versus NCR1 dot plots both $CD3^-NCR1^+$ and $CD3^+NCR1^+$ cells were identified, though not in all dogs (Fig. 3b and c and Supplementary table 2). A positive

Fig. 1 Amino acid sequence alignment of *canine (cf)* NCR1 compared to human and *bovine (bt)* NCR1. *Bars* (–) indicate identical amino acids to canine NCR1, empty spaces are gaps. *Grey* shaded Cs indicate cysteine residues forming intra domain disulfide bridges. GenBank accession numbers: JN790861.1 (canine NCR1), NM_004829.6 (human NCR1) and AF422181.1 (bovine NCR1)

```
                    Signal sequence                    Ig-like domain 1
Canine  NCR1  MTSTLTALLF LGLYLSQTTS TQKQILSKPI IWVKPNFMIP KGMPVLIWCQ GTPEAIEYQL  60
Human   NCR1  -S---P---C V--C---RI- A-Q-T-P--F --AE-H--V- -EKQ-T-C-- -NYG--V---  60
Bovine  NCR1  -P---AVF-V ---G---RS- ----M----A ---R-S---- --RSAT-G-R -AG--M----  60

Canine  NCR1  HLEGHLYAQQ SPKQLERRNT VMFRIPKMTL LTAGQYRCFY RIKELWSKPS DPLDLVVTGM  120
Human   NCR1  -F--S-F-VD R--PP--I-K -K-Y--D-NS RM----S-I- -VG----E-- NL------E-  120
Bovine  NCR1  -F--G-S-LK R--SSPVM-W -K-P--A--S HT------S- -SG----EL- D--E-----L  120

                        Ig-like domain 2
Canine  NCR1  YDTPTLSVHP GNKVISGKNV TFSCRLDTAT NKFFLLKEGR SSHPQHRYGN IQVEFPIGPV  180
Human   NCR1  ---------- -PE-----K- --Y-------- SM-L------ ---V-RG--K V-A---L---  180
Bovine  NCR1  --------E- RSE-T--E-- --H-Q-A--- ST-----K-- --RP-L---- LRA--HL---  180

                                                Stem
Canine  NCR1  TTAHRGTYRC FGSYNNHAWS FPSKPVKLLV T  EGVGDTS LVPTEHTSFS EPWDTYLLAT  240
Human   NCR1  ----------- ---------- ---------- -  GDIEN-- -A-EDP-FPA DT-G----T-  240
Bovine  NCR1  -P--G-----  ---------- ---------- KVPGDA---T FA------ S DY--S-E-T-  240

                          Transmembrane          Cytoplasmic
Canine  NCR1  EAEFQQAPAF WDHTAHNILQ IGLAFLVLMA LMCLLVDYWL CRKRIQEQAN RASPQECRRR  300
Human   NCR1  -TGL-KDH-L -----Q-L-R M-------V- -VWF--ED-- S---TR-R-S ---TW-----  300
Bovine  NCR1  GTQS-KDHLL -N-MIQ-LIR L---V---V- --GF-LED-- H--KS--R-H ---RRG----  300

Canine  NCR1  FRTQRTPGQM TKRCAGHS  316
Human   NCR1  LN--TL  304
Bovine  NCR1  L-A--ALET  308
```

population was defined as a distinct population of cells counting 0,1 % or more. In 13 out of the 14 dogs (93 %) analyzed a circulating $CD3^{-}NCR1^{+}$ cell population was detected and in 11 of 13 dogs (79 %) a $CD3^{+}NCR1^{+}$ population was detected. Figure 3d shows the distribution of percentage of gated cells for both $CD3^{-}$ $NCR1^{+}$ and $CD3^{+}$ $NCR1^{+}$ cells, indicating that the $CD3^{+}NCR1^{+}$ measurements have a more spread distribution in healthy dogs ranging from 0 to almost 4, 8 % compared to CD3-$NCR1^{+}$ cells ranging from 0 to 2, 5 %. The $CD3^{+}$ cells had a weaker expression of $NCR1^{+}$ than the $CD3^{-}$ cells as measured by mean fluorescence intensity (MFI) (Fig. 3c). In 9 of the 14 dogs measurements were repeated at 2 to 9 time points throughout a period of 1 year revealing a substantial variation within the same healthy individual (Fig. 3e).

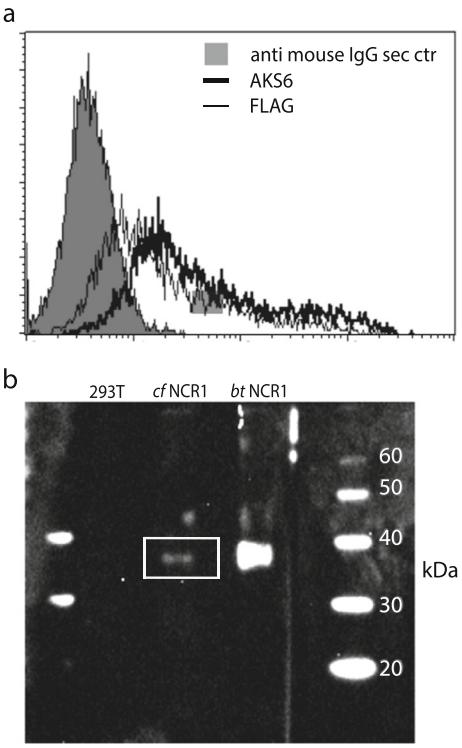

Fig. 2 Flow cytometric analysis of 293 T cells transfected with: **a** *cf* NCR1-FLAG construct, stained with anti-FLAG antibody (M2) and cross reacting anti bovine NCR1 mAb (AKS6). **b** Western Blot of 293 T cells transfected with *cf* NCR1-FLAG and *bt* NCR1-FLAG construct incubated with rabbit anti-FLAG polyclonal Ab. Relative molecular masses in kDa are indicated

$NCR1^{+}$ cells in healthy individuals were characterized further combining CD3 and NCR1 with monoclonal antibodies against CD56, Granzyme B and CD8 respectively. 8 of 14 healthy dogs (57 %) had a population of $CD56^{+}$ cells and all of the $CD56^{+}$ cells were $CD3^{+}$ as shown in Fig. 4a. Co staining cells with the NCR1 and CD56 antibody showed that all $NCR1^{+}$ cells, including the cells expressing $CD3^{+}$, were $CD56^{-}$ as shown in Fig. 4b. The percentage of $CD3^{-}CD56^{+}$ and $CD3^{+}CD56^{+}$ cells among the cells in the lymphocyte gate are shown in Fig. 4c. Stainings with CD3, NCR1 and Granzyme B showed a clear population of $NCR1^{+}$Granzyme B^{+} cells among the $CD3^{-}$ cells as shown in Fig. 5a. Between 73 and 98 % of the $CD3^{-}NCR1^{+}$ cells expressed Granzyme B. Stainings with CD3, NCR1 and CD8 showed that 93–97 % of $CD3^{-}NCR1^{+}$cells were $CD8^{-}$ (Fig. 5b)

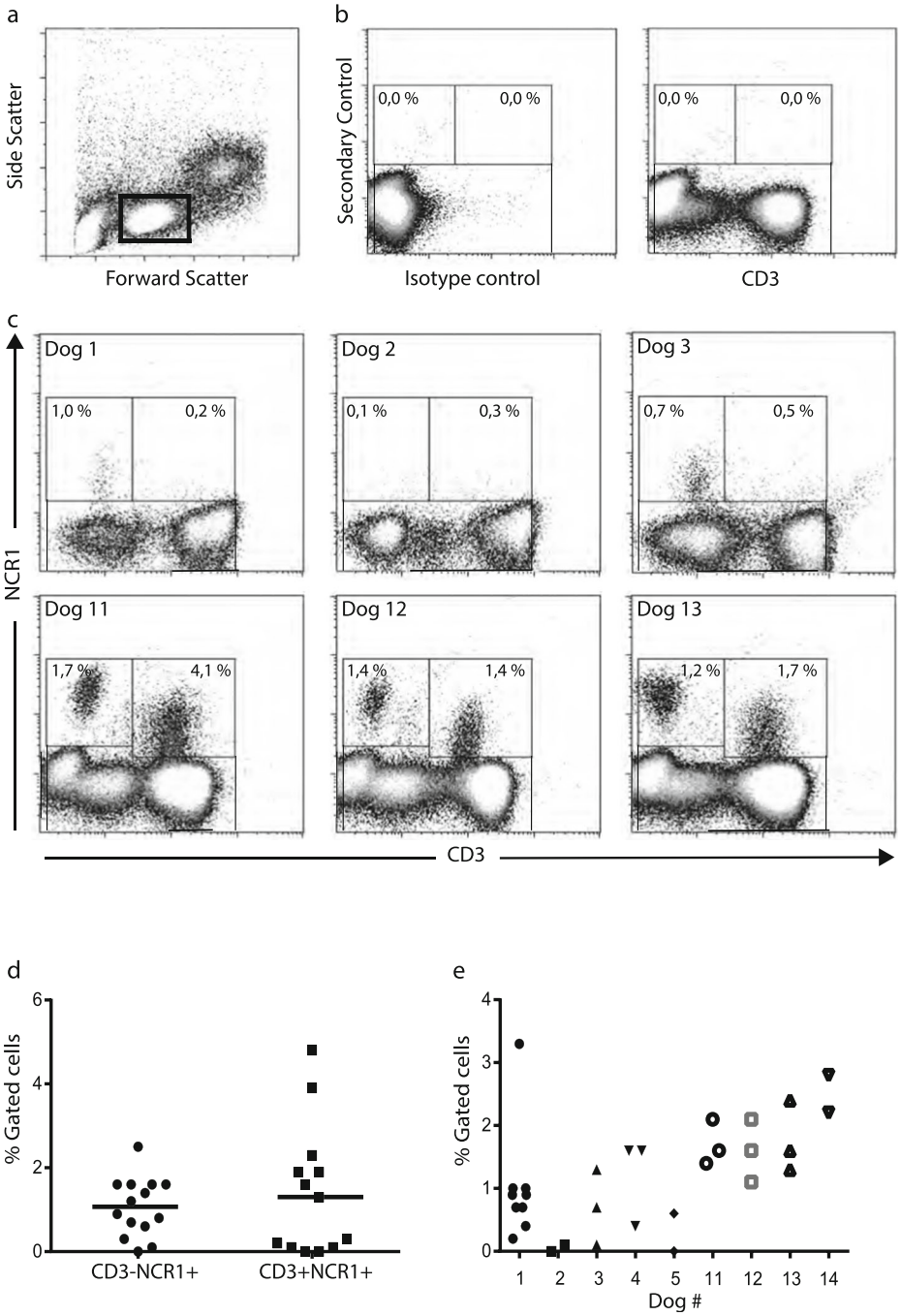

Fig. 3 CD3 and NCR1 two color flow cytometric analysis of PBMC from healthy dogs. **a** Forward vs. side scatter plot showing the lymphocyte gate. All further plots are gated lymphocytes. **b** Isotype control and secondary control. **c** CD3 and NCR1 double staining from 6 different healthy dogs gated on lymphocytes. The plots are representative for the 14 healthy dogs included in the study. **d** Percentage of $CD3^{-}NCR1^{+}$ and $CD3^{+}NCR1^{+}$ cells in the lymphocyte gate from all 14 healthy dogs studied. Median is presented. **e** Variation in percentage of $CD3^{-}NCR1^{+}$ cells in the lymphocyte gate in 9 individual dogs sampled between 2 and 9 times. For detailed information on individual dogs see Supplementary table 1

$NCR1^{+}$ cells in dogs with various diagnoses

PBMC from 36 dogs of various breeds and ages with various diagnoses were analyzed using flow cytometry and 1 rottweiler with LGL leukemia (dog # 47) were analyzed using flow cytometry and immunofluorescence. 34 out of 36 (94 %) dogs had circulating $CD3^{-}NCR1^{+}$ cells ranging from 0, 1 to 6, 8 %. The dogs were grouped in 8 different groups according to the diagnosis. Median for each group and the distribution of values within the groups are presented in Fig. 6. Two of the dogs belonging to the group diagnosed with gastrointestinal disease had a remarkably high $CD3^{-}NCR1^{+}$ percentage measured to 3, 2 % (dog # 25) and 6, 8 % (dog # 23). The latter represented the highest measurements of $CD3^{-}NCR1^{+}$ cells in PBMC registered in our material. The other dogs had levels comparable to healthy dogs. Results from $CD3^{+}NCR1^{+}$

