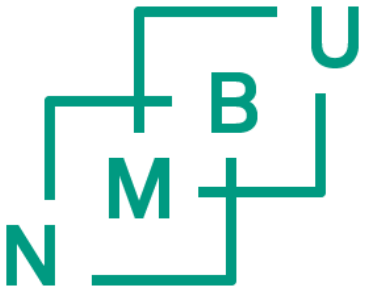
Activation and recirculation of bovine natural killer cells in steady state and inflammation

Philosophiae Doctor (PhD) Thesis

Hege Lund



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Oslo, March 2015
Hege Lund

ABBREVIATIONS

AL	afferent lymph
APC	antigen-presenting cell
CD	cluster of differentiation
DC	dendritic cell
HEV	high endothelial venule
IFN	interferon
IL	interleukin
ILC	innate lymphoid cell
ISCOMs	immune stimulating complexes
ITAM	immunotyrosine activation motif
ITIM	immunotyrosine inhibitory motif
KIR	killer immunoglobulin-like receptor
LN	lymph node
mAb	monoclonal antibody
MHC	Major histocompatibility complex
MPS	mononuclear phagocyte system
NCR	natural cytotoxicity receptor
NK	natural killer
PBMC	peripheral blood mononuclear cell
PB	peripheral blood
S1P	sphingosine 1-phosphate
S1PR	sphingosine 1-phosphate receptor
T_H1	T helper 1
TLR	toll-like receptor
TNF	tumour necrosis factor

LIST OF PAPERS

Paper 1.

Interleukin-15 activated bovine natural killer cells express CD69 and produce IFN-γ

Hege Lund, Preben Boysen, Gregg A. Dean, William C. Davis, Kun Taek Park, Anne K. Storset

Veterinary Immunology and Immunopathology 2012; 150:79 - 89

Paper 2.

Natural killer cells in afferent lymph express an activated phenotype and readily produce IFN-γ

Hege Lund, Preben Boysen, Jayne C. Hope, Siri K. Sjurseth, Anne K. Storset

Frontiers in Immunology 2013; 4:395

Paper 3.

Adjuvant injection leads to transient recruitment of intermediate monocytes and activation of lymphocytes in the draining lymph node

Hege Lund, Preben Boysen, Caroline Piercey Åkesson, Anne K. Storset

Manuscript

SUMMARY

Natural killer (NK) cells are innate lymphocytes that act as early responders in the immune response by means of cytotoxicity and production of immunoregulatory cytokines. NK cells in cattle were described a decade ago, and since then, significant progress has been made in the characterization of NK cells. However, NK cell activation and recirculation *in vivo* remains largely unknown. This thesis describes the properties of activated NK cells at steady-state and inflammation, and investigates routes of NK cell recirculation in the bovine model.

In paper 1, we demonstrated that CD69 is an appropriate marker for detection of early NK cell activation in cattle, by the use of a monoclonal antibody (mAb) specific for bovine CD69. NK cells in culture rapidly up-regulated CD69 on their surface, followed by an increase in expression of the classical activation marker CD25, regardless of which of the cytokines bovine IL-2, or bovine or human IL-15 used for stimulation. CD69 was also expressed by a proportion of blood NK cells *ex vivo*.
In paper 2, properties of NK cells in skin-draining afferent lymph (AL) were described, by the use of a pseudo-afferent lymph vessel cannulation technique in calves. Afferent lymph NK cells expressed an activated phenotype with the majority of cells being $CD25^{+}$ and $CD44^{bright}$, and with an enhanced capacity for interferon (IFN)-γ production. In contrast to blood NK cells, only a low percentage of NK cells in afferent lymph were CD69 positive.
In paper 3, we studied the activation of NK cells in an *in vivo* inflammation, by injecting calves subcutaneously with a saponin-based adjuvant. Our results demonstrated an activation of NK cells *in vivo*; NK cells up-regulated CD69 in the draining lymph node (LN), had an increased mean fluorescence intensity (MFI) of CD25 expression, and readily produced IFN-γ. Furthermore, an increase in $CD69^{+}$ and $CD25^{+}$ NK cells was demonstrated in blood. Finally, we showed that adjuvant injection in calves resulted in a massive recruitment of inflammatory monocytes to the draining LN.

To summarize, results obtained in papers 1 – 3 showed that NK cells in cattle had a varying activation status depending on their localization. Whereas a proportion of NK in blood were primed rather than naïve, afferent lymph NK cells expressed an activated phenotype. In an

inflammation, NK cells in lymph nodes were activated, and were most likely able to egress from the LN early after the onset of inflammation. Furthermore, we found that the afferent lymph appears to be an important migration route of tissue-activated NK cells that are likely to home to the lymph node.

The results presented in this thesis are of value for the general understanding of NK cell recirculation and activation, and may have important implications in future adjuvant/vaccine strategies that aim to target NK cells.

SAMMENDRAG (SUMMARY IN NORWEGIAN)

NK-celler (naturlige dreperceller) er lymfocytter i det medfødte immunforsvaret som bidrar tidlig i en immunrespons ved hjelp av celle-drap og produksjon av immunregulatoriske cytokiner. NK-celler hos storfe ble beskrevet for et tiår siden, og siden den gang har det vært en betydelig fremgang i karakteriseringen av disse cellene. Aktiveringen og resirkuleringen av NK celler *in vivo* er likevel i stor grad fortsatt ukjent. Denne avhandlingen beskriver egenskaper ved aktiverte NK-celler under steady-state (stabil tilstand) og ved inflammasjon, og undersøker NK celle resirkulering i en storfe modell.

I artikkel 1 viste vi at CD69 er en passende markør for påvisning av tidlig NK-celle aktivering hos storfe, ved hjelp at et storfe-spesifikt antistoff mot CD69 molekylet. NK-celler i kultur hadde en rask oppregulering av CD69 på overflaten, etterfulgt av en økt CD25 ekspresjon, og disse resultatene var like om NK cellene ble stimulert med bovint IL-2, eller bovint eller humant IL-15. CD69 var også uttrykt av en andel NK celler *ex vivo* i blod.
I artikkel 2 beskrev vi egenskaper ved NK celler i afferent lymfe, ved hjelp av en teknikk der man kannulerer pseudo-afferente lymfekar. NK celler i afferent lymfe hadde en aktivert fenotype, der majoriteten av cellene var $CD25^{+}$og $CD44^{bright}$, og med en økt evne til interferon (IFN)-γ produksjon. I motsetning til NK celler i blod, var kun en lav prosentandel av NK celler i lymfe CD69 positive.
I artikkel 3 studerte vi NK celle aktivering *in vivo* i en betennelses situasjon, ved å injisere kalver med en saponin-basert adjuvanse. Våre resultater viste en tydelig aktivering av NK-cellene *in vivo*; NK celler i den drenerende lymfeknuten oppregulerte CD69, hadde en økt intensitet av CD25 fluorescens på overflaten, og produserte villig IFN-γ. Samtidig ble det observert en økning i $CD69^{+}$ og $CD25^{+}$ NK-celler i blod.
Vi kunne også rapportere en massiv rekruttering av inflammatoriske monocytter til drenerende LN etter adjuvanse injeksjon.

For å oppsummere: resultater i artikler 1 – 3 viser at NK-celler hos storfe viser ulik grad av aktivering avhengig av deres vevs lokalisering. Mens en andel av NK celler i blod var mer primede enn naive, hadde NK celler i afferent lymfe en aktivert fenotype. NK celler ble aktivert

i lymfeknuten ved en betennelse, og kunne trolig raskt forlate lymfeknuten. Videre kunne vi vise at afferent lymfe ser ut til å være en viktig rute for vandring av vevs-aktiverte NK-celler som trolig vil home til lymfeknuten.

Resultatene som presenteres i denne avhandlingen er av verdi for forståelsen av generell NK celle resirkulering, og kan ha viktige implikasjoner i fremtidige adjuvanse/vaksine strategier som målrettet ønsker å aktivere NK celler.

INTRODUCTION

Innate and adaptive immunity

The main functions of the immune system are to prevent disease and to eradicate established infections. The immune response is mediated by two broad systems that cooperate, where the innate system represents an initial and general response and the adaptive responses are slower and highly specific. The interaction of these two systems is essential in the protection against infection and for establishing protection upon vaccination.

The recirculation of immune cells is a key characteristic of the mammalian immune system, and provides a continuous immunosurveillance of the body for foreign antigens. The immune response is initiated by innate leukocytes, which recognize pathogens and infected cells trough germline encoded receptors, thus by definition belonging to the innate immune system. Dendritic cells (DCs) survey tissues looking for antigen, and upon activation mature DCs can migrate to secondary lymphoid tissues and serve as professional antigen presenting cells (APCs). Phagocytic cells like granulocytes and monocytes are quickly recruited to a site of inflammation through extravasation from post-capillary venules, and neutrophil granulocytes and tissue-resident macrophages can phagocytose and eliminate microbes at the inflamed site. Monocytes are a heterogeneous population of circulating cells that function as precursors of tissue macrophages and DCs, and may also have effector functions on their own.

NK cells are innate lymphocytes that can kill infected and cancerous cells, and like other innate cells, NK cells participate in the regulation of the adaptive immune response, mainly by secretion of immunoregulatory cytokines. If NK cells like adaptive monocytes can recirculate through tissues is currently not known.

The hallmarks of the T and B lymphocytes of the adaptive immune system are recombination-activating gene (RAG) –dependent somatic recombination of receptor genes, enabling the recognition of a large array of antigens. Immunological memory is based on clonal expansion of antigen-specific cells and an amplified response upon subsequent antigen exposure.

General NK cell biology

Development of NK cells

The bone marrow is essential for the development and function of NK cells (1). NK, B and T cells all develop from pluripotent common lymphoid precursor cells (2), which in turn originate from $CD34^+$ hematopoietic precursor cells. There is also evidence for distinct populations of NK cells developing from hematopoietic cells in other organs like lymph nodes and thymus (3, 4), although it is likely that the hematopoietic precursor originated from the bone marrow, as discussed in (5). NK cell development consists of several stages characterized by sequential acquisition of NK cell specific receptors and functional characteristics (3). The earliest NK cell progenitor in mice expresses CD122, the shared ß-chain of the IL-2 and IL-15 receptors (6), and the IL-15 cytokine is essential for early NK cell development in the bone marrow (4, 7, 8).

NK cells express activating and inhibitory receptors, however a single NK cell express only some of the inhibitory NK receptors present in the genome. The acquisition of these receptors is believed to be stochastic and independent of MHC class 1. The selection of functionally competent NK cells are based on their interactions with various MHC class I ligands, a process that is central to ensure that mature NK cells do not attack self-tissue. If a NK cell is not able to interact with MHC I through its inhibitory receptors, or if activating NK receptors ligate with self-molecules, the NK cell becomes anergic or hyporesponsive. Central NK cell functions such as cytotoxicity and cytokine secretion are acquired during the last developmental stages (9).

A comparative view on NK cell definition

The name natural killer originates from the initial definition of these cells based on their ability to spontaneously kill virally infected and tumor cells by lysis (10-12). In humans, $CD3^-$ NK cells are traditionally divided into two functional populations based on the expression of CD56 and CD16, a division which is still widely used. CD16 is a low affinity receptor for the Fc part of IgG and can mediate NK cell antibody-dependent cellular cytotoxicity (ADCC) (13). The CD56 molecule (neural cell adhesion molecule, N-CAM) belongs to the Ig superfamily and its function on NK cells is unknown. Most circulating human NK cells are defined as cytotoxic

$CD3^{-}$ $CD56^{dim}$ $CD16^{+}$, whereas the majority of NK cells residing in secondary-lymphoid organs are $CD56^{bright}$ $CD16^{-}$ cytokine-producing cells that become cytotoxic only after prolonged activation (14-16).
Mouse NK cells do not express CD56, and functionally distinct subsets in this species are defined by the expression of CD11b and CD27 (17). Murine NK cells share many functional characteristics with human NK cells, and the mouse has by far been the most widely used animal model in immunological research.

The natural cytotoxicity receptor 1 (NCR1) or NKp46 is evolutionary conserved in mammals, and recognized as the most applicable pan-species NK cell marker, including NK cells in humans, primates, mice and several mammalian species (18-21). Following the description of major NK cell receptor gene families in cattle (22, 23), a monoclonal antibody (mAb) raised against the NKp46 receptor defined NKp46 as a highly appropriate marker for bovine NK cells (19). Furthermore, it was demonstrated that CD56 is not expressed by bovine NK cells, neither at the gene transcription level (24) nor on their surface (25). Since then, significant progress has been made in the characterization of natural killer cells in the cow (26). Positively selected $NKp46^{+}$ cells are $CD3^{-}$, large granular lymphocytes that possess all the major characteristics of typical NK cells after in vitro proliferation with recombinant bovine IL-2 (19). Bovine NK cells are highly cytotoxic towards murine, human and bovine cellular targets (19, 25), contain intracellular perforin, and produce significant amounts of Interferon (IFN)-γ in response to cytokines and/or microbial structures (25, 27-29). Bovine NK cells can be divided into two basic subsets based on the expression of CD2 (25, 28). The majority of NK cells in blood, liver and lung are $CD2^{+}$, whereas a $CD2^{-/low}$ subset dominates in spleen and lymphoid tissues. The $CD2^{-/low}$ subset has a distinct $CD44^{bright}$ $CD25^{+}$ phenotype, indicative of an activated state, and is functionally more responsive with a high IFN-γ production.

Although bovine NK subsets are not directly comparable to human subsets; in terms of distribution and function the bovine $CD2^{-/low}$ NK cells to a large extent resemble the $CD56^{bright}$ subset that dominates in human lymphoid tissue. However, in contrast to human NK cells, CD16 is equally expressed by the two bovine subsets. It has been suggested that human NK cell subsets are not developmentally independent, but rather stages of developing NK cells on a

linear scale, where $CD56^{dim}$ cells represent fully differentiated NK cells and $CD56^{bright}$ cells are less mature, as reviewed in (30). In cattle however, studies of the functional capabilities of the $CD2^-$ NK cell subset do not support an immature status (28).

Parallel to the unveiling of NK cells in cattle, the focus on these innate cells increased in several domestic animal species, especially with the development of novel monoclonal antibodies against NKp46. As in cattle, sheep NK cells defined by NKp46 expression possessed all the major characteristics of conventional NK cells (18). In pigs, the definition of NK cells is more complex, and NK cells are defined as $CD3^-$ $CD8\alpha^+$ cells, which can be further divided into subsets of $NKp46^+$ and $NKp46^-$ cells (31). Recently, also a subset of $CD8\alpha^{dim/-}$ $NKp46^{high}$ cells was described (32).

Other innate lymphoid cells

It has recently been suggested that NK cells should be included in the ever expanding family of innate lymphoid cells (ILCs) (33). ILCs include several distinct populations and are divided into three groups based on their ability to produce T helper cell-associated cytokines (34). NK cells have been placed in group 1, which comprises cells that produce the Th1 cytokines IFN-γ and tumor necrosis factor (TNF). However, conventional NK cells have additional properties such as cytotoxicity, which distinctly separates them from the other ILCs.

Although NKp46 has been suggested as strictly NK cell specific (5, 35), populations of other ILCs that express NKp46 have been identified in human and mouse gut (36-38). In sheep however, the majority of $NCR1^+$ cells in gut associated lymphoid tissues (GALT) are most likely classical NK cells (39), and in humans, gut non-NK ILCs were found to express barely detectable level of NKp46 (40). Although ILCs are sparse and mainly prevalent on mucosal surfaces (41, 42), it is important to keep these novel populations in mind when identifying NK cells based on NKp46 expression, especially in mucosal tissues. Interestingly, the presence of a novel population of $NKp46^+$ cells which also expressed CD3 was recently reported in cattle, and these cells were distinct from conventional NK, T and NKT cells (43).

Other innate cells that do not belong to the ILC family, but are functionally linked to NK cells, are the γδ T cells. In human and most animal species, γδ T cells represent only a minor part of

the circulating T lymphocyte compartment. In ruminants however, they represent a major lymphocyte subset in both blood and lymph, and especially in young animals (44, 45). It has been proposed that γδ T cells are the most actively recirculating lymphocyte subset in ruminants (46).

Activation of NK cells

The fundamentals of NK cell activation

There are two modes of natural killer cell activation recognized. NK cells can be directly activated by interacting with somatic cells that lack the expression of MHC class I molecules, the ligand for many NK inhibitory receptors (47), or indirectly by accessory cells secreting cytokines and upregulating co-stimulatory receptors (48, 49). Normal healthy cells have an abundant expression of MHC class I on their surface as a marker of self-tissue affiliation. Ligation of NK inhibitory receptors by MHCI delivers a dominant negative signal to the NK cell and protects healthy cells from lysis (50, 51). When a mature NK cell encounters cells lacking MHCI, like infected or tumor cells, inhibitory receptors will not be engaged and activating signals will dominate, a process known as the "missing self" recognition of NK cells (47, 52). The absence of MHCI is however not sufficient in most instances to initiate target cell killing by NK cells, and triggering of activating receptors is also a requirement.

NK cells possess a limited repertoire of germline-encoded receptors for target recognition, and have no somatic rearrangement of antigen receptor genes. Instead, NK cells express different combinations of a variety of receptors, and reactivity is dependent on the balance of signals produced by activating and inhibitory receptors, leading to either NK triggering if activating signals dominate or inhibition if negative signals override positive ones (reviewed in (48)). However, NK cell responses appear to be not just the outcome of a simple balance of activation and inhibition, but rather involve the integration of signals from multiple receptors. The complete topic of NK cell receptor repertoire in human (5) and cattle (26) has been reviewed extensively elsewhere, and is outside the scope of this thesis. However, a brief overview of the main receptor families will be given below.

Inhibitory NK receptors

The most extensively studied inhibitory NK cell receptors are the Killer cell immunoglobulin (Ig)-like receptors (KIRs) in humans (53) and the Ly49 receptors in rodents (54), the latter being functionally similar to the human KIRs.

Humans and primates have polymorphic KIR receptors encoded by multiple genes, which can recognize a variety of MHC1 molecules. Human inhibitory KIRs consist of two extracellular Ig-like domains and a long intracytoplasmic tail with two immunoreceptor tyrosine-based inhibition motifs (ITIM), generating a negative signal to the NK cell. The only non-primate mammal discovered to also possess diversity in the KIR gene repertoire is cattle (22, 23, 55). Sanderson and co-workers recently found eight expressed KIR genes in cattle, of which seven encoded inhibitory receptors and one encoded an activating receptor (55). However, it is not yet established if cattle KIRs recognize MHC I molecules.

The non-polymorphic NK group 2 member A receptors (NKG2A) can bind to non-classical MHC I molecules, and is also present on cattle NK cells (24, 26), but the ligands are still unknown.

Activating NK receptors

NK cells express a variety of activating receptors and co-receptors recognizing ligands on target cells and "stressed" cells, primarily tumor or virus infected cells (reviewed by (5)).

The group of NCRs includes NKp46 (NCR1), NKp44 (NCR2) and NKp30 (NCR3). NCRs are important mediators of NK cell cytotoxicity and highly involved in NK cell killing of tumor targets (56). NKp44 is not normally expressed on human NK cells, but induced after NK cell activation (57).

CD16 is an activating NK receptor, and in human NK cells CD16 ligation is sufficient on its own to initiate full activation and degranulation (13). *In vitro* expanded bovine NK cells are strongly cytotoxic in redirected lysis assays with CD16 mAb (25).

Both the NCRs and CD16 signal through immunoreceptor tyrosine-based activation motif (ITAM)-bearing signaling molecules.

The NKG2D receptor recognizes the MHC class-I-related chain A and B (MICA/MICB) molecules that are up-regulated on infected cells or stressed tumor cells (58), and signals

through the transmembrane molecule DAP10 (53). NKG2D expression has been described on cattle NK cells (59), but the function is still unknown.
Other groups of NK activating receptors include activating KIRs and Toll-like receptors (TLRs) (60).

Activation of resting NK cells to produce immunoregulatory cytokines requires the simultaneous engagement of several NK receptors, working in synergy. The requirement of more than one receptor to be triggered may serve as a protective mechanism to prevent uncontrolled activation of NK cells. For NK cell chemokine secretion, however, activation of an individual receptor was shown to be sufficient (61).
There are only a few examples of known ligands for NK activating receptors. The MICA and MICB molecules have already been mentioned, and the B7-H6 ligand expressed by many tumors is recognized by NKp30 (62). Murine cytomegalo virus infected cells express the m157 viral protein on their surface, which is specifically recognized by the Ly49H receptor (63). Another example is the influenza hemagglutinin which has been recognized as a ligand for the NKp46 receptor in humans, implicating NK cells in the control of *Influenza* (64). Swine NKp46 has also been shown to bind to *Influenza* infected cells (Forberg et al., 2014). Recently, also an activating tumor cell-associated ligand for NKp44 was identified (65, 66).

Indirect activation of NK cells: cross-talk with accessory cells

An optimal activation of NK cells requires in most instances an interaction with accessory cells that produce cytokines and express membrane bound molecules (49, 67, 68). For IFN-γ production, NK cells are dependent on IL-12 produced by DCs and monocytes/macrophages. The NK-DC interaction is bi-directional, and results in mutual regulation (reviewed by (69)). These interactions occur primarily in secondary lymphoid organs, but also at the site of inflammation or tumor infiltration (70, 71), and studies in mouse and cattle have indicated that direct cell-to-cell contact is necessary (72-74).

Major cytokines involved in NK cell homeostasis are IL-2, IL-15, IL-12, IL-18, IL-21 and the type I interferons IFN-α and IFN-ß (reviewed in (75, 76)). The role of T-cell derived IL-2 in NK cell activation is widely established from early *in vitro* studies (15). More recently, antigen-

specific T cell derived IL-2 activation of NK cells was also demonstrated *in vivo* (77, 78). The IL-12 cytokine is the most potent inducer of NK cell IFN-γ production (79), however, most accessory cell derived cytokines do not induce potent IFN-γ production from resting human NK cells alone, and a synergic effect is needed. For instance, IL-18 can potentiate the effect of IL-12 by inducing NK cell IL-12 receptor expression (80).

IL-15 is a multifunctional cytokine necessary for development, proliferation and activation of innate immune cells, including NK cells, γδ T cells and NK-T cells (81, 82). IL-15 mRNA is found in multiple tissues including the placenta, skeletal muscle, spleen, kidney, lung and heart, and the IL-15 cytokine is secreted by dendritic cells, monocytes and macrophages (83). Although the IL-15 cytokine shares the β chain of the IL-2 receptor and the common γ chain with IL-2 and therefore mediates many similar functions as IL-2 *in vitro* (84-86), IL-15 plays distinct roles *in vivo*. IL-15 can act on its target cells as a free soluble molecule or be presented in *trans*, a process that involves the expression of the cytokine in complex with its unique IL-15 receptor α-chain on an accessory cell to the intermediate affinity βγ receptor on NK cells or T cells (87, 88) **(Figure 1)**. The IL-15 trans-presentation is essential for NK cell survival (89, 90), and has been shown to be central in the process of NK cell priming *in vivo* in response to infection (91, 92). The IL-15/IL-15Rα complex is also critical in survival and maintenance of memory $CD8^+$ T cells (Burkett et al., 2004).

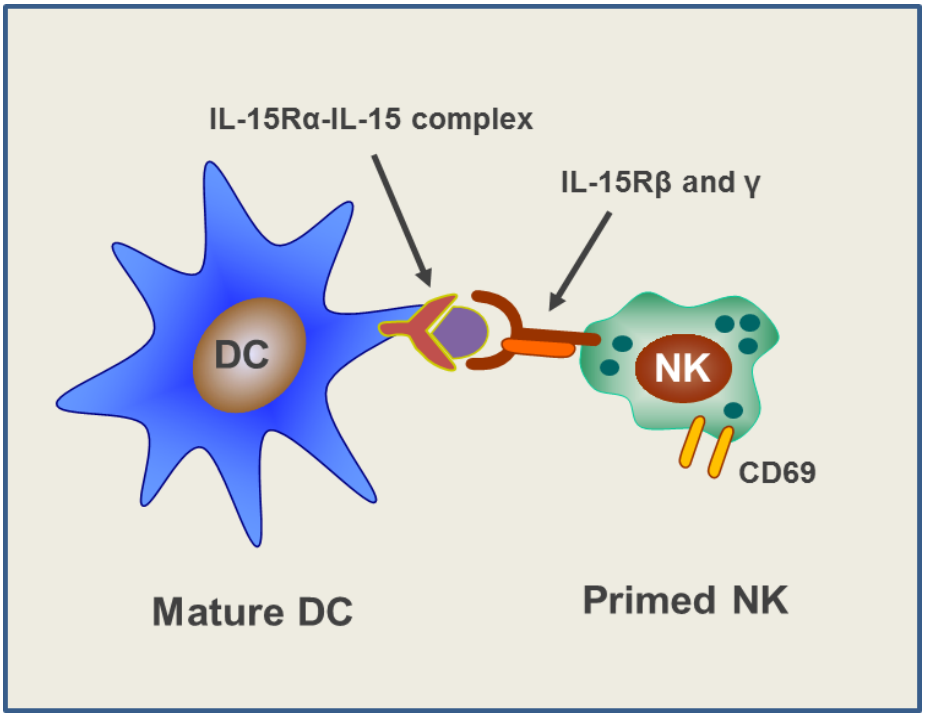

Figure 1. Schematic drawing of IL-15 trans-presentation in the lymph node.
Priming of NK cells in lymph nodes requires the trans-presentation of IL-15 in complex with the IL-15 receptor α unit by mature DCs. Primed NK cells express the early activation molecule CD69 on their surface.

NK cells were initially described as naturally active cells that could provide a rapid response upon microbial challenge (10, 11). However, Lucas *et al*. showed that naïve murine NK cells did nor acquire effector functions without an initial priming step (92). This priming step involved the trans-presentation of IL-15 in lymph nodes, and primed NK cells expressed CD69 on their cell surface (91, 92). CD69 is a type II transmembrane glycoprotein, inducible on lymphocytes and NK cells *in vitro* in response to cytokines, mitogens or TLR ligands (93, 94). As CD69 is not expressed on resting murine NK cells (92) and on circulating human NK cells only in some healthy donors (93, 95), it is a useful marker of NK cell priming and activation in these species.

Functions of NK cells

Control of infections and tumor growth

Down-regulation of MHCI is a common mechanism of virus-infected and tumor cells to evade recognition by adaptive lymphocytes, but render these cells as targets for direct NK cell-mediated killing (96). Ligands for NK activating receptors are mainly expressed on "stressed" cells like infected and tumor cells, hence favoring killing of such cells. NK cells alone cannot clear the body of a viral infection, but play a major role in the control of virus titers until adaptive responses can take over. Activated NK cells kill target cells by the formation of an immunological synapse (97), which culminates in granule exocytosis with the release of perforin and granzymes (98). NK cells can also kill target cells by induction of apoptosis through the FAS ligand (99).

Human NK cells are implicated in the control of several microbial infections, including viruses, bacteria and protozoa, as reviewed in (100). NK cell derived IFN-γ can trigger phagocyte mediated killing of infected cells, which promotes rapid clearance of many protozoan infections (100, 101). In cattle, NK cells are important in the immune response against a variety of intracellular pathogens; infections where a Th1-biased immune response is required. These include *Neospora* (27, 102), *Babesia* (103, 104), *Theileria* (105), and *Mycobacterium bovis* (24, 106, 107). Bovine NK cells also produce IFN-γ in response to microbial structures (25, 27-29, 104, 107).

The ability of NK cells to target and kill cancerous cells has led to the research into human cancer therapy based on adoptive transfer of NK cells (108, 109). In this aspect, the molecular specificity of NK cell receptors in tumor cell recognition is extensively investigated.

