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# Bovine neonate natural killer cells are fully functional and highly responsive to interleukin-15 and to NKp46 receptor stimulation

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**Abstract** – Natural killer (NK) cells are key components of the innate immune system with their killing and cytokine producing abilities. Bovine NK cells have been characterized as NKp46<sup>+</sup>/CD3<sup>-</sup> lymphocytes, but little is known about these cells in neonatal calves. As the newborn calf, with an insufficiently developed acquired immunity, has to employ the innate immune system, we wanted to investigate whether neonate NK cells had the same characteristics as cells from older calves. Freshly isolated neonate and calf NK cells presented the same resting CD2<sup>+</sup>/CD25<sup>low</sup>/CD8<sup>-/low</sup> phenotype. Neonates less than 8 days old had one third of the circulating NKp46<sup>+</sup> cells of older calves, but the NK cells proliferated more actively *in vitro* in the presence of interleukin (IL)-2 or IL-15. Moreover, neonate NK cells were more cytotoxic both in an NKp46 mediated redirected lysis assay and in direct killing of a bovine cell line MDBK when cultured in the presence of IL-15. Neonate and calf NK cells cultured in the presence of IL-2 and then stimulated with IL-12 produced similar dose-dependent interferon (IFN)- $\gamma$  amounts, while IL-15 cultured NK cells did not give such a response whatever the age. However, neonatal NK cells cultured in IL-15 and stimulated by IL-12 concomitantly with cross-linking of NKp46, produced 4 to 5 times more IFN- $\gamma$  than calf NK cells. These data suggest that although present in lower number at birth, neonate NK cells are fully functional and are more responsive to IL-15 and activation through the NKp46 receptor than NK cells from older calves.

**natural killer cell / neonate / bovine / cytotoxicity / interleukin**

## 1. INTRODUCTION

A general feature of neonates is that they have an incompletely developed immune system which leads to an increased susceptibility to infections and a weak response to most vaccines [27, 33, 42, 53, 61]. However, in response to BCG vaccination, neonates can mount a potent T<sub>H</sub>1 cell response [34] and acquire

significant protection against the disease. In early life, smaller numbers of immune cells are present in peripheral lymphoid tissues, and qualitative and quantitative differences in cells of the innate and adaptive immune systems have been described [2, 16, 31, 46]. Moreover, at birth, cellular components of the adaptive immune system are in a naive state and several days are required to develop an adaptive response. Therefore, the protection of neonates in the first days of life relies on maternal

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transmitted immunity through colostrum and their own innate immune system [11, 25, 43]. We have recently demonstrated that mesenteric lymph node or spleen cells from goat neonates stimulated with various toll-like receptor ligands produce much more interleukin (IL)-12 and interferon (IFN)- $\gamma$  than adult node or spleen cells [58]. This shows that ruminants may have developed some mechanism to compensate for the immaturity of their adaptive immune system during the neonatal period.

Natural killer (NK) cells represent important early effector cells of the innate immune system. These cells can lyse infected and tumour cells without prior sensitisation and are also able to secrete immunomodulatory and antiviral cytokines like IFN- $\gamma$ , making them important members of the immune system first line of defence. In addition to their role in innate immunity, NK cells can assist T-cells in  $T_H1$  polarization and promote dendritic cell (DC) maturation. Unlike T and B lymphocyte activation which involves re-arranged receptors, NK cell activation is controlled by a limited repertoire of germ line-encoded receptors that do not undergo somatic recombination [40]. To date, in all species, the cell surface phenotype defining NK cells is the absence of CD3 and the expression of the receptor NKp46, a member of the natural cytotoxicity receptor family [6, 17, 22, 54, 56, 63].

In veterinary species, NK cell studies have been delayed because of the lack of antibodies specific for this cell population. Since we generated the first antibody against NKp46 in cattle [56], the majority of studies have been carried out on NK cells from calves about three months old. As in other species, cattle NK cells have been found in blood, liver, lung, spleen and lymph nodes [10] and share characteristics with NK cells from humans, rats and mice. Neonate NK cells have been studied mainly in humans [16, 18, 21, 30, 48, 57]. However, to date, there has been no study on neonatal NK cells from farm animals.

We conducted a comparative study on peripheral blood NK cells from neonates and older calves, to understand better the innate response of newborn calves. Most in vitro studies performed with human or rodent NK cells

show that their activation can be modulated by various cytokines [41, 45, 64, 65]. IL-15 and IL-2 are cytokines with overlapping effects on NK cells. They are best known for their effects on development, proliferation, survival and activation of these cells [5]. However, in bovine NK cells, their various effects have not been well documented. IL-12 known to cause NK cells to produce IFN- $\gamma$  effectively [59], is an appropriate cytokine to demonstrate the difference in IFN- $\gamma$  producing capability between neonatal and calf NK cells. We therefore analysed the proliferation, cytotoxic activity and IFN- $\gamma$  production in response to IL-12 of NK cells expanded in the presence of IL-2 or IL-15. Although less numerous, NK cells from neonates are more responsive to stimulating factors, especially IL-15 and NKp46 triggering.

## 2. MATERIALS AND METHODS

### 2.1. Animals and blood sampling

Clinically healthy female Holstein cattle of various ages reared in conventional but sanitary protected facilities (PFIE, INRA F-37380 Nouzilly; Lycée agricole, F-37230 Fondettes, France) were used in this study. To evaluate the relative numbers of NK cells, blood samples were collected from several animals ranging from 2 days to 3 years old. In the following experiments, we collected blood from 6–7 day-old neonates and 4–10 month-old calves, to standardize the blood sampling conditions.

### 2.2. Isolation and culture of bovine NK cells

Blood samples were collected on EDTA (BD vacutainers). Peripheral blood mononuclear cells (PBMC) were recovered after centrifugation on a density gradient (Histopaque  $d = 1.77$ ; Sigma-Aldrich, Lyon, France). NK cells were isolated from PBMC and cultured as previously described [56]. Briefly, NK cells were positively selected using mAb against NKp46 (AKS1; IgG1) and immunomagnetic anti-mouse pan IgG beads (Dyna, Invitrogen, Cergy Pontoise, France) and cultured in RPMI medium supplemented with 60  $\mu\text{g}/\text{mL}$  penicillin, 100  $\mu\text{g}/\text{mL}$  streptomycin, 1 mM sodium pyruvate, non-essential amino acids, 50  $\mu\text{M}$  2-mercaptoethanol (all GIBCO, Invitrogen), with 10% Fetal Calf Serum (FCS) and 100 U/mL recombinant bovine IL-2

(rbIL-2) or 10 ng/mL recombinant human IL-15 (rhIL-15) (Immuno Tools, Germany). Cytokine doses used to promote NK cell growth are based on previous experiments for IL-2 (100 U/mL) [8] or have been optimized for IL-15 (10 ng/mL). After 24–48 h, beads were spontaneously released from the cells and removed. NK cells were then cultured for the appropriate length of time in medium containing rbIL-2 or rhIL-15.

### 2.3. Cell labelling and flow cytometry

NK cell surface receptors were single or double labelled on PBMC or cultured NK cells as already described [56] using antibodies against the following molecules: CD2 (MUC2A; IgG2a), CD25 (CACT108A; IgG2a) and TcR1 (GB21A; IgG2b) from VMRD (USA), CD8 (38.65; IgG2a) and NKp46 or NKp46-AF488 (clone AKS1, IgG1) from Serotec (Oxford