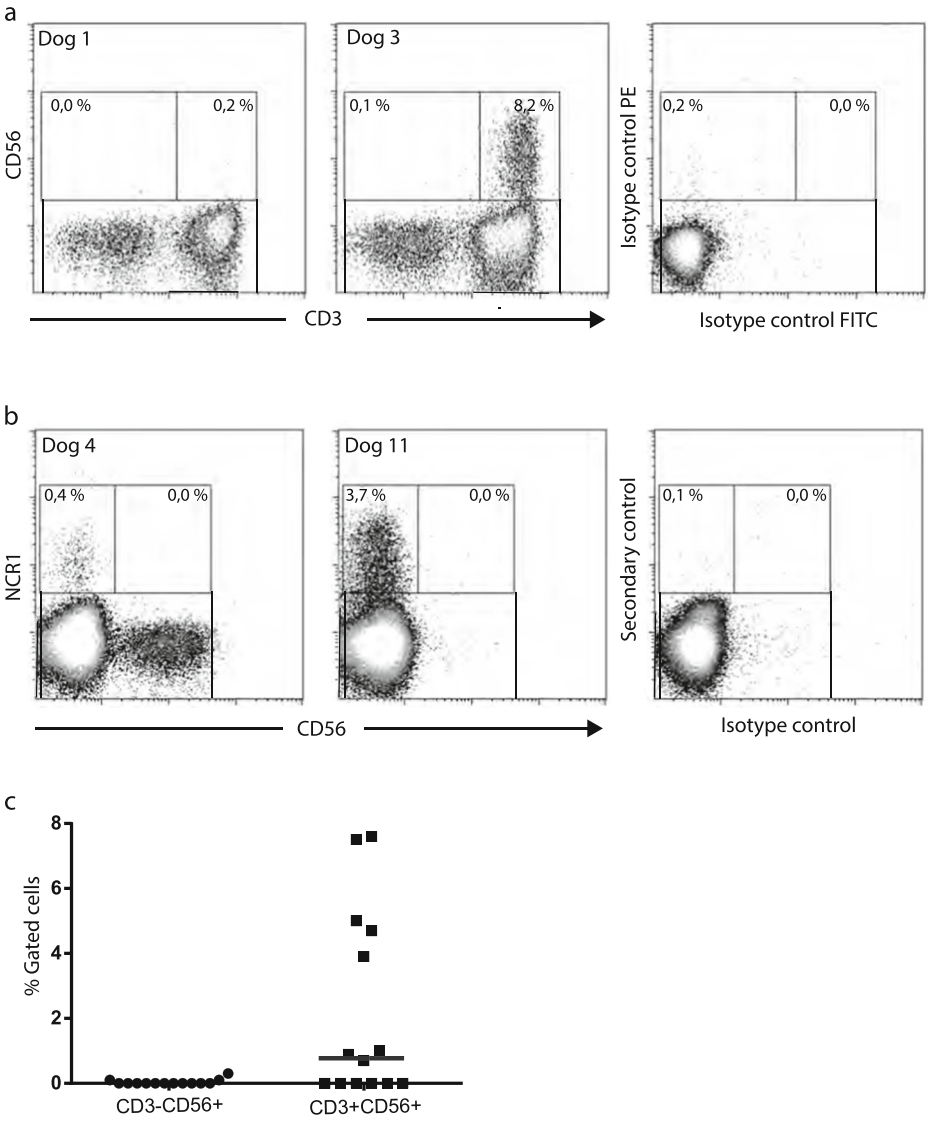

Fig. 4 CD56 expression in PBMC. Two color flow cytometric analysis of PBMC from healthy dogs gated on lymphocytes. **a** CD3 and CD56 double staining. **b** CD56 and NCR1 double staining. Plots are representative for the 14 healthy dogs included in this study. **c** Variation in the percentage of $CD3^{-}CD56^{+}$ and $CD3^{+}CD56^{+}$ cells in PBMC from the 14 healthy dogs. Median is presented

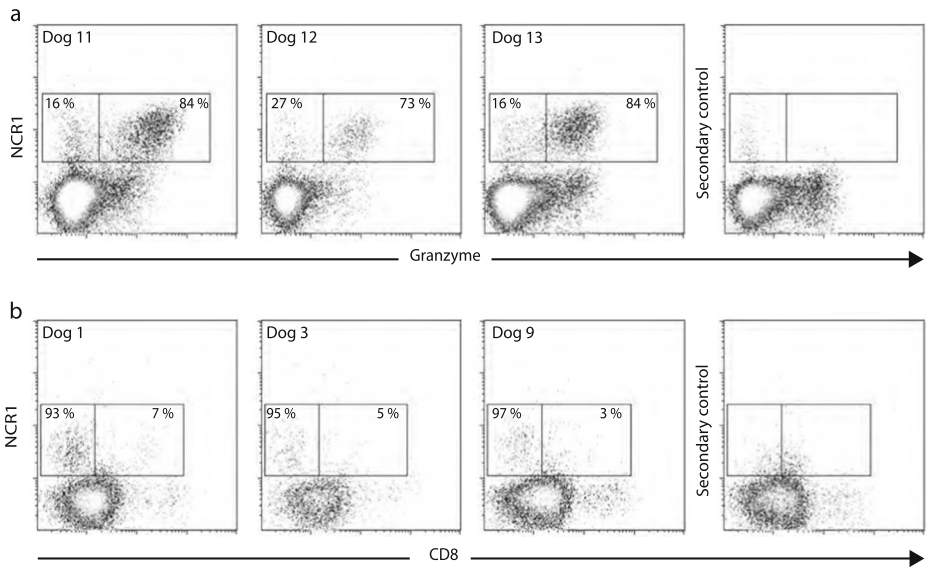

Fig. 5 Granzyme and CD8 staining of PBMC from healthy dogs gated on lymphocytes. **a** CD3, Granzyme and NCR1 triple staining. Cells shown have been gated on lymphocytes and further on $CD3^{-}$ cells. Percentages in gates refer to percent of $CD3^{-}NCR1^{+}$ cells. **b** CD3, CD8 and NCR1 triple staining. Cells shown have been gated on lymphocytes and further on $CD3^{-}$ cells. Percentages in gates refer to percent of $CD3^{-}NCR1^{+}$ cells

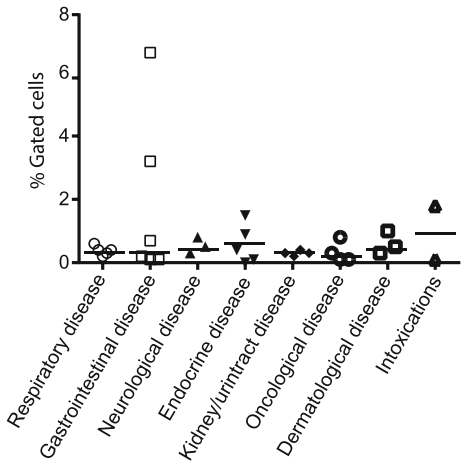

Fig. 6 Percentages of $CD3^{-}NCR1^{+}$ cells in the lymphocyte gate in dogs with various diagnoses. A total of 36 dogs divided in 8 disease categories were analyzed. Median is presented

cells were only available for 4 of the dogs with a clinical diagnosis. The $CD3^{+}NCR1^{+}$ populations ranged from 1, 8 to 26, 5 % of gated cells. 29 of the 34 dogs with a clinical diagnosis were also tested for CD56 expression. 21 of 29 dogs had $CD56^{+}$ cells ranging from 0, 1 to 9, 7 % and these cells were all $CD3^{+}$ (data not shown).

Dog # 47 was diagnosed with LGL leukemia. On a blood smear stained with modified Wright's stain the percentage of neoplastic cells containing granules were counted to 11 % (Fig. 7a). The leukemia was phenotyped to be $CD3^{+}NCR1^{+}CD8^{+}$ as shown in Fig. 7b using flow cytometry and showed granular staining of Granzyme B^{+} in bloodsmears (Fig. 7c). The granzyme B^{+} granules were polarized on one side of the cells as also seen on blood smears stained with modified Wright stain. The percentage of neoplastic

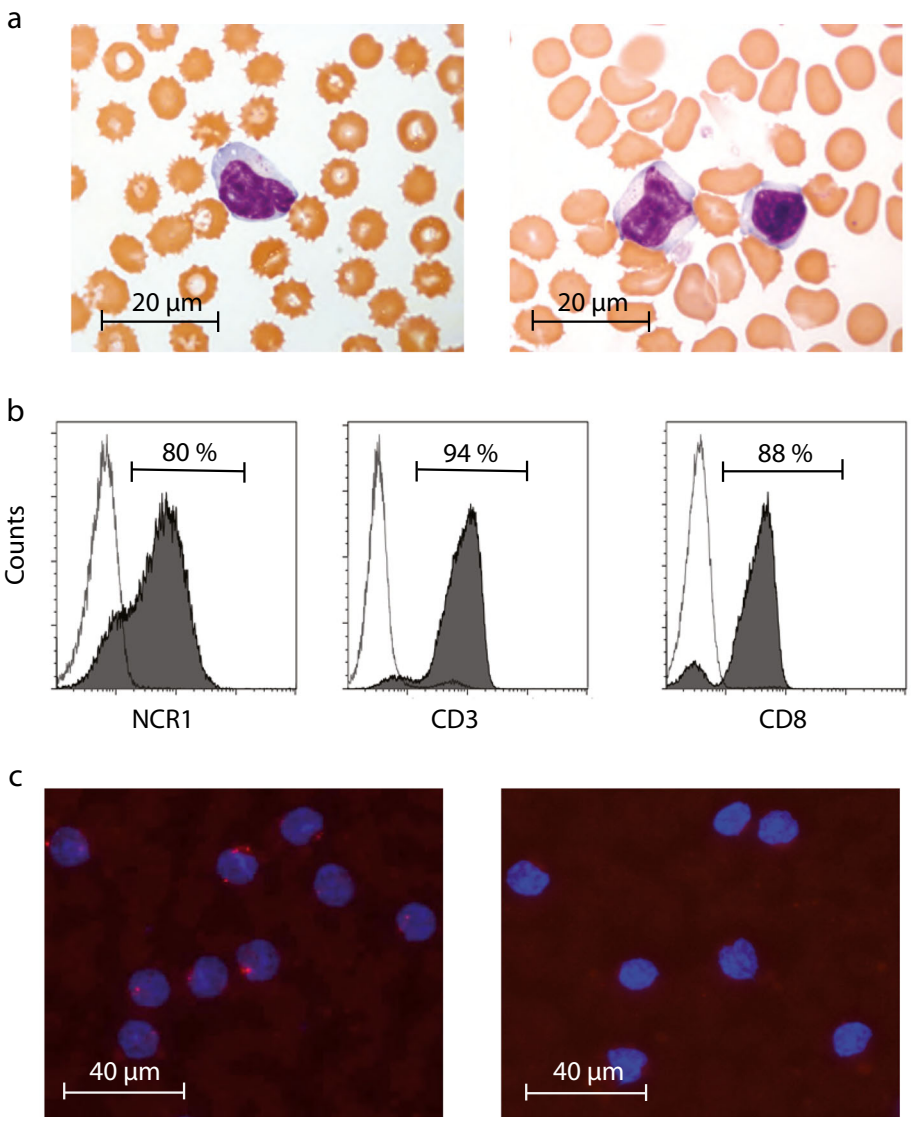

Fig. 7 Characterization of PBMC from dog #47 with leukemia. **a** Neoplastic cells in a blood smear air dried and stained with a modified Wright's stain. **b** Results from flow cytometric analysis of PBMC. Solid line, empty histograms show controls and shaded histograms show NCR1, CD3 and CD8 respectively. **c** Blood smears stained with anti Granzyme B in *red* and nucleus stained *blue* in left panel

cells containing granzyme B was counted to 72 %, a results that coincide with the number of neoplastic cells being $NCR1^+$ as shown in Fig. 7b. Additional flowcytometric analysis showed that the neoplastic cells also had a $CD4^-MHCII^+CD21^-$ phenotype (results not shown).

NCR1+ cells found in liver, spleen and lymph nodes

Mononuclear cells from liver, spleen and lymph nodes from 8 dogs belonging to various breeds and ages with various diagnoses were analyzed using flow cytometry. $NCR1^+$ cells were found in the liver, spleen and peripheral and intestinal lymph nodes as shown in Table 1. Dog # 55 had no circulating $CD3^-NCR1^+$ cells, though 1, 3 % of cells from an intestinal lymph node had these characteristics, indicating that dogs with no circulating $CD3^-NCR1^+$ cells may have this cell population located in other body sites.

Discussion

Several reports have detected natural killer activity in canine PBMC (Knapp et al. 1993; Krakowka 1983; Miyata et al. 2013) and lymphokine-activated killer cell (LAK) cultures (Huang et al. 2008; Lin et al. 2010; Shin et al. 2013) but there exists no satisfactory phenotypic definitions of canine NK cells. Since the early days of human and murine NK cell biology, NK cells have been defined as $CD3^-TCR^-$ large granular lymphocytes (Hercend and Schmidt 1988), and this definition has also been used in domestic animals (Mair et al. 2012; Storset et al. 2004). Canine NK cells should therefore be sought among $CD3^-$ cells. Shin et al. have recently characterized canine LAK cell cultures with natural killer cell activity. These cells were, however, uniformly expressing CD3. LAK cultures have been described as IL-2 activated lymphocytes with natural killer activity that can comprise NK cells, T cells or usually both and the relative contribution depends on the source of the lymphocytes (Hercend and Schmidt 1988).

We here report the presence of a population of $CD3^-NCR1^+$ cells in blood and tissues in dogs. As NCR1 has been regarded as a reliable NK cell marker on $CD3^-$ cells in other species, these cells may represent canine NK cells, although present in lower numbers than reported in other species. Among the $CD3^-NCR1^+$ cells 73–98 % of the cells expressed Granzyme B indicating that these cells have a killing capability. As positive selection of $NCR1^+$ cells using AKS6 has not been successful, culturing non-cancerous CD3-$NCR1^+$ canine cells is a challenge. We can therefore not determine if the $CD3^-NCR1^+CD56^-GranzymeB^+CD8^-$ population described here has the functional characteristics of NK cells.

From the initiation of this study until time of publication, no annotated canine NCR1 transcript based on cDNA sequencing has been published in GenBank. Neither has any canine Expressed Sequence Tags representing NCR1 been identified by blast searches. The number of EST sequences with match to NCR1 is less than ten for both *homo sapiens* and *bos taurus*. This indicates that NCR1 expressing cells/tissues in general are not abundantly represented in GenBank. The canine NCR1 transcript identified in this study (JN790861) has been used by GenBank to generate a NCBI Reference sequence for canine NCR1 (NM_001284448.1). A canine NCR1 transcript is computationally predicted by genebuid in Ensembl (ENSCAFT00000004152). This predicted transcript is identical in nucleotide sequence to our JN790861, except for coding for a shorter protein of 307 amino acids.

As CD56 has been used to define canine NK cells, we investigated the expression of this marker on $CD3^-NCR1^+$ cells, and found that these populations did not overlap. We found that $CD56^+$ cells in all dogs examined were expressing $CD3^+$. Others have reported that $CD56^+$ cells in the dog are $CD3^+$ (Otani et al. 2002). Furthermore CD56 mRNA is not found in tissues where one should expect to find NK cells (Lin et al. 2010) It is therefore likely that canine NK cells do not express CD56.