Immunoregulation

During the past decades, it became clear that NK cells play a fundamental role in shaping the downstream adaptive immune response by production of immunoregulatory cytokines and chemokines. For these reasons, the functional links between NK cells and accessory cells, in particular DCs, have been widely investigated. As already mentioned, accessory-cell derived cytokines promote NK cell priming and activation, cytokine release, proliferation and

cytotoxicity (110). On the other hand, NK cell derived IFN-γ and TNF and/or engagement of activating receptors can affect DC maturation (111). NK cells can kill immature DCs expressing lower levels of MHC class, while mature DCs are spared (112).

Memory-like functions of NK cells

Exciting recent data is challenging the conventional view of NK cells as innate cells that respond consistently to infection, regardless of previous exposure. During the last decade, NK cells have revealed themselves as lymphocytes that function at the interface of innate and adaptive immunity, with a more dynamic nature than previously anticipated.

Several studies of viral infections in mice have provided evidence of memory-like functions of NK cells (113, 114). Sun and colleagues showed that murine NK cells bearing the Ly49 receptor specifically recognized a murine cytomegalovirus (MCMV) -encoded antigen, and proliferated and resided in the body for several months following infection (115). Upon a secondary challenge with MCMV, these long-lived "memory" NK were rapidly triggered to degranulate and produce cytokines. In another study, NK cells activated *in vitro* with cytokines exhibited prolonged survival after adoptive transfer into naïve hosts (116). Transferred cells were phenotypically similar to naïve NK cells, but showed an enhanced capacity for IFN-γ production upon re-stimulation suggesting a "memory-like" property. A hepatic NK cell population with antigen-specific memory responses has also been described (117).

Clear evidence of human NK cell memory is lacking, and specific phenotypic markers defining NK cell memory would facilitate further studies.

NK cell distribution and traffic

Distribution and the chemokine system

The presence of NK cells in lymph nodes was demonstrated not more than a decade ago in humans (15, 16), cattle (19) and mice (72, 110). In mice, NK cells are found widely distributed in lymphoid and non-lymphoid organs (118, 119), although at a very low frequency in LNs of non-immunized animals. Recent studies have also increased our knowledge on NK cell distribution in human tissues (40, 120). Bovine NK cells display varied tissue distribution, and the highest NK percentages as part of mononuclear cells have been described in liver and lung

(4 – 29 %), followed by spleen and peripheral blood (6 – 7 %), non-mucosal LNs (5 - 13 %) and mucosal LNs (1 – 6 %) (25). In bovine LNs, NK cells were found residing in the T-cell rich paracortex and the medulla (25), corresponding well to the gross localization of NK cells in in human and murine lymph nodes (15, 72).

Details concerning NK cell migration between tissues and back to the circulation are not fully understood. The traffic of immune cells during homeostasis and disease is largely orchestrated by the chemokine system, a complex system that relies on the interaction of chemokines (cytokines) and chemokine receptors; a family of seven-transmembrane domain proteins. Movements of NK cells are most likely orchestrated by combinations of chemokines, and NK cells are recruited to healthy and diseased tissues by the presence of a unique chemokine environment in specific tissues, which is suggested to also shape the local NK phenotype and organ-specific features (119, 121).

Chemokines and non-chemokine factors involved in NK cell homing to lymph nodes have particularly received attention, as the acquired immune response is largely initiated in lymph nodes. In particular, the chemokine receptor CCR7 has been given a central role in NK cell homing to and egress from LNs (118), and cells expressing CCR7 migrate in direction of the major CCR7 ligands CCL19 and CCL21. Human LN-resident $CD56^{bright}$ NK cells express CCR7 (16, 122), whereas the $CD56^{dim}$ NK subset in blood is CCR7 negative. Murine NK cells do not appear to express CCR7 (123).

Interestingly, XCL1 produced by NK cells and XCR1 expressed by certain subsets of DCs have been identified to play a role in reciprocal interactions between NK cells and DCs in secondary lymphoid organs in both human and sheep (124). NK cells are also reported to produce CCL3, CCL4, and CCL5 (125).

Among the non-chemokine factors, the Sphingosine 1-Phosphate (SIP) receptor (S1PR) has a pivotal role in lymphocyte traffic.

The comprehensive topic of NK chemokine receptors in different species is outside the scope of the current thesis, has been reviewed in detail elsewhere (118, 122, 123, 126).

Studies of the afferent lymphatics

The afferent lymph (AL) compartment constitutes the physical connection between peripheral tissues and the draining lymph node, and continuously transports protein, lipids and immune cells to the lymph node, thereby being central in the cellular recirculation from the periphery and back to the blood (127). Lymph nodes are interspersed along the lymph vessels and serve to filter the lymph for the presence of exogenous antigen. The lymph leaves the lymph node via efferent vessels to ultimately get mixed back with the blood circulation.

The surgical technique of lymph node removal followed by catheterization of pseudo-afferent vessels has been established in various animal models. The pseudo-afferent vessel is made up of the reconnected afferent and efferent vessels **(Figure 2)**, and can provide long-term access to afferent lymph (128, 129).

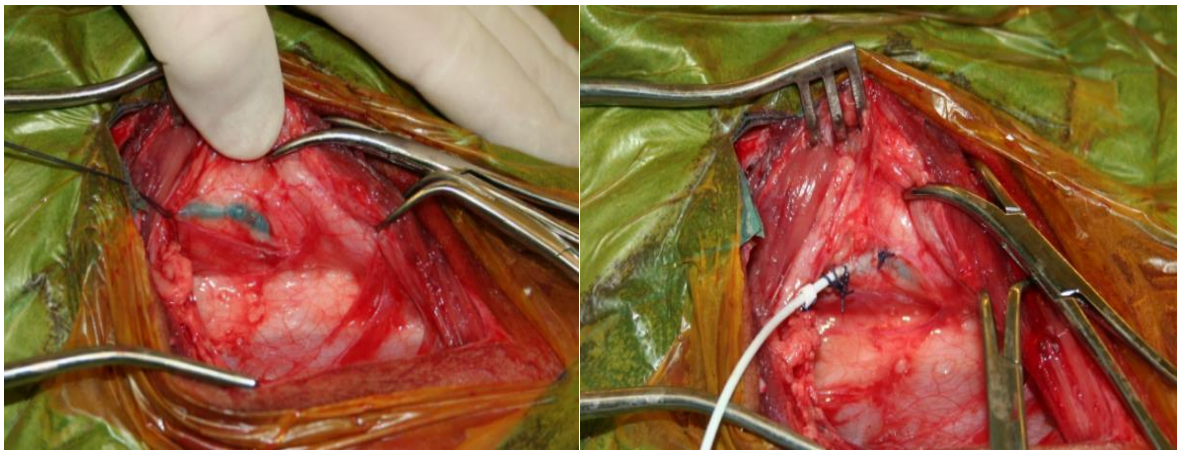

Figure 2. Catheterization of a pseudo-afferent lymphatic vessel

Photos of pseudo-afferent lymph vessel catheterization in a calf. The afferent vessel is visualized by the injection of coloured dye (Patent Blue V) into an area that drains into the afferent lymphatics (left photo). The catheter is inserted into the vessel and fixated in position with ligatures (right). The catheter is then passed externally via a skin-incision. Photos by Siri K. Sjurseth (Norwegian Veterinary Institute).

Pioneer studies of afferent lymphatics were carried out as early as the beginning of the 70's in sheep (130), followed by cattle studies in the late 80's (131). In sheep, immune cells have been collected from lymph draining head tissues (129, 132), skin (133) and gut (134). In cattle, studies have mainly focused on skin draining lymph in a prescapular cannulation model (45, 128, 135). Other animal models include the pig (136-138) and rat (139-141).

There is a continuous flow of immune cells circulating via the afferent lymphatics, even at steady state (recently reviewed in (142)), and the technique provides access to large numbers of *in vivo* generated DCs migrating in lymph (143, 144). Other leukocytes reported to be present in afferent lymph of ruminants are T and B lymphocytes, γδ T cells, monocytes and granulocytes (131, 132, 135, 145-147), and plasmacytoid DCs (148). As for natural killer cells, a presence in afferent lymph has been reported for ruminants (124, 128, 135) and humans (149, 150), and recently a population of $CD3^-$ $CD56^{bright}$ $NKp46^+$ cells were found in human seroma fluid collected after LN removal during breast cancer surgery (151). However, these cells have never been further characterized.

Afferent lymph studies in large animals have provided novel insight into the field of leukocyte traffic, and generated a large body of our general knowledge on cellular migration from peripheral tissues to the draining lymph node (126, 127, 142, 152). However, despite a large number of studies on leukocytes in afferent lymph in both homeostatic and stimulated conditions, the complete picture of *in vivo* leukocyte recirculation, including NK, is incompletely understood.

AIMS OF STUDY

Main objective

The main aims of the current work were to characterize the phenotype and activation status of NK cells in different anatomical compartments, and to investigate aspects of NK cell recirculation in homeostasis and inflammation.

Sub goals

1) Describe the expression of early activation molecule CD69 on cattle NK cells *in vitro* and *in vivo* (papers 1 – 3).
2) Characterize the phenotype and IFN-γ producing capacity of bovine NK cells that are
    a. stimulated *in vitro* with cytokines (paper 1).
    a. activated *ex vivo* with cytokines, from peripheral blood and in afferent lymph at steady state (paper 2).
    b. activated *in vivo* in an inflammatory setting (paper 3).
3) Provide novel insight into general NK cell recirculation based on findings in subgoals 1 - 3.

SUMMARY OF PAPERS

Paper 1. Interleukin-15 activated bovine natural killer cells express CD69 and produce IFN-γ

Interleukin-15 is an essential cytokine for the function of natural killer cells. Previous studies had indicated a need for homologous bovine IL-15 to adequately stimulate NK cell IFN-γ production in cattle (104, 153). The production of recombinant bovine IL-15 in mammalian cells was achieved by the use of a modified expression vector. NK cells stimulated with bovine IL-15 proliferated in culture and produced IFN-γ in a dose-dependent manner in the presence of IL-12. In contrast to previous findings, we could also demonstrate potent IFN-γ production from bovine NK cells stimulated with human IL-15 and IL-12.

An appropriate marker for detection of early NK cell activation in the cow was previously lacking. A monoclonal antibody specific for bovine CD69 was developed by the immunization of mice with purified bovine CD69 protein. CD69 was found to be expressed on a substantial proportion of blood NK cells *ex vivo*. Bovine IL-15 and IL-2 were similar in their capacity to induce surface expression of CD69 and CD25 on NK cells. NK cells had a peak expression of CD69 12 h after cytokine stimulation, demonstrating that the novel mAb against bovine CD69 is an appropriate marker for detection of early NK cell activation. As expected, the induction kinetics of CD25 expression was slower than for CD69. The bovine specific CD69 mAb will be an important tool in further studies of NK cell activation in cattle.

Paper 2. Natural killer cells in afferent lymph express an activated phenotype and readily produce IFN-γ

The afferent lymph compartment is central to the recirculation of leukocytes from peripheral tissues to the draining lymph node. Although the presence of natural killer cells in ruminant (124, 128, 135) and human (149-151) afferent lymph had been reported, these cells had never been characterized. By the application of a well-established method of pseudo-afferent lymphatic vessel cannulation, the phenotype and cytokine producing capacity of NK cells in skin-draining afferent lymph at steady state was described in calves. NK cells constituted 2 – 11 % of mononuclear cells in afferent lymph, and the majority expressed a $CD16^+$, $CD8\alpha^+$ and

$CD2^{-/low}$ phenotype. Several findings indicated that afferent lymph NK cells were in an activated state: the majority of cells expressed CD25 and CD44, and readily produced the effector cytokine IFN-γ when stimulated with IL-12 cytokine only. However, a lower percentage of NK cells were positive for the early activation marker CD69 than NK cells in blood. The lymph node homing molecule L-selectin (CD62L) was expressed on around half of the NK population in afferent lymph, whereas NK cells were found to be negative for surface CCR7 expression. NK cells in afferent lymph had a similar phenotype as lymph node resident NK cells, and were in a more activated state than NK cells in blood. This indicates that the afferent lymph may be an important migration route of tissue-activated NK cells that are likely to home to the lymph node, thus providing novel information on NK cell recirculation.

Paper 3. Adjuvant injection leads to transient recruitment of intermediate monocytes and activation of lymphocytes in the draining lymph node

NK cells are implicated in the early response to infection and vaccination. In paper 2 we found that NK cells are present in skin-draining afferent lymph. To study the recruitment and activation of leukocytes including NK cells in an adjuvant induced inflammatory setting, calves were injected subcutaneously with Matrix-Q™ adjuvant (Novavax AB). Phenotypic and histopathological findings demonstrated a potent but transient recruitment of cells to subcutaneous tissues and the draining lymph node. Recruited cells were predominately CD14 positive monocytes, and expressed a $CD14^{bright}$ $CD16^{dim}$ phenotype consistent with intermediate, inflammatory monocytes. The cells did not express phenotypic markers indicative of a differentiation into dendritic cells. Furthermore, a significant increase in granulocytes and B cells was demonstrated in the draining lymph node, whereas the percentages of T-cells and natural killer (NK) cells were reduced. However, all lymphocyte classes carried signs of activation, and several results indicated an activation of NK cells: NK cells rapidly up-regulated CD69 in the lymph node, which was followed by an increase in $CD69^{+}$ NK cells in circulation. Furthermore, an increased percentage of NK cells in blood expressed the high affinity IL-2 receptor CD25. Finally, NK cells in the draining lymph node showed an enhanced capacity for IFN-γ production. Altogether, this study provides evidence of in vivo NK cell activation in inflammation. Also, novel information on monocyte recruitment and migration is presented.

DISCUSSION

Methodological considerations

Production of recombinant bovine IL-15

Paper 1 describes the production of bovine IL-15 in mammalian cells, which was to be further used in functional studies of NK cells. However, the bovine IL-15 production became a greater challenge than anticipated. IL-15 is a multifunctional cytokine involved in several aspects of immune regulation, and *in vivo* IL-15 expression is a tightly regulated process, which is likely necessary due to the high potency of the cytokine (81).

Bamford *et al.* could not demonstrate IL-15 protein in the supernatants of cells that clearly expressed IL-15 mRNA, and initially suggested a post-transcriptional regulation of IL-15 translation (154). Additional studies revealed a multifaceted regulation, with negative control elements at the level of mRNA transcription, translation, intracellular trafficking and protein secretion (155, 156).

Bovine IL-2 was cloned and expressed in 1986 (157), and we have produced recombinant bovine IL-2 (19) and ovine IL-2 (18) in mammalian cells by the use of a traditional expression vector. The production of bovine IL-15 was however not achieved by the use of the same vector (paper 1). Finally, production of bioactive bovine IL-15 was accomplished by the use of a modified expression vector, which contained elements that addressed several of the negative regulatory bottlenecks of IL-15 production. This vector had previously been used to produce bioactive feline IL-15 (158).

However, a low transfection yield indicated that alternative strategies were necessary to produce an optimal bovine IL-15, a topic which has been further discussion in paper 1.

Pseudo-afferent lymph cannulation

In paper 2, we described the presence of NK cells in afferent lymph by the catheterization of pseudo-afferent lymphatic vessels in calves. The large animal cannulation model has proven to be highly valuable for studies of leukocyte traffic from peripheral tissues to the draining lymph

node, both at steady state and inflammation (142, 152). It opens up for the direct study of cells *ex vivo*, making it an ideal model system for studies of responses to infection and vaccination (128, 129). The model also permits the collection of large amounts of cells over extended periods of time.

Ruminant models should be no less valid for general assumptions than experimental rodent species; perhaps contrary due to their more relevant size and, arguably, living conditions. As the afferent lymph compartment is virtually inaccessible in humans and rodents, large animal cannulations offer a unique model for comparative research. Studies of rat lymph have been performed by accessing the thoracic lymphatic duct to sample lymph from the intestine and liver (139, 141), however, the thoracic duct sampling often consists of a mixture of afferent and efferent lymph, as reviewed by (142). In humans, early studies of afferent lymph was performed by direct microsurgical cannulation of a superficial lymphatic vessel (159, 160). The major limitation to the cannulation model is the technical challenge of the procedure that requires specialized surgical skills, and an average success rate of 1 in 3 has been estimated for both the bovine and the ovine model (128, 129). The main causes of failure are the presence of residual lymph nodes, failed anastomosis of afferent and efferent vessels after lymph node removal, too small vessels for catheterization, or clogging and dislodging of the catheter.

We performed removal of superficial cervical lymph nodes in three Norwegian Red calves (paper 2). Unfortunately, due to the generation of small pseudo-afferent vessels in two out of three calves, we only had access to paired samples of afferent lymph and blood from one animal (paper 2). The majority of afferent lymph samples were therefore obtained from Holstein-Fresian calves from another research facility. As the ideal situation would have been to have paired samples of blood and lymph from all individuals and of the same cattle breed, we looked for breed influences on parameters measured in PBMC from both breeds, and no such differences were detected, as further discussed in paper 2.

In the end we do find that the main conclusions of paper 2, that NK cells are present in afferent lymph and are more activated than NK cells in peripheral blood, are well founded regardless of the breed limitation.

The method in cattle is well established, and no apparent induced effects of surgery on cellular activation status has been reported (128). Precautions were nevertheless taken to avoid a possible inflammatory effect of the surgery itself, and sampling of afferent lymph was initiated after a certain time interval, allowing cellular alterations to stabilize after surgery. An increased presence of monocytes and granulocytes in afferent lymph has been reported for the first few days after surgery in an ovine model, however, these elevations stabilized at a lower percentage after 10 days and remained stable throughout the sampling period (132). In our study (paper 2), the features of afferent lymph NK cells were fairly constant, even though the lymph was sampled from several animals and at different time-points after cannulation. In addition, the phenotype of afferent lymph NK cells showed a high degree of similarity to the phenotype of NK cells in lymph nodes of healthy cattle (25), indicating that the activated phenotype of afferent lymph NK cells that we observed is a physiological trait rather than an induced one. Thus, while the effects of surgery cannot be totally avoided, it is likely that the NK cell presence and activation we consistently observed over a long period represents normal cell recirculation and not the effect of local inflammation.

The expression of CCR7 on bovine NK cells

The interaction of chemokines and chemokine receptors has a central role in NK cell distribution in a homeostatic context, enabling NK cells to migrate to and exist within tissues, as well as initiating the re-distribution of cells to effector sites during an inflammatory immune response. However, little is known regarding the mechanisms that regulate entry of cells into LNs from the afferent lymph.

The chemokine receptor CCR7 is a central mediator for LN-homing of lymphocytes (161), and human effector/memory T cells are recruited to LNs via the afferent lymph in a CCR7 dependent manner (162, 163). CCR7 is present on most human $CD56^{bright}$ NK cells that home to the LN, but absent on the $CD56^{dim}$ subset that dominates in blood (122, 164). The role of CCR7 on cattle NK cells is unknown. We found CCR7 to be seemingly absent on bovine NK cells in blood and afferent lymph (paper 2), when applying an anti-human CCR7 antibody reported to be cross-reactive to bovine cells (45). In contrast to our observations, human afferent lymph NK cells were demonstrated to be strongly CCR7 positive (121). When

attempting to use the same antibody on lymph node-derived NK cells in paper 3, we found inconsistent results with regards to CCR7, and at several instances the CCR7 isotype-control was positive. We therefore find it difficult at the current time to predict the CCR7 expression on bovine NK cells, and most likely a bovine specific CCR7 antibody will be necessary to conclude on the matter.

Administration of matrix-adjuvant to calves

Safety aspects of saponin-based adjuvants

In paper 3, the local recruitment and activation of leukocytes was studied in calves following subcutaneous administration of Matrix-Q™ adjuvant (Novavax AB, Uppsala, Sweden). Matrix adjuvants are based on the technology of the Immune stimulating complexes (ISCOMs), and are homogenous formulations consisting of *Quil-A* saponin, phospholipids and cholesterol (165, 166). Unlike the ISCOMs, which incorporate adjuvant and antigen in the same particle, matrix adjuvants are simply mixed with antigen. *Quil-A* saponins in free form are potent adjuvants, but have a high molecular instability and are associated with hemolytic properties that may cause adverse effects in the recipient (165, 167). Matrix adjuvants are therefore made of well-defined fractions of saponins, and a high stability is provided by the strong affinity between saponin and cholesterol, which also eliminates the hemolytic activity of the saponin.

An *in vivo* toxicity evaluation of Matrix-Q™ has previously been carried out in one week old piglets (167). No adverse reactions were observed, and the authors concluded that the adjuvant was well tolerated in young piglets. The same conclusion was reached after intramuscular injection of Matrix-M™ (Novavax AB) to eleven week old pigs. In our study, the adjuvant injection induced a strong inflammation locally at the injection site and in the draining lymph node, and a potent recruitment of monocytes. However, based on the clinical examination of animals and histopathological findings, we found that the applied dosage induced an appropriate inflammatory reaction.

Characterization of mononuclear phagocytes

Perhaps the most surprising result of the study described in paper 3, was the massive recruitment of intermediate monocytes to the draining lymph node after adjuvant injection. A more detailed description of monocyte subset classification in different species has been summarized in **Box 1**.

Box 1 Monocyte subsets in different species

Circulating monocytes are a heterogeneous population of cells, which can differentiate into a range of tissue macrophages and DC subsets, as well as exert effector functions on their own. Human monocytes are classified into three subsets based on their surface expression of CD14 and CD16 (168, 169). The CD16-negative, classical monocytes are $CD14^{bright}$ $CD16^{-}$ and form the major population in human blood (90 %). CD16-positive monocytes are subdivided into a $CD14^{bright}$ $CD16^{dim}$ intermediate monocyte subset, and a $CD14^{dim}$ $CD16^{bright}$ nonclassical subset. A recent study in cattle defines bovine blood monocytes as CD172a-positive cells, which can be further subdivided into the three subsets described in the human system by the use of cross-reactive anti-human antibodies against CD14 and CD16 (170). In mice, monocytes subsets are defined by their differential expression of Ly6C and the presence of various chemokine receptors (171). Pig monocytes are $CD14^{+}$ and can be further subdivided into a CD163-negative and a CD163-positive subset (172).

The number of CD16-positive monocytes can increase under various inflammatory conditions (169), and in human and cattle intermediate monocytes are described as inflammatory, due to a high capacity to produce TNF, IL-12 and reactive oxygen species (170).

The classical view of monocytes during inflammation is that these differentiate into DCs in the inflamed tissue, which can subsequently travel to the draining LN. It was therefore of importance in the current study to investigate whether a differentiation of recruited monocytes to a DC phenotype had taken place. Characterization and definition of DCs in ruminants is based on a large body of research from *in vitro* studies and from lymph draining the skin and gut (144, 173, 174), and the topic of cattle DC heterogeneity is beyond the scope of the current thesis, however a brief description of molecules used to characterize DCs will be given below, followed by an overview of primary antibodies applied in paper 3 to identify cells of the MPS in cattle (**Table 1**).

In paper 3, we used CD11c and DEC205 in immunofluorescent stainings for a DC identification in calves.

Monocyte differentiation *in vitro* and *in vivo* is characterized by the loss of CD14 and the upregulation of CD11c, DEC205 and MHCII (168, 175-178). Monocyte derived DCs or inflammatory DCs in mice have been identified based on the high surface expression of CD11c, however in humans, CD11c defines all cells that are part of the MPS and is considered to be non-specific (179), except for DCs in lymphoid organs (180).

DEC205 (CD205) is an endocytic receptor on DCs, which mediates antigen uptake and directs antigen to intracellular compartments for processing and presentation (178). DEC205 is up-regulated during maturation of DCs from monocytes *in vitro* and on certain DC populations *in vivo* (177, 178). Dendritic cells draining the skin in cattle express high levels of DEC-205, independent of their origin, and this marker can be used together with size (forward scatter) to identify DCs in this species (128, 181). Cattle $CD14^{+}$ cells are negative for DEC205. A moderate expression of DEC205 has also been described on T and B cells in cattle blood, and on B cells and follicular dendritic cells in the lymph node follicles (178). This latter aspect was also described in paper 3.

Molecule	Cellular expression	Antibody	Clone	References
CD14	Monocytes, granulocytes	Mouse anti-human	TÜK4[a]	(145, 182)
CD11b	Macrophages, monocytes, granulocytes	Mouse anti-bovine	MM12A[b]	(145, 183)
CD11c	Dendritic cells, macrophages	Mouse anti-bovine	BAQ153A[b]	(184)
DEC205	Dendritic cells, moderately on B cells and T cells, some epithelial cells	Mouse anti-bovine	MCA1651G[a]	(178)
CD16	NK cells, NK-T cells and monocytes	Mouse anti-human	KD1[a]	(25)
Gran marker	Granulocytes	Mouse anti-bovine	CH138A[b]	(19, 185)

Table 1. Primary antibodies used in paper 3 for characterization of bovine leukocytes of the MPS system

[a] AbD Serotec

[b] Monoclonal Antibody Center, Washington State University, Pullman, USA.

The mononuclear phagocyte system (MPS) is a heterogeneous family of cells comprising bone marrow precursors, circulating blood monocytes, and tissue macrophages (186). Lymphoid tissue DCs, plasmacytoid DCs and monocytes/macrophages derive from the same progenitor (187), and share many functional characteristics. The phenotypic discrimination between different mononuclear phagocytes is also a challenge in several species, including cattle, due to a high degree of plasticity in the expression of surface markers among these cells (180). Different markers are also expressed depending on stages of cell development, activation status, tissue localization, species and function (188). These aspects have led to several authors criticizing the division of mononuclear phagocytes into distinct populations, and it has been suggested that dendritic cells and macrophages are only different subsets of mononuclear phagocytes instead of separate lineages (179, 189, 190).

Results and general discussion

Cytokine activation of NK cells

Cytokines of the 4-alpha helix family, in particular IL-2 and IL-15, have been used for *in vitro* expansion and differentiation of human NK cells (8, 85). Bovine NK cell do not proliferate in response to human IL-2 (19, 24), and although human IL-15 has stimulated proliferation of bovine NK cells in culture (24, 103, 104), NK cells were shown to produce far less IFN-γ *in vitro* when stimulated with human IL-15 and IL-12 compared to bovine IL-2 and IL-12 (104, 153).

In paper 1 we described the production of recombinant bovine IL-15 in mammalian cells. We demonstrated that the IL-15 containing supernatant from transfected cells stimulated NK cell proliferation in culture, induced surface expression of CD69 and CD25, and stimulated IFN-γ production in the presence of IL-12, and to the same extent as bovine IL-2. In contrast to previous findings, we also found potent IFN-γ production from bovine NK cells stimulated with human IL-15 and IL-12, when changing the supplier of the commercial human IL-15 cytokine. This latter aspect has been discussed more in detail in paper 1. Interestingly, Pillet *et al.,* has demonstrated that human NK cells in culture are only transiently responsive to free IL-15, which is in later stages replaced by a dependency on IL-2 and trans-presented IL-15 (191, 192).Translated to an *in vivo* setting, this would indicate that NK cells can be initially activated by a variety of IL-15 producing cells at the beginning of an immune response, whereas activation in later stages is restricted to T cell derived IL-2 and mature DCs that can trans-present IL-15.