Table 1 Age, breed, diagnosis and percentage of $CD3^-NCR1^+$ in tissue samples from organs of 8 dogs. Gating was set as in Fig. 3

Dog #	Age (years)	Breed	Diagnosis	Liver	Spleen	*P.lnn.*	*I.lnn.*	PBMC
48	0, 3	Greyhound	Heart disease	–	–	1,7	–	–
49	0, 4	Cairn terrier	PSS	23,8	4,6	0,9	–	–
50	1,5	Mixed breed	Kidney disease	0,9	3,0	1,5	2,3	2,1
51	5	Mixed breed	Kidney disease	1,0	1,0	0,9	–	0,9
52	1,5	Mixed breed	Kidney disease	1,0	1,1	1,4	0,3	0,9
53	0,3	Mixed breed	Kidney disease	–	4,1	3,0	2,6	–
54	2	Mixed breed	Kidney disease	–	–	3,1	2,2	–
55	9	Mixed breed	Mast cell tumor with metastasis	–	–	–	1,3	0,0

P.lnn popliteal/prescapular lymph nodes, *I. lnn* intestinal lymph nodes, − tissue not analysed. *PSS* portosystemic shunt

The level of $CD3^-NCR1^+$ cells in blood of healthy dogs was observed over time and variations in $CD3^-NCR^+$ cell population size within the same healthy dogs was observed. As the healthy dogs in this study did not go thorough physical examinations on each blood sampling, they may have had minor subclinical infections not noticed by the owner. Substantial fluctuations in NK cells in peripheral blood over time has also been seen in cattle (Kampen et al. 2006). A trivial challenge to the immune system may have an effect on the percentage of circulating NK cells and this may account for the time variation seen in $CD3^-NCR1^+$ cells.

In this study a population of $CD3^+NCR1^+$ cells was observed in canine PBMC. $CD3^+NCR1^+$ cells have been described in mice and humans, both in *in vitro* and *in vivo* studies (Hudspeth et al. 2013). Recently $CD3^+NCR1^+$ cells were also described in PBMC from cattle as a distinct population of lymphocytes expressing a rearranged T cell receptor (Connelley et al. 2014). NCR1 has been shown to be up regulated on certain subgroups of T cells upon activation, for example on intestinal αβ T cells during celiac disease in humans (Meresse et al. 2006) The $CD3^+NCR1^+$ cell population is presented here in unstimulated PBMC from the dog is most likely a subgroup of T cells, but this needs to be confirmed.

We also describe a leukemia with a $CD3^+NCR1^+CD8^+$Granzyme B^+ phenotype indicating that NCR1 expressing neoplasia may exist in the dog. A large degree of discrepancy between neoplastic cells containing granules evaluated on a modified Wright stained blood smear and neoplastic cells containing granzyme B evaluated using a immunofluorescence technique was observed. It is known that NK cell granules can be difficult to visualize using standard staining techniques and hence alternative staining methods have been used (Storset et al. 2004). A modest number of case reports of LGL neoplasia have been reported in the dog reflecting the rarity of this disease (Maruo et al. 2009; Snead 2007; Takahashi et al. 2007; Wellman et al. 1989; Williams et al. 2008). In humans LGL neoplasia is a heterogenous group of neoplasias ranging from benign conditions to aggressive disease. Both the aggressive and chronic diseases are split into T cell LGL neoplasia ($CD3^+CD56^+$) and NK cell LGL neoplasia ($CD3^-CD56^+$) (Sokol and Loughran 2006). NCR1 is known to be expressed on neoplasias with a NK cell origin, but also 20 % of T cell tumors evaluated in a study from 2013 expressed NCR1 (Freud et al. 2013). These were particularly T cell large granular lymphocytic leukemias showing that NCR1 can be upregulated in malignantly transformed cells in humans.

To conclude we have described a $CD3^-NCR1^+$ $CD56^-$GranzymeB$^+CD8^+$ cell population that may represent NK cells in dogs. These cells were found in the circulation in more than 90 % of the dogs examined and in the liver, spleen and lymph nodes in all dogs in the study. However, they were present in lower numbers that NK cells in other species and since a population of $CD3^-NCR1^-$GranzymeB$^+$ were also present in some animals, NCR1 may not be expressed on all NK cells in the dog as reported in swine (Mair et al. 2012). A $CD3^+NCR1^+$ cell population was also detected in PBMC in healthy dogs. This cell population likely represents a subset of T cells but this needs further investigation. Neoplastic cells from PBMC from a Rottweiler with a leukemia had morphological characteristics of LGLs and a $CD3^+NCR1^+CD8^+$Granzyme B^+ phenotype.

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Ethical standards The authors declare that the experiments comply with the current laws of the country in which they were performed.

Conflict of interest statement The authors declare that no financial or commercial conflict of interest exists in relation to the content of this article.

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Supplementary Table 1

Dog #	Age (years)	Breed	Tissue samples	Diagnosis
1	3	Kleiner münsterlender	PBMC	Healthy
2	7	Standard poodle	PBMC	Healthy
3	5	Springer spaniel	PBMC	Healthy
4	6	Mixed breed	PBMC	Healthy
5	6	Golden retriever	PBMC	Healthy
6	7	Flatcoated retriever	PBMC	Healthy
7	2	Miniature poodle	PBMC	Healthy
8	14	Fox terrier	PBMC	Healthy
9	9	Boxer	PBMC	Healthy
10	-	Vorsteh	PBMC	Healthy
11	3	Etnahound	PBMC	Healthy
12	0,4	Etnahound	PBMC	Healthy
13	2	Etnahound	PBMC	Healthy
14	10	Mixed breed	PBMC	Healthy
15	0,5	Tamaskan dog	PBMC	Respiratory disease
16	1	Nova Scotia duck tolling retriever	PBMC	Respiratory disease
17	3	Standard poodle	PBMC	Respiratory disease
18	-	Mixed breed	PBMC	Respiratory disease
19	6	Gordon setter	PBMC	Neurological disease
20	1	Standard poodle	PBMC	Gastrointestinal disease
21	3	Standard poodle	PBMC	Neurological disease

22	6	Schnauzer	PBMC	Endocrine disease
23	4	Italian greyhound	PBMC	Gastrointestinal disease
24	11	Italian greyhound	PBMC	Gastrointestinal disease
25	4	Gordon setter	PBMC	Gastrointestinal disease
26	2	Jack russel terrier	PBMC	Intoxication
27	7	Rottweiler	PBMC	Dermatological disease
28	0,6	Mixed breed	PBMC	Kidney/Urintract disease
29	3	Pug	PBMC	Kidney/Urintract disease
30	8	Boxer	PBMC	Oncological disease
31	9	West Highland white terrier	PBMC	Oncological disease
32	2	Whippet	PBMC	Endocrine disease
33	9	German shepherd	PBMC	Oncological disease
34	6	Mixed breed	PBMC	Endocrine disease
35	5	Mixed breed	PBMC	Gastrointestinal disease
36	5	Flatcoated retriever	PBMC	Dermatological disease
37	2	Scottish deerhound	PBMC	Dermatological disease
38	6	Dachs hound	PBMC	Gastrointestinal disease
39	4	Cocker spaniel	PBMC	Endocrine disease
40	6	Shar pei	PBMC	Oncological disease
41	1	Whippet	PBMC	Intoxication
42	0,4	Miniature poodle	PBMC	Kidney/Urintract disease
43	4	Hygen hound	PBMC	Kidney/Urintract disease
44	0,8	Gordon setter	PBMC	Neurological disease

45	7	Miniature poodle	PBMC	Respiratory disease
46	8	Cairn terrier	PBMC	Endocrine disease
47	13	Rottweiler	PBMC	LGL leukemia
48	0,3	Greyhound	Lnn	Heart disease
49	0,4	Cairn terrier	Liver, Spleen, Lnn	Liver disease
50	1,5	Mixed breed	PBMC, Liver, Spleen, Lnn	Kidney/Urintract disease
51	5	Mixed breed	PBMC, Liver, Spleen, Lnn	Kidney/Urintract disease
52	1,5	Mixed breed	PBMC, Liver, Spleen, Lnn	Kidney/Urintract disease
53	0,3	Mixed breed	Spleen, Lnn	Kidney/Urintract disease
54	2	Mixed breed	Lnn	Kidney/Urintract disease
55	9	Mixed breed	PBMC, Lnn	Oncological disease

Overview of the 55 dogs used in this study showing number assigned for each dog, age, breed, tissue samples taken and health status.

Supplementary Table 2

Dog #	Age (years)	Breed	$CD3^{-}NCR1^{+}$	$CD3^{+}NCR1^{+}$
1*	3	Kleiner münsterlender	0,9	0,1
2*	7	Standard poodle	0,1	0,1
3*	5	Springer spaniel	0,7	0,2
4*	6	Mixed breed	1,6	0,0
5*	6	Golden retriever	0,3	0,3
6	7	Flatcoated retriever	1,2	4,8
7	2	Miniature poodle	0,0	0,0
8	14	Fox terrier	0,6	-
9	9	Boxer	0,8	1,6
10	-	Vorsteh	1,4	1,9
11*	3	Etnahound	1,6	3,9
12*	0,4	Etnahound	1,6	2,3
13*	2	Etnahound	1,6	1,3
14	10	Mixed breed	2,5	1,9

Age, breed and the percentage of $CD3^{-}NCR1^{+}$ and $CD3^{+}$ $NCR1^{+}$ cells measured in EDTA blood of the 14 healthy dogs included in the study. For dogs sampled multiple times (*) a median of these measurements is presented in the table. Gating was set as shown in Fig 3a.

NCR1 is an activating receptor expressed on a subset of canine NK cells

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Abstract

Defining NK cells has been challenging in many veterinary species. Although several groups have described putative NK cell populations, there is still no consensus on a definition of NK cells in the dog. In the present study, canine NK cells are characterized as $CD3^-GranzymeB^+$ cells, further divided into a $NCR1^+$ and a $NCR1^-$ subset. All dogs examined displayed both subsets in blood, although of quite variable magnitude. Following vaccination an increase was observed in the $CD3^-$ $NCR1^-$ cell population in blood, but not in the $CD3^-$ $NCR1^+$ population. Non-B non-T cell cultures stimulated with IL-2 and IL-15 were dominated by $CD3^-GranzymeB^+$ cells after approximately 2 weeks and a large proportion of the $CD3^-$ $GranzymeB^+$ cells expressed NCR1. IL-12 stimulation lead to a further upregulation resulting in an almost uniform expression of NCR1. The cultured cells expressed MHC class II, showed a variable expression of CD8 and were negative for CD4 and CD21. The cultures were able to kill known NK cell targets, and NCR1 was shown to be a major activating receptor. A large proportion of the $NCR1^+$ cells, but none of the $NCR1^-$ cells, produced IFNγ in response to IL-12 stimulation. These results show that NCR1 defines two subsets of canine NK cells, likely to represent different activation stages, and that NCR1 acts as an activating receptor on canine NK cells.

Keywords: NCR1/NKp46; dog; natural killer cell; Granzyme B;

Introduction

Natural killer (NK) cells have been defined as large granular lymphocytes (LGLs) with a cytotoxic and immune regulatory function. NK cells recognize damaged, infected and

cancerous cells by a repertoire of germ line encoded inhibitory and activating receptors. The balance in the stimulation of these receptors decides the final outcome of the cell-to-cell contact. Natural killer cells as well as other lymphocytes like $CD8^+$ T cells and NKT cells, exert their cytotoxic function by releasing granules containing perforin and granzyme. Granzymes are serine proteases (Wensink et al., 2015) which induce apoptosis through activation of caspase-driven cell death pathways. Granzyme B is most extensively studied and is considered the most powerful pro-apoptotic granzyme (Voskoboinik et al., 2015). NK cells are important producers of cytokines, most important is the production of IFNγ (Schroder et al., 2004) which may activate other immune cells and contribute to the development of Th1 response in infections as well as vaccine responses (Horowitz et al., 2010; Martín-Fontecha et al., 2004). Although considered a part of the innate immune system, recent discoveries show that NK cell responses may have features reminiscent of adaptive immunity, like antigen specificity and memory (Geiger and Sun, 2016).

NCR1 (NKp46, CD355) is described as a consistent NK cell marker in many species examined (Walzer et al., 2007) such as humans (Sivori et al., 1997), monkeys (De Maria et al. 2001), rats (Westgaard et al., 2004), mice (Biassoni et al., 1999), cattle (Storset et al., 2004) and sheep (Connelley et al., 2011). However only about half of the NK cells in swine express NCR1 (Mair et al., 2012) demonstrating that this receptor cannot be regarded as a pan species NK cell receptor across mammals. Furthermore NCR1 was originally thought to be expressed by NK cells only, but it is now clear that also certain subgroups of T cells express NCR1 constitutively (Connelley et al., 2014; Hudspeth et al., 2013) or upregulate NCR1 under certain conditions (Meresse et al., 2006).

Triggering of NK cells is generally dependent on multiple signals but it has been shown that NK cell cytotoxicity could be mediated by NCR1 alone (Moretta et al., 2001) showing the importance of this receptor. Specific cellular ligands for NCR1 have still not been described but it probably binds hemagglutinin of influenza, suggesting that it may be involved in direct pathogen recognition (Mandelboim et al., 2001).

Canine non-B non-T cells have been the main focus of many studies, but there is still no agreement on how to define NK cells in the dog. NK cell activity against a canine thyroid adenocarcinoma cell line (CTAC) has been described at several occasions (Huang et al., 2008; Knapp et al., 1993; Lin et al., 2010; Ringler and Krakowka, 1985). Canine cells forming conjugates with CTAC have been described morphologically as cells with a kidney bean shape nucleus, and electron-dense cytoplasmic granules (Knapp et al., 1995). mRNA for NK associated markers have been quantitatively measured by real time PCR in non-B non-T

cell cultures showing an increase in expression of mRNA for several NK cell receptors compared to control cells (Lee et al., 2015; Shin et al., 2015). A method of establishing non-B non-T cultures with NK cell features has been described by depletion of $CD5^+$ cells from peripheral blood mononuclear cells (PBMC) and subsequent stimulation of cultures with recombinant human IL-2 and IL-15 (Michael et al., 2013). These cultures displayed NK cell characteristics but lacked the distinctive granules typical for NK cells.
We recently identified the presence of $CD3^-GranzymeB^+NCR1^+$ cell population in canine PBMC (Grøndahl-Rosado et al., 2015). However, $CD3^-NCR1^+$ cells appeared in substantial lower numbers in canine PBMC compared to what is reported in other species, and a considerable individual variation was observed including a virtual absence in some individuals. Notably, a distinctive $CD3^-GranzymeB^+NCR1^-$ population was observed in this study. Assuming that a total absence of circulating NK cells are unlikely, we hypothesized that dogs either possess two different subsets of NK cells with respect to NCR1 expression or that NCR1 may only be expressed on NK cells primed by inflammatory stimuli such as vaccination or adjuvants as previously shown in mice (Lucas et al., 2007).

In this study, we performed *ex vivo* and *in vitro* studies on canine NK cells. To encompass the possibility of $NCR1^-$ NK cells we based these studies on the absence of CD3 and presence of Granzyme B. We here show that dogs both have a $CD3^-GranzymeB^+NCR1^+$ and a $CD3^-GranzymeB^+NCR1^-$ cell population. NCR1 appears to be present in a majority of activated NK cells and this activating receptor participate in a major part of functional NK cell responses.

Materials and Methods

Animals and blood samples

Sixteen healthy dogs were included in this study with the consent of the owner. Ten dogs belonged to staff and students at the Norwegian University of Live Sciences and 6 dogs belonged to The Norwegian Armed Forces. The dogs were evaluated as healthy based on a clinical examination. Age, breed and vaccination status of the dogs included in the study are summarized in Table 1. Blood samples were taken before and 48 hours after a routine vaccination (Rabisin ®, Merial, Lyon, France). 20-50 ml EDTA blood was collected from the cephalic vein and processed within 3 hours. Blood collection was approved by the The Norwegian Animal Research Authority.

Table 1

Dog #	Breed	Age (years)	Vaccination status
1	English Setter	6	Revax
2	Standard Poodle	3	1st vax
3	Great Dane	3	1st vax
4	Mixed Breed	6	Revax
5	Flat Coated Retriever	5	1st vax
6	Mixed Breed	Au	Revax
7	Labrador Retriever	9	1st vax
8	Boxer	2	1st vax
9	English Springer Spaniel	2	Revax
10	German Shepard	6	Revax
11	German Shepard	8	Revax
12	Belgian Sheepdog	6	Revax
13	English Springer Spaniel	8	Revax
14	Belgian Sheepdog	Au	Revax
15	Kleiner Münsterländer	3	Revax
16	English Springer Spaniel	6	Revax

All 16 dogs included in the study. Breed, age and vaccination history are shown. Revax = revaccination, 1st vax = the dog had not previously been vaccinated, Au = age unknown

Processing of EDTA blood and isolation of PBMC

PBMC was isolated using Lymfoprep ® (Axis-Shield, Dundee, Scotland). The interphase was removed and washed 3 times with Dulbecco`s Phosphate Buffered Saline (DPBS) supplemented with 2mM EDTA. The cells were counted and viability evaluated using trypan blue expression analysed by the Countess ® Automated Cell Counter (Thermo Fisher Scientific, Carlsbad, CA, USA).

Establishing $CD5^-CD3^-$ cultures

A depletion was performed using mAbs against canine CD5 (YKIX322.3) (AbDSerotec, Oxford, UK) and MACS immunomagnetic beads coated with an anti-rat pan IgG mAb and a LD column (both from Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. The $CD5^-$ cells were cultured in RPMI 1640 supplemented with 60 μg/ml penicillin, 100 μg/ml streptomycin, 1 mM sodium pyruvate, nonessential amino acids, 50 μM 2-mercaptoethanol, (all Invitrogen, Carlsbad, CA, USA), 10% fetal calf serum (FCS) (PAA Laboratories, Pasching, Austria) (NK cell medium) with 200 U/ml recombinant human (rh) IL-2 and 10 ng/ml rhIL-15 (both eBioscience, San Diego, CA, USA). Cultured cells were split when needed and new medium containing 100 U/ml rhIL-2 and 5 ng/ml rhIL-15 was added. The cultured cells were monitored for CD3 expression. In a vast majority of cultures a subpopulation of $CD3^+$ cells appeared. These were removed by depletion using mAbs against canine CD3 (Ca17.2A12, AbDSerotec) and MACS immune magnetic beads coated with anti-mouse pan IgG (Miltenyi Biotec). CD3 depletion was conducted 1-2 days before functional assays were performed to assure an absence of $CD3^+$ cells. Cultured cells were analysed between day 12 and 17.

Cytospin preparation

Cytospin preparations were made using a Thermo Shandon Cytospin 4 (Thermo Fisher Scientific). Briefly, 200μL of cell suspension harvested from cell cultures were spun at 600 rpm/min for 6 minutes. The slides were stained with modified Wright´s stain using an automatic stainer, Hematek 2000 ® (Siemens, Munich, Germany). The percentage of granulated lymphocytes was determined by light microscopy (Olympus BX60) using 100x oil lens.

Flow cytometry

Flow cytometric analysis was performed on peripheral blood mononuclear cells (PBMC) on the day of blood sampling and on the cultures before functional assays were performed. The cultures were also monitored with regards to CD3 and NCR1 expression. The antibodies used in this study are shown in Table 2. They were all used according to manufactures recommendations. 5 $x10^5$ cells re-suspended in 25 μl PBS with 1 % bovine serum albumin (BSA) and 10 mM NaN_3 (flow buffer) were incubated with appropriate antibodies on ice. For all stainings involving mouse anti-bovine NCR1 (AKS6) the antibodies were incubated

individually. The AKS6 antibody was incubated first, then the appropriate secondary antibody followed by the remaining directly conjugated antibodies. All cells marked with extracellular staining were analysed without previous fixation. For intracellular staining for granzyme B and IFNγ the BD Cytofix/Cytoperm ^{TM} kit (BD Biosciences, San Jose, CA, USA) was used according to the manufactures instructions. After the last washing, the cells were dissolved in 200 µl flow buffer and kept at 4 C° until analysis. Data was collected using Gallios and processed in Kaluza (both Beckman Coulter, Brea, CA, USA). Dead cells were excluded using LIVE/DEAD® Fixable Aqua Dead Cell Stain Kit (Thermo Fisher Scientific). Doublets were excluded by a FS INT vs. FS PEAK gate. All results presented from PBMC are gated on lymphocytes. Results presented from cell cultures are gated on the entire culture.

Table 2

Antibody	Conjugate	Clone	Supplier
Mouse anti-canine CD3 (IgG1)	Unconjugated FITC	CA17.2A12	AbDSerotec
Rat anti-canine CD4 (IgG2a)	Al647	YKIX302.9	AbDSerotec
Rat anti-canine CD5 (IgG2a)	Unconjugated	YKIX322.3	AbDSerotec
Mouse anti-canine CD8 (IgG1)	Al700	YCATE55.9	AbDSerotec
Mouse anti-canine CD21 (IgG1)	FITC	CA2.1D6	AbDSerotec
Mouse anti-canine CD25 (IgG1)	FITC	P4A10	eBioscience
Mouse anti-human CD94 (IgG2a)*	Al647	HP-3D9	eBioscience
Rat anti-canine MHCII (IgG2a)	FITC	YKIX334.2	AbDSerotec
Mouse anti-human Granzyme B (IgG1)**	PE	GB11	eBioscience
Mouse anti-bovine IFNγ (IgG1)***	Al647	CC302	AbDSerotec
Mouse IgG1 negative control	FITC	-	AbDSerotec
Mouse IgG1 negative control	PE	-	AbDSerotec
Mouse anti-bovine NCR1 (IgG2b)**	Unconjugated	AKS6	In house production

Antibodies used in the study. Cross reactivity to the canine equivalent reported * in (Schuberth et al., 2007). ** in (Grøndahl-Rosado et al., 2015). *** by the supplier.

Cytotoxicity assay (51Cr release)

The cytotoxic activity of rhIL-2/rhIL-15 activated canine $CD5^-CD3^-$ cells against the canine thyroid adenocarcinoma cell line (CTAC) (Sigma-Aldrich St Louis, MO, USA) (Kasza, 1964; Knapp et al., 1993; Krakowka, 1983) and the Fc expressing murine cell line P815 were tested in a standard ^{51}Cr release assay. Target cells (5×10^6cells) were incubated with 100 µCi $Na_2{}^{51}CrO_4$ (Amersham Biosciences, Piscataway, NJ, USA) in 1 ml RPMI 1640 (Thermo Fisher Scientific) supplemented with 10% FCS at 37°C for 1h and then washed three times in PBS supplemented with 2% FCS. Two-fold dilutions of effector cells ($CD5^-CD3^-$ cell cultures) were added to 1×10^4 ^{51}Cr-labeled target cells in 96-well round-bottom microtiter plates and incubated 37°C for 15h. The supernatant was harvested and radioactivity counted on a microplate scintillation counter (Packard Top10 Count). Specific ^{51}Cr release was calculated on the basis of the ratio [(sample release – spontaneous release)/(total release – spontaneous release)]. The results were expressed as the average of three parallel samples. In blocking experiments with CTAC targets and in redirected lysis assays of the $Fc\gamma R^+$ target cell line P815 the effector cells were pre incubated for 60 min with 1 µg/ml mAb against NCR1 (AKS6).

IL-12 stimulation of cultured cells for detection of IFNγ production

To assess the ability of the cultured cells to produce IFNγ in response to stimulation by rhIL-12 (eBioscience) the cells were cultured with and without rhIL-12 in addition to rhIL-2/rhIL-15. Cells cultured without rhIL-12 contained few IFNγ positive cells and was used as baseline. Four different concentrations of IL-12 were used to evaluate the effect of rhIL-12 stimulation. $1x10^6$ cultured cells in 1.5 ml NK cell medium supplemented with rhIL-2 (200 U/ml) and rhIL-15 (10 ng/ml) were incubated with appropriate amount of IL-12 overnight. 4 hours before analysis Brefeldin A (Sigma-Aldrich) was added according to the manufacturer's instructions. The cells were then washed and stained for flow cytometric analysis as described earlier.

Statistical analysis

Differences between non-vaccinated and vaccinated dogs were evaluated by non-parametric Wilcoxon rank-sum test for matched pairs using JMP Pro 11.0 (SAS, Cary, NC, USA). P values $p<0.05$ were considered significant.

Results

Presence of CD3$^-$GranzymeB$^+$NCR1$^+$ and CD3$^-$GranzymeB$^+$NCR1$^-$ cells in blood before and after vaccination

In order to evaluate if individual differences in NK cell numbers could be explained by minor subclinical inflammation, PBMC was analysed using flow cytometry before and 48 hrs after routine vaccination against rabies. GranzymeB$^+$NCR1$^+$ and GranzymeB$^+$NCR1$^-$ cell populations were detected among CD3$^-$ cells both pre and post vaccination (Fig 1A).

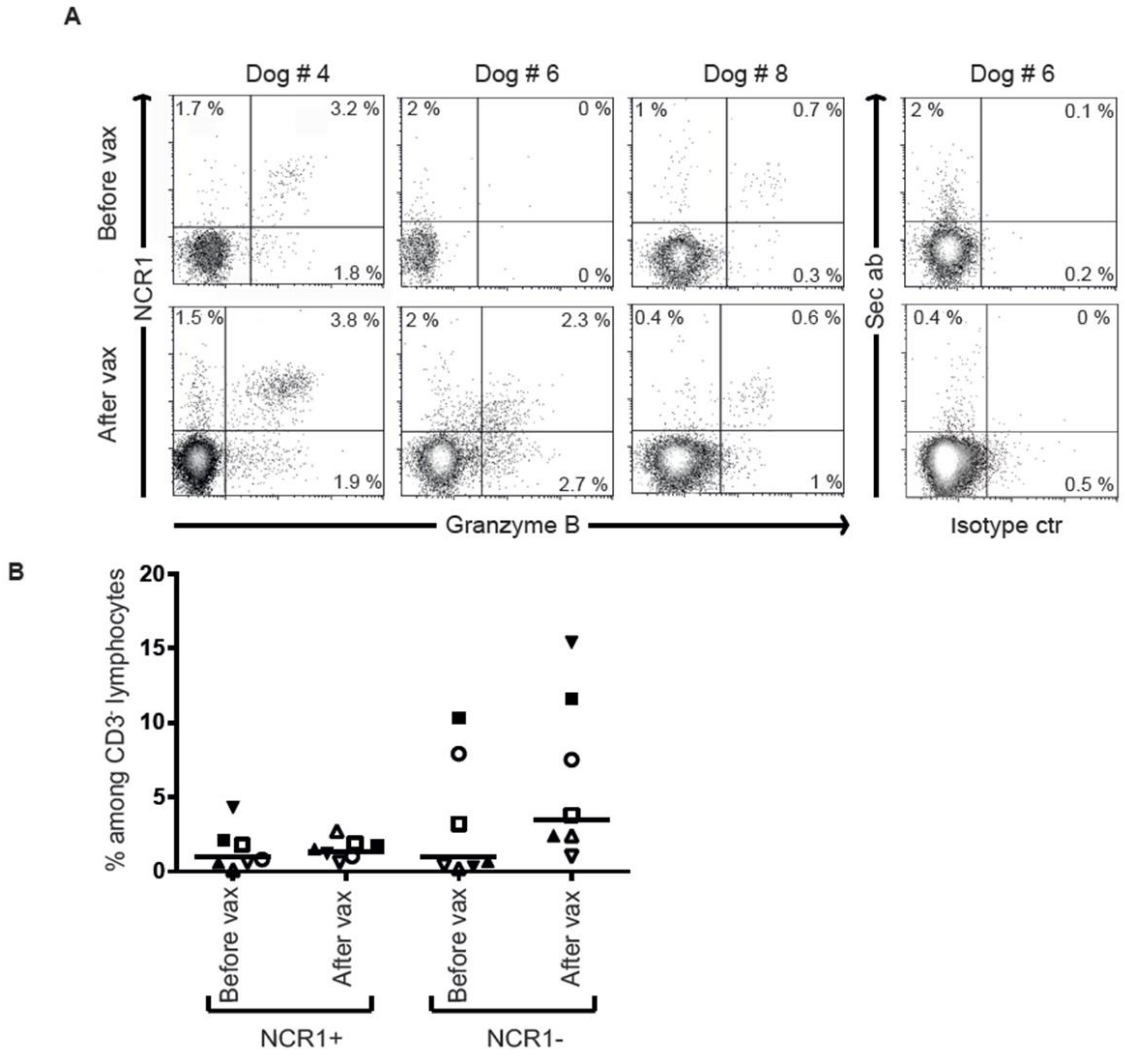


Fig. 1 A Granzyme B vs. NCR1 dot plots gated on CD3$^-$ cells from 3 representative dogs from before and 48 hours after vaccination. **B** Scatterplot showing the distribution of GranzymeB$^+$ NCR$^-$ and GranzymeB$^+$ NCR1$^+$ cells among the CD3$^-$ cells before and after vaccination for 7 dogs.