Due to a suboptimal transfection yield of the produced bovine IL-15, as discussed above, bovine IL-2 and human IL-15 were applied in subsequent studies of NK cell activation in cattle (paper 2 – 3). These studies demonstrated an equal capacity of the two cytokines together with IL-12 in stimulating IFN-γ expression in blood, afferent lymph and LN NK cells. NK cells in bovine afferent lymph (paper 2) and the inflamed draining LN (paper 3) had an enhanced capacity for IFN-γ production, as shown by a potent IFN-γ production also upon stimulation with IL-12 cytokine alone, or IL-2 or IL-15 alone. In contrast, NK cells from blood and LNs of non-injected animals produced significantly less IFN-γ when stimulated with IL-12 alone. The

enhanced capacity for IFN-γ production is reminiscent of memory-like properties of NK cells reported by Cooper and co-workers (116). They showed that previously activated, adoptively transferred NK cells responded with a robust IFN-γ production upon subsequent restimulation with cytokines. An enhanced response upon restimulation is a key trait of immunological memory. However, these memory-like cells were phenotypically similar to naïve cells.

CD69 as a marker to define early activation of bovine NK cells

The CD69 molecule is expressed on the surface of activated human and murine NK cells, and is an accurate marker of early NK cell activation *in vitro* and *in vivo* (92, 93, 191). The expression of CD69 on bovine NK cells has until now not been described due to the lack of monoclonal antibodies.

Paper 1 describes the production of an antibody recognizing the bovine CD69 molecule. By the use of this novel antibody, we demonstrated that bovine blood NK cells had a rapid induction of surface CD69 expression *in vitro* when stimulated with IL-2 or IL-15. A peak CD69 expression was reached at 12 h after cytokine stimulation. This experiment showed that CD69 is an appropriate marker for early bovine NK cell activation *in vitro*.

A surprisingly large proportion of bovine blood NK cells were $CD69^+$ *ex vivo* (paper 1), contrasting the general knowledge from other species that CD69 is absent on most resting NK cells (92, 193). In comparison to lymph node resident NK cells, NK cells in bovine blood carry lower levels of the activation markers CD25 and CD44, and have been characterized as having a naïve or non-activated phenotype (25, 28). The expression of CD69 on about half of blood NK cells in paper 1 indicates that cattle may have an enduring state of primed NK cells in circulation, as suggested in human (91, 92). In paper 2 we demonstrated that the phenotype of NK cells in afferent lymph is strikingly different from the phenotype of blood NK cells. The majority of afferent lymph NK cells were $CD25^+$ and $CD44^{bright}$, indicative of being in an activated state, although significantly fewer NK cells in afferent lymph expressed CD69. However, when activated *in vivo* in an adjuvant –induced inflammatory setting, NK cells in skin-draining lymph nodes rapidly upregulated CD69 on their surface (paper 3).

Summarizing the results from studies of NK cell CD69 expression in papers 1 – 3 **(Figure 3)**, it is likely that a subset of NK cells in bovine blood are primed rather than naïve, whereas afferent lymph NK cells are representative of a later phase of activation. With the appropriate stimulation, NK cells can be further triggered to rapidly upregulate CD69 on their surface in the lymph node, for instance in an infection or vaccination setting. Moreover, the results show that CD69 expression on bovine NK cells is a highly dynamic process, likely influenced by tissue localization and changing degrees of activation resulting from pathogen encounters and cellular interactions, as discussed below.

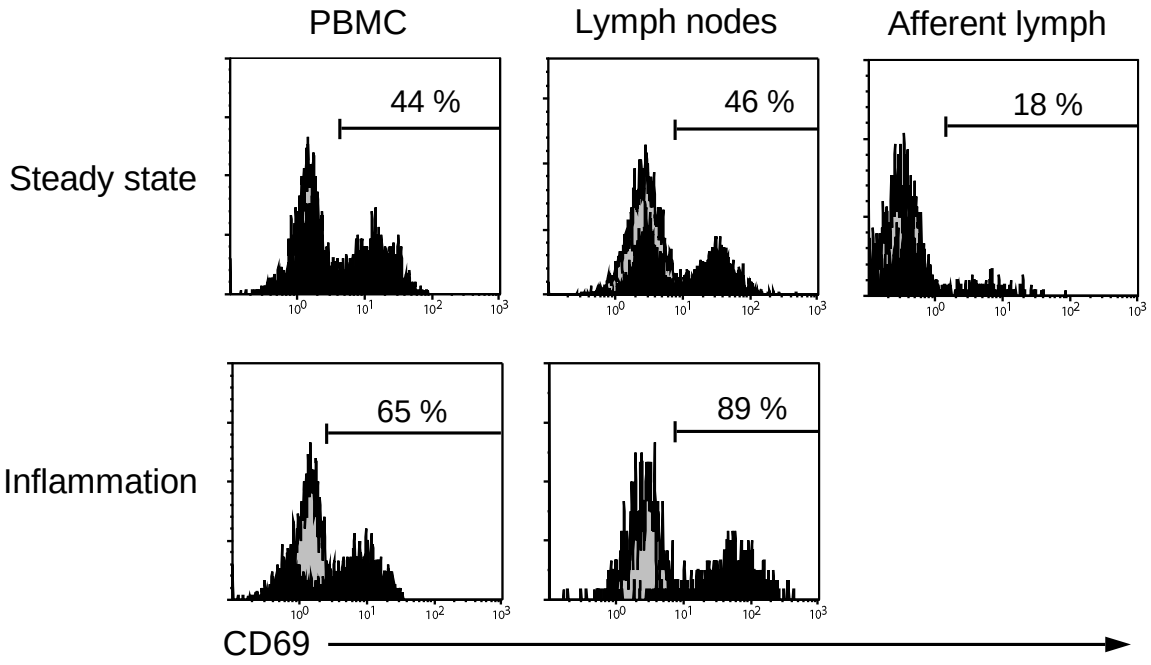

Figure 3. CD69 expression on bovine NK cells in steady state and inflammation

Histograms displaying surface expression of CD69 on NK cells (black) and secondary antibody control (grey). Representative plots from PBMC, lymph nodes and afferent lymph are shown. Percentages within each panel indicate $CD69^+$ NK cells of total NK cells.

It is widely accepted that the upregulation of CD69 indicates cellular activation. It has also been shown that CD69 expression can persist on the surface of lymphocytes for a period after stimulation (194), and a population of memory-like NK cells in mice expressed CD69 for up to

a week after activation (116). However, the functional role of the CD69 molecule is not known. A role in immunoregulation has been suggested (195), as well as a function in NK cell mediated cytotoxicity (196). The ligation of CD69 on human, but not murine, NK cells has also been involved in lysis of tumor cells (197, 198). However, the physiological ligand of CD69 is still unknown (199).

The dynamic nature of CD69 expression

A possible role for the CD69 molecule might lie within its tight linkage to the Sphingosine-1 phosphate (S1P) receptor (S1PR). Studies in mice have demonstrated that CD69 can associate with and inactivate the S1P receptor, and inhibit the exit of T cells from lymph nodes (200). It would be fruitful to decipher the roles of S1P-receptors on bovine NK cells, but this is not possible at present due to lack of reagents recognizing cattle receptors.

S1P is a secreted lysophospholipid bound to plasma proteins, and therefore found in high concentrations in extracellular fluids and in low concentrations in tissues (201-203). A S1P gradient allows the egress of S1P receptor expressing cells from tissues and into the circulation. In the S1P-rich lympatic vessels, a down-regulation and internalization of the receptor on lymphocytes is accompanied by a down-regulation of CD69 (204). This mechanism provides a likely explanation for the low CD69 expression of NK cells in afferent lymph demonstrated in paper 2. Following entry into lymph nodes, S1P receptor expression on lymphocytes increases, and theoretically, this should be followed by a lymphocyte migration towards blood. However, the S1P receptor mediated egress is inhibited by the presence of CD69 on primed lymphocytes (205, 206). This CD69-mediated retention of lymphocytes is probably advantageous, as it provides time for cellular interactions and clonal expansion of antigen-reactive lymphocytes, at least in a situation of microbial challenge. In relevance to this, we found CD69 to be markedly upregulated on B cells in the draining LN after adjuvant injection (paper 3). In blood, however, B cell percentages did not increase and the majority of B cells were CD69 negative, indicating a possible CD69-mediated retention of B cells in the inflamed LN in our study.Following several cell divisions, an increased S1P receptor level overrides the CD69 inhibition, and the cells can leave the lymph node.

S1P can bind to five distinct G-protein coupled receptors in mice; S1PR1 – S1PR5. Murine NK cells specifically express the S1PR5, which appears to be resistant to CD69 inhibition (207). We found CD69 to be expressed by a substantial proportion of blood NK cells (paper 1), suggesting that CD69 is not capable of preventing NK cell egress from lymph nodes in cattle, and that NK cells are likely to exit the lymph nodes in a primed state, as already suggested. In further support of this notion, in paper 3 we found that NK cells rapidly up-regulated CD69 in the LN during inflammation, followed by an increase in $CD69^+$ NK cells in circulation (paper 3). In contrast to T- and B- lymphocytes, a rapid exit of NK cells from lymph nodes is probably advantageous for their role as early effector cells. Other effector cells such as the $CD8^+$ T cells also express S1P receptor 5, and are likely to leave the LN in an activated state (207).

NK cell activation in different species

The concept that NK cells need an initial phase of priming in the lymph nodes, largely defined by the upregulation of CD69, was initially described from experiments with laboratory mice (91, 92). According to this model, naïve NK cells are recruited to lymph nodes upon microbial exposure, where they acquire the ability to rapidly respond to infections, i.e. they are "primed". Lymph nodes of experimental mice living in a pathogen free environment harbor few NK cells, and CD69 appear consistently absent on the NK cell surface (72, 110). NK cell reactivity in laboratory animals is dependent on a pre-stimulation by TLR-agonists, cytokines or infection. In contrast, a significantly higher percentage of NK cells were recently found in LNs of mice captured in the wild (Boysen et al., 2011). Furthermore, NK cells from wild-mice were phenotypically activated, and responded rapidly to cytokine stimulation. Human lymph nodes are also abundant in NK cells (15, 16), and in cattle, lymph node-resident (25) and afferent lymph NK cells (paper 2) show characteristics of being activated.

The different levels of NK activity in LNs of different species could be explained by the levels of microbial burden that laboratory mice, wild mice, humans and cattle normally live under: whereas the microbial load of a cow is greater than that of a human, the protected living environment of laboratory rodents probably represent the other end of the scale. The near absence of NK cells in LNs of laboratory mice might therefore have environmental causes rather than representing a natural situation for this species. Similarly, the presence of NK cells in LNs and the maintenance of a pool of primed NK cells in circulation of humans, wild-mice

and cattle is likely due to a constant exposure to microbes from the environment. This could be advantageous, as primed NK cells are able to respond more rapidly than naïve cells upon pathogen encounter (91).

Recirculation of bovine NK cells

The recirculation of adaptive lymphocytes can in general follow two pathways; lymphocytes can enter lymph nodes from blood through high endothelial venules (HEVs), or recirculate through extralymphoid tissues, like the skin, and migrate into the afferent lymph (reviewed by (208)). Most available data on NK cell traffic originate from experimental mouse models (119, 120, 123). Although the use of knock-out mice deleted for chemokines or chemokine receptors has improved the general knowledge on molecular mechanisms of NK traffic, the *in vivo* recirculation of NK cells under steady state and pathological conditions are not fully understood.

Similar to humans and mice, NK cells in cattle are present in many lymphoid and non-lymphoid organs, with subset specific features depending on their tissue localization (19, 25, 104). Common to all three species is the clear distinction between the phenotype of NK cells found in the blood and spleen compared to NK cells in lymph nodes. This distinction raises the question of whether subsets of NK cells home to and reside in specific tissues, develop locally from tissue specific precursors, recirculate between organs, or most likely, a combination of all above.

As for naïve T and B lymphocytes, NK cell entry into LNs was until recently believed to be mainly from the blood circulation through HEVs (72, 110). In paper 2, we describe the presence of NK cells in bovine skin-draining afferent lymph, providing evidence that NK cells can exit the skin and enter afferent lymphatics, and indicate that NK cells can recirculate through peripheral tissues. It also suggests that NK cells can be recruited to lymph nodes not only from blood via HEV, but also from the AL. In support of this notion, the LN homing molecule L-selectin (CD62L) was expressed on around half of the NK population in afferent lymph.

NK cells in afferent lymph showed an activated phenotype. This finding raises the question of where and how these cells have been activated. Although the majority of blood NK cells express low levels of activation molecules, a minor proportion of NK cells in blood are indeed $CD25^{+}$ $CD44^{bright}$ ((25) and paper 2), implicating the possibility that a subset of activated NK cells ($CD25^{+}$ $CD44^{bright}$) might leave the blood compartment to enter peripheral tissues and subsequently afferent lymph. Indeed, early studies of human and sheep lymphatics showed that whereas naïve T cells preferentially homed to lymph nodes directly from blood and were largely excluded from peripheral tissues, memory and effector T cells migrated to peripheral tissues and further into the lymph (44, 150, 209, 210). Also for B lymphocytes, it has been suggested that different subsets recirculate through different tissues (211). It is therefore likely that subsets of NK cells also migrate according to different homing preferences, although this has not been verified *in vivo*. However, subsets of human NK cells do express unique chemokine receptor repertoires (122), and Carrega and co-workers recently published extensive data on the chemokine gene expression level in different human tissues, which indicated that specific tissues are oriented towards recruiting specific NK cell subsets (121). In cattle, the $CD2^{-}$ subset, which dominates in lymph nodes and afferent lymph, was found to transcribe significantly higher levels of inflammatory chemokine receptors than their $CD2^{+}$ blood counterparts (74). NK cells do appear to be far more heterogenous than previously appreciated. Sun and co-workers recently suggested a model in which mature NK cells in different stages of differentiation coexist within the same compartment (114). In relevance to this, it is possible that cattle may have coexisting subsets of both naïve, primed and activated NK cells in the blood circulation, and that these subsets have different homing preferences. Moreover, LN-resident NK cells may consist of a combination of naïve NK cells recruited from blood, and activated NK cells arriving via the afferent lymph. Shortly after the characterization of NK cells in bovine afferent lymph (paper 2), a descriptive study of human afferent lymph NK cells was published (121). NK cells were $CD56^{bright}$ $perforin^{low}$, thus resembling the NK cell subset dominating in human LNs and many other organs, and were strongly positive for the lymph node-homing molecules CCR7 and CD62L. Human AL NK cells expressed low levels of CD16 and KIRs, and did not express the activation molecules HLA-DR or NKp44, indicating a non-activated phenotype. However, the expression of CD69 and CD25 on these NK cells was not studies.

Finally, activation of bovine NK cells may also take place in peripheral tissues, even at steady state. Interactions of NK cells with dendritic cells or other myeloid cell populations in the periphery have been demonstrated; $CD56^{+}$ $CD3^{-}$ NK cells were detected in healthy human skin (70, 212), and in diseased skin affected by atopic dermatitis NK cells were found in close contact with DCs (70). Della Chiesa and co-workers suggested that innate effector mechanisms are initiated in inflamed tissue upon encounter of NK cells and DCs, by the involvement of toll-like receptors on both cell types (213).

Although progress has been made in understanding aspects of NK cell recirculation, with novel contribution from the current study describing NK cells in afferent lymph, further work is necessary to understand the processes that mediate NK cell migration in cattle and other species.

MAIN CONCLUSIONS

- The activation molecule CD69 is a useful marker for studies of early NK cell activation in cattle.
- A proportion of NK cells expressed CD69 in blood at steady state, indicative of a primed phenotype of a subset of NK cells in bovine blood.
- NK cells in bovine afferent lymph were in an activated state characterized by a high expression of CD25 and CD44 and a capacity to readily produce IFN-γ. A significantly lower percentage of NK cells in bovine afferent lymph were $CD69^+$ than in blood.
- NK cells in afferent lymph had a similar phenotype to lymph node-resident NK cells, and a proportion of the cells expressed CD62L, indicating that NK cells may home to lymph nodes from the afferent lymph.
- NK cells were activated in the skin-draining lymph node following the injection of a saponin-based adjuvant. This activation was characterized by an increased CD69 expression, an enhanced MFI of CD25 on NK cells, and a greater capacity to produce IFN-γ. Furthermore, activation of NK cells in LNs was accompanied by an increase of activated NK cells in circulation.

FUTURE PERSPECTIVES

The afferent lymph cannulation technique in large animals, and experimental post-mortem analysis combining histopathology and flow cytometry, are methods used herein that offer powerful tools for studies of NK cell recirculation. Knowledge on the initiation of immune responses in cattle can form a basis for development of new vaccines in this species, as well as being important to understand processes in other species, including humans.

Further studies of NK cell subset distribution and migration pattern are needed, to decipher how the effector capacities of this innate population cell population can be targeted in infection and vaccination. For cattle studies, an important future task will be to expand the bovine immunology tool box with the production of bovine-specific reagents that facilitate studies of cellular traffic (e.g. anti-bovine CCR7 mAb, S1PR antibodies). A lack of such reagents is currently limiting research within this field.

Finally, it would also be of great interest to further study inflammatory monocytes in cattle. The afferent lymph compartment in particular can provide direct access to monocytes for more detailed studies of the phenotype and cytokine production of these cells.

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Research paper

Interleukin-15 activated bovine natural killer cells express CD69 and produce interferon-γ

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ABSTRACT

Interleukin (IL)-15 is an essential cytokine in natural killer (NK) cell development and survival. In humans, IL-15 shows overlapping properties with IL-2 due to partly shared receptors and signal transduction and both cytokines synergize equally well with IL-12 in the induction of interferon (IFN)-γ production from NK cells. Bovine NK cells however, have been reported to produce less IFN-γ after in vitro IL-12 stimulation when exposed to human IL-15 in comparison to bovine IL-2. We therefore wanted to determine if homologous IL-15 is needed for adequate stimulation of bovine NK cells. Biologically active recombinant bovine IL-15 (rbIL-15) produced in mammalian cells by the use of a modified expression vector stimulated NK cells to a dose-dependent IFN-γ production in the presence of IL-12. In contrast to earlier findings, we also detected potent IFN-γ production from bovine NK cells stimulated by human IL-15 and IL-12.

Finally, we describe a monoclonal antibody recognizing bovine CD69 and show the expression of this early activation marker on bovine NK cells ex vivo and following rbIL-15 stimulation.

1. Introduction

Interleukin-15 was initially described as a cytokine stimulating the proliferation of T-lymphocytes (Grabstein et al., 1994), but it is today recognized to have multiple immunoregulatory functions. NK cells, which are central effectors against pathogens and tumors in the early innate response, are highly dependent on IL-15 for development in the bone marrow (Leclercq et al., 1996; Mrozek et al., 1996), effector functions and survival (Becknell and Caligiuri, 2005; Carson et al., 1994). IL-15 is a 4 α-helix type molecule like IL-2, and although these cytokines have low sequence homology they show overlapping properties due to their shared signal transduction. Both utilize the intermediate-affinity β and γ subunits of the IL-2 receptor (Carson et al., 1994; Giri et al., 1994; Grabstein et al., 1994), whereas specificity is provided by distinct receptor α-chains which together with the two other subunits make up high-affinity receptors for IL-2 or IL-15. Important sources of IL-15 are monocyte/macrophages and dendritic cells (DC). IL-15 mRNA expression is found in multiple tissues but not in T-lymphocytes (Grabstein et al., 1994), in contrast to IL-2 which is mainly produced by antigen activated T-cells (Smith, 1988).

IL-15 can act as a free cytokine or in a trans-presented form. Upon infection, activated dendritic cells can present IL-15 in complex with the IL-15Rα to a neighboring NK or

Abbreviations: UTR, untranslated region; HCMV, human cytomegalovirus; TPA, tissue plasminogen activator; ORF, open reading frame; MFI, mean fluorescence intensity.

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CD8+ T cell bearing the β and γ subunits of the receptor (Dubois et al., 2002). This trans-presentation of IL-15 is necessary for the priming of murine NK cells in vivo (Koka et al., 2004; Lucas et al., 2007), but additional signals are needed for NK cells to gain full activation and mediate effector functions such as cytotoxicity and IFN-γ production (Koka et al., 2004). Primed or activated NK cells express CD69, an early activation marker which is absent on most resting lymphocytes (Lanier et al., 1988). The bovine CD69 molecule has been cloned and characterized (Ahn et al., 2002), but the CD69 expression in cells of cattle and other domestic animals has not been known due to lack of reactive antibodies. On the other hand, the IL-2 receptor α-chain, CD25, has been widely used as an activation marker on ruminant T-cells, and is also expressed on a population of CD4+ regulatory T-cells (Sakaguchi et al., 1995). A minor part of bovine NK cells in peripheral blood express CD25 (Boysen et al., 2006), but CD25 is upregulated following in vitro activation with bovine IL-2 (Boysen et al., 2006; Storset et al., 2004) or human IL-15 (Elhmouzi-Younes et al., 2009).

Both recombinant bovine IL-2 and recombinant human IL-15 are used for in vitro expansion of bovine NK cells, but previous studies did not detect proliferation in response to human IL-2 (Endsley et al., 2006; Storset et al., 2004). Although both human cytokines synergize equally well with IL-12 in the production of IFN-γ from human NK cells in vitro (Carson et al., 1994), we have previously observed that bovine NK cells produce far less IFN-γ after in vitro IL-12 stimulation when stimulated with human IL-15 in comparison to bovine IL-2 (Elhmouzi-Younes et al., 2009). Also, bovine splenic NKp46+ cells activated by human IL-15 were described to produce IFN-γ in significant amounts only when given both IL-12 and IL-18 (Goff et al., 2006). In contrast, Endsley and colleagues found the intracellular IFN-γ expression of cattle NK cells to be enhanced upon stimulation with human IL-15, with or without IL-12 (Endsley et al., 2006). To address these contradictory findings, we wanted to determine if homologous IL-15 would give optimal IFN-γ production by bovine NK cells. The gene for bovine IL-15 has previously been described, and IL-15 expressed in bacteria was able to stimulate the proliferation of bovine PBMC (Canals et al., 1997). Here we present a strategy for expression of recombinant bovine IL-15 in mammalian cells, and have performed studies of surface phenotype, IFN-γ secretion and proliferation of NK cells under the influence of bovine and human IL-15 and bovine IL-2. We also describe the production and specificity of an anti-bovine CD69 mAb and show for the first time the expression of this important early activation marker on bovine NK cells.

2. Materials and methods

2.1. Cloning and expression of recombinant bovine IL-15

Bovine PBMC were isolated by density gradient centrifugation on lymphoprep (Axis-Shield) at 2210 × *g* for 30 min. After stimulation by ConA for 24 h, messenger RNA was extracted from PBMC by μMACS kit (Miltenyi Biotec, Bergisch Gladbach, Germany), followed by complementary DNA synthesis by SuperScript™ III reverse transcriptase (Invitrogen). The bovine IL-15 sequence was amplified from cDNA by PCR (35 cycles, 30 s at 95 °C, 30 s at 60 °C, 1 min at 72 °C) using Easy-A enzyme (Stratagene, La Jolla, CA, USA) and the forward primer 5′-GGATCCACTCTGGCATTGAGTA**ATG**A-3′ including the IL-15 start codon and reverse 5′-CTCGAGTTTGGCAGCACATTTGAAAT-3′ recognizing a sequence in the 3′ UTR (untranslated region) 78 bp downstream the encoding sequence. Both primers included restriction sites (underlined). The 5′ and 3′ UTR sequences were found by screening the bovine EST database (NCBI) with the bovine IL-15 sequence (GenBank NM_174090). The full-length bovine IL-15 gene sequence was cloned into 2.1-TOPO vector, released by BamHI and Xho1 digestion and inserted into the pcDNA1 expression vector (all Invitrogen). The construct was verified by sequencing (GATC Biotech, Konstanz, Germany).

To achieve a better expression of the IL-15 protein, the sequence encoding the mature protein only was cloned into the pND14 plasmid (a kind gift from Dr. Gary Rhodes, University of California, Davis, USA) that consists of the HCMV (Human cytomegalovirus) promoter, the TPA (tissue plasminogen activator) signal peptide replacing the IL-15 sp, a sequence from the TPA ORF (open reading frame) in the 3′end to disrupt any cis-acting negative regulatory elements, the simian retrovirus constitutive transport element and bovine growth hormone poly A. The sequence encoding the mature IL-15 protein was amplified from the existing btIL-15/pcDNA1 construct by PfuUltra™ (Stratagene, La Jolla, CA, USA) at 35 cycles (30 s at 95 °C, 30 s at 56 °C, 1 min at 72 °C) using the forward primer 5′-GGATCCAACTGGCAGTATGTAATAAA-3′ and reverse primer 5′-GAATCCTCAGAAGTGTTGATGAACA-3′. This resulted in a product of 357 bp that was cloned into the 2.1-TOPO vector, released by BamHI and EcoR1 digestion and subcloned into the similarly digested pND14 plasmid. The correctness of the construct was verified by sequencing.

RbIL-15 was produced by transfection of 293T cells. Cell culture flasks were coated with 100 μg/ml poly-L-lysine hydrobromide (Sigma–Aldrich) in PBS for 16–20 h, followed by plating of 18×10^6 293T cells in the precoated flask in RPMI with 10% inactivated FBS Gold (PAA, Pasching, Austria) for 24 h. For transfection, 37.5 μg plasmid DNA purified from *E. coli* cultures by endotoxin free Nucleobond® Xtra Maxi EF (Macherey-Nagel, Dóren, Germany) was diluted in PBS and 193.5 μg polyethyleneimine (Polysciences, Eppelheim, Germany) was diluted in sterile distilled water, the two components were subsequently mixed and incubated for 30 min at room temperature, and this hypotone solution was added drop wise to the 293T cells in 30 ml RPMI + 10% FBS. After 10 h the cells were washed and the medium changed to RPMI + 10% FBS + 1% P/S. The supernatant containing IL-15 was harvested at 48 h post transfection, neutralized with 5 M NaOH to pH 7.5 and filtered through 0.45 μM filters. Pierce protein concentrators (Thermo scientific, Rockford, USA) with a molecular-weight cut-off at 9 K was used for the concentration of the transfection supernatant at 3000 × *g* at 4 °C for 35 min according to the manufacturer's instructions. The approximately 7× more concentrated supernatant was stored at −70 °C.

2.2. Western blot analysis

For Western blot analysis, the transfection supernatant containing rbIL-15 was concentrated as described above, diluted in NUPAGE sample buffer with reducing agent (both BioRad Laboratories AB), boiled at 95 °C for 5 min, and separated by 12% Precast Bis–Tris polyacrylamide gel at 200 V for 1 h. Recombinant human IL-15 (rhIL-15, cat. no. 14-8159, eBioscience) diluted to a total amount of 5 ng was included as a positive control and cell culture medium only as a negative control. Molecular weight markers used were MagicMark Xp Standard (BioRad) for development by chemoluminiscence and SeeBlue Plus2 Pre-Stained Standard (Invitrogen) for colorimetric visualization. The product was transferred to a pre-activated Immuno-blot PVDF membrane by wet transfer at 100 V for 20 min. The membrane was blocked for 1 h with PBS-Siff/0, 1% Tween 20/5% dry-milk, and incubated with polyclonal goat IgG anti-human IL-15 antibody (AF 315, R&D systems) diluted 1:500 in PBS/0, 1% Tween 20/1% dry-milk for 18 h at 4 °C. The membrane was washed four times at room temperature and incubated with peroxidase-conjugated rabbit anti-goat IgG (BioRad) diluted 1:1000 for 1 h at RT. The membrane was washed four times, before the HRP-signal was developed by ECL Plus substrate (GE Healthcare) and detected by BioRad Chemidoc XRS.