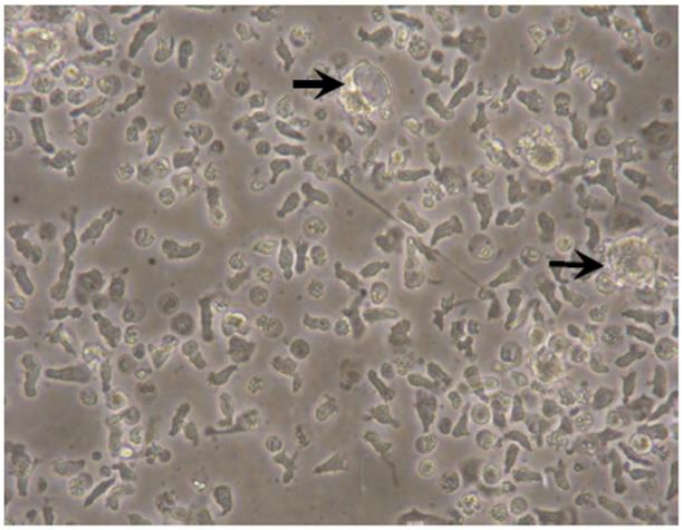
Before vaccination NCR1$^+$ cells ranged from 0.1 % to 4.3 % and NCR1$^-$ cells ranged from 0.2 % to 10.3 % of CD3$^-$ cells. A significant relative increase of Granzyme B$^+$ NCR1$^-$ cells was observed following vaccination, the increase being slight to moderate in most of these individuals. One dog did not show an increase after vaccination but this dog had an already high level of GranzymeB$^+$NCR1$^-$ cells before vaccination. No significant change was detected in the NCR1$^+$ cell population (Fig. 1B). Thus while this type of inflammatory episode seemed to increase the circulating levels of Granzyme B$^+$ non-T cells, it did not influence NCR1$^+$ expression among these cells.

CD5$^-$CD3$^-$ cell cultures had a variable expression of NCR1

Since Granzyme B is an intracellular protein that cannot be used for sorting of living cells, we established non B non T cell cultures based on CD5 depletion (Michael et al., 2013). CD5 depletion resulted in a 94-100 % purity of CD5$^-$ cells. The CD5$^-$ cells proliferated vigorously when stimulated with IL-2 and IL-15. However, CD3$^+$ cells appeared after several days in a majority of these cell cultures (constituting 7-64 % of the cultured cells), and additional CD3 depletion was performed 1-2 days before further analysis, resulting in 95-98 % purity of CD3$^-$ cells. The dominant cell type in the CD5$^-$CD3$^-$ cultures had a morphological appearance consistent with Large Granular Lymphocytes (LGLs) evaluated by phase-contrast microscopy (Fig. 2A) and by assessment of cytospin preparations (Fig. 2B). A differential count of granulated cells correlated with the portion granzyme B positive cells measured with flow cytometry. Using phase-contrast microscopy also rounded accessory cells of a larger size then the LGL cells could be identified (Fig. 2A).

At day 12-17 granzyme B was expressed on 82 to 100 % of the cultured cells. The percentage of NCR1$^+$ cells in CD5$^-$CD3$^-$ cell cultures showed a gradual increase throughout the culture period peaking around 2 weeks (Fig. 3A). All NCR1$^+$ cells expressed Granzyme B. A CD3$^-$ GranzymeB$^-$NCR1$^-$ cell population was identified in all cultures (Figure 3B). Further flow cytometric analysis of the cultures at the time of functional tests revealed a MHCII$^+$CD8$^{+/-}$ CD25$^{+/-}$ CD4$^-$phenotype (Fig. 3C). The cells were negative for the B cell marker CD21 (data not shown). An anti-human CD94 antibody reported to cross react with canine CD94 did not stain these cells convincingly (data now shown). Cultures established from PBMC collected before and 2 days after vaccination proliferated with the same intensity evaluated by subjective assessment.

A



B

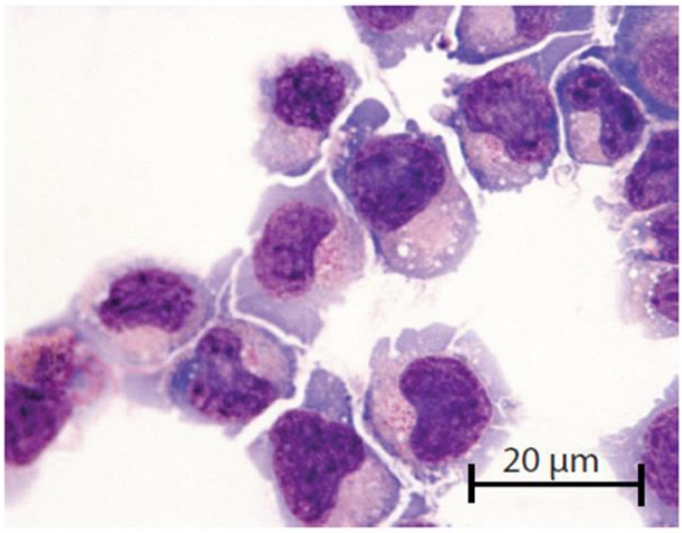


Fig. 2 A Phase-contrast microscopy image of a 12 day NK cell culture from dog # 5. Arrows indicate accessory cells. **B** Cytospin preparation stained with modified Wright´s stain of the same culture as shown in A.

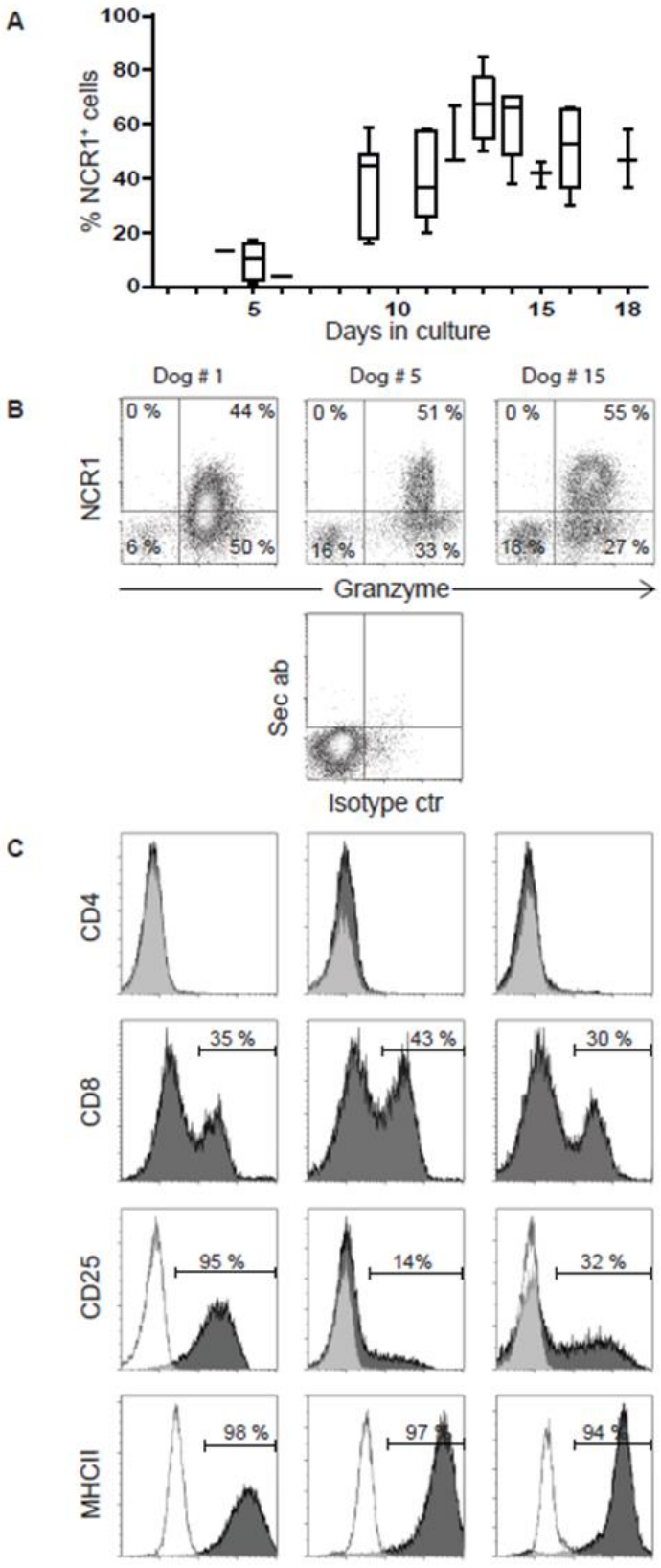


Fig. 3 A Graph with box plots showing the proportion of cultured cells expressing NCR1 between day 0 and 18. 1-2 cultures from 13 different dogs were analysed at 1-4 time points. The graph indicates maximum and minimum values and 5-95 percentile. **B** NCR1 vs. Granzyme dot plots from 12 day old cultures from 3 representative dogs. **C** Histograms of the same cultures as presented in 3B.

$CD5^-CD3^-$ cell cultures showed cytotoxic activity towards NK cell targets and NCR1 served as an activating receptor

IL-2 /IL-15 stimulated $CD5^-CD3^-$ cultures were tested for cytotoxic activity towards the canine thyroid adenocarcinoma (CTAC) cell line known to be a canine NK cell target and the Fc expressing murine cell line P815. All tested cultures showed spontaneous cytotoxic activity towards both CTAC and P815. Pre-incubation with the AKS6 antibody, leading to a blocking of NCR1 resulted in a reduced cytotoxic effect towards the CTAC cell line (Fig. 4A). In a redirect lysis assay against P815, pre-incubation of the NK cell cultures with AKS6 mediated a higher degree of killing compared to when no mAb was added (Figure 4B).

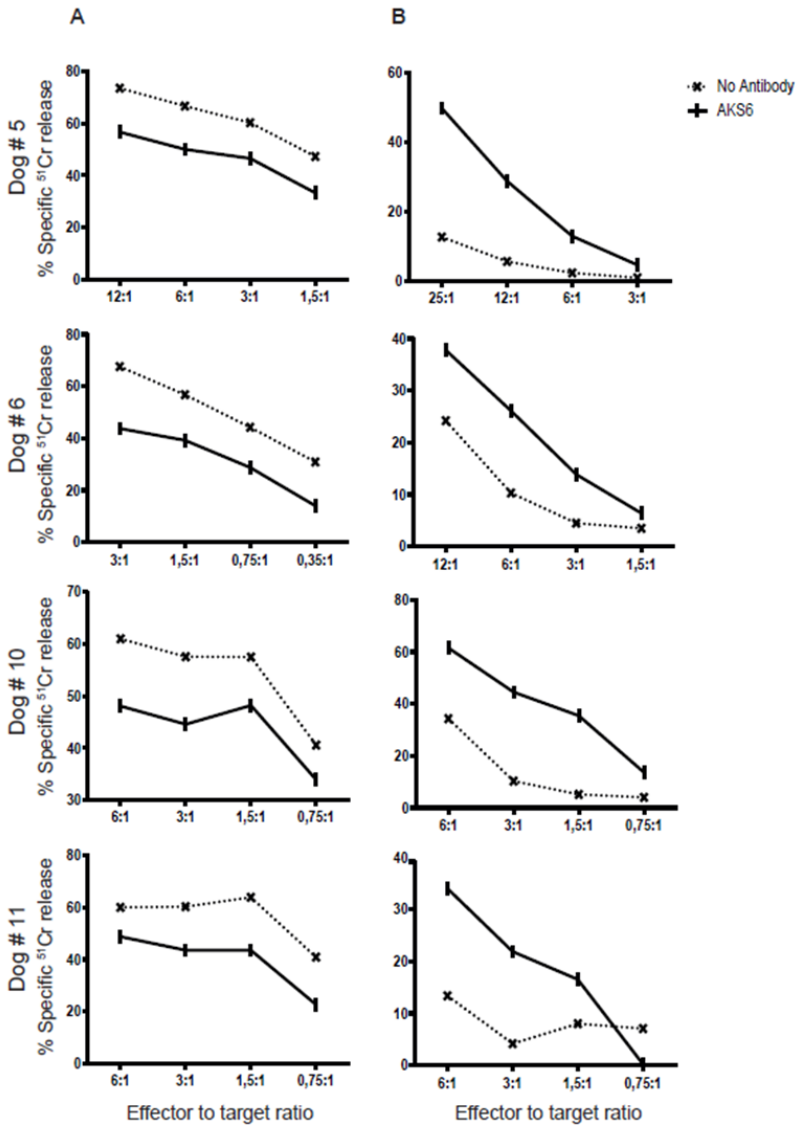


Fig. 4 Cytotoxic activity of $CD5^-CD3^-$ cultures from 4 representative dogs tested towards **A** CTAC and **B** P815. All cultures shown contains between 40-50 % $NCR1^+$ cells.

This demonstrates that NCR1 in dogs, like in other species, is an activating receptor as cross binding of the receptor increased and blocking of the receptor decreased the cultured cells cytotoxicity against respective target cells.

$CD3^{-}GranzymeB^{+}NCR1^{+}$ cells produced IFNγ in response to IL-12 stimulation and IL-12 stimulation gave an upregulation of NCR1.

About 30 % of the cultured cells produced IFNγ in response to IL-12 stimulation and all the IFNγ producing cells were $NCR1^{+}$ (Fig. 5A). A positive correlation was observed between concentration of IL-12 and the number of IFNγ producing cells (Fig 5B). In the control cultures stimulated with IL-2 and IL-15 only, NCR1 was expressed on 50-60 % of the cells. When cultures were stimulated with IL-12 in combination with IL-2 and IL-15, a dose dependent upregulation of NCR1 was observed reaching more than 90 % with the highest concentration of IL-12 (Figure 5C).

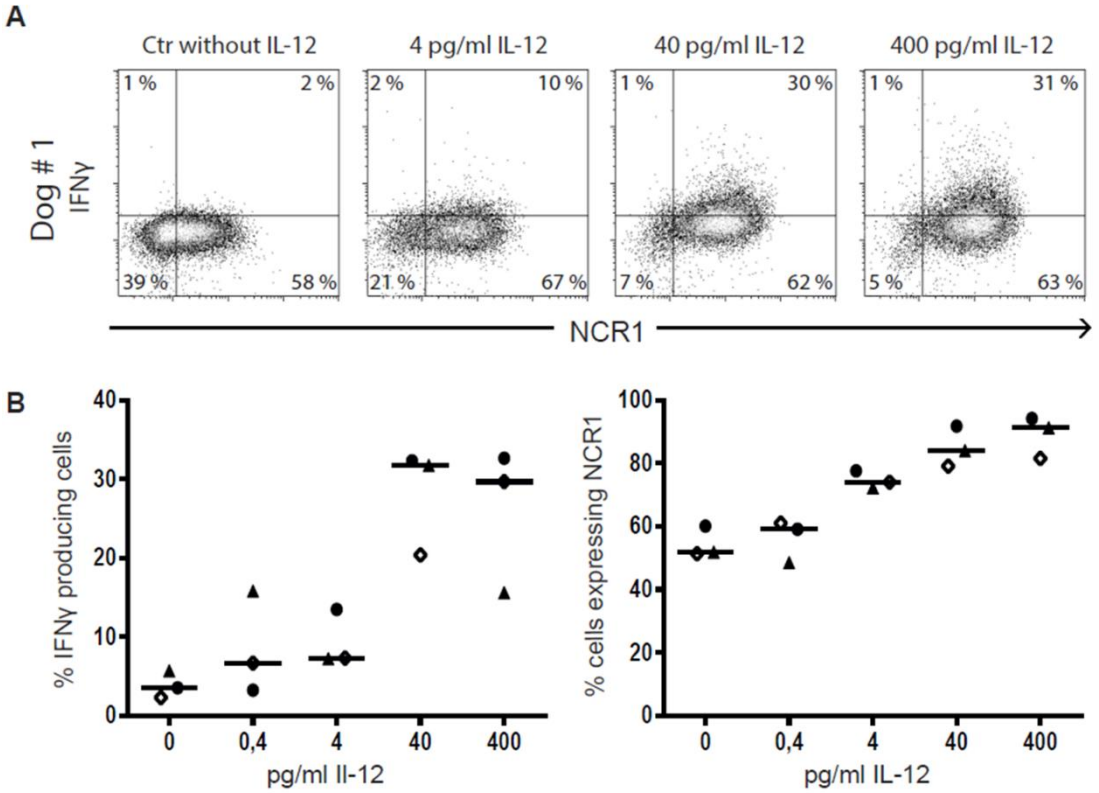


Fig. 5 A Representative IFNγ vs. NCR1 dot plots from dog # 1 after IL-12 stimulation. **B** Scatter plot showing percentage of the $NCR1^{+}$ cells producing IFNγ after overnight IL-12 stimulation of a 12 day old $CD5^{-}CD3^{-}$ culture from 3 dogs. **C** Scatter plot showing percentage of NCR1 expressing cells in $CD5^{-}CD3^{-}$ cultures when stimulated with IL-12 overnight.

Discussion

NCR1 has previously been demonstrated in the dog and a highly variable percentage of $NCR1^+$ cells in the circulation of healthy dogs was found (Grøndahl-Rosado et al., 2015), suggesting that not all NK cells in the dog express this marker. In most animal species examined, NCR1 is expressed on all NK cells and NCR1 has therefore been suggested a valid NK cell marker across species (Walzer et al., 2007). In humans NCR1 expression can however vary greatly within the NK cell population (Caligiuri, 2008; Sivori et al., 1997). In swine both a $NCR1^+$ and a $NCR1^-$ NK cell population has been characterized in healthy individuals (Mair et al., 2012). In the present study, the definition of canine NK cells was expanded to comprise $CD3^-GranzymeB^+$ cells, and we show that within this circulating cell population, healthy dogs have $NCR1^+$ and $NCR1^-$ cells. The $NCR1^+$ and $NCR1^-$ cell populations were of similar sizes except for 2 dogs displaying as much as 7.9 and 10.3 % $NCR1^-$ cells in PBMC. Together these two cell populations comprised between 1 and 12.4 % of $CD3^-$ lymphocytes. In adult humans NK cells comprise 7-31 % of the circulating lymphocytes (Comans-Bitter et al., 1997) while in cattle 0.5-10 % NK cells has been reported (Kulberg et al., 2004). In numbers, the sum of canine $NCR1^+$ and $NCR1^-CD3^-GranzymeB^+$ cells would thus be consistent with NK cells in other mammals.

To obtain cell cultures comprising both of these subsets we followed the $CD5^-CD3^-$ cell selection strategy described by Michaels et al. (Michael et al., 2013). After 2 weeks of IL-2 and IL-15 stimulation, these cultures were largely dominated by $CD3^-GranzymeB^+$ cells, of which an increased proportion expressed NCR1. After additional IL-12 stimulation, a further upregulation of NCR1 could be detected, resulting in around 90 % of the cultured cells expressing NCR1. Cytokine-induced NCR1 expression has been demonstrated in other species, as IL-2 stimulation has been shown to upregulate NCR1 on NK cells from uninflamed human tonsils (Ferlazzo et al., 2004) and IL-12 together with IL-2 or IL-15 has been shown to upregulate NCR1 on human circulating NK cells (Loza and Perussia, 2004). Also in cattle NCR1 is upregulated on γδ T cells after IL-15 stimulation (Johnson et al., 2008). Swine $NCR1^-$ NK cells upregulate NCR1 in response to IL-2, IL-12 and IL-18 and NCR1 is considered a marker of activation in this species (Mair et al., 2013, 2012). As we here see a similar inducible NCR1 in the dog, we find it highly likely that circulating $NCR1^-CD3^-GranzymeB^+$ also belong to the NK cell population, presumably at a lower stage of activation. We did not succeed in separating the $NCR1^+$ and $NCR1^-$ cell population for

isolated studies due to low cell numbers in the cultures and because the AKS6 antibody does not perform well in immunomagnetic cell sorting of canine cells.

In order to see if a similar upregulation of NCR1 occurred in an *in vivo* situation, we examined if an inflammatory episode would cause relative changes in these two subsets since injection of adjuvant has been shown to prime NK cells in mice. (Lucas et al., 2007). Routine rabies vaccination lead to a relative increase of $NCR1^-$ cells but not in $NCR1^+$ cells in the blood. Some dogs with underlying pathology have a relative increase in $NCR1^+$ cells in different body compartments showing that $NCR1^+$ cells might be recruited or upregulated during certain diseases (Grøndahl-Rosado et al., 2015). However, in the present study a minor intervention like rabies vaccination did not provoke such a response in blood. It should be noted that the alum adjuvant used here, is not considered ideal for the induction of cellular immune response, and more optimal adjuvants should be tested in further studies before drawing a firm conclusion.

Functionally, the $CD5^-CD3^-$ cell cultures showed spontaneous cytotoxic activity both towards the known canine NK cell target CTAC and the murine cell line P815. A baseline killing of the murine cell line P815 has also been reported in bovine NK cells (Storset et al., 2004). In a redirect lysis assay, cross-binding of the NCR1 receptor gave a marked increase of cytotoxic activity indicating that crosslinking and subsequent activation of this receptor is sufficient to initiate killing of target cells. The degree of increase however, varied between cultures. As all NK cell cultures applied in cytotoxic assays displayed NCR1 on approximately 50 % of the cells, differences in NCR1 expression between cultures could not explain this variation. Blocking of NCR1-ligand binding resulted in a decrease in cytotoxic activity against CTAC cells but some activity remained. Overall this shows NCR1 as a triggering receptor in the dog similar to what is seen in other species (Mair et al., 2012; Moretta et al., 2000; Storset et al., 2004). Since not all killing was blocked or mediated by NCR1, other activating receptors are probably also involved in killing of target cells, but it remains unresolved if the $NCR1^-$ cell population contributed to the detected cytotoxic activity.

IFNγ production was observed in $CD3^-GranzymeB^+NCR1^+$ cells but not in $CD3^-GranzymeB^+NCR1^-$ cells after IL-12 stimulation. This shows that NCR1 defines functionally different subsets of canine NK cells. Differences in IFNγ production has been associated with different subsets of NK cells in other species (Boysen et al., 2006; Cooper et al., 2001) but it

has not been strongly associated with NCR1 expression before it was described in the pig (Mair et al., 2012).

As the cultured NK cells were highly IL-2 responsive it was expected that they uniformly expressed CD25 (Abrams et al., 2010). Our results showed a highly variable expression of CD25 in these cell cultures, for unknown reasons. An anti-human CD94 antibody reported as cross-reactive to dog (Schuberth et al., 2007) did not stain these cultured cells, contrary to what should be expected since CD94 is expressed on the surface of a large subset of NK cells in humans and mice (Borrego et al., 2006). The reactivity of this antibody to canine cells may consequently require more detailed investigation. All the cultured cells uniformly expressed MHC class II, similar to reports in swine and human NK cell cultures (Mair et al., 2012; Nakayama et al., 2011; Roncarolo et al., 1991). In contrast, in the mouse MHC class II expression is absent on pure NK cell cultures but may appear in mixed cultures due to trogocytosis from accessory cells, mediating a T-cell suppressive function (Nakayama et al., 2011). The function of MHC class II on canine NK cells is unknown.

In a majority of established cultures, a distinct $CD3^+$ population emerged. These cells probably represent $CD5^{low}CD3^+$ cells described elsewhere (Huang et al., 2008). A proportion of these $CD3^+$ cells were also $NCR1^+$. $CD3^+NCR1^+$ cells has earlier been demonstrated in canine PBMC (Grøndahl-Rosado et al., 2015) showing that NCR1 is not exclusively expressed on NK cells in dogs. It is likely that $CD3^+NCR1^+$ cells represent NKT-like cells. $CD3^+NCR1^+$ cells have also been identified in other species like humans, mice (Hudspeth et al., 2013) and cattle (Connelley et al., 2014). These canine $CD3^+NCR1^+$ cells warrant further characterization, which is beyond the scope of this work. Finally, up to 18 % of the cells cultured using the $CD3^-$ $CD5^-$ strategy were $CD3^-GranzymeB^-NCR1^-$, probably representing monocytes as judged by visual evaluation. The presence of monocytes in NK cell cultures have previously been shown to enhance proliferation (Miller et al., 1992; Pierson et al., 1994). Hence, more precise selection markers would be desirable, but with the presently available reagents, we conclude that this is the currently best strategy to obtain canine NK cell cultures.

To summarize, we here present *in vivo* and *in vitro* studies of canine NK cells defined as $CD3^-GranzymeB^+$ lymphocytes, further divided into $NCR1^+$ and $NCR1^-$ subsets. *In vitro* studies of IL-2 and IL-15 stimulated $CD5^-CD3^-$ cell cultures showed that NCR1 was

increased, and that additional stimulation with IL-12 lead to an almost uniform expression of NCR1, indicating that $NCR1^-$ cells are also NK cells. IFNγ production was detected in $NCR1^+$ cells only after IL-12 stimulation, showing that NCR1 defines functionally different subsets of NK cells in dogs. Furthermore, NCR1 was shown to be an activating receptor contributing to a large part of the cytotoxic activity. In conclusion, since the dog has a large $NCR1^-$ subset of circulating NK cells, NCR1 does not define all NK cells in this species, although it can be used as a marker for activation.

Research Highlights

- Dogs have $CD3^-GranzymeB^+NCR1^+$ and $CD3^-GranzymeB^+NCR1^+$ lymphocyte subsets in the blood. These two cell populations probably represent canine NK cells in different stages of activation.
- NCR1 can be upregulated on $NCR1^-$ cells upon *in vitro* cytokine stimulation and is as an activating receptor.
- $CD3^-GranzymeB^+NCR1^+$ cells produce IFNγ in response to IL-12.

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Ethical standards

The authors declare that the experiments comply with the current laws of Norway.

Conflict of interest

The authors declare that no financial or commercial conflict of interest exist in relation to the content of this article.

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Lymphoproliferative Large Granular Lymphocyte (LGL) disorders in three dogs

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Abstract

Large granular lymphocytes (LGLs) is a morphological designation describing cytotoxic T cells and NK cells. Lymphoproliferative LGL disorders are a heterogeneous group of diseases affecting both humans and dogs characterized by an increase of LGLs in blood. Clinical, haematological and immunophenotypic findings in three dogs with lymphoproliferative LGL disorders are presented. Two dogs were diagnosed with T LGL leukaemia displaying a $CD8^+MHCII^+NCR1^-$ phenotype, of which one was additionally diagnosed with a non-B non-T cutaneous lymphoma. These two dogs had an acute clinical course and were euthanized shortly after initial presentation. The last dog presented most likely had a reactive lymphocytosis concurrent to a carcinoma.