2.3. Development of anti-bovine CD69 antibody

An anti-bovine CD69 antibody was raised against recombinant protein. A cDNA fragment of bovine CD69 (392 bp) was amplified by PCR using a specific primer set designed using GenBank sequences (GenBank accession no. AF272828.1, forward primer 5′-CCATGGCAGGACAATATGCATCATCA-3′ and reverse primer 5′-CTCGAGTTTGGAGGGTTTGCTACATAT-3′). The amplified fragments were restricted with NcoI and XhoI restriction enzymes and ligated into corresponding sites of pET-30a plasmid (Novagen). The recombinant protein was expressed in *E. coli* BL21 (DE3) (Invitrogen) and purified using His-Select Nickel Affinity Gel (Sigma) according to the manufacturer's recommendation. The purified His-tagged protein was used for immunization of mice for developing an anti-bovine CD69 monoclonal antibody named KTSN7A, by conventional techniques as previously described (Hamilton and Davis, 1995). To determine the specificity of the produced mAb, cell lysates from *E. coli* BL21 (DE3) untransformed (control) and transformed with pET-30a-CD69 plasmid were resolved by SDS-PAGE gels, and stained by Coomassie blue, or transferred to a membrane and probed by anti-CD69 antibody (KTSN7A) in Western blotting as described above.

2.4. Animals, cell isolation and culture

Blood donors were clinically healthy Norwegian red dairy cattle of both sexes at 2–5 months of age, unless otherwise stated. Peripheral blood samples were collected in EDTA-containing tubes, followed by isolation of NKp46+ cells by immunomagnetic separation from PBMC as previously described (Storset et al., 2004). NK cells were cultured for 7–10 days in RPMI 1640 with L-glutamine supplemented with 60 μg/ml penicillin, 100 μg/ml streptomycin, 1 mM sodium pyruvate, 50 μM 2-mercaptoethanol and non-essential amino acids (all Gibco/Invitrogen) and 10% FBS, and in the presence of previously determined optimal concentrations of rbIL-2 (200 U/ml) (Boysen et al., 2006) and rhIL-15 (rhIL-15, cat. no.14-8159, eBioscience) (10 ng/ml). RbIL-15 was used at a 1:10 or 1:15 dilution of the concentrated cytokine. Cultured cells were split when needed.

2.5. Flow cytometry

Flow cytometry (FCM) of surface markers was performed on isolated PBMC or NK cells stained with primary monoclonal antibodies against bovine NKp46: AKS1 (IgG1) (Storset et al., 2004) or AKS6 (IgG2b), produced against recombinant bovine NKp46 in the same manner as AKS1, and CD2 (MUC2A, IgG2a), CD8 (CACT80C, IgG1), CD3 (MM1A, IgG1) and CD25 (CACT108A, IgG2a) (all Washington State University Monoclonal Antibody Centre, Pullman, WA, USA), and CD69 (KTSN7A, IgG1). Secondary antibodies used were polyclonal goat anti-mouse and were either PE-conjugated (Southern Biotech, Birmingham, USA) or Alexa Fluor 647- or 488-conjugated (Invitrogen, Eugene, USA). Two- or three-colored flow cytometric analysis was performed with a FACS Calibur flow cytometer and the CellQuest Pro software (BD, Franklin Lakes, USA).

To determine if the mAb against bovine CD69 was useful for detecting early NK cell activation, we looked at the expression kinetics of CD69 as well as CD25 ex vivo and after cytokine stimulation. PBMCs isolated from 5 animals were seeded in 24-well plates at 2×10^6 cells/well and incubated with medium only or in the presence of 25 U/ml rbIL-2 or 1:10 dilution of rbIL-15 for different time periods of 0, 2, 6, 12 or 24 h. For FCM, cells were labeled with mAbs against NKp46, CD3, CD69 and CD25, followed by isotype-specific secondary Ab labeling. Secondary mAb controls were included.

For intracellular labeling of recombinant bovine IL-15 in cells transfected with plasmid DNA, cells were released by 0.05% trypsin–EDTA (Gibco/Invitrogen) and stained with monoclonal mouse IgG1 anti-human IL-15 Ab (1 μg/ml) (MAB 247, R&D systems), and secondarily by PE-conjugated Ab (1 μg/ml). Transfected cells labeled with secondary Ab only were included.

For the detection of intracellular IFN-γ in NK cells, isolated PBMCs from three calves at 2–5 months and two heifers at 18 months of age were added to 24-well plates at a concentration of 10^6 cells/well in 1 ml RPMI + 10% FBS + 1% P/S and incubated for 20 h in medium only or in the presence of rbIL-2 (100 U/ml), rhIL-15 (10 ng/ml) or rbIL-15 (1:7.5 dilution), and in the absence or presence of rhIL-12 (400 pg/ml) (eBioscience). Brefeldin A (10 μg/ml) (Sigma) was added to cells 4 h prior to staining for FCM. Cells were surface stained with AKS1 and secondary Alexa Fluor 647-conjugated Ab. Following permeabilization and fixation procedures

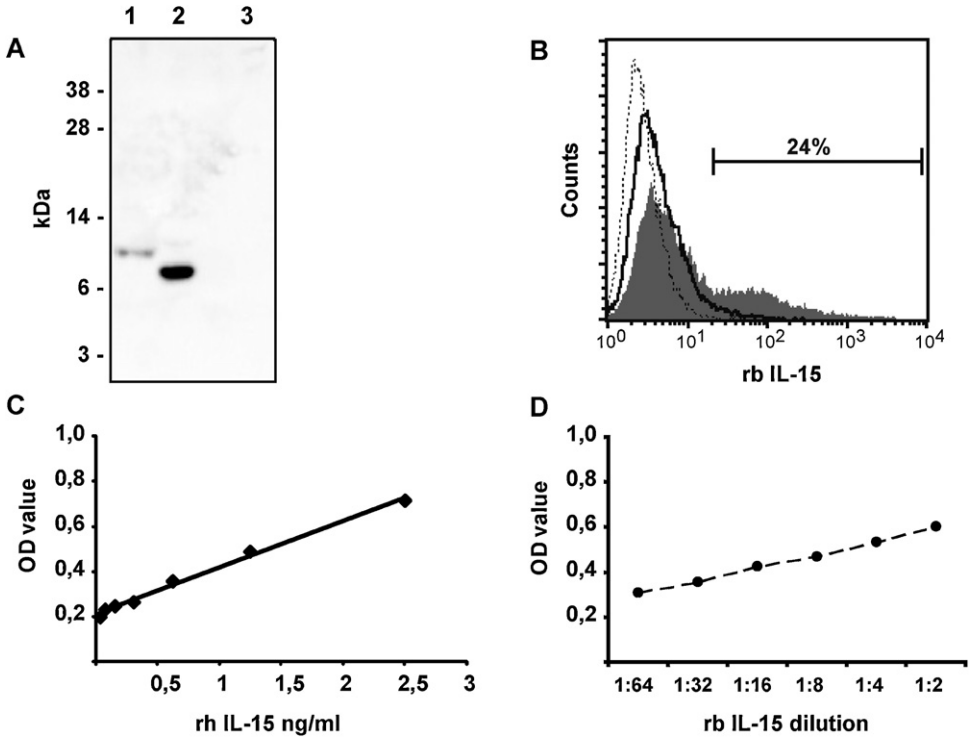

Fig. 1. Production of recombinant bovine IL-15. (A) Western blot staining of rbIL-15 (1), positive control rhIL-15 (2) and cell culture medium only (3) with an anti-human IL-15 polyclonal Ab. Relative molecular masses are indicated. (B) Intracellular labeling of rbIL-15 (solid gray histogram) in pND14/btIL-15mp transfected 293T cells with anti-human IL-15 mAb. Mock-transfected cells (dotted line) and transfected cells labeled with secondary antibody only (black line). (C) Concentration of rhIL-15 plotted against OD-value as measured by ELISA. (D) Different dilutions of rbIL-15 transfection supernatant plotted against OD-value as measured by the same ELISA as in (C).

(Cytofix/Cytoperm, BD Biosciences, San Diego, CA, USA), intracellular labeling of IFN-γ was performed on cells as described before (Olsen et al., 2005) using anti-bovine IFN-γ mAb (clone 6,19, IgG2a) (a kind gift from Gregers Jungersen at the Technical University of Denmark) and secondary PE-conjugated Ab. Cells were analyzed by FCM.

2.6. Analysis of proliferation

To study proliferation, NK cells were expanded in culture for 7–10 days on rbIL-2, rbIL-15 or rhIL-15, then washed three times and seeded at 5×10^5 NK cells per well and stimulated for additional 24 h with 25 U/ml rbIL-2 or 1:10 dilution of rbIL-15, or with two-fold dilutions of rbIL-2, rbIL-15 or rhIL-15. Proliferation was measured as 16 h uptake of 3H-thymidine (Perkin-Elmer, Waltham, USA) as previously described (Storset et al., 2001).

2.7. ELISA detection of cytokine

For evaluation of secreted IFN-γ, bovine NK cells were expanded as described above, then further stimulated for 24 h in triplicates of 10^5 cells in the presence of different concentrations of rbIL-2 (50, 25, 10 U/ml), rbIL-15 (1:7.5, 1:15) or rhIL-15 (10, 5, 1 ng/ml), and in the absence or presence of various rhIL-12 concentrations (400, 200, 100 pg/ml). The assay supernatants were analyzed in a bovine IFN-γ ELISA (Mabtech AB, Nacka Strand, Sweden) following the manufacturer's instructions. Briefly, a maxisorp ELISA plate was coated with anti-bovine IFN-γ mAb (2 μg/ml) at 4 °C for 16 h, followed by washing and blocking steps and incubation of samples and standards for 2 h. After washing, biotinylated detection mAb (PAN-biotin, 0.1 μg/ml) was added to wells for 1 h, wells were washed again and added streptavidin-HRP at a 1:1000 dilution. The end product was developed using tetramethylbenzidine substrate (Sigma, Saint Louis, USA) according to the manufacturer's instructions, the reaction was stopped by 2 M H_2SO_4 and read spectrophotometrically at 450 nm (Titertek Multiscan Plus Elisa reader). Concentrations were calculated from the obtained curve using the bovine IFN-γ standard supplied by the manufacturer.

An ELISA was performed to estimate the rbIL-15 concentration in the transfection supernatants, by using the protocol of the bovine IFN-γ ELISA kit, as explained above. Plates were coated with polyclonal anti-human IL-15 antibody (0.5 μg/ml) and added the rbIL-15 transfection supernatant in two-fold dilutions. Bound rbIL-15 was detected by monoclonal anti-human IL-15 antibody (1 μg/ml), biotinylated goat anti-mouse IgG1 (1:2000) (Southern Biotechnology, Birmingham, USA), streptavidin-HRP (1:1000) and read spectrophotometrically at 450 nm. Recombinant human IL-15 in two-fold dilutions was used as the basis for the standard curve.

3. Results

3.1. Production of biologically active bovine IL-15 by the use of a modified vector

No proliferation of NK cells in culture was observed when cells were given the concentrated supernatant from 293T cells transfected with the pcDNA1 vector containing the entire IL-15 coding region (not shown). The sequence encoding only the bovine IL-15 mature protein was cloned into the pND14 expression vector that contains the HCMV promotor and TPA signal peptide and transfected into 293T cells. In Western blot analysis of the concentrated transfection supernatant a band of about 13 kDa was visible (Fig. 1A). The recombinant human IL-15 produced in *E. coli* has a predicted molecular mass of 12.7 kDa, and migrates as an 11 kDa polypeptide under reducing conditions (as stated by the manufacturer). The theoretical masses of human and bovine IL-15 are both about 14–15 kDa. The difference in mass between IL-15 produced in *E. coli* and the bovine IL-15 produced in eukaryotic cells is likely due to glycosylation as the IL-15 sequence contains two functional N-glycosylation sites (Kurys et al., 2000).

Intracellular staining of rbIL-15 was performed on 293T cells transfected with the pND14/btIL-15mp construct. An anti-human IL-15 mAb labeled rbIL-15 in 24% of transfected 293T cells (Fig. 1B). No labeling was observed in mock-transfected cells or in transfected cells labeled with secondary antibody only.

An ELISA was performed with anti-human IL-15 antibodies to estimate the content of rbIL-15 in the concentrated transfection supernatant. Recombinant human IL-15 in two-fold dilutions was the basis of the standard curve (Fig. 1C) and different dilutions of the rbIL-15 transfection supernatant were plotted against OD-value measured by the same ELISA (Fig. 1D). Similar results were obtained from three other transfection supernatants (not shown). Unfortunately, the results cannot be used for exact measurements of rbIL-15 content as a linearity of dilution was not achieved in the rbIL-15 ELISA. The amount of applied

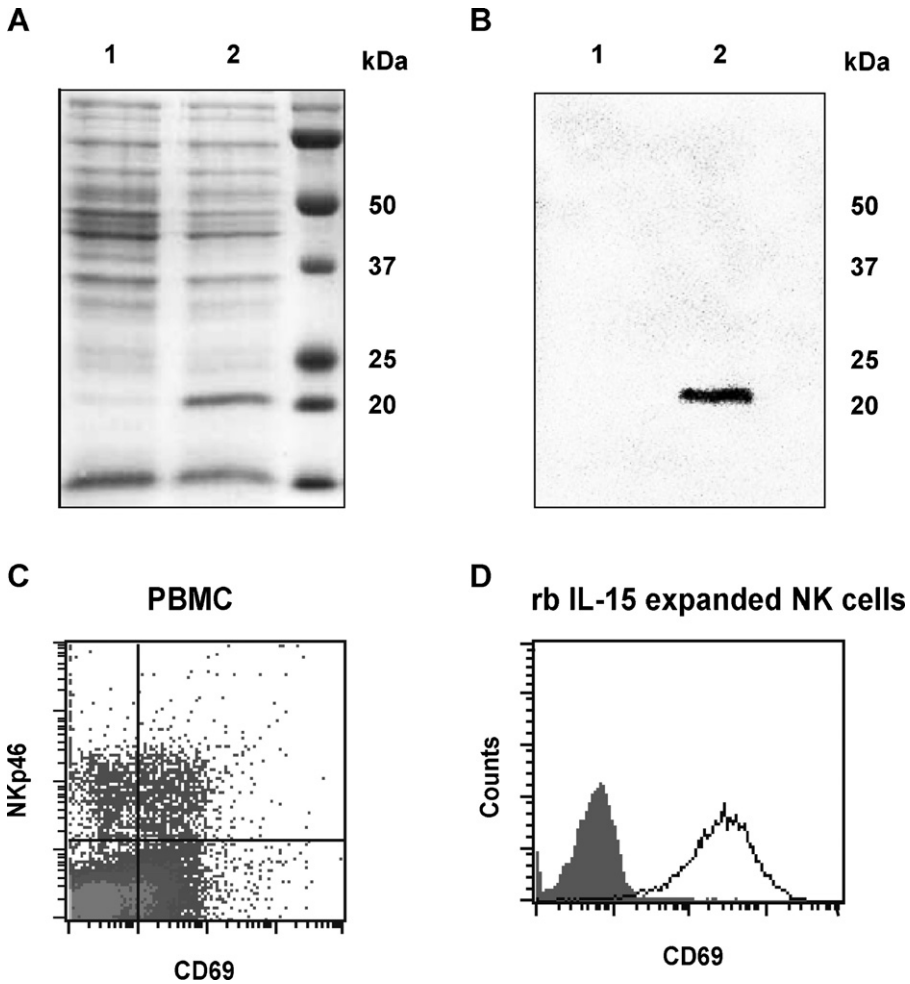

Fig. 2. Specificity of CD69 and its expression on NK cells. (A) SDS-PAGE gel stained by Coomassie blue and (B) Western blot analysis with anti-CD69 antibody (KTSN7A) of whole lysate from *E. coli* BL21 (DE3) expressing bovine CD69. Cell lysate from *E. coli* BL21 (DE3) untransformed (control) lane 1 and transformed with pET-30a-CD69 plasmid lane 2. (C) Two-color FCM of CD69 and NKp46 on ex vivo bovine PBMC. (D) Histogram displaying surface expression of CD69 (black line) and secondary antibody control (solid gray) on NK cells expanded for 7–10 days in culture with rbIL-15. Results shown are representative for experiments with eight (C) and four (D) different animal, respectively.

Table 1
Expression of CD69 and CD25 on NK cells ex vivo and after 7–10 days of expansion in culture with rbIL-2, rbIL-15 or rhIL-15.

	Ex vivo	rb IL-2	rb IL-15	rh IL-15
CD69				
Median MFI	37	218	286	240
Range	(29–59)	(136–365)	(237–528)	(118–351)
n	8	7	4	6
CD25				
Median MFI	16	212	274	288
Range	(12–19)	(118–529)	(109–416)	(96–1316)
n	5	7	4	6

MFI: mean fluorescence intensity; rb: recombinant bovine; rh: recombinant human.

rbIL-15 cytokine is therefore given as dilutions of the concentrated transfection supernatant throughout the assays.

3.2. Activation of NK cells in vitro

The specificity of the monoclonal antibody against bovine CD69 was confirmed by Western blot analysis (Fig. 2A and B). The calculated size of the target protein was 20.8 kDa.

In peripheral blood (ex vivo), 42% (33–70) of cattle NK cells expressed CD69, with a median mean fluorescence intensity (MFI) of 37 (29–59) ($n = 8$) (Fig. 2C, Table 1). NK cell cultures at day 7–10 consisted of >96% NKp46+ CD3− cells, of which CD2 was expressed on 20–30% and CD8 on 40–80%, irrespective of which of the cytokines rbIL-2, rbIL-15 or rhIL-15 were used for expansion (not shown). After expansion on rbIL-15 NK cells expressed CD69 and CD25 to a similar degree as cells expanded on rbIL-2 or rhIL-15 (Fig. 2D, Table 1). The expression kinetics of both activation markers upon cytokine stimulation was measured by FCM of NKp46+ cells (Fig. 3). CD69 was rapidly upregulated on the NK cell surface, mainly between 6 and 12 h of stimulation with up to a 5-fold increase of the MFI. At 24 h the MFI was similar to or lower than at 12 h, indicating that a maximum CD69 expression was reached at about 12 h of stimulation. The upregulation of CD25 expression was slower than for CD69, with a steady increase in MFI up to 24 h. As the median MFI of CD25 was higher in NK cells after 7–10 days of culture (Table 1), the CD25 expression, unlike CD69, had not reached it maximum during the first 24 h. These results did not reveal any difference between rbIL-2 and rbIL-15 stimulated cells in expression of either CD25 or CD69.

3.3. Proliferative response

Studies of proliferation were performed on NK cells expanded for 7–10 days with rbIL-2, rbIL-15 or rhIL-15, followed by an additional 24 h of in vitro stimulation with 25 U/ml rbIL-2 or 1:10 dilution of rbIL-15, or two-fold dilutions of rbIL-2, rbIL-15 or rhIL-15. The degree of proliferation, measured as 16 h uptake of 3H-thymidine, showed

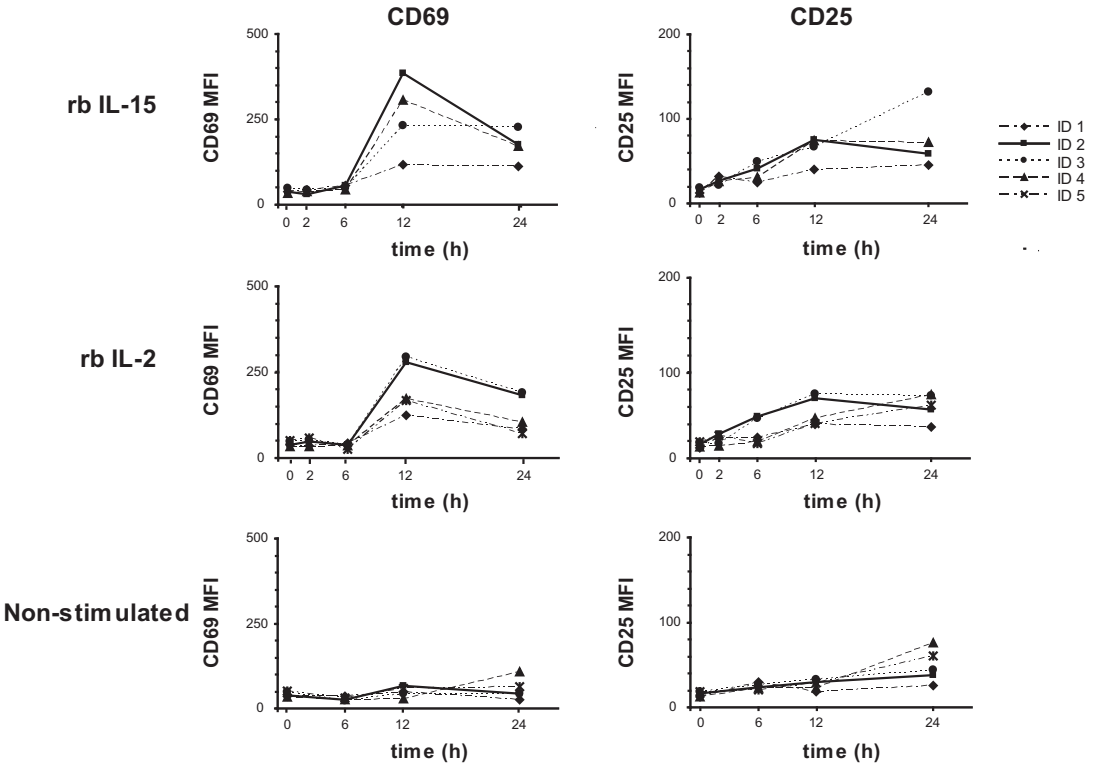

Fig. 3. Induction of activation markers on bovine NK cells. Bovine PBMC were cultured in the presence of rbIL-15, rbIL-2 or in medium only for 2, 6, 12 or 24 h. The expression kinetics of activation markers was measured ex vivo and at the given time-points by flow cytometry of NKp46+ cells. Mean CD69 and CD25 MFI of all individuals are shown as a function of the duration of cytokine treatment. Four or five animals were used in each experiment.

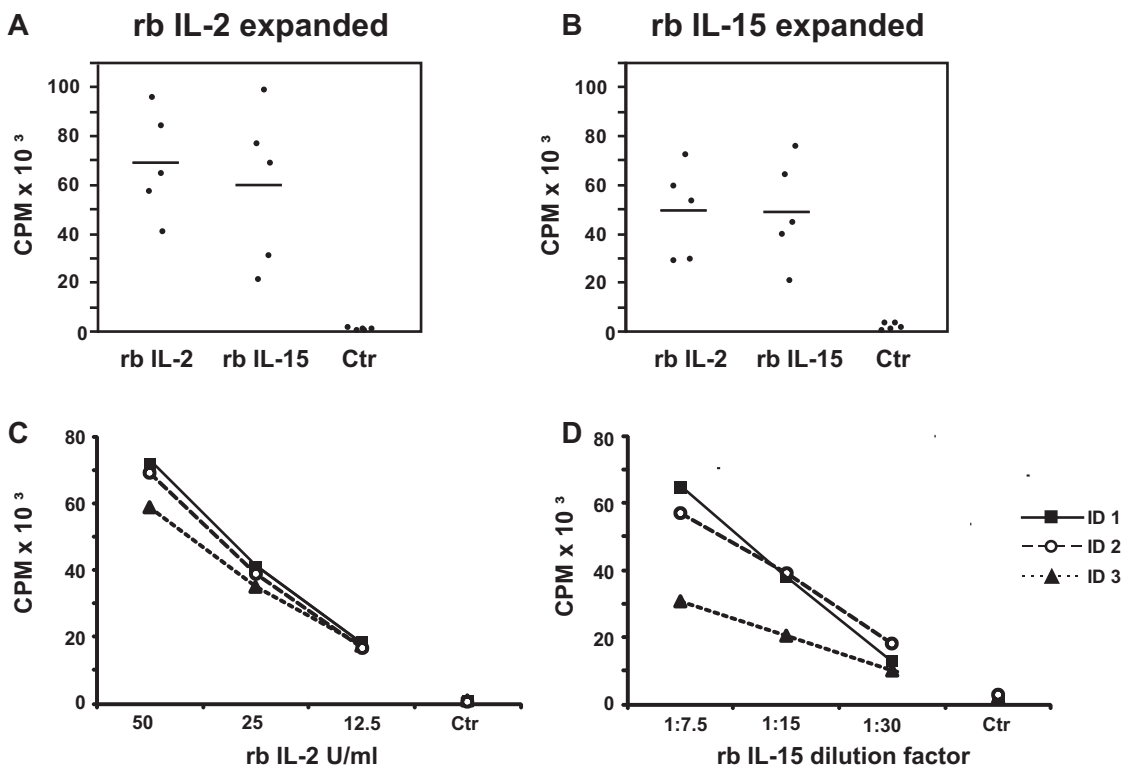

Fig. 4. Proliferative response of bovine NK cells. NK cells were expanded for 7–10 days in culture with rbIL-2 or rbIL-15, and then further stimulated for 24 h with rbIL-2 or rbIL-15. The degree of proliferation was measured as 16 h uptake of 3H-thymidine and is given in counts per minute (cpm). (A and B) Proliferation of NK cells stimulated with either 25 U/ml rbIL-2 or 1:10 dilution of rbIL-15 or with medium only (Ctr). Individual values ($n = 5$) and medians are presented. (C and D) Proliferation of NK cells to two-fold dilutions of rbIL-2 or rbIL-15. Three individual animals are presented.

a dependency on the dose of given cytokine (Fig. 4C and D), but no differences between rbIL-2, rbIL-15 (Fig. 4A and B) and rhIL-15 (not shown) were detected at the given concentrations. In the absence of cytokines NK cells did not proliferate.

3.4. IFN-γ production by cytokine activated NK cells

The level of IFN-γ secreted by activated NK cells was assessed in an ELISA. NK cells expanded on rbIL-2, rbIL-15 or rhIL-15 were stimulated for 24 h in the presence of different concentrations of the same cytokines and in the absence or presence of IL-12. A dose-dependent IFN-γ production was observed in the presence of rhIL-12 (Fig. 5A–C). Bovine NK cells also produced IFN-γ when stimulated with human interleukin-15. In the absence of IL-12, low or no IFN-γ production was seen.

The IFN-γ production by non-expanded NK cells was measured by intracellular staining. Bovine PBMCs were cultured overnight in the presence of rbIL-2, rbIL-15 or rhIL-15 and in the absence or presence of IL-12. Cells were analyzed by FCM for intracellular IFN-γ in NK cells (Fig. 6A and B). In the absence of added cytokines 1–6% of peripheral blood NK cells were positive for IFN-γ. A large proportion of NK cells were positive for IFN-γ following incubation with IL-12 and either IL-2 or IL-15. The response seemed to be age-dependent, as calves had approximately twice the values of two heifers, but to similar proportions between cytokines (Fig. 6B). In the absence of IL-12, all cytokines induced only marginally more IFN-γ^+ NK cells compared to controls in all animals.

4. Discussion

The use of heterologous cytokines may be the only choice when homologous reagents are not available. However, it may lead to ambiguities as it is not possible to distinguish true species differences from suboptimal stimulation due to incorrect receptor binding of heterologous cytokines. We and others have previously reported that stimulation of bovine NK cells with recombinant human IL-15 in combination with IL-12 only leads to minimal IFN-γ production (Elhmouzi-Younes et al., 2009; Goff et al., 2006). In this study we describe the production of recombinant bovine IL-15 and show that bovine NK cells stimulated by this cytokine produce IFN-γ in a dose-dependent manner in the presence of IL-12. However, when changing the supplier of recombinant human IL-15, we also detected a similar response to the human cytokine. We have observed that bioactivity per weight unit may differ between different products according to declarations by the manufacturer. Most commercially available recombinant human IL-15 is *E. coli*-derived, and unfolding and refolding steps that are necessary to purify *E. coli*-derived proteins (Ward et al., 2009) may lead to such differences in bioactivity. Furthermore, IFN-γ levels in bovine NK cells

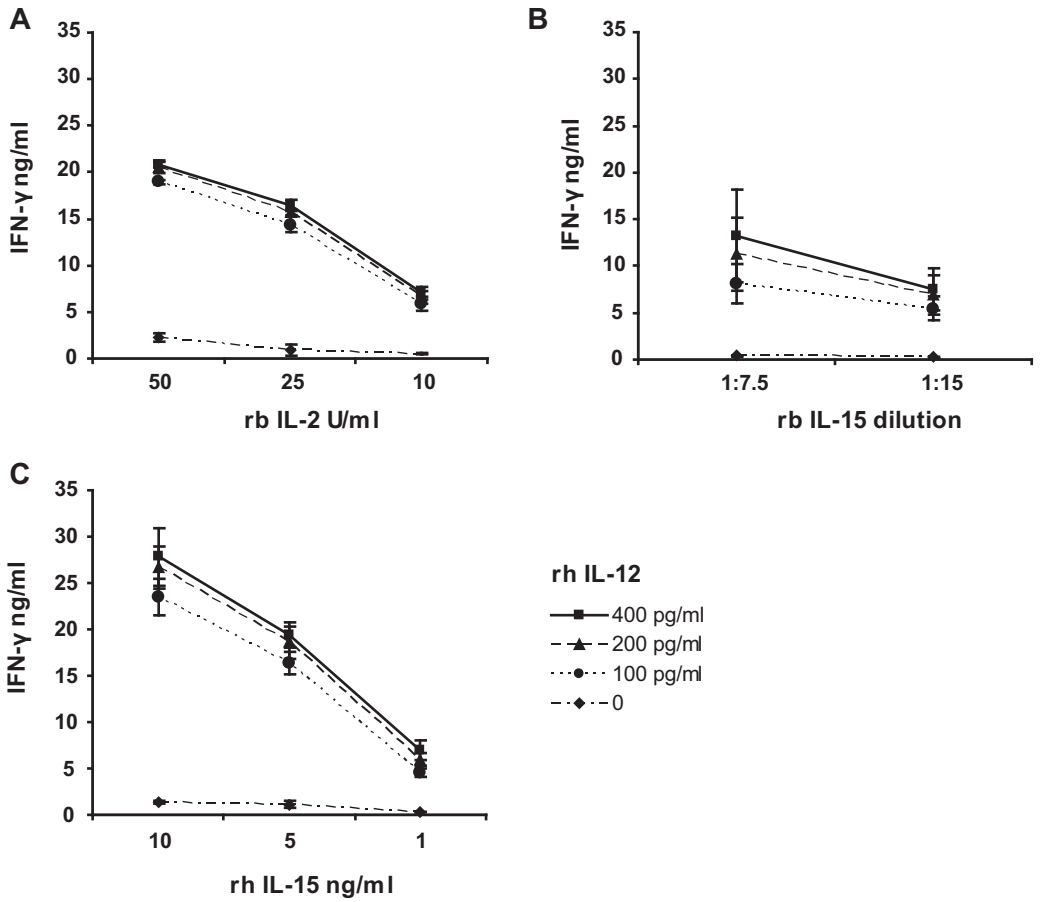

Fig. 5. IFN-γ measured in supernatant of bovine NK cells by ELISA. Cells were expanded with (A) rbIL-2, (B) rbIL-15 or (C) rhIL-15 for 7–10 days, followed by additional stimulation for 24 h in the presence of different concentrations of the same cytokines and in the absence or presence of rhIL-12. Results shown are mean values from three animals ±SD.

were higher in this study compared to our earlier work. We have previously used recombinant bovine IL-12, titrated and applied at optimal doses to trigger IFN-γ production (Boysen et al., 2006; Olsen et al., 2005). In this study, human IL-12 was used, with only marginal differences in IFN-γ production observed between given concentrations, indicating that IL-12 was used in excess. This again draws attention to the origin of the cytokine; although it cannot be excluded that optimization of the intracellular staining method may also have contributed. In conclusion, these results emphasize that the expression system and bioactivity per weight unit are at least as important considerations as species origin when choosing cytokines for in vitro stimulation.

We here demonstrate ex vivo expression of CD69 in a proportion of cattle lymphocytes, including NK cells, seemingly contrasting the general knowledge from other species that CD69 is absent in resting NK cells. However, some healthy human donors do carry proportions of circulating CD69+ NK cells (Lanier et al., 1988; Newman et al., 2006); perhaps not surprisingly since various factors such as vaccination, acute infections and exercise may lead to upregulation of CD69 on NK cells (Azeredo et al., 2006; Jost et al., 2011; Newman et al., 2006; Timmons and Bar-Or, 2007). In contrast, CD69 appears consistently absent in NK cells of laboratory mouse strains, but inducible by TLR (Toll-like receptor) ligand stimulation in vivo and in vitro (Lucas et al., 2007; Tsujimoto et al., 2005). We recently demonstrated that wild-caught mice express elevated CD69 on their NK cells compared to C57BL/6 laboratory mice (Boysen et al., 2011). It has been shown that CD69 expression persist on lymphocytes for a relatively long period after stimulation (Lopez-Cabrera et al., 1993), and a recently characterized memory-like NK cell population maintained CD69 one week after pre-activation (Cooper et al., 2009). Thus, the observed expression of CD69 in cattle may rather be a result of unknown activating factors in the animals' recent history rather than a true species difference.

When stimulated with cytokines, bovine NK cells start to express increased levels of CD69 on their surface as early as 6 h after stimulation and reach a peak of CD69 expression after 12 h. This shows that this novel mAb against bovine CD69 is a useful marker to detect early NK cell activation in this species. The kinetics of induction was found to be similar for IL-2 and IL-15 stimulated cells. In human NK cells, CD69 was induced more efficiently by IL-15 than IL-2

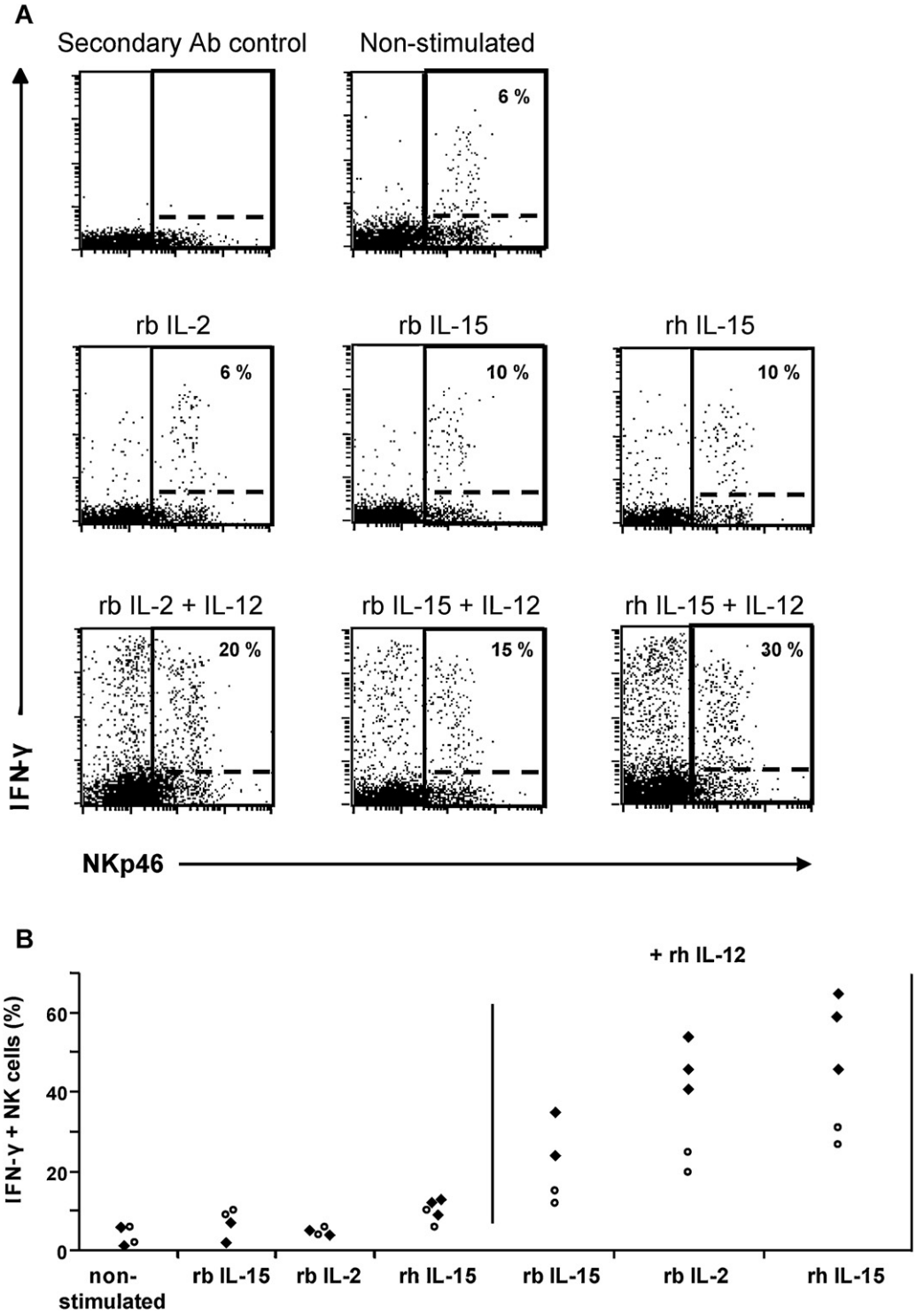

Fig. 6. (A) Two-color flow cytometric analysis of intracellular IFN-γ in NK cells. Bovine PBMC were incubated for 20 h with rbIL-2, rbIL-15, rhIL-15 or in medium only, and in the absence or presence of rhIL-12. Cells were surface labeled for NKp46, followed by permeabilization and labeling for intracellular IFN-γ. Numbers indicate IFN-γ^+ NK cells as percent of all NK cells. The results shown are representative for experiments with 4–5 different animals. (B) IFN-γ^+ NK cells as percent of all NK cells following cytokine stimulation as in A. Individual values of calves (squares) and heifers (open circles) are presented.

when cells were given suboptimal cytokine concentrations (Pillet et al., 2009). At high concentrations, however, the expression level was comparable between the two types of stimulations, which correspond to our findings.

The production of IL-15 in eukaryotic cells has proven difficult, as it is controlled by a complex post-transcriptional regulation, found mainly at the level of mRNA translation. The translation is poor due to multiple upstream start codons in the 5′ untranslated region, an unusually long 48 amino acid signal peptide, as well as negative regulatory elements in the 3′ coding sequence (Bamford et al., 1996, 1998). In the feline species, the production of biologically active IL-15 in mammalian cells was accomplished by the use of a modified expression vector (Dean et al., 2005). After failure using a traditional expression vector, production of biologically active bovine IL-15 was accomplished by the use of a similar modified vector. Two anti-human IL-15 antibodies have been reported to cross react with feline IL-15 (Dean et al., 2005), and we were here able to use the same antibodies in intracellular labeling and Western blot detection of bovine IL-15, and in an ELISA to estimate the approximate rbIL-15 content of the transfection supernatants. The relatively low percentage of IL-15 producing cells indicates a low transfection efficiency or low production of IL-15 protein in transfected cells. We could not measure the exact content of the recombinant protein as a linearity of dilution was not achieved for the rbIL-15 ELISA, probably due to interfering factors in the concentrated transfection supernatant. The supernatant had to be diluted in the functional studies due to toxic effects of the undiluted cell culture supernatant on NK cells. Thus, we did not obtain sufficiently high concentrations to make a fully dose-adjusted comparison to human IL-15. However, comparative results presented herein indicate that the bovine IL-15, if applied in a more concentrated form, would probably be as potent as the human homologue.

To obtain functional concentrated bovine IL-15, alternative strategies should be attempted, e.g. by applying a polyhistidine-tag to facilitate protein purification. This would also enable the determination of the exact content of recombinant protein. Alternatively, potent IL-15 may be produced by co-transfecting cells with the genes for both IL-15 and IL-15 receptor alpha. Soluble human IL-15/IL-15Rα complexes were shown to be more stimulatory on NK cells than IL-15 alone (Rubinstein et al., 2006), probably because it is mimicking the biological trans-presentation of IL-15 by dendritic cells. The co-expression also resulted in increased IL-15 secretion and a higher stability of both components of the complex (Bergamaschi et al., 2008).

In conclusion, this work demonstrates that bovine as well as human recombinant IL-15 stimulates bovine NK cells to proliferate, produce IFN-γ and express activation markers, including CD69, here presented for the first time in the bovine system. While functional recombinant bovine IL-15 was achieved in a mammalian expression system, the presence of toxic byproducts indicated that alternative strategies are necessary in order to obtain optimal concentrations. One such strategy would be to co-express IL-15 with its α-receptor. An optimal recombinant bovine IL-15 cytokine and the presence of a CD69 mAb will be important tools in future studies in bovine immunology.

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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Natural killer cells in afferent lymph express an activated phenotype and readily produce IFN-γ

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Natural killer (NK) cells are motile cells that migrate between peripheral blood (PB), lymph nodes (LNs), and various organs. Domestic animals have frequently been used to study cellular migration, and offer unique opportunities for such studies. The aim of this study was to characterize the phenotype and cytokine producing capacity of NK cells in bovine skin-draining lymph. NKp46/NCR1$^+$ CD3$^-$ cells constituted 2–11% of mononuclear cells in afferent lymph (AL), a majority of cells were CD16$^+$, CD8α$^+$, and CD2$^{-/low}$, and elevated CD25 and CD44 expression indicated an activated phenotype. Interestingly, significantly fewer AL NK cells expressed the early activation marker CD69 compared to PB NK cells. A large proportion of lymph and blood NK cells produced interferon (IFN)-γ following stimulation with IL-2 and IL-12. Notably, in AL, but not blood, a similar amount of IFN-γ$^+$ NK cells was observed when cells were stimulated with IL-12 alone. Overall, AL NK cells were more similar to LN-residing NK cells than those circulating in PB. We conclude that AL appears to be an important migration route for tissue-activated NK cells, and may represent an alternative route for NK cell traffic to LNs. These findings may have important implications in the development of adjuvant strategies that aim to target NK cells in a vaccine response.

Keywords: natural killer cells, afferent lymph, bovine, trafficking, activation, interferon-γ

INTRODUCTION

Natural killer (NK) cells are innate lymphocytes that act as early responders during infection or inflammation by means of cytotoxicity and production of immunoregulatory cytokines. Although NK cells are found widely distributed in non-lymphoid and lymphoid tissues in human, mice, swine, and cattle (1–4), much is still unknown regarding their recirculation and the chemokines and homing molecules controlling these movements (5, 6).

During infection or inflammation, murine NK cells gain increased entry from peripheral blood (PB) into lymph nodes (LNs) via high-endothelial venules (HEVs). Interaction with activated dendritic cells (DCs) in the LN is believed to be important for the priming of NK cells (7), upon which NK cells express the early activation marker CD69 (8), but additional signals are needed for NK cells to reach full activation and mediate effector functions such as cytotoxicity and interferon (IFN)-γ production (9). NK cells are believed to provide an early source of IFN-γ required for a Th1 polarization of the immune response *in vivo* in mice (10, 11). These events may be essential for the promotion of efficient Th1 targeting of vaccines (12). Since many vaccines are delivered to the skin (intradermal or subcutaneous) understanding the function and phenotype of cells draining from this site can provide important mechanistic insights to assist effective vaccine design and delivery. Whilst a number of studies have focused on DC in this context little is currently understood about other innate cells draining from the periphery.

The migration of NK cells from LNs via the efferent lymph, into the PB and subsequent entry into inflamed tissue has been demonstrated (2), but little is known of NK cell trafficking after entry into tissues. Afferent lymphatics drain T cells and DCs from tissues (13). The presence of NK cells has been reported in afferent lymph (AL) draining the skin in humans (14, 15) and domestic animals (16–18), but these cells have never been further characterized. In human, AL was accessed by direct microsurgical cannulation of a superficial lymphatic vessel (19, 20), giving access to cell populations originating from healthy normal skin (15, 21) and allergic contact dermatitis affected skin (14). CD56$^+$ cells were observed in human AL at a significantly lower percentage than in PB (14, 15). A recent paper describes the presence of NKp46/NCR1$^+$ cells in seroma fluid believed to be an accumulation of AL (22). To the authors' knowledge, the three latter studies provide the only observations of NK cells in human AL to date.

The method of pseudo-afferent lymphatic vessel cannulation has been established in various animal models, including sheep (23–25), cattle (17, 26), swine (27), and rat (28), and provides a unique model that has generated a large body of our general knowledge of lymphatic cellular migration from peripheral tissues to the draining LN (23). The application of this model in cattle enables the study of NK cells, which have been relatively well characterized in this species (29, 30). Bovine NK cells, defined as NKp46/NCR1$^+$ CD3$^-$ lymphocytes, can be divided into a CD2$^+$ subset dominating in PB and a CD2$^{-/low}$ subset dominating in LNs

Abbreviations: AL, afferent lymph; FCM, flow cytometry; HEVs, high-endothelial venules; LNs, lymph nodes; MFI, mean fluorescence intensity; PB, peripheral blood; $S1PR_1$, sphingosine-1-phosphate receptor 1.

(31). The latter subset carry higher levels of CD16, CD8α, and the activation markers CD25 and CD44, are particularly strong IFN-γ producers, and dominate following *in vitro* stimulation with IL-2 or IL-15 (32). Although the bovine subsets are not directly comparable to humans, bovine $CD2^{-/low}$ NK cells to a large extent resemble the $CD56^{bright}$ phenotype that dominates in human lymphoid tissues in terms of function and distribution (33, 34).

We here examined the phenotype and cytokine producing capacity of NK cells in skin-draining AL under homeostatic conditions, using a bovine cannulation model. AL NK cells were found in a highly activated state and of a similar phenotype as LN-residing NK cells, indicating that these cells may home to the LN. More knowledge of the functional capacity and trafficking pattern of NK cells in AL may help to illuminate central unanswered questions about NK cell recirculation and their role in vaccine responses.

MATERIALS AND METHODS

ANIMALS

Animal experiments were carried out according to guidelines of the UK Home Office and the Norwegian Animal Research Authorities, with full ethics approval.

PB was taken from Norwegian Red (NR) dairy calves (Bos taurus) of both sexes and 6–8 months of age, and collected in EDTA-containing tubes. Animals were clinically healthy cattle from a commercial Norwegian dairy farm. PB from Holstein–Friesian calves at the Institute for Animal Health (IAH) was collected by jugular venepuncture into sodium heparin (Leo Pharma, UK).

Pseudo-AL vessel cannulations at IAH were carried out on conventionally reared British Holstein–Friesian male calves. All animals at IAH were aged between 6 months and 1 year. Paired samples of PB and AL were obtained from one individual NR male calf of 6–8 months of age. Repeated phenotyping and intracellular IFN-γ analysis was performed on material collected from this individual at 2–4 weeks after cannulation, and representative results are included in this study.

SURGERY AND AFFERENT LYMPH COLLECTION

Pseudo-AL vessels were generated by surgical removal of superficial cervical LNs and cannulations were performed essentially as previously described (17). Briefly, approximately 8 weeks post-lymphectomy, pseudo-AL vessels were surgically cannulated with sterile, pre-siliconized, and heparinized portex tubing (Portex Ltd.). Catheters were fixed in position, passed externally via a skin incision and adequate flow of lymph was ensured. Lymph was collected at various time points from day 3 to 28 post-surgery into sterile plastic bottles containing heparin (10 U/ml), penicillin (60 μg/ml), and streptomycin (100 μg/ml) (Gibco/Invitrogen), and bottles were replaced every 8–12 h. Animals were injected subcutaneously twice daily with heparin (0.5 ml, 2500 IU, GP Pharmaceuticals) into a site draining to the catheterized lymph vessel. The lymph collected was centrifuged ($300 \times g$, 8 min), and AL cells were either used immediately in phenotypic or functional studies or resuspended in FBS Gold (PAA, Pasching, Austria) and 10% DMSO for storage in liquid nitrogen. Bovine PBMC were isolated from EDTA or heparinized blood by density gradient centrifugation ($2210 \times g$, 30 min) on lymphoprep (Axis-Shield), and used immediately in phenotypic or functional studies.

FLOW CYTOMETRY

Three-color flow cytometric (FCM) analysis of surface markers or intracellular proteins was performed on isolated PBMC or on fresh or previously frozen AL cells. Cells were first stained with LIVE/DEAD® fixable far red dead cell stain for 633 excitation (Invitrogen), following the manufacturer's instructions. Subsequently, cells were surface labeled with in house produced primary monoclonal antibodies (mAbs) against bovine NKp46/NCR1 [AKS1, IgG1 or AKS6, IgG2b; Ref. (32)], alone or in combination with mouse anti-bovine mAb against one of the following molecules: CD3 (MM1A, IgG1), CD2 (MUC2A, IgG2a), CD8α (BAQ111a, IgM), CD25 (CACT108A, IgG2a), CD44 (BAG40a, IgG3), CD62L (BAQ92A, IgG1), CD69 (KTSN7A, IgG1) (all Monoclonal Antibody Center, Washington State University, Pullman, WA, USA), or mouse anti-human CD16 (KD1, IgG2a) (a kind gift from Daniela Pende, ISTGE, Italy) or PE anti-human CCR7 (3D12, rat IgG2a; BD Biosciences, USA). Secondary antibodies used were polyclonal goat anti-mouse and were either PE-conjugated (Southern Biotech, Birmingham, AL, USA) or Alexa Fluor 488-conjugated (Invitrogen, Eugene, OR, USA). Cells surface labeled with AKS1 were permeabilized and fixed (Cytofix/Cytoperm; BD Biosciences), and further incubated with mouse anti-human perforin (delta g9, IgG2b; BD Biosciences), followed by a PE-conjugated secondary antibody. The method for intracellular staining has been described in detail elsewhere (32). Gating was based on stainings with secondary antibodies only or on non-stimulated controls. Flowcytometry was performed with a FACS Calibur flow cytometer and the CellQuest Pro software (BD Biosciences), and expression was measured as % positive NK cells for bimodal distributions and as mean fluorescence intensity (MFI) for other distributions.

INTRACELLULAR IFN-γ ANALYSIS

For the detection of intracellular IFN-γ in NK cells, PBMC and AL cells were added to 24-well plates at a concentration of 10^6 cells/well in 1 ml RPMI (Gibco/Invitrogen), with added penicillin, streptomycin, and 10% FBS. Cells were incubated at 37°C and with 5% CO_2 for 24 h in medium only, or in the presence of rbIL-2 (100 U/ml), rhIL-12 (400 pg/ml, eBioscience) or a combination of the two cytokines, or in the presence of rhIL-15 (10 ng/ml, eBioscience) alone or in combination with rhIL-12. Brefeldin A (10 μg/ml, Sigma) was added to cells for the final 4 h of stimulation. Cells were stained with LIVE/DEAD® fixable far red dead cell stain, followed by surface staining against NKp46/NCR1 (AKS6) and a secondary PE-conjugated Ab. Permeabilized and fixed cells were incubated with anti-bovine IFN-γ mAb (clone 6, 19, IgG2a) (a kind gift from Gregers Jungersen at the Technical University of Denmark) and secondary Alexa 488-conjugated antibody. Cells were analyzed by FCM on a FACS Calibur.

STATISTICS

Differences between the groups were assessed by the non-parametric Wilcoxon rank-sum test.

RESULTS

PHENOTYPE OF *EX VIVO* NK CELLS

By using a model of bovine pseudo-AL vessel cannulation, we examined the NK cell number and phenotype in skin-draining lymph during the steady state. PBMC and AL cells were gated on viable cells in a FSC/LIVE/DEAD plot and further gated on mononuclear cells in a FSC/SSC plot (**Figure 1A**). Bovine NK cells were defined as NKp46/NCR1$^+$ CD3$^-$ cells (**Figure 1B**). NK cells constituted 2–11% of mononuclear cells in AL, with a median value of 4.6% (**Figure 1C**). In PBMC NK cells were present at 3–18%, with a median value of 7.1%. These results show that PB was significantly more NK cell rich than AL in cattle ($p < 0.01$).

Viable mononuclear cells from PBMC and AL were further analyzed by FCM for the expression of NKp46/NCR1 in combination with other surface molecules. In AL, the majority of NK cells were CD2$^{-/low}$ while around one third were CD2^{high} (**Figure 2A**). In contrast, a significantly higher proportion of the CD2^{high} NK cell population was found in PB ($p < 0.01$). Significantly more ($p < 0.05$) NK cells from AL were CD8α^+ compared to NK cells from PB, although percentages of positive NK cells in AL were highly variable between individuals (**Figure 2B**). The majority of NK cells in AL and PB expressed CD16 (**Figure 2C**); however there were significantly more CD16$^+$ NK cells present within PB compared to lymph ($p < 0.001$).

The expression of activation molecules CD44, CD25, and CD69 on viable NK cells from the mononuclear cell fraction was analyzed by FCM. The vast majority of NK cells in AL were CD44^{bright} with no NK cells being completely negative. By contrast in PB significantly fewer NK cells were CD44$^+$ (median 40%, $p < 0.001$), with the remaining population being CD44 dim to negative (**Figure 3A**). Striking differences in CD25 expression were observed between the two compartments. A major CD25$^+$ NK population was present in AL, while significantly fewer ($p < 0.001$) CD25$^+$ NK cells were present in PB where less than a third of the NK cells expressed this molecule (**Figure 3B**). In AL, 14% (10–40%) of cattle NK cells expressed the early activation marker CD69, whereas in PB the median percentage of CD69 positive NK cells was found to be significantly higher ($p < 0.001$) at 53% (26–68%) (**Figure 3C**).

To determine whether skin-draining NK cells are equipped with molecules that allow for LN recruitment, we assessed expression of L-selectin (CD62L) and CCR7. CD62L was expressed on 48% (24–73%) of AL and 59% (39–67%) of PB NK cells, and differences between the groups were not significant (not shown). CCR7 expression was not detected on bovine NK cells in PB or AL (not shown).

Without prior stimulation, AL NK cells expressed intracellular perforin with a median MFI value of 24 (21–28), whereas in PB the median MFI value was found to be significantly higher at 49 (20–110, $p < 0.05$) (**Figure 3D**).

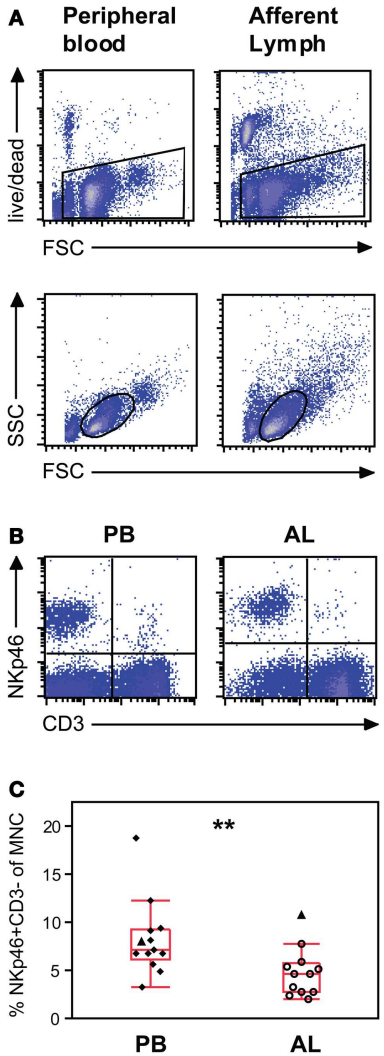

FIGURE 1 | Natural killer (NK) cell distribution in peripheral blood and afferent lymph from calves *ex vivo*. (A) Gating strategy illustrated by representative density plots. Peripheral blood (PB) mononuclear and afferent lymph (AL) cells were gated on viable cells in a FSC/LIVE/DEAD plot (upper panels) and further gated on mononuclear cells in a FSC/SSC plot (lower panels). **(B)** Representative density plots showing NKp46$^+$/CD3$^-$ NK cells in the upper left quartile and **(C)** distribution of the results showing NK cells as a percentage of mononuclear cells in PB ($n = 13$) and AL ($n = 12$). Quadrants were based on control staining with secondary antibodies alone. Symbols represent individual animals, where open symbols are Holstein–Friesian and filled symbols are Norwegian Red. Filled triangles identify one paired NR individual. Box plots show median and quartiles, and whiskers indicate data points within the 1.5* (interquartile range). Statistically significant differences between groups indicated as $^{**}p < 0.01$.

INTRACELLULAR IFN-γ PRODUCTION OF NK CELLS *IN VITRO*

To determine whether NK cells in AL were capable of producing the effector cytokine IFN-γ, we stimulated PBMC and AL cells *in vitro* with cytokines and stained for intracellular IFN-γ after 24 h. Viable mononuclear cells were analyzed by FCM. A large proportion of PB (20–71%) and AL (32–86%) NK cells produced IFN-γ following stimulation with IL-2 and IL-12 (**Figure 4A**). Similar results were obtained when cells were stimulated with IL-15 and IL-12 (not shown). In AL, a similar amount of IFN-γ$^+$ NK cells were observed upon stimulation with IL-12 *only* (24–81%), whereas PB NK cells produced significantly less IFN-γ under this stimulatory condition (9–52%, $p < 0.05$). This difference is illustrated in **Figures 4B,C** which show the percentage of

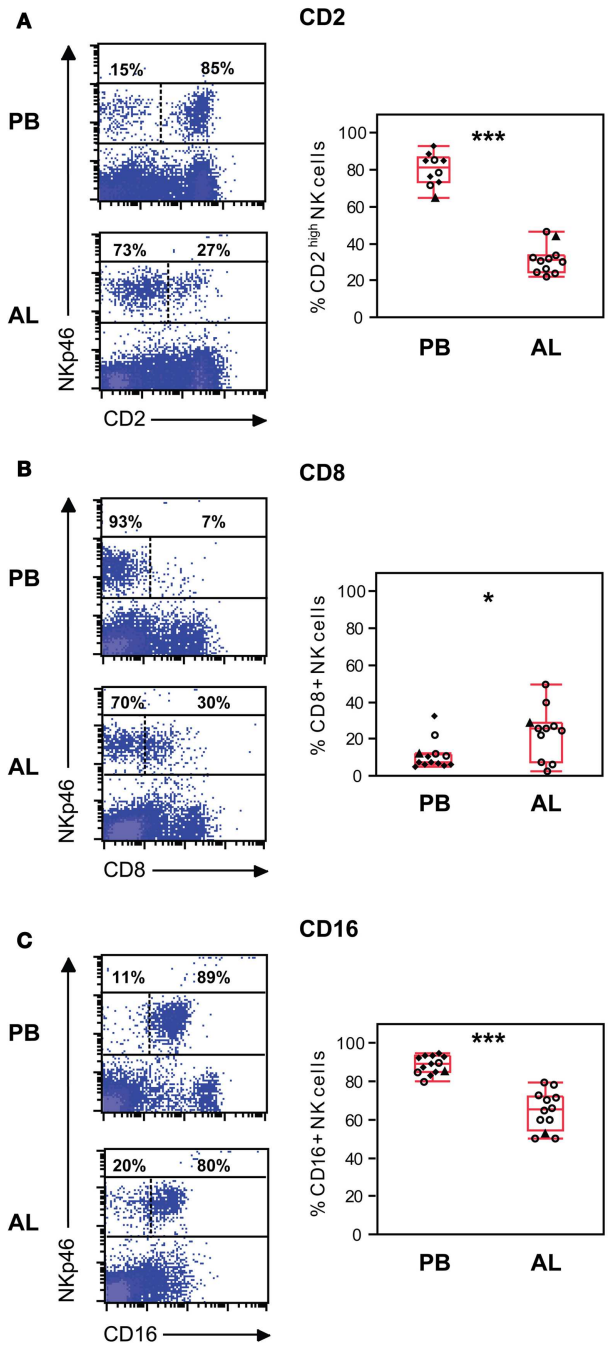

FIGURE 2 | Phenotype of NK cells *ex vivo*. NK cell expression of the surface molecules CD2 **(A)**, CD8α **(B)**, and CD16 **(C)**. Gating of cells as in **Figure 1**. Density plots (left panels) of representative animals from peripheral blood (PB) and afferent lymph (AL). Numbers indicate the percentage of NKp46$^+$ cells above or below an expression threshold (dashed line) for the indicated molecule. Distribution of the results (right panels) showing % positive NK cells in PB ($n = 10$–13) and AL ($n = 11$–12). Symbols and statistics as in **Figure 1** (*$p < 0.05$, ***$p < 0.001$).

NKp46/NCR1$^+$ IFN-γ$^+$ cells in the mononuclear cell fraction in all individuals (**Figure 4B**). IL-2 or IL-15 alone induced only marginally more IFN-γ$^+$ NK cells compared to non-stimulated cells in PB. AL NK cells, however, produced higher amounts of IFN-γ compared to non-stimulated cells when stimulated with IL-2 or IL-15 alone, although at a non-significant level (data not shown).

DISCUSSION

Although NK cells show a wide tissue distribution (2, 4, 35), the mechanisms by which NK cells traffic through tissues at steady state and following infection are not well characterized. In this study we report the presence and phenotype of NK cells in skin-derived AL from healthy cattle. Our results indicate an alternative route of NK cell recruitment to LNs under physiological conditions, not only from PB via HEV (10, 11), but also from the AL.

Natural killer cells in AL showed a more activated (CD25$^+$ and CD44$^+$) phenotype than NK cells in PB, and readily produced IFN-γ upon *in vitro* stimulation, raising the question of where and how these cells have been stimulated. The presence of CD56$^+$ CD3$^-$ NK cells have been reported in human healthy (36, 37) and lesional skin (38), and NK cells were observed in close contact with DCs *in vivo* (36). A close cellular interaction of NK cells and DCs or IL-2 producing T cells in tissues may possibly lead to a further activation of NK cells recruited from PB. It cannot be totally excluded that the activation observed in the current study could be caused by inflammatory stimuli due to the invasive technique used, but cells were only included after stabilization in the cellular composition and animals were carefully monitored for the absence of clinical signs of inflammation; conditions were in accordance to standardized protocols for this technique (17, 24). Thus, the CD2$^{-/low}$ CD25$^+$ CD44$^+$ phenotype of NK cells in bovine AL, and the resemblance of these with LN-residing NK cells, suggest that under non-inflammatory conditions, NK cells are activated in the tissues, migrate through AL and enter the LNs, where they constitute a large proportion of the residing NK cells.

Natural killer cells in the AL had a significantly lower expression of the early activation marker CD69 than NK in PB, despite the presence of CD25 and potent IFN-γ production which indicate that these cells are not naïve. They may represent a late phase of activation, since *in vitro*, bovine NK cells express high levels of CD69 after 6–12 h of stimulation, followed by a down-regulation in later stages (32). It should also be noted that in T- and B-cells in other species, CD69 is tightly linked to sphingosine-1-phosphate receptor 1 (S1PR$_1$) (39). The usage of S1P-receptors in bovine lymphocytes is not known and was not studied here due to limitation of reagents for cattle. However, the observed expression pattern of CD69 would be consistent with reports that this receptor is internalized together with S1PR$_1$ in the S1P-rich lymphatic vessels (13), followed by CD69 up-regulation in the S1P-free environment in LNs, where CD69 inhibits S1PR$_1$-mediated egress (40, 41). While the mechanisms behind entry of NK cells from AL to LNs remain to be studied, NK cell egress has been shown dependent on S1PR$_5$ rather than S1PR$_1$ in the mouse, in a process apparently resistant to CD69 inhibition (42, 43). In line with this, we here and previously (32) observed that CD69 is present on a substantial proportion of cattle PB NK cells, although the migration history of these CD69$^+$ PB NK cells needs to be clarified.

In human NK cells, CCR7 is present on the CD56^{bright} subset, and alleged responsible for their LN-homing, but absent on

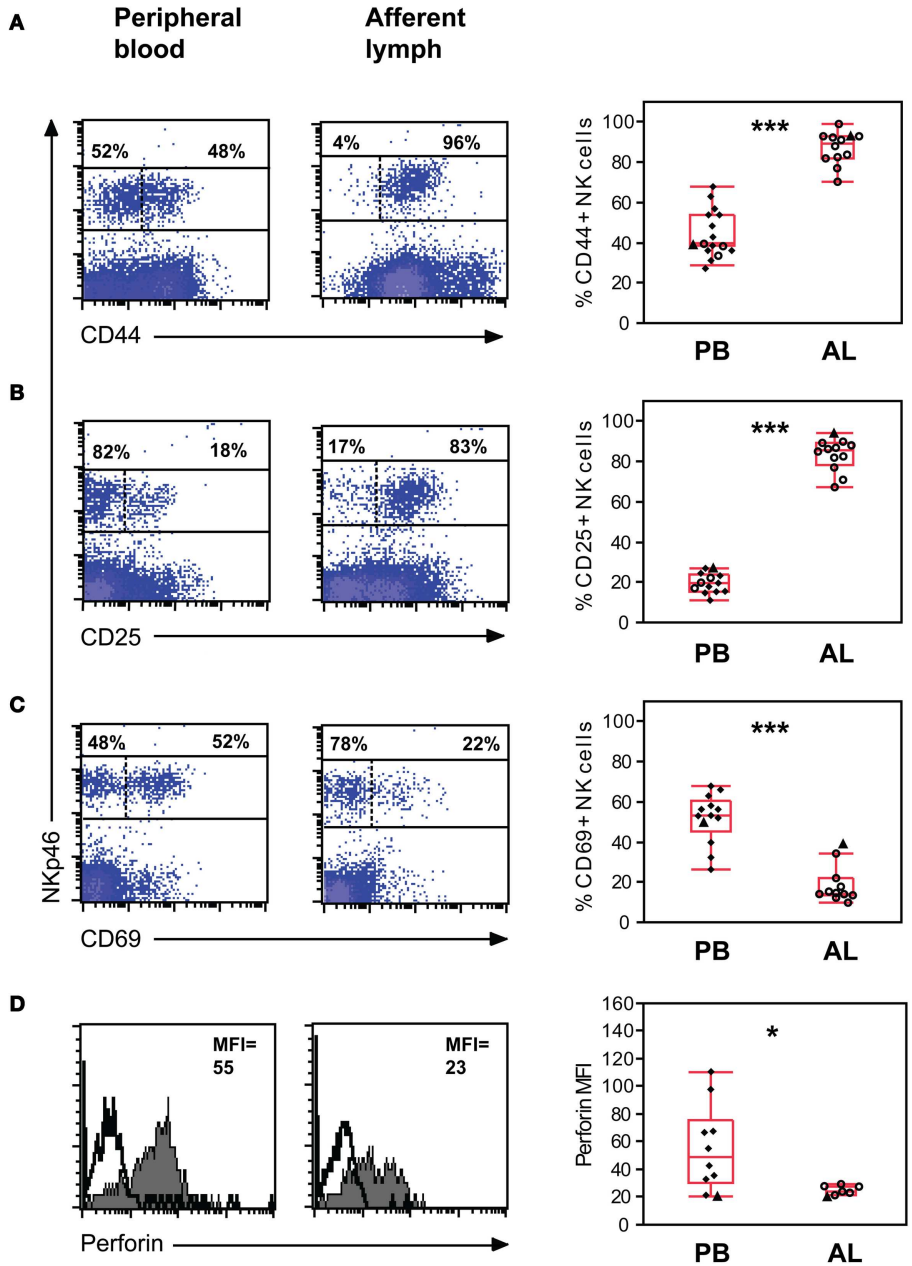

FIGURE 3 | Expression of activation molecules and intracellular perforin in NK cells *ex vivo*. Expression of the activation molecules CD44 **(A)**, CD25 **(B)**, and CD69 **(C)** on NK cells. Gating of cells as in **Figure 1**. Density plots of representative animals from peripheral blood (PB, left panels) and afferent lymph (AL, middle panels). Numbers indicate the percentage of NKp46^{+} cells above or below an expression threshold (dashed line) for the indicated molecule. Distribution of the results (right panels) showing % positive NK cells in PB ($n = 10$–13) and AL ($n = 11$–12). **(D)** Intracellular perforin in NK cells, calculated as mean fluorescence intensity (MFI) of the NK cell population. Histograms displaying representative animals from PB (left panel) and AL (middle panel). Filled histograms indicate perforin expression and solid histograms indicate the secondary control staining. Distribution of the results (right panel) showing MFI of NK cells in PB ($n = 10$) and AL ($n = 7$). Symbols and statistics as in **Figure 1** ($^{*}p < 0.05$, $^{***}p < 0.001$).

the CD56^{dim} subset that dominates in PB (44). Here we could not detect CCR7 expression on bovine NK cells in either PB or AL, or on NK cells from LNs (Lund, unpublished observations) when applying an anti-human CCR7 antibody cross-reactive to bovine cells (45), even though we have previously found moderate expression of mRNA transcripts for CCR7 by bovine NK cells in PB (46) and high expression in LN-resident NK cells (Siddiqui and Hope, unpublished observations). It remains a possibility that post-transcriptional modification of CCR7 halts the surface expression or interferes with antibody binding, and ideally these results should be confirmed with a bovine-specific CCR7 antibody. It was recently shown that bovine γd T cells egress from skin into

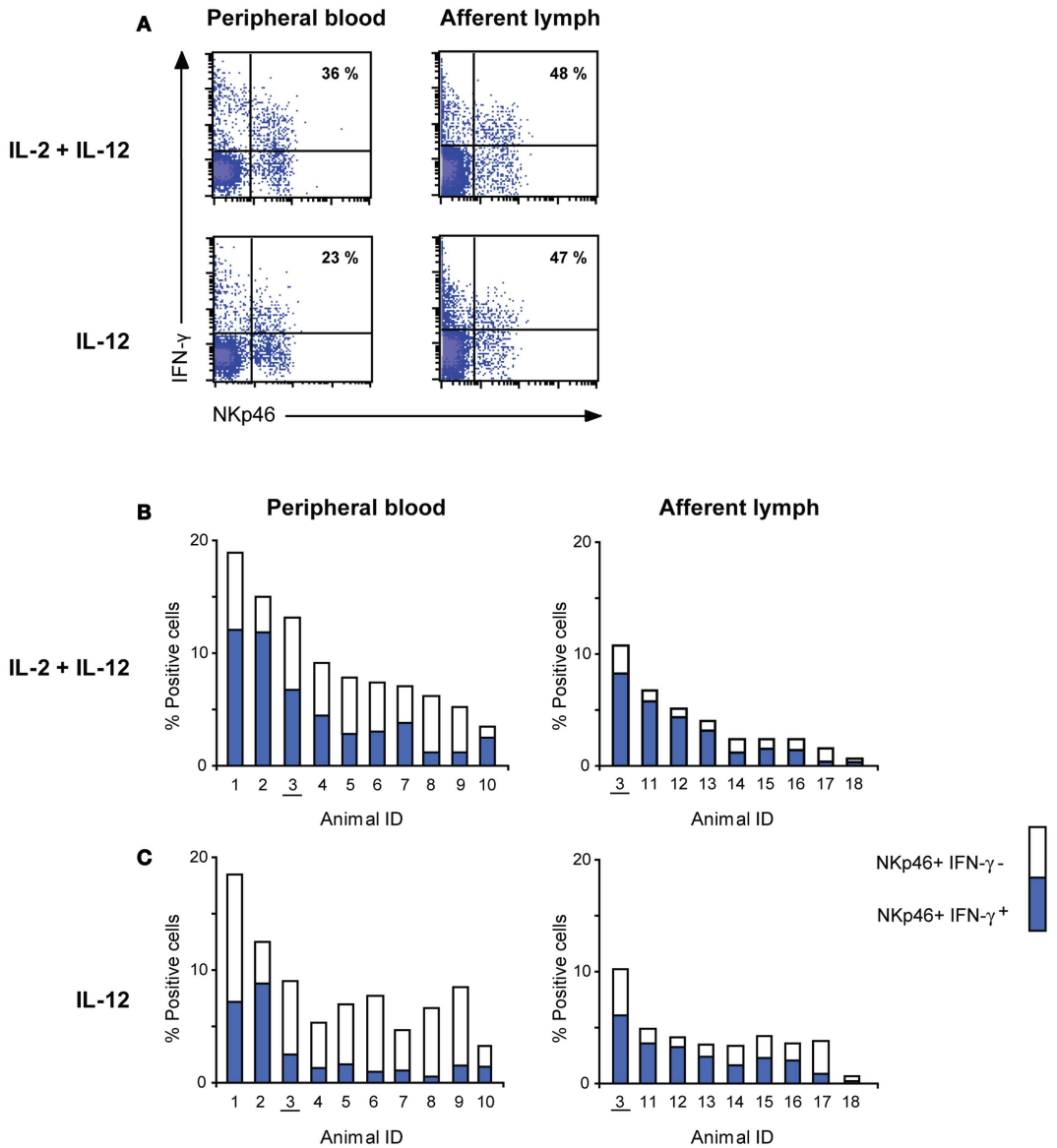

FIGURE 4 | Interferon-γ producing capacity of NK cells from peripheral blood and afferent lymph. **(A)** Flow cytometric analysis of intracellular IFN-γ after 24 h of *in vitro* cytokine stimulation. Gating of cells as in **Figure 1**. Density plots of representative animals from peripheral blood (left panels) and afferent lymph (right panels). Numbers indicate IFN-γ$^+$ NK cells (upper right quartile) as percentage of all NK cells. **(B,C)** IFN-γ production of NK cells following cytokine stimulation as in A with IL-2 and IL-12 or only IL-12. Complete columns represent the total percentage of NKp46$^+$ cells in the mononuclear cell fraction, while the inner blue columns indicate the percentage of NKp46$^+$ IFN-γ$^+$ cells in the same fraction. All individual animals are presented with a unique ID, where numbers 1–10 are Norwegian Red and 11–18 are Holstein–Friesian. One paired NRF calf is underscored.

lymphatic vessels in a CCR7-independent manner (45), whilst the entry of conventional murine and ovine αβ T cells into initial lymphatic vessels relies on CCR7 (47). The inability to detect CCR7 on bovine NK cells suggest that similar to bovine γd T cells, NK cells may migrate in a CCR7-independent manner.

Natural killer cells are responsive to adjuvants (48) and may be important players in vaccine responses (12, 49), underscoring the relevance for harnessing the stimulation of NK cells when designing vaccines. Examining afferent lymphatic NK cells draining the sites of cutaneous vaccination will provide information as to the induction of innate immune cell activation. This could be particularly important in the context of BCG vaccination and infection with *Mycobacteria* where reciprocal interactions between DCs and NK cells lead to enhanced Th1 bias and CD8$^+$ T cell activation that has been linked to vaccine efficacy (11, 50). Recent evidence suggests that in humans effective BCG vaccination is dependent upon innate (NK and gamma delta T) cell derived IFN-γ (51, 52), and ongoing studies assessing BCG vaccination and M. bovis infection in cattle have revealed key roles for NK cells [Ref. (53) and unpublished].

The cost and complexity of the cannulation technique, which in the present study was performed at two research sites, resulted in a data material that may contain biasing factors. Steps were taken to limit such confounders: Laboratory analyses were carried out using standardized protocols by the same person, often repeated several times. All animals were recruited from a similar age group since age has proven to be a significant variable in NK cell biology (54–56). Finally, no breed influence was detected in parameters measured in PBMC (**Figures 2** and **3**). Only one NR dairy calf was successfully catheterized, and since this individual had a high number of NK cells in AL (**Figure 1**), attention should be paid to a possible breed bias for this parameter. However, its exclusion did not affect statistical or overall conclusions and it was thus included here as an identifiable animal.

In conclusion, we here describe the presence of activated NK cells in AL, suggesting a novel migration route for NK cells from tissues into LNs. LNs may therefore not only be a site for priming of naïve NK cells recruited from PB at the initiation of an immune response (7), but also a site for tissue-activated NK cells arriving via AL that may contribute substantially to the shaping of the adaptive immune response. Further studies of NK cell recirculation under vaccination or infection conditions are needed to fully reveal mechanisms that can be utilized for optimal adjuvant strategies in vaccine development.

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Paper 3

Adjuvant injection leads to transient recruitment of intermediate monocytes and activation of lymphocytes in the draining lymph node

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Abstract

The dynamics of skin-draining cells following infection or vaccination provide important insight to the initiation of immune responses. In the current study, the local recruitment and activation of leukocytes was studied in calves following subcutaneous administration of a saponin-based adjuvant. A marked but transient recruitment of leukocytes to subcutaneous tissues and the draining lymph node was demonstrated by flow cytometry and immunofluorescent techniques. Recruited cells in lymph nodes were predominately $CD14^{bright}$ and $CD16^{dim}$, consistent with intermediate monocytes that have been characterized as inflammatory, and did not express markers that indicated differentiation into dendritic cells. An increase in granulocytes and B cells was demonstrated, whereas the percentages of T-cells and natural killer cells were reduced. However, all lymphocyte classes were activated, as CD69 was upregulated on B cells and NK cells, while CD25 expression was increased on T-cells and NK cells. Furthermore, NK cells in lymph nodes showed an enhanced capacity for IFN-γ

production. In conclusion, this study provides novel information on monocyte recruitment and migration during inflammation, and demonstrates that a saponin-based adjuvant appears to be an efficient immune stimulator in calves.

Keywords: Intermediate monocytes, natural killer cells, draining lymph node, activation, migration, adjuvant

Abbreviations: AL, afferent lymph; PB, peripheral blood; FCM, flow cytometry; LNs, lymph nodes; MFI, mean fluorescence intensity

Introduction

A protective immune response to infection or vaccination is dependent on the recruitment of immune cells to the inflamed tissue, their activation and subsequently the movement of cells and antigens to the draining lymph node. Antigen-loaded dendritic cells (DCs) and recirculating lymphocytes migrate in afferent lymph (AL), and DCs from this compartment have been extensively characterized in ruminants [1-4]. Monocyte differentiation *in vitro* and *in vivo* has been characterized by the loss of CD14 and the upregulation of CD11c, DEC205 and MHCII [5-8]. It is likely that this process *in vivo* is dependent on a combination of factors in the tissue environment, such as chemokines, cytokines or administered adjuvant or antigen [9, 10]. Studies of afferent lymph in animals have shown that leukocyte populations other than DCs and lymphocytes migrate in afferent lymph. The presence of monocytes in sheep [11] and rat [12] lymph was described a decade ago, and more recently, adoptively transferred monocytes were shown to migrate from skin to the draining LN in mice [13]. Natural killer (NK) cells are another group of early innate effector cells that were recently described in afferent lymph [14, 15]. In cattle, AL NK cells were activated and phenotypically similar to LN-residing cells, suggesting AL as an important migration route of tissue activated NK cells to the draining LN [15].

Circulating monocytes have classically been regarded as precursors of tissue macrophages and DC, which are recruited to tissues for maintenance of these cell populations during homeostasis and expansion during inflammation. [16, 17]. However, recent evidence suggest that certain monocyte subsets may have other distinct roles than replenishment [13, 18, 19]. The classical

monocytes are $CD14^{bright}$ $CD16^{-}$ cells that form the major population in human and cattle blood [20, 21]. The minor population of CD16-positive monocytes have been subdivided into nonclassical $CD14^{dim}$ $CD16^{bright}$ monocytes and intermediate $CD14^{bright}$ $CD16^{dim}$ monocytes, and the latter subset has been described as inflammatory in both humans and cattle [22, 23]. NK cells are involved in early responses to infection and vaccination, and participate in the regulation of the immune response by providing IFN-γ for a T helper 1 (Th1) polarization [24]. The interaction and mutual regulation of NK cells and myeloid cells like DCs and monocytes/macrophages has been extensively investigated [25, 26], especially as Th1 responses are dependent on the production of IL-12 by myeloid cells and NK cell-derived IFN-γ. These interactions occur primarily in secondary lymphoid organs, but also at the site of inflammation or tumor infiltration [27-29].

The aim of this study was to characterize the recruitment and activation of immune cells in the skin draining LNs of cattle in inflammation. We show that the administration of an adjuvant based on saponins from the tree *Quillaja saponaria* Molina (*Quil-A*) [30, 31] to calves, resulted in a potent recruitment of cells to subcutaneous tissues and the draining LN. The recruitment was highly dominated by inflammatory monocytes. Furthermore, major lymphocyte classes including NK cells were activated. The current study provides novel information on monocyte recruitment and migration.

Materials and methods

Animals and experimental design

Animals included in the study were clinically healthy Norwegian Red dairy calves (Bos taurus) of both sexes, and 8 - 9 weeks of age. Fourteen animals were distributed into four experimental groups that were kept in separate pens: Six animals were left untreated in a control group, whereas 8 calves were injected with 500 µg Matrix-Q™ (a kind gift from Novavax AB, Uppsala, Sweden) The Matrix-Q™ adjuvant was suspended in 2 ml sterile Hanks' balanced salt solution (Gibco, Life Technologies) prior to injection and administered as a single subcutaneous dose in the left dorsal posterior flank, in an area drained by the subiliac lymph node. The contralateral skin with subcutaneous tissue and subiliac LN were left untreated. Calves were given acidified milk, water ad lib and access to straw, and the health status of the animals was examined twice daily. All experimental procedures were conducted in accordance

with the laws and regulations controlling experiments using live animals in Norway; the Norwegian Animal Welfare Act of 28 December 2009 and the Norwegian Regulation on Animal Experimentation of 15 January 1996.

Tissue collection and preparation

EDTA blood samples were collected prior to adjuvant injection (pre-injected samples), and from the three experimental groups at 24 h, 48 h and 96 h post-injection. In addition, the 96 h group was sampled for blood at 72 h. Hematological differential analysis was performed on blood (Advia® 2120 Hematology System, Siemens AG, Erlangen, Germany). Bovine PBMCs were isolated by density gradient centrifugation (2210 × g, 30 min) on lymphoprep (Axis-Shield, Norway), and either analyzed immediately by flowcytometry or subjected to stimulation assays, or added freezing medium (Recovery™ cell culture freezing medium, Gibco) for further storage in liquid nitrogen.

Subiliac lymph nodes from calves in the non-injected group (n=6) were collected at a conventional slaughterhouse. Injected calves groups were stunned by captive bolt and exsanguinated and subjected to post-mortem examination. Samples were collected at 24 (n=3), 48 (n=3), and 96 hours (h) (n=2) after injection.Tissue samples for flow cytometry were collected immediately and included the subiliac LN draining the injected side, in addition to the contralateral LN. LNs were excised vertically in half, and the anterior half of the LN towards the injection site was subjected to tissue preparation. LN tissue was minced mechanically by scissors in the presence of PBS/EDTA buffer. Subsequently, the LN cell suspensions were filtered through a Cell Strainer™ (BD Falcon), secondly through a cotton filter pad soaked with PBS/EDTA, and finally washed in PBS/EDTA before direct analysis or freezing as described above.

Skin with subcutaneous tissue and the draining lymph node at the injected side and from the contralateral flank were collected and fixed for light and fluorescence microscopy. The formalin fixed samples were embedded in paraffin wax and prepared by standard procedures before staining with Haematoxylin and Eosin (HE) for light microscopy. Skin and LN specimens were frozen in chlorodifluoromethane (Isceon™) chilled with liquid nitrogen, and stored at -70ºC until further preparation.