Keywords: LGL; dog; NCR1/NKp46;

Introduction

Large granular lymphocyte (LGL) is a collective description of medium to large lymphoid cells with eccentric nuclei and abundant cytoplasm containing azurophilic granules. Both cytotoxic T cells and NK cells present as LGLs and specific markers are necessary to differentiate between

these two functionally different populations of lymphocytes [1]. Lymphoproliferative disorders of LGLs encompass all conditions with an increased level of LGLs in the blood, both benign and malignant [2]. The WHO classification divides tumours of mature NK cells and T LGLs into four groups: 1) T cell LGL leukaemia 2) chronic lymphoproliferative disorders of NK cells (CLPD-NK) 3) aggressive NK-cell leukaemia and 4) extranodal NK/T cell lymphoma, nasal type [3]. T cell LGL leukaemia and CLPD-NK are considered chronic diseases [2] while aggressive NK cell leukaemia is one of the most fatal malignancies described in human oncology with a short survival ranging from days to weeks [4]. T LGL leukaemia is the most common presentation and represents about 85 % of the total caseload of LGL diseases. T cell LGL leukaemia and CLPD-NK are often seen concurrent to other diseases like autoimmune diseases, viral infections and haematological as well as non-haematological malignancies [5].

The WHO classification system is based on human disease but is also relevant in veterinary medicine. Similarities between haematological malignancies in humans and dogs have been observed both on a morphological [6] and a cytogenetic level [7]. The clinical behavior of many cancers is comparable between dog and man [8]. Around 40 cases of LGL disorder have been reported in the dog [9–19]. The majority of expanded LGL populations described are of T cell origin. Canine LGL disorders have a variable clinical course ranging from indolent to highly aggressive disease like observed in humans. Also, canine LGL disorder is observed concurrent to other diseases like infections and other forms of cancers but has not been reported concurrent to autoimmune disease commonly seen in people.

Morphological evaluation of atypical cells is an important part of the diagnostic work up of all haematological disorders. Additional analyses are, however, necessary to specify the diagnosis further. Both phenotypic expression of markers evaluated by flow cytometry and genomic studies are widely used in human medicine. LGL disorders can grossly be divided into $CD3^+$ T cells and $CD3^-$ NK cells but numerous other markers are used to sub classify LGL disorders. Today no specific marker for NK cells has been identified in the dog making it hard to confirm canine malignancies of NK cell origin. Separating polyclonal expansions of lymphoid cells from monoclonal haematopoietic cancers can also represent a challenge, especially early in the disease or in chronic cases. Clonality in T cell malignancies can be confirmed using PCR amplification of variable regions of the T cell receptor (TCR) as each T cell express a unique TCR. This is

referred to as a PARR test (PCR for Antigen Receptor Rearrangement) and is also developed for use in dogs [11,20]. NK cells do not express a rearranged antigen receptor and clonality can therefore not be assessed using the same method. An aberrant expression of inhibitory NK cell receptors has been associated with malignant transformation of human NK cells and atypical expression of inhibitory receptors show potential as a marker of malignancy in humans [21,22]. Expression of inhibitory receptors on canine NK cells are not characterized although one study has identified an inhibitory receptor on a genomic level [23].

NCR1 (Natural Cytotoxicity Receptor) has traditionally been considered a pan species marker for NK cells across species [24] but has been reported to be expressed on subsets of T cells in humans, mice [25], cattle [26], swine (Mair et al., unpublished results) and dogs [27]. Also $NCR1^-$ NK cells have been demonstrated in swine [28] and dogs (Grøndahl-Rosado et al., unpublished results). In humans $CD3^+NCR1^+$ cells are shown to be susceptible to leukaemic transformation [25] and NCR1 is probably commonly expressed by T LGL disease although more studies are needed for confirmation. Non-LGL T cell malignancies have also been reported to express NCR1 [29]. NCR1 is reported to be downregulated on certain NK cell neoplasia [22]. Consequently, NCR1 seems to be dysregulated in many categories of malignant disease. No studies in human nor veterinary medicine have investigated the connection between NCR1 expression on cancerous cells and clinical outcome to the authors knowledge.

Recently a case of chronic T LGL leukaemia expressing NCR1 was reported in a Rottweiler dog [27] showing that NCR1 can be expressed in chronic canine T LGL leukaemia. We further wanted to investigate if NCR1 expression, or lack thereof, can be used as a prognostic indicator in canine lymphoproliferative disorders of LGL.

Materials and methods

Dogs

Dogs with an aberrant level of LGLs in the circulation were included in the study through the Central Laboratory at NMBU, School of Veterinary Medicine from 2010 to 2015. 2 ml EDTA blood was collected from the cephalic vein.

Laboratory tests

Analyses used for diagnostic workup was performed at NMBU, School of Veterinary Medicine (Complete Blood Count (CBC), serum biochemistry, cytology and flow cytometry). Histopathology was performed at REST Associates (Norfolk, UK) (dog 2) and IDEXX Laboratories (Maine, USA) (dog 3) using established methods.

Flow cytometric analysis

PBMC was isolated using Lymfoprep ® (Axis-Shield, Dundee, Scotland). The cells were counted and viability evaluated using trypan blue expression analyzed by the Countess ® Automated Cell Counter, Life Technologies (Thermo Fisher Scientific, Carlsbad CA, USA). The antibodies used in this study are shown in Table 1. They were all used according to manufactures recommendations. 5 x10^5 cells resuspended in 25 µl DPBS with 1 % bovine serum albumin (BSA) and 10 mM NaN_3 (flow buffer) were incubated with appropriate antibodies on ice. For all stainings involving mouse anti-bovine NCR1 (AKS6) the antibodies were incubated individually as described elsewhere [27]. For intracellular staining for granzyme B the BD Cytofix/Cytoperm ™ kit (BD Biosciences, San Jose, CA, USA) was used according to the manufactures instructions. After the last washing the cells were diluted in 200 µl flow buffer and kept at 4 C° until analysis. Data was collected using Gallios (Beckman Coulter, Brea, CA, USA). Data was processed in Kaluza (Beckman Coulter). Doublets were excluded by a FS INT vs. FS PEAK gate.

Table 1

Antibody	Conjugate	Clone	Supplier
Mouse anti-canine CD3 (IgG1)	FITC	CA17.2A12	AbDSerotec
Rat anti-canine CD4 (IgG2a)	Al647	YKIX302.9	AbDSerotec
Mouse anti-canine CD8 (IgG1)	Al700	YCATE55.9	AbDSerotec
Mouse anti-canine CD21 (IgG1)	FITC	CA2.1D6	AbDSerotec
Mouse anti-human CD94 (IgG2a)*	Al647	HP-3D9	eBioscience
Rat anti-canine MHCII (IgG2a)	FITC	YKIX334.2	AbDSerotec
Mouse anti-human Granzyme B (IgG1)**	PE	GB11	eBioscience
Mouse IgG1 negative control	FITC	-	AbDSerotec
Mouse IgG1 negative control	PE	-	AbDSerotec
Mouse anti-bovine NCR1 (IgG2b)**	Unconjugated	AKS6	-

Antibodies used in the study. Cross reactivity to the canine equivalent is reported by *[30] **[27].

Presentation of cases

Dog 1 was an eight-year-old female spayed Bernese Mountain Dog, dog 2 was a ten-year-old male Old Tyme Bulldog and dog 3 was a female spayed mixed breed imported from Hungary evaluated to be middle aged. Dog 1 presented with fever, lethargy, anorexia, and diarrhoea lasting for three weeks. The dog had firm and slightly enlarged peripheral lymph nodes, a mildly enlarged spleen and nodulated lesions in the lungs. Dog 2 presented with generalized non pruritic multiple nodular 3 to 10 mm lesions in the skin (Fig. 1A). Dog 3 presented with fever, lethargy, anorexia and enlarged mandibular lymph nodes. Changes in size of liver and spleen was not evaluated in dog 2 and 3.

Fig. 1 Photographs taken of dog 2 to illustrate the neoplastic lesions and their distribution. **A** Skin lesions at initial presentation. **B + C** Skin lesions three weeks after initial presentation showing worsening of the skin lesions and secondary damage due to itching. (Photo: Karin Stormoen)

Serum biochemistry and CBC were done in all three dogs. In dog 1 serum biochemistry showed increased AST (56 U/L, ref 0-40) CK (408 U/L, ref 0-200), amylase (2024 U/L, ref 0-1050), lipase (428 U/L, ref 0-150), creatinine (114 µmol/L, ref 65-110) and chloride (120 mmol/L, ref 99-115) and decreased total protein (49 g/L, ref 54-75), albumin (25 g/L, ref 32-44) and fructosamine (192 µmol/L, ref 250-315). CRP was measured with a two-day interval showing an increase from 51.4 mg/L to 72.5 mg/L (ref 0-15). In dog 2 serum biochemistry showed mild increase in amylase (1100 U/L, ref 0-1050) and globulin (32 g/L, ref 22-31) and a decrease in albumin (25 g/L, ref 32-44). In dog 3 only an in-house

(machine of unknown manufacturer) biochemistry was done showing mildly increased AP and globulins.

Aberrant findings in CBC at the time of presentation are presented in Table 2.

Table 2

	Dog 1	**Dog 2**	**Dog 3**	**Referance interval**
RBC	5.22 x 10^{12}/L	3.98 x 10^{12}/L	7.4 x 10^{12}/L	5.1-8.5 x 10^{12}/L
WBC	36.44 x 10^{9}/L	284.1 x 10^{9}/L	35.5 x 10^{9}/L	6.0-18.0 x 10^{9}/L
Lymphocytes	14.71 x 10^{9}/L	270 x 10^{9}/L	12.9 x 10^{9}/L	0.8-0.6 x 10^{9}/L
% LGLs of lymphocytes	50 %	20 %	35 % (19-85%)**	-
Neutrophils	18.06 x 10^{9}/L	8.5 x 10^{9}/L	20.6 x 10^{9}/L	3.6-13.0 x 10^{9}/L
Toxic neutrophils	No	No	11-30 %	-
Leftshift	No	No	Mild/moderate	-
Monocytes	2.21 x 10^{9}/L	4.3 x 10^{9}/L	0.1 x 10^{9}/L	0-1.6 x 10^{9}/L
Trombocytes	12 x 10^{9}/L *	263 x 10^{9}/L	Platelet clumps	180-500 x 10^{9}/L

* Small platelet clumps found on blood smear ** LGL was monitored over a period of 40 days

All dogs had leukocytosis primarily caused by a marked to extreme lymphocytosis. Dog 1 had a thrombocytopenia. Dog 1 and 3 had a moderate neutrophilia. In dog 3 the neutrophils were left shifted and showed toxic changes indicating an inflammation or infection. Dog 2 presented with a mild to moderate regenerative anaemia. Morphological evaluation revealed acanthocytes and keratocytes. In all three dogs an abnormally high number of large granular lymphocytes was detected in blood smears as illustrated in Fig. 2. CBC was repeated in dog 2 11 days after initial presentation showing an increase in lymphocyte count to 384 x 10^{9}/L. Dog 1 had about 50 % LGLs while dog 2 had 20 % LGLs of the total number of lymphocytes. The presence of LGLs in blood smear from dog 3 was monitored over a 40-day period finding a variation between 19 and 85 % but at initial presentation 35 % LGLs were found.

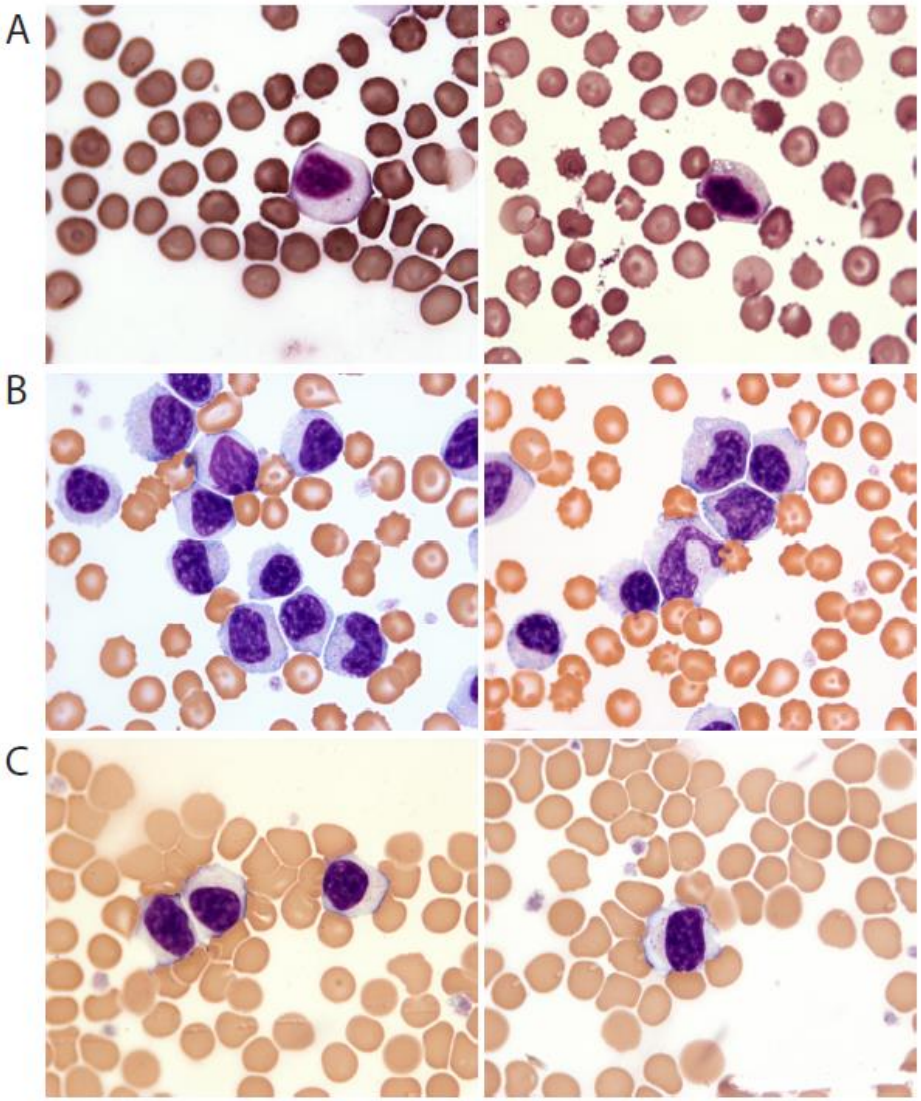

Figure 2 Blood smears from all three dogs. **A** Dog 1: The lymphocytes were 9-10 µm up to 16-18 µm in diameter with moderate amounts of light blue-grey cytoplasm. Approximately 50 % of the cells contained a variable amount of very faintly stained violet granules in the cytoplasm or a variable number of small, clear vacuoles with close proximity to each other. The nuclei were round to mildly indented with clumped/hyperchromatic chromatin. **B** Dog 2: The lymphocytes were 10-12 µm up to 15-16 µm in diameter with moderate amounts of light blue cytoplasm. Approximately 20 % of the cells contained a variable number of pink granules. Round to clefted to irregular, sometimes bizarre, nuclei with clumped chromatin. **C** Dog 3: The lymphocytes were 7-8 µm up to 13-14 µm in diameter with sparse to moderate amount of a light blue cytoplasm. Approximately 85 % of the cells contained a variable number of pink granules in the cytoplasm. The nuclei were round to mildly indented with clumped chromatin.