Immunofluorescence

Immunofluorescent staining was performed essentially as previously described [32]. Briefly, 7 µm cryostat sections were fixed in acetone and treated with 20% BSA/TBS in order to block non-specific binding. One of the following two mixtures of three primary antibodies was added to the sections: Mouse anti-human CD14 (Tük4, IgG2a), mouse anti-bovine DEC205 (MCA1651G, IgG2b) (both AbD Serotec) and mouse anti-bovine CD11c (BAQ153A, IgM) (VMRD), or Mouse anti-human CD14, mouse anti-bovine CD21 (DU2-74-25, IgG2b) (Hein et al 1998) and polyclonal rabbit anti-ki67 (Abcam). Secondary antibodies were isotype-specific Alexa Fluor (350, 594 and 488) (Molecular Probes, Inc., USA). All incubations were done in a slowly rotating humid chamber for 1 h at room temperature. Slides were mounted in polyvinyl alcohol and stored at 4 ºC until examination. Control sections were included. All tissue sections were examined in a Carl Zeiss Axio Imager M2 microscope equipped with a conventional camera (Axiocam HRc Rev. 3) and fluorescence camera (Axiocam HRm Rev. 3).

Flow cytometry

Flowcytometric analysis of surface or intracellular molecules was performed on fresh or previously frozen LN cell suspensions or PBMCs. Cells were first stained with LIVE/DEAD® Fixable Aqua dead cell stain kit for 405 nm excitation (Invitrogen), following the manufacturer's instructions. Primary unconjugated monoclonal antibodies (mAb) applied in the current study were mouse anti-bovine against the following molecules: NCR1/NKp46 (CD335; AKS1, IgG1 or AKS6, IgG2b; in house produced [33, 34]; CD3 (MM1A, IgG1), CD8α (BAQ111a, IgM), CD2 (MUC2A, IgG2a), CD25 (CACT116a, IgG1), CD62L (BAQ92A, IgG1), CD69 (KTSN7A, IgG1), granulocyte marker (CH138A, IgM), TCR1(GB21A, IgG2b), WC1 (CC108, IgG2a), and CD11b (MM12A, IgG1), or cross-reactive mouse anti-pig CD44 (BAG40a, IgG3) (all Monoclonal Antibody Center, Washington State University, Pullman, USA), and mouse anti-bovine CD4 (MCA1653G, IgG2a) and DEC205 (MCA1651G, IgG2b) (both AbD Serotec/BioRad). Directly conjugated antibodies were cross-reactive anti-human CD16-FITC (KD1, IgG2a) or CD14-Pacific blue (Tük4, IgG2a) (both AbD Serotec/BioRad). Secondary isotype-specific reagents were either PE-conjugated or APC-conjugated (Southern Biotech, Birmingham, USA), or Alexa Fluor 488 -or 647-conjugated (Molecular Probes/Life

Technologies) polyclonal goat-anti-mouse antibodies, or PerCP- eFluor 710-conjugated rat anti-mouse antibodies (eBioscience/Affymetrix).
For intracellular antigens, surface staining was followed by permeabilization and fixation (Cytofix/Cytoperm; BD Biosciences), and further incubated with cross-reactive mouse anti-human Ki67-FITC (IgG1, BD Pharmingen), or perforin (delta g9, IgG2b; BD Biosciences), or rat anti-human CD3-Pacific blue (CD3-12, IgG1; AbD Serotec/BioRad). Flow cytometry was performed with a 3-laser Gallios flowcytometer (Beckman Coulter), and gating based on staining with secondary antibodies only or isotype controls. Data was analyzed using Kaluza 1.2 software (Beckman Coulter), and expression of molecules measured as % positive cells for bimodal distributions and as mean fluorescence intensity (MFI) for other distributions.

Intracellular staining for IFN-γ

Bovine LN cell suspensions and PBMCs were washed twice in PBS without EDTA prior to the functional intracellular IFN-γ assay. Cells were added to 24-well plates at a concentration of 10^6 cells/well in 1 ml RPMI with penicillin and streptomycin (Gibco/Invitrogen), and 10 % FBS Gold (PAA, Pasching, Austria). Cells were incubated at 37 °C and with 5 % CO2 for 24 h in medium only, or in the presence of rbIL-2 (100 U/ml, in house produced), rhIL-12 (400 pg/ml, eBioscience) or a combination of the two cytokines, or in the presence of rhIL-15 (10 ng/ml, eBioscience) and rhIL-12. Brefeldin A (10 μg/ml, Sigma) was added to cells for the final 4 h of incubation. Cells were stained with LIVE/DEAD® Fixable Aqua stain, followed by surface staining against NCR1 (AKS6) and CD3 (MM1A) with the appropriate secondary antibodies. Permeabilized and fixed cells were incubated with anti-bovine IFN-γ mAb (clone 6, 19, IgG2a) and an Alexa-488 conjugated secondary antibody, and 5 x 10^5 cells were analyzed by flow cytometry for the expression of IFN-γ by $NKp46^+$ $CD3^-$ cells.

Statistics

Data were analyzed in JMP 11 statistical software (SAS Institute) using the non-parametric Wilcoxon rank-sum test. Statistical significance was assigned at a p-value less than 0.05.

Results

Adjuvant injection leads to a substantial recruitment of innate cells to the draining LN

Injection of Matrix-Q™ adjuvant to calves induced a local inflammation, characterized by subcutaneous edema and a 2-3 fold enlargement of the draining LN, most prominent at 24 h post-injection. Histopathology of the injection site revealed a diffuse, locally extensive, and moderate to severe inflammatory reaction in deeper cutaneous and subcutaneous tissues. In the draining LNs, the germinal centers of the follicles were larger, and the cells herein were larger and lighter stained compared to the contralateral LN. In addition mitoses were observed more often in the draining LN germinal centers than in the contralateral LN (Fig 1A). At 48 h post-injection, the germinal center changes were less evident.

Flow cytometry analysis revealed a large increase in monocyte percentages in the draining LN at 24 h post-injection, with these cells constituting a major proportion of total cells (Fig 1B and 1C). In contrast, no changes in cellular composition were observed in the contralateral LN. The granulocyte percentage was also significantly increased, while the overall lymphocyte population was were reduced in the draining LN. Changes in cellular composition in the draining node appeared to be transient, with a return to near baseline (non-injected) values at 48 h post-injection. Results from the individual animals are presented in supplementary table 1.

Hematological differential analysis demonstrated an increase in the absolute number of granulocytes in peripheral blood at 24 h post-injection, with a mean fold increase of 2.8 from pre-injected levels (Fig 1D). At 72 h post-injection, the absolute number of monocytes was also increased to two fold. Individual results are presented in supplementary table 2.

Monocytes recruited to the draining LN were $CD14^{bright}$ and $CD16^{dim}$

Numerous CD14+ monocytes were present in the subcapsular and peritrabecular sinuses in immunofluorecently labelled histologic sections, as well as in the T cell zones in the cortex of the draining LNs, at 24 h post-injection (Fig 2A). Positive cells were also found at a moderate to large amount in the medullary sinuses and chords. CD14+ cells did not co-label with Ki67, indicating that monocytes did not proliferate after reaching the LN. CD14+ cells were not detected in the contralateral LNs (Fig 2B). At 48 h post-injection, CD14+ cells were present, but had decreased in the cortex compared to 24 h post-injection (Fig 2C). However, a moderate

to large amount were present in the medulla, including the area around efferent vessels (Fig 2D).

The major population of monocytes in blood before adjuvant injection were $CD14^{bright}$ $CD16^{-}$ cells representing classical monocytes. At 24 h post-injection, an increase in percentages of $CD14^{bright}$ $CD16^{dim}$ and $CD14^{dim}$ $CD16^{bright}$ cells were detected in PBMC (Fig 3A). The vast majority of monocytes recruited to the draining LN were $CD14^{bright}$ $CD16^{dim}$ cells. They were $CD11b^{bright}$, and did not express the dendritic cell marker DEC205, granulocyte antigen (CH138A), the T cell markers CD3, CD4, CD8, or γδ T cell receptor (TCR1), or the NK cell marker NCR1 (not shown). Furthermore, the majority were $CD44^{bright}$ and $CD62L^{bright}$ (not shown). After 48 h, the $CD14^{bright}$ cells were only present at low numbers in the draining LN (Fig 3B). However, there was a significant increase in the total number of $CD14^{bright}$ monocytes in PBMC (Fig 3C).

To further investigate whether a differentiation of CD14+ cells towards a DC phenotype had taken place, immunofluorescent triple labelling of skin and lymph node tissue were performed with CD14, and the DC-markers DEC205 and CD11c. Numerous CD14+ cells were present in the deep cutis and sub cutis on the injected side at 24 h post-injection (Fig 4A). A few DEC205+ cells were also present, possibly representing macrophages. Very few CD14+ cells were present in the skin at the non-injected side (Fig 4B). A low to moderate amount of CD11c+ DEC205+ DCs were observed in both the draining and the contralateral lymph node at 24 h post-injection (Fig 4C and D). Only a few CD14+ cells in the draining LN were found to be CD11c+, while none were DEC205+ (Fig 4C, insert). DEC205 also labelled cells within the lymphoid follicles, most likely B lymphocytes, as previously demonstrated in cattle [6]. Taken together, phenotypic and immunohistological findings demonstrated that the recruited CD14+ cells corresponded to monocytes rather than DCs. Furthermore, the $CD14^{bright}$ $CD16^{dim}$ phenotype was consistent with inflammatory monocytes in cattle [22].

B cells but no other lymphocytes increased in numbers in the draining LN

A population of $CD3^{-}$ $NCR1^{-}$ within the lymphocyte gate, corresponding to bovine B cells, showed a significant increase in the draining LN at 24 h post-injection (Fig 5A). This population remained at moderately elevated percentages throughout the sampling period. There was no significant increase in B cells observed in blood (not shown). The percentage of NK

cells was significantly reduced, and NK cells remained at reduced percentages in the draining LN and the blood throughout the sampling period (Fig 5B). Also the percentage of T cells was reduced in the draining LN at 24 h post-injection (Fig 5C). This reduction was most prominent for $CD4^+$ T cells (Fig 5D), while the reduction in $CD8^+$ (Fig 5E) and γδ T cells (Fig 5F) were not significant. Except for the 24 h reduction in lymphocyte percentages, the total percentage of T cells did not change significantly throughout the sampling period, neither in LNs nor in blood.

Activation of lymphocytes and NK cells in the draining LN

The activation status of lymphocytes and NK cells was determined by the expression of CD69 and CD25, as well as intracellular IFN-γ expression of NK cells and T cells.

At 24 h post-injection, the percentage of CD69 positive NK cells was increased in the draining LN (Fig 6A and B). There was also a marked increase in surface CD69 expression of B cells after 24 h, with a median percentage of 78 % of these cells being positive for CD69 in the draining LN as compared to 12 % in LN of non-injected animals (Fig 6A and C). CD69 expression remained elevated on the surface of B cells at 48 h. Parallel to these findings in LNs, an increase in $CD69^+$ NK cells was demonstrated in PBMC (Fig 6D), while the majority of B cells in blood were negative for surface CD69 expression at all time points (not shown). At 48 h post-injection, the percentage of NK cells expressing CD69 had returned close to the baseline, both in the draining LN and in blood. No significant increase in surface CD69 expression was found on T cells, $CD14^{bright}$ monocytes or cells within the granulocyte gate (not shown).

The majority of bovine NK cells in LNs expressed CD25. However, at 24 h post-injection the CD25 MFI on NK cells was increased in the draining LN (Fig 7A and B). An increase in surface CD25 expression was also demonstrated on T cells, with a median of 88 % $CD25^+$ $CD3^+$ cells at 24 h (Fig 7A and C). The percentage of $CD25^+$ NK cells also increased in PBMC, where twice as many $CD25^+$ NK cells were present at 48 h than before adjuvant injection (Fig 7D). The vast majority of B cells did not express CD25 (not shown).

When stimulated with IL-2 and IL-12 in vitro, a higher percentage of NK cells from both the draining LN and the control LN expressed IFN-γ at 24 h post injection in comparison to NK cells from non-injected LNs (Fig 8A). Similar results were seen when NK cells were stimulated with IL-15 and IL-12 (not shown). Near similar percentages of IFN-γ+ NK cells were

demonstrated after stimulation of LN NK cells with IL-12 only, whereas NK cells from LNs of non-injected animals produced significantly less IFN-γ under these conditions (Fig 8B). There were no significant changes in IFN-γ production of blood NK cells after adjuvant injection (not shown). Only a low percentage (2 – 5 %) of total T cells stained positively for intracellular IFN-γ in the draining LN at 24 h (not shown). NK cells in the draining LN showed a bimodal distribution of perforin expression, with a significantly increased MFI of the perforin^{+} population in comparison to the non-injected group (not shown).

Discussion

Understanding the dynamics of innate cell recruitment and activation in inflammation provides important insight on how to specifically target desired cell populations during vaccination. We demonstrate that the subcutaneous administration of a saponin-based adjuvant to calves resulted in a strong recruitment of inflammatory monocytes to the injection site and to the draining LN. Within the LN, CD14 expressing cells were present in the sinus and perifollicular T-cell area and did not enter the B-cell follicles. Monocyte recruitment appeared to be transient, as CD14^{bright} cells were detectable only in low numbers at 48 h post-injection in the draining LN. However, immunofluorescent staining revealed a distinct local population of CD14 positive cells in the medulla after 48 h, in the area surrounding efferent lymphatic vessels, suggesting an internal monocyte migration within the LN towards an exit through efferent lymphatic vessels. In support of this, we found a significant increase in CD14^{bright} monocytes in PBMC at 48 h post-injection.

Although the recruitment of monocytes to LNs from blood across high endothelial venules has been reported [35], the predominant route of monocyte travel to LNs is believed to be via the afferent lymph [8, 20, 36], and CD11b^{+} CD14^{bright} monocytes have previously been demonstrated in the AL and the draining LN of sheep after vaccine administration [11]. Recruited monocytes in the current study were CD14^{bright} and CD16^{dim}, thus resembling inflammatory monocytes, which have been characterized as potent cytokine producers in human [23, 37], and cattle [22]. Furthermore, the transport and presentation of antigen by monocytes has been documented [11, 13, 18, 19, 38, 39], functions that were previously designated to DCs only. We found that the majority of recruited cells did not express the DC-markers CD11c and DEC205, thus contrasting the general view that only monocyte derived

DCs will migrate to lymph nodes in an inflammatory setting. The differentiation of monocytes can however be influenced by various factors. For instance, differentiation was blocked in the presence of LPS [19, 40], and in sheep, a liposomal adjuvant/antigen formulation induced the maturation of naïve blood monocytes into mature DCs in peripheral tissues, but apparently only if monocytes had taken up administered antigen [41]. Indeed, the discrimination between dendritic cells and monocytes/macrophages has proven to be a challenge. It has been suggested that inflammatory DCs in LNs are in fact inflammatory monocytes, which have merely upregulated MHCII in the node [13], and that DCs and macrophages are only different subsets of mononuclear phagocytes instead of separate lineages of myeloid cells [16, 42].

We found a significant increase in B cells in the draining LN after 24 h. This was paralleled by a marked drop in $CD3^+$ T cells and NK cells, likely due to the concurrent B cell dominated influx to the lymph node, as previously described [18, 43]. However, all major lymphocyte classes showed characteristics of activation, as CD69 was upregulated on B cells and NK cells, and CD25 expression increased on $CD3^+$ lymphocytes and NK cells. CD69 is a marker of early activation of hematopoietic cells, including T cells, B cells, monocytes and NK cells, and NK cells in cattle [33]. In human and mice, the increased expression of CD69 on T –and B cells promotes cellular retention in LNs, as the Sphingosine-1 phosphate receptor 1 mediated egress of activated lymphocytes is inhibited in the presence of CD69 [44-46]. In the current study, CD69 was markedly upregulated on B cells in the draining node, however in blood the percentage of B cells did not increase significantly and the majority of B cells in blood were CD69 negative. We therefore speculate that the increase of B cells observed was likely due to a combination of recruitment and CD69-mediated retention in the lymph node.

Several findings in the current study demonstrated an activation of NK cells. We have recently shown that natural killer cells in bovine AL had a lower CD69 expression than NK cells in blood [15]. Here we demonstrate that NK cells rapidly up-regulated CD69 in the LN during inflammation, followed by an increase in $CD69^+$ NK cells in circulation. In contrast to adaptive lymphocytes, NK cell exit from LNs is apparently not inhibited by CD69 [47, 48], suggesting that NK cells can exit in a primed state. We also found that an increased percentage of NK cells in blood expressed the high affinity IL-2 receptor CD25. We have previously shown that bovine NK cells upregulated CD25 *in vitro* following activation with IL-2 [34] or IL-15 [49]. It is therefore conceivable that NK cells in the current study had recently been exposed to activating

factors such as IL-15 or T cell derived IL-2. Finally, NK cells in LN had an enhanced capacity for IFN-γ production.

In the current study, a saponin-based adjuvant appears to be an efficient immune stimulator in calves. Saponins have been used with success in animal vaccines for decades [50, 51], and some formulations have also recently entered human clinical trials [52, 53]. Matrix-M™ (Novavax AB) has been shown to enhance cell traffic, activate immune cells, and induce a balanced Th1/Th2 response, even in the absence of antigen [54, 55]. The Matrix-Q™ formulation is intended for use in veterinary vaccines and is currently only available for experimental applications.

Most studies looking into basic mechanisms of adjuvants have been performed in small laboratory animals, although a large part of our knowledge on leukocyte recirculation derives from large animal models (recently reviewed in [3]). More knowledge on the initiation of immune responses in cattle can form a basis for new vaccines in this species, as well as being important to understand processes in other species, including humans. Thus, experimental post-mortem analyses as herein, as well as lymph cannulation models in large animals [15, 56, 57] offer powerful tools for future studies of dynamics and recirculation of immune cells, in the steady state as well as following adjuvant and vaccine injection or other inflammatory conditions.

Conflict of interest statement

The authors declare that no financial or commercial conflict of interest exists in relation to the content of this article. The authors have no financial involvement in Novavax AB.

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Fig 1

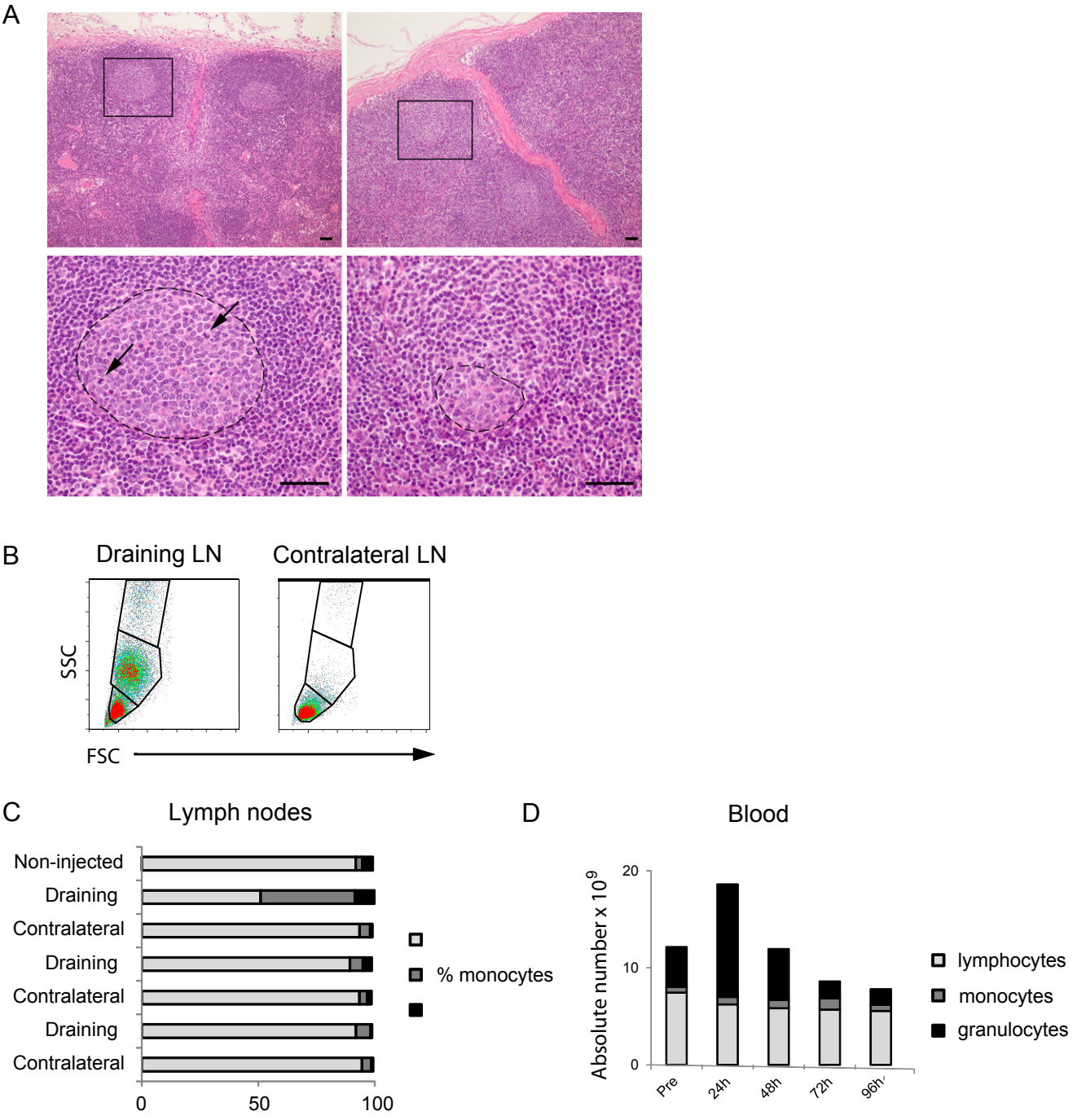

Figure 1. **Cellular recruitment to lymph nodes (LNs) and peripheral blood**

(A) HE stained sections of draining (left panels) and contralateral (right panels) LN, 24 h post-injection. Bottom panels are enlarged areas of outlined areas in upper panels. The germinal centers of the LN follicles were generally larger in the draining compared to the contralateral LN. Mitoses were observed more frequently in the germinal centers of draining LNs (arrows bottom left panel). Scale bar: 50 µm.

(B) LN cells were prepared for flow cytometric analysis and gated on FSC/ SSC characteristics. Plots from one representative animal are presented. Panels illustrate the gating of lymphocytes, monocytes and granulocytes in the draining LN (left) and the contralateral LN (right) at 24 h post-injection.

(C) Percentages of cell populations in LNs, as gated in A. Horizontal columns show mean percentages of lymphocytes (dark grey), monocytes (grey) and granulocytes (black) of the total live cell population at different time points after adjuvant injection.

(D) Cellular differential counts in peripheral blood. Vertical columns show mean absolute numbers (x 10^9) of lymphocytes (dark grey), monocytes (grey) and granulocytes (black).

Fig 2

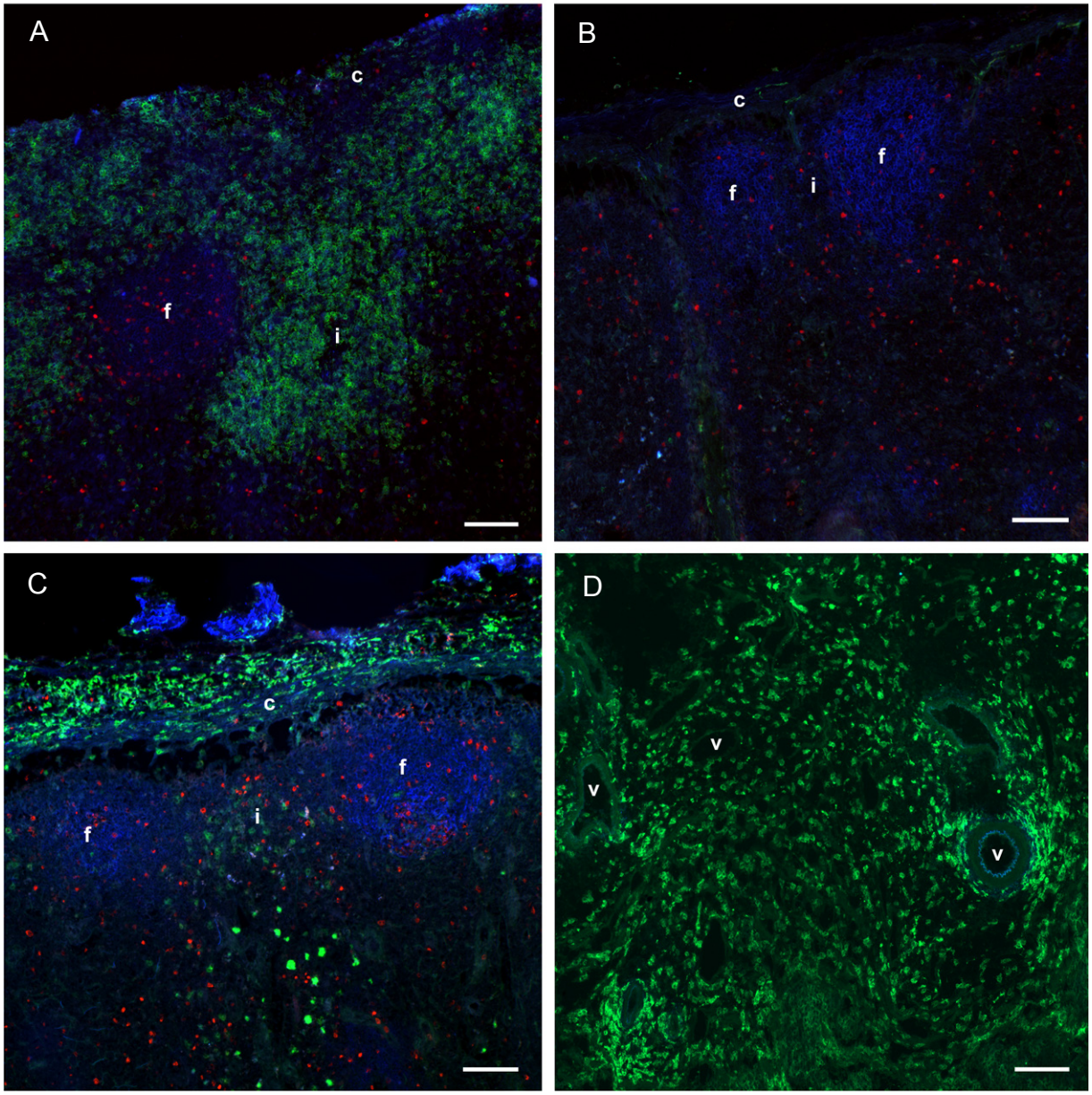

Figure 2. **Distribution of recruited monocytes**

Immunofluorescent labelling of LNs with antibodies against CD14 (green), CD21 (blue), and Ki67 (red). **(A)** Abundant amounts of CD14+ cells were present in the capsule, subcapsular sinus, peri-trabecular sinus, and interfollicular T cell areas of the draining LN at 24 h post-injection. **(B)** The contralateral LN was mainly devoid of CD14+ cells. **(C)** CD14+ cells were still abundant in the capsule, but decreased in number in the sinus and interfollicular area of the cortex at 48 hours post-injection. **(D)** Abundant CD14+ cells were present in the medulla of LNs at 48 hours post-injection, and were particularly dense around vessels.

Follicle (f), interfollicular area (i), capsule (c), vessel (v). Scale bars: 100 μm.

Fig 3

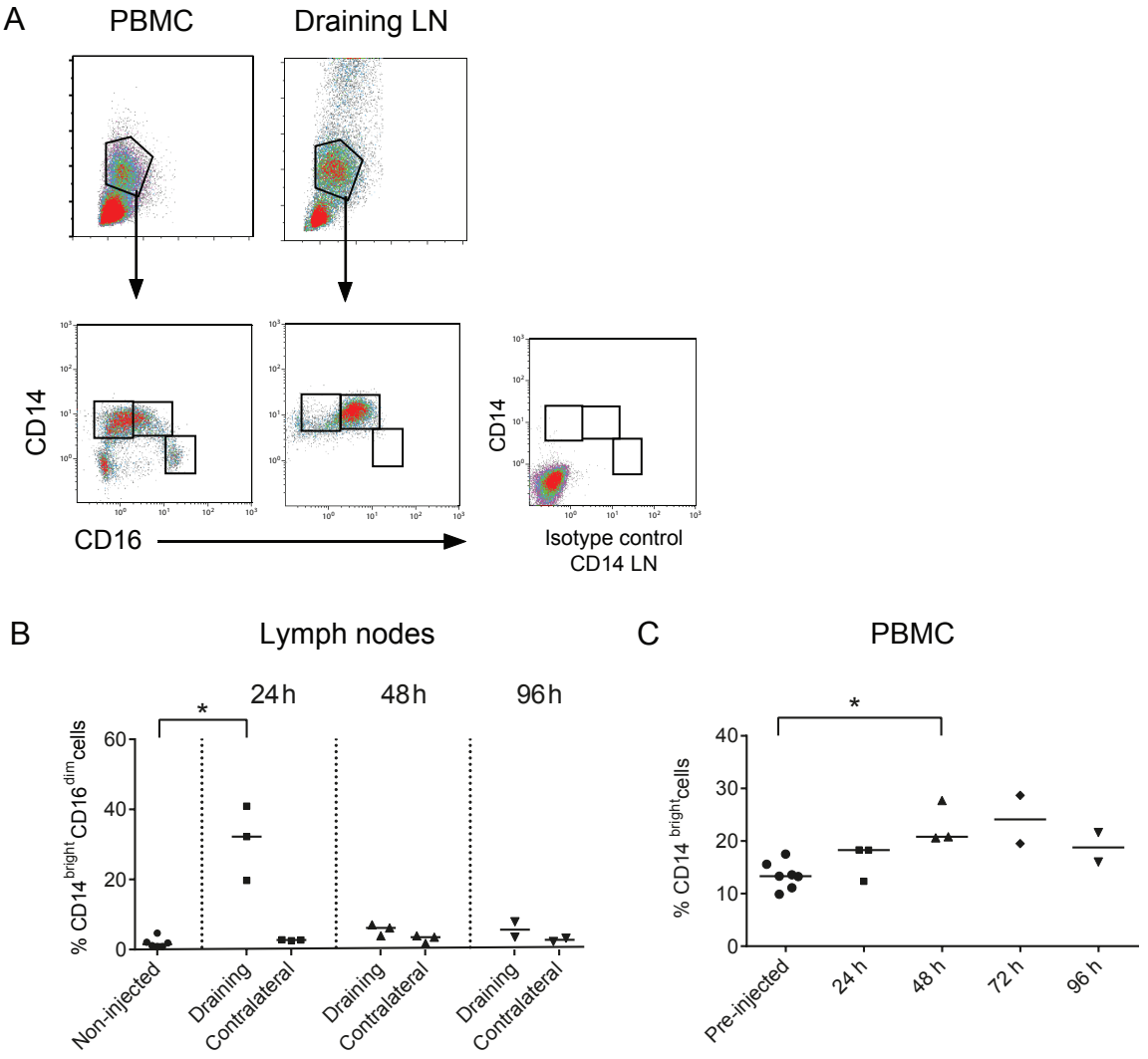

Figure 3. **Phenotype of recruited monocytes**

(A) Density plots of live cells from PBMC (left panels) and draining LN (right panels) at 24 h post-injection from one representative animal. Monocytes are further gated into subsets based on their expression of CD14 and CD16 (lower panels). Isotype control for CD14 in LN is presented in far right panel.

(B) Percentage of $CD14^{bright}CD16^{dim}$ cells of total live cells in LNs of non-injected animals, and in draining and contralateral LNs at 24 h, 48 h and 96 h post-injection. Symbols represent individual animals and the median value within each group is illustrated with a line. Statistical significant differences between injected groups and the non-injected group using the non-parametric Wilcoxon rank-sum test is indicated as *$P < 0.05$.

(C) Percentage of $CD14^{bright}$ cells from PBMC at pre-injection, and at 24 h, 48 h, 72 h and 96 h post-injection. Symbols and statistics as in B (*$P < 0.05$).

Fig 4

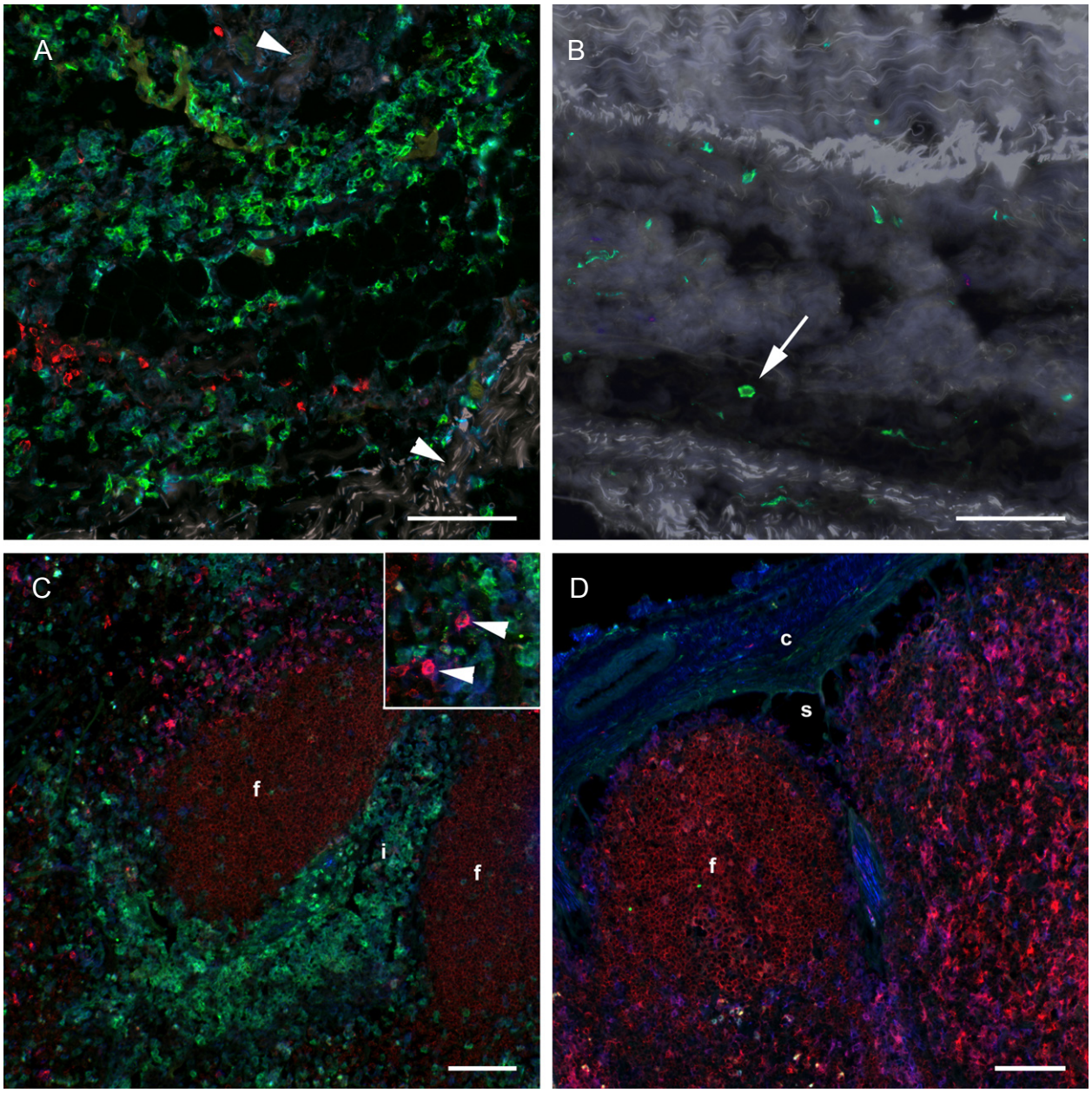

Figure 4. Distribution of monocyte and DC markers in skin and LNs

Immunofluorescent staining of subcutaneous tissues and LNs with CD14 (green), DEC205 (red) and CD11c (blue), at 24 h post injection.

(A) CD14+ cells infiltrated the subcutaneous connective tissue, and pressed the collagen fibers aside (grey auto fluorescent, and arrowheads). In addition, CD205+ and CD11c+ cells were present, CD205+ cells did not co label CD11c+ nor CD14+ cells. **(B)** Very few CD14+ cells (arrow) were observed in the contralateral non-injected skin. **(C)** CD14+ cells infiltrated the peritrabecular and interfollicular T cell areas of the cortex of the draining LN, 24 h post-injection. A moderate amount of double positive CD205+/CD11c+ cells were observed (arrow heads in insert), but did not co-label CD14+ cells. **(D)** CD14+ cells were not observed in the contralateral LN. CD205+ follicles were surrounded by CD11c single labelled and CD11c+/CD205+ double labelled cells. Note the empty sub capsular sinus area (s).

Follicle (f), interfollicular area (i), capsule (c), sinus area (s). Scale bars: 100 μm.

Fig 5 Lymph nodes

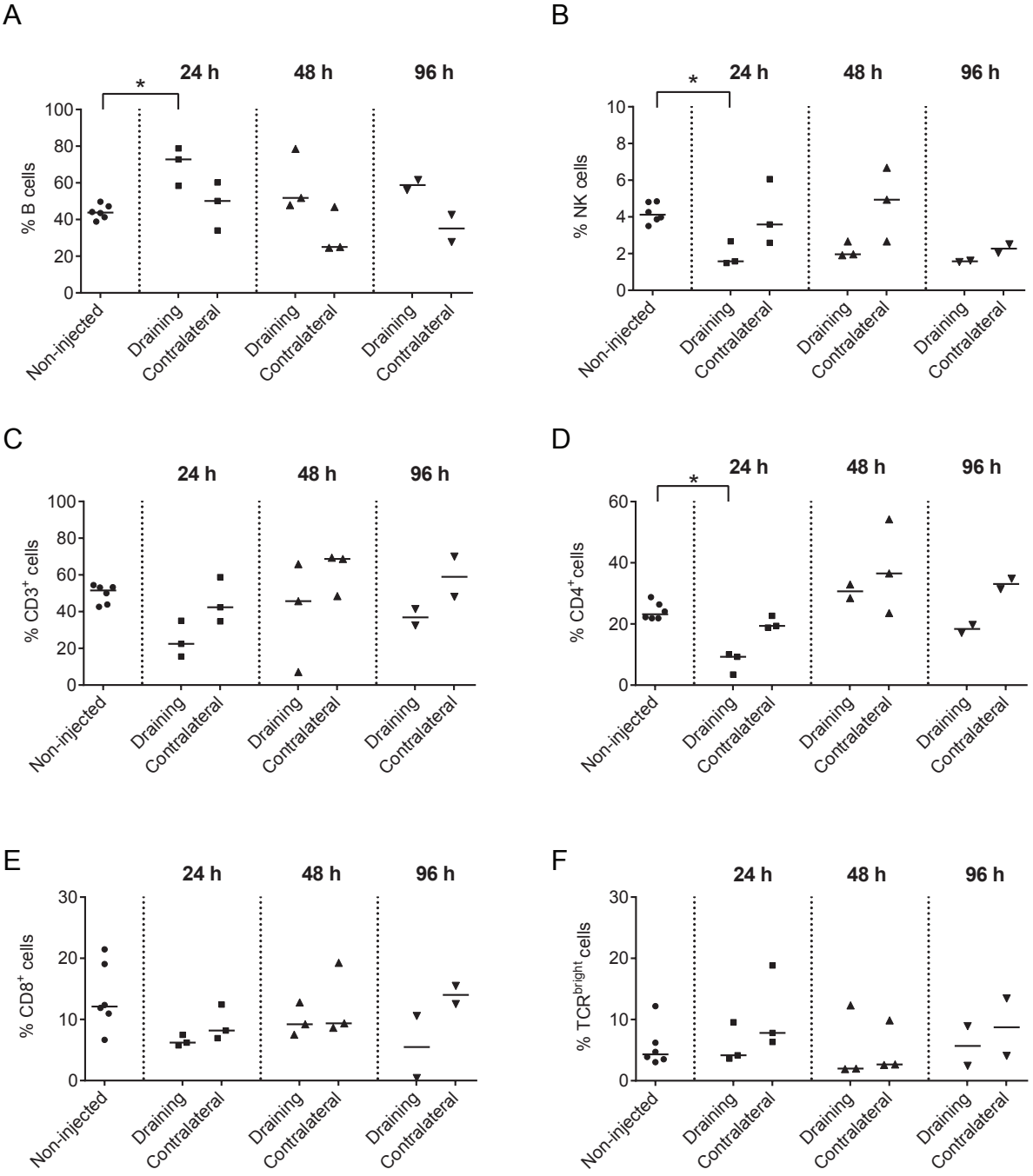

Figure 5. Lymphocyte subsets and NK cells in lymph nodes

Percentages of different lymphocyte subsets and NK cells in lymph nodes after adjuvant injection, determined by flow cytometry with subset specific markers. Percentage of **(A)** B cells, **(B)** NK cells, **(C)** CD3+ cells, **(D)** CD4+ cells, **(E)** CD8+ cells and **(F)** TCR1^{bright} cells of total lymphocytes in LNs of non-injected animals, and in draining and contralateral LNs at 24 h, 48 h and 96 h post-injection. Symbols and statistics as in Fig. 3B (*P < 0.05).

Fig 6

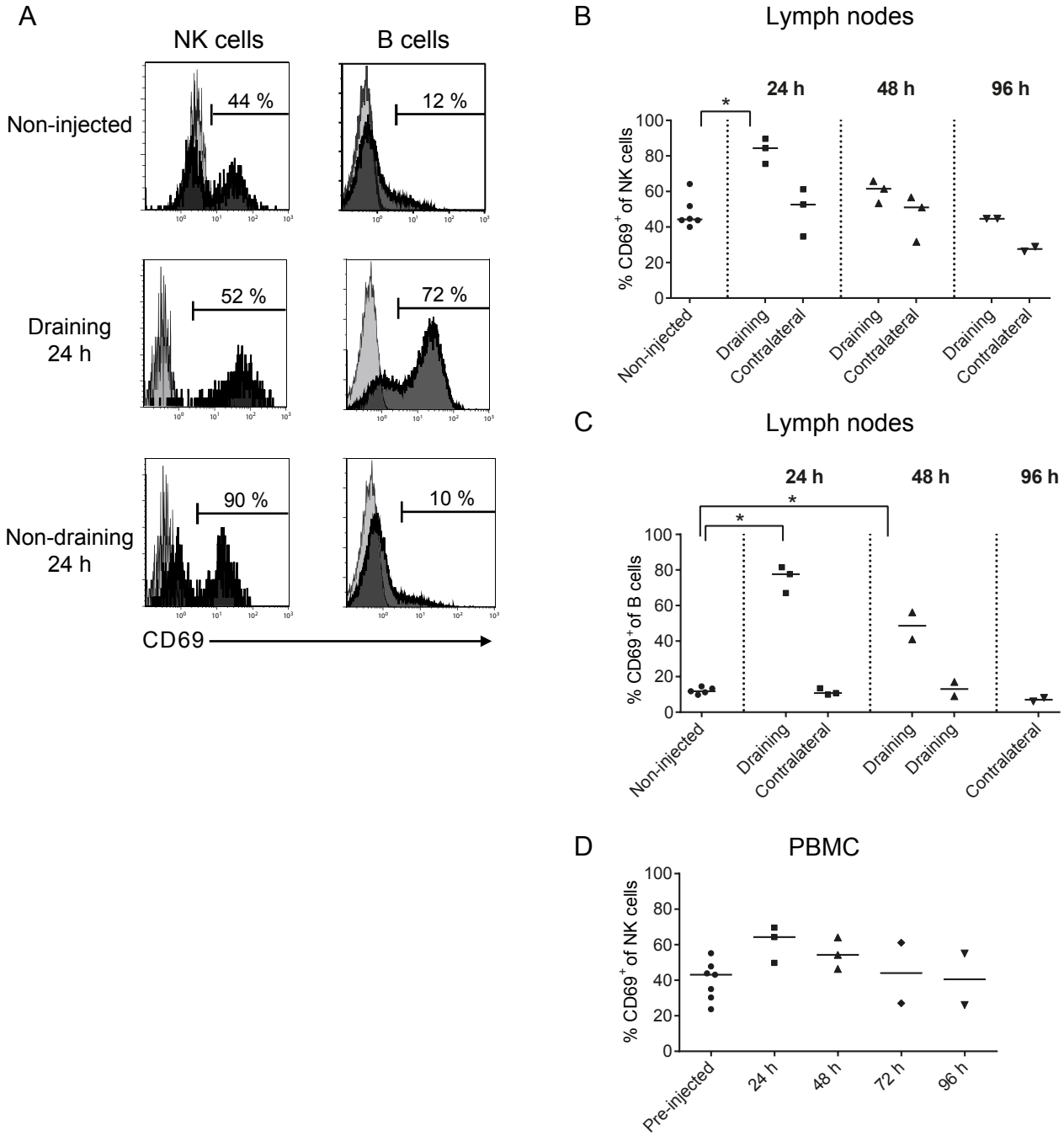

Figure 6. Expression of CD69 on NK cells and B cells

(A) Histograms displaying CD69 expression of NK cells (left panels) and B cells (right panels) in LNs of non-injected animals, and in draining and contralateral LNs at 24 h post-injection. CD69 expression is shown as dark grey histograms and the secondary control staining as light grey histograms. Percentage of positive cells is indicated within each histogram. **(B)** Distribution of results presented in A (left panels). Percentage of $CD69^+$ NK cells of all NK cells in LNs of non-injected animals, and in draining and contralateral LNs at 24 h, 48 h and 96 h post-injection. Symbols and statistics as in Fig. 3B (*$P < 0.05$). **(C)** Distribution of results presented in A (right panels). Percentage of $CD69^+$ B cells of all B cells in LNs of non-injected animals, and in draining and contralateral LNs at 24 h, 48 h and 96 h post-injection. Symbols and statistics as in Fig. 3B (*$P < 0.05$).**(D)** Percentage of $CD69^+$ NK cells of all NK cells in PBMC at pre-injection, and at 24 h, 48 h, 72 h and 96 h post-injection. Symbols as in Fig. 3B.

Fig 7

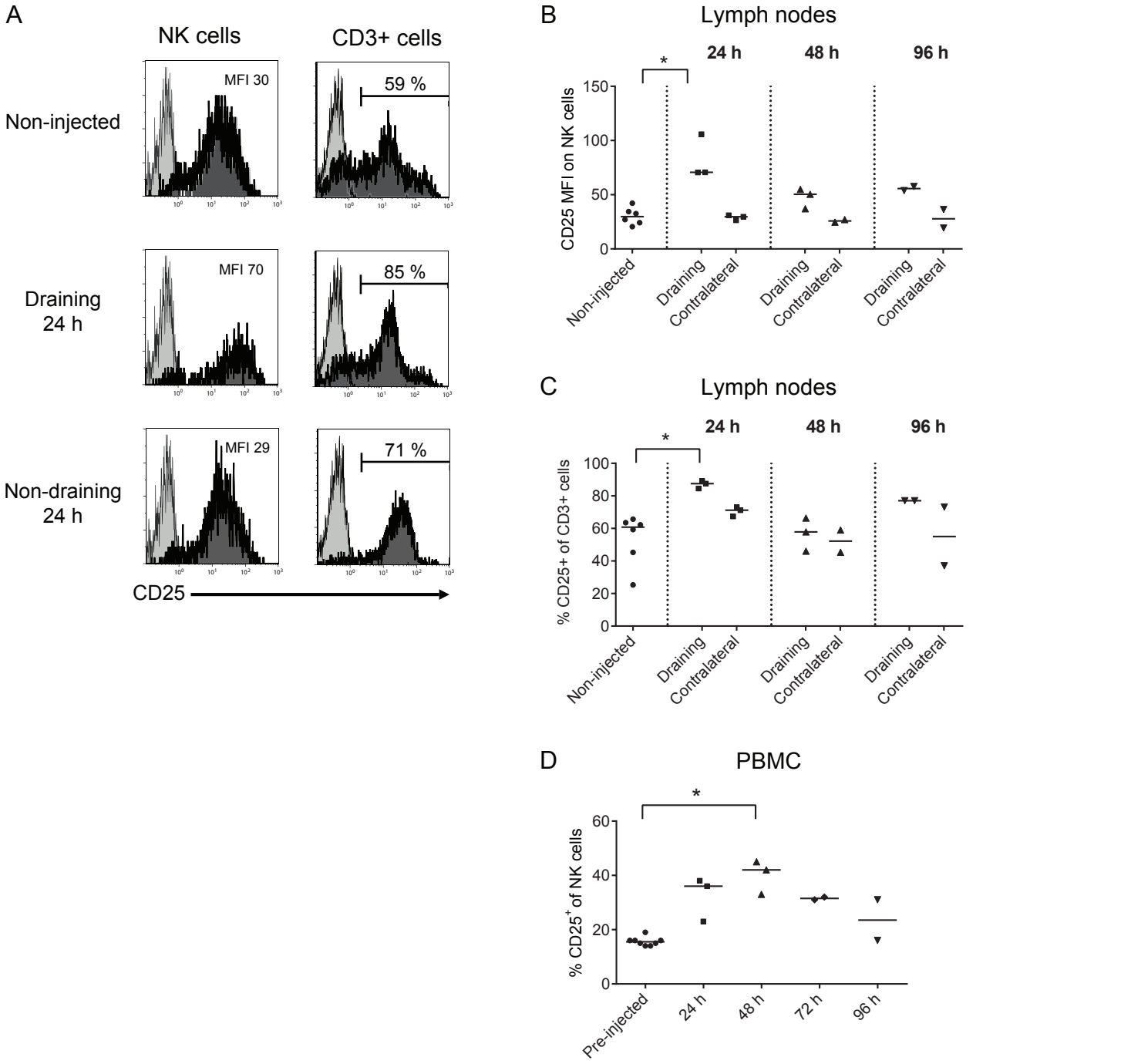

Figure 7. **Expression of CD25 on NK cells and CD3+ lymphocytes**

(A) Histograms displaying CD25 expression of NK cells (left panels) and $CD3^+$ cells (right panels) in LNs of non-injected animals, and in draining and contralateral LNs at 24 h post-injection. CD25 expression is shown as dark grey histograms and the secondary control staining as light grey histograms. The mean fluorescence intensity (MFI) or the percentage positive cells is indicated within each histogram. **(B)** Distribution of results presented in A (left panels). MFI of CD25 on NK cells in LNs of non-injected animals, and in draining and contralateral LNs at 24 h, 48 h and 96 h post-injection. Symbols and statistics as in Fig. 3B (*$P < 0.05$). **(C)** Distribution of results presented in A (right panels). Percentage of $CD25^+$ $CD3^+$ cells of all $CD3^+$ cells in LNs of non-injected animals, and in draining and contralateral LNs at 24 h, 48 h and 96 h post-injection. Symbols and statistics as in Fig. 3B (*$P < 0.05$).

(D) Percentage of $CD25^+$ NK cells of all NK cells in PBMC at pre-injection, and at 24 h, 48 h, 72 h and 96 h post-injection. Symbols and statistics as in Fig. 3B.

Fig 8

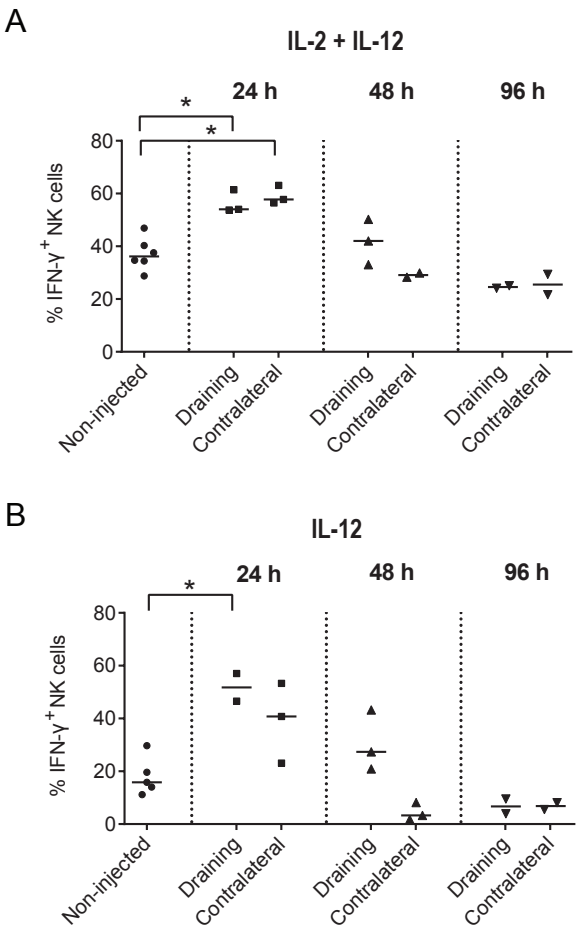

Figure 8. IFN-γ producing capacity of NK cells from LNs

(A and B) Flow cytometric analysis of intracellular IFN-γ in NK cells from LNs after 24 h of in vitro stimulation with the indicated cytokines. Values are from LNs of non-injected animals, and in draining and contralateral LNs at 24 h, 48 h and 96 h post-injection are shown. Symbols and statistics as in Fig. 3B (*$P < 0.05$).

		N	**% lymphocytes**	**% monocytes**	**% granulocytes**
	Non-injected	6	92 (88 – 97)	3 (1 - 4)	4 (1 - 9)
24h	**Draining**	3	49 (41 - 66)	41 (31 - 49)	8 (8 - 9)
	Non-draining	3	93 (93 - 94)	5 (4 - 5)	1 (1 - 2)
48h	**Draining**	3	90 (83 - 95)	6 (3 - 8)	3 (1 - 8)
	Non-draining	3	93 (92 - 94)	3 (3 - 4)	2 (1 - 3)
96h	**Draining**	2	(91 - 93)	(5 - 7)	(0 - 1)
	Non-draining	2	(94 - 95)	(4 - 4)	(1 - 1)

Supplementary table 1. Numbers are median percentage and range of cells within the indicated immune cell populations in lymph nodes, defined by the gating indicated in Figure 1B. N=number of animals investigated.

	N	**lymphocytes**	**monocytes**	**granulocytes**
Pre-injected	8	7 (6 - 11)	0,6 (0,4 - 0,8)	4 (3- 6)
24h	3	5 (5 - 8)	0,8 (0,6 - 0,9)	13 (7 - 15)
48 h	3	7 (5 - 7)	1,0 (0,5 - 1,1)	5 (3 - 8)
72h	2	(5 - 6)	(0,9 - 1,4)	(1 - 3)
96h	2	(5 - 6)	(0,5 - 0,8)	(1 - 2)

Supplementary table 2. Numbers are median and range of cells from the indicated immune cell populations in peripheral blood, and are given as absolute number x 10^9. N=number of animals investigated.