Peripheral blood mononuclear cells (PBMC) from all dogs were phenotyped using flow cytometry (Fig. 3). Dog 1 and 2 displayed a homogenous lymphocyte population. Cells from both dogs were $CD3^+CD4^-CD8^+MHCII^+NCR1^-$. Dog 2 was also tested for granzyme B, CD21 and CD94 expression. All cells were granzyme B^+ (Fig. 3) but they were negative for CD94 and CD21 expression (data not shown). In dog 3 a biphasic lymphocyte population was observed. Roughly 30 % of the cells displayed a $CD3^+CD8^+MHCII^+$ phenotype. No $CD4^+$ or $CD21^+$ cells were found in the circulation. In dog 3, 4 % $NCR1^+$ cells were demonstrated, of which 2.5 % was $CD3^+$ and 1.5 % was $CD3^-$ (data not shown).

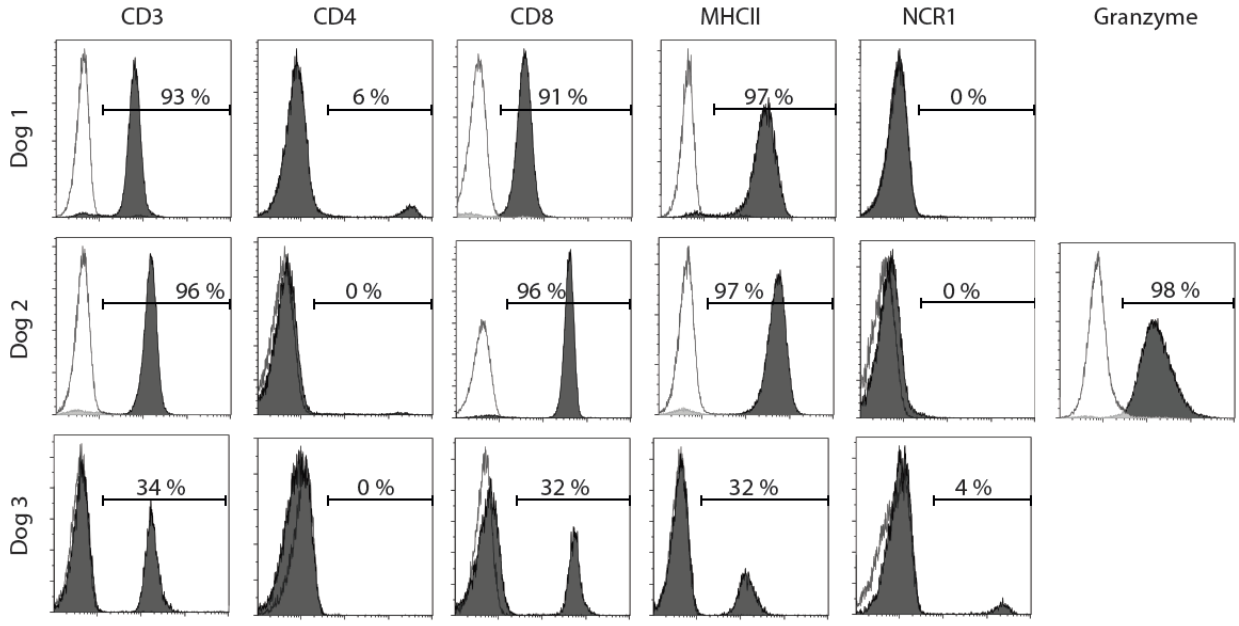

Fig. 3 Histogram from flow cytometric analysis of PBMC from all three dogs showing negative control as a transparent graph.

A fecal sample from dog 1 tested negative for intestinal parasites (*Isospora* sp. *Giardia* and *Cryptosporidium* and parasitic eggs).

Dog 3 was tested for tick borne disease. Pending the results, the dog was put on doxycycline resulting in a reduction in the size of mandibular lymph nodes. The analysis was mildly positive for *Anaplasma phagocytophilium* antibodies (1:100, negative value < 1:50).

Otherwise the analysis was negative for Borrelia, *Ehrlichia canis*, *Babesia canis* and leishmanial antibodies.

Fine needle aspirates were taken from enlarged peripheral lymph nodes from dog 1 and 3. A reactive hyperplasia was found in both cases.

Skin and subcutis of a representative lesion from dog 2 was examined histologically by REST Associates. The mass was multinodular and was defined as dermal and hypodermal. Dense infiltrates of large round cells extending to the dermo-epidermal junction and rarely infiltration the epidermis and follicular epithelium was observed. The cells had ovoid to oblong, sometimes cleaved nuclei with scanty cytoplasm. Nucleoli were mildly enlarged and mitoses were frequent. The round cells were examined immunohistochemically revealing a $CD3^{-}CD79a^{-}CD18^{-}PAX5^{-}$ phenotype, concluding with the diagnosis of a cutaneous epitheliotrophic lymphoma of non-B non-T origin. During the course of a couple of days the dog developed an intense itch and a deterioration of dermatological signs (Fig. 1B). The dog was treated with prednisone with questionable effect.

During a three-month period, a rapidly growing mass became apparent in the mandibular region of dog 3. In this period the dog developed undulating fever that resolved without treatment. After three months the mass was so extensive that the dog had difficulties swallowing. A thoracic radiograph was taken and no metastasis were detected. A biopsy of the mass was evaluated by IDEXX Laboratories. Neoplastic cells were detected within fibrocollagenous tissue showing an infiltrative pattern. The tumour cells were cuboidal to columnar and were arranged in small and irregular tubules. Neoplastic cells had scant eosinophilic cytoplasm and round to oval hyperchromatic nucleus with inconspicuous nucleoli. Anisocytosis and anisokaryosis were moderate. The tumour cells were embedded in the moderate fibrovascular stroma with a prominent inflammatory infiltrate composed of mainly macrophages, lymphocytes and plasma cells. Granules were not observed in the infiltrating lymphocytes. A diagnosis of malignant apocrine carcinoma was made.

Dog 1 had an aggressive disease and was euthanized three days after initial presentation due to worsening of generalized clinical signs. Dog 2 was euthanized three weeks after initial presentation because of acute onset of itching refractory to treatment. Dog 3 had a chronic course and was euthanized three months after initial presentation because of invasive local disease.

None of the dogs underwent surgery or was treated with chemotherapy or radiotherapy. A necropsy was not performed in any of the dogs.

Discussion

Here we present three cases of canine lymphoproliferative disorders of LGLs. Two dogs had a neoplastic expansion of LGLs and one most likely had a reactive lymphocytosis secondary to malignant disease. NCR1 expression was not detected on abnormal cells from any of the three dogs.

Dog 1 was diagnosed with an aggressive T cell LGL leukaemia. Flow cytometry revealed a $CD8^{+}MHCII^{+}CD4^{-}$ homogenous phenotype, a frequently observed phenotype for T LGL leukaemia in dogs [10]. Splenomegaly and nodular lesions in the lung tissue suggest a disseminated disease though this was not confirmed with biopsies. Lung disease with LGL infiltrations of the pulmonary tissue has been reported in aggressive cases of T LGL leukaemia in humans [31]. This dog also had a diarrhoea. The dog was currently on carprofen treatment for a ruptured cruciate ligament and the diarrhoea could have been a side effect associated with non-steroidal anti-inflammatory drugs (NSAIDs). A markedly increased lipase and amylase was also detected in this dog indicating a possible pancreatic disorder.

Dog 2 was diagnosed with a non-B non-T cutaneous lymphoma with a concurrent T cell LGL leukaemia. The leukaemic cells from this dog had a $CD8^{+}MHCII^{+}CD4^{-}$ phenotype as observed in dog 1. Neoplastic cells were additionally tested for granzyme B using flow cytometry detecting granzyme B expression in all the abnormal cells as opposed to morphological evaluation concluding with around 20 % granulated cells. This discrepancy between morphological and flow cytometric evaluation shows that morphological assessment might underestimate the number of cells containing granula. It has been shown that visualization of granula could represent a challenge and therefore care should be taken when evaluating granules using standard staining methods [32]. The AKS6 antibody used in this study to detect canine NCR1 does not work well in analysis of formalin fixated material and therefore the neoplastic cutaneous cells were not investigated for NCR1 expression. The difference in phenotype between the neoplastic cells in the skin and circulation could indicate two unrelated malignancies. Another possible explanation is a downregulation of CD3 in neoplastic cells in the skin during malignant transformation. Loss

of CD3 and CD8 has been reported in malignant transformation of a T LGL leukemia in a dog going from a chronic to an acute clinical course with infiltrations of the skin among others [33]. Consequently, care should be taken when concluding with NK cell origin based on lack of CD3 only, as also expression of NK cell markers should be verified. A case of LGL disease in a 10 year old mixed breed dog with a concurrent cutaneous lymphoma of a non-T non-B cell origin has been described [18]. In this case the phenotype of the cutaneous malignant cells and leukaemic cells coincided suggesting a common origin. In humans, skin lesions have been reported concurrent to LGL leukaemia.

Dog 3 was diagnosed with a malignant apocrine carcinoma in the mandibular region and a reactive LGL lymphocytosis. Clinical signs and a response to doxycycline treatment could suggest an infectious process. The dog tested weakly positive for antibodies towards *Anaplasma phagocytophilum* but was negative for negative for Borrelia, *Ehrlichia canis, Babesia canis* and leishmania antibodies. A second blood sample to investigate a rise in *Anaplasma* antibodies to confirm an active infection was not taken. Two reports connect reactive LGL lymphomatosis to increase in *Ehrlichia* antibody titer in the dog [10,34]. Doxycycline and other tetracyclines have been demonstrated to have an cytostatic and cytotoxic activity [35] and the positive response seen in this dog after doxycycline treatment was initiated could be due to this effect. Also clinical signs as fever and changes in haemogram could represent a paraneoplastic syndrome. Therefore, the observed clinical signs could all be caused by the neoplasia diagnosed later in the course of the disease.

Phenotypic characterization of PBMC revealed around 30 % of the cells to be $CD3^+CD8^+MHCII^+$, and no $CD4^+$ or $CD21^+$ cells were detected. $CD4^+$ T cells are normally more numerous than $CD8^+$ T cells in canine blood and reference values for B cells range between 5.6 and 21.3 % [36]. The biphasic lymphocyte population indicated a reactive process though the atypical composition of the lymphocyte population could suggest a neoplastic etiology. A reversed CD8:CD4 ratio has been associated with a case diagnosed with a non-neoplastic reactive LGL lymphocytosis [34]. A PARR analysis would have been informative but was unfortunately not performed on in any of the three dogs.

The etiology of human LGL disorders has been suggested to involve a long lasting antigen stimulation resulting in an uncontrolled clonal expansion due to unknown secondary factors [37].

This is supported by many human patients simultaneously suffering from autoimmune disease, virus infections or other cancers. Two dogs presented in this study also had concurrent malignant disease. LGL lymphocytosis has previously been observed concurrently to $CD3^-$ intravascular lymphoma [12], $CD3^-$ intestinal lymphoma [15], histiocytic sarcoma [13] a cranial mediastinal mass [9] and a thymoma [17] in the dog.

Dog 1 and dog 2 both had LGL leukaemia of T cell origin displaying a $CD3^+$ phenotype. A comprehensive study presenting 25 dogs with LGL lymphocytosis reported over 90 % of the cases to have a $CD3^+$ phenotype illustrating that T cell LGL leukemia, like in humans, are the most common presentation [10]. In this study chronic disease was the most frequently observed clinical course and progressive disease was only observed in three of 25 dogs, of which two was $CD3^+$ and one was $CD3^-$. In our material the two dogs diagnosed with neoplastic disease were both categorized as having an acute disorder and was euthanized shortly after initial presentation. However, dog 2 was euthanized because of symptoms connected to concurrent disease and not because of symptoms connected to the leukaemia itself. In veterinary medicine it could be difficult to evaluate clinical course of LGL leukaemia if only evaluating time from initial presentation to euthanasia. It is possible that other disorders are present being the main cause of euthanasia. In dog 2 an increase of lymphocyte count was observed and the neoplastic cells showed signs of malignancy and this case was therefore categorized as acute.

Many breeds have been presented with LGL disorders in the literature but Golden Retrievers and German Shepherd Dogs may be overrepresented [10]. In humans, an ethnical predisposition is seen in aggressive NK cell leukemia and extranodal NK/T cell lymphoma suggesting a genetic predisposition. There is no previous description of LGL disorders in Bernese Mountain Dogs or in Old Tyme Bulldog.

None of the three dogs presented expressed elevated levels of NCR1 in the circulation. Neoplastic cells from a dog with chronic T LGL leukaemia has previously been reported to express NCR1 [27]. In the present study two cases of acute T LGL leukaemia was found to be negative for NCR1. No $CD3^-$ LGL leukaemia was demonstrated and therefore the occurrence of NCR1 on a possible NK cell LGL leukaemia was not assessed. Too few individuals were tested to give any conclusions about clinical significance of NCR1 expression in LGL leukaemia.

To conclude we here present three cases of canine lymphoproliferative disorders of LGLs. Dog 1 and 2 were diagnosed with malignant disease and dog 3 most likely had a polyclonal expansion secondary to a carcinoma. Dog 1 and 2 had an acute LGL leukaemia of T cell origin and dog 2 had a concurrent non-B non-T cutaneous neoplasia. Increased NCR1 expression was not detected in any of the cases.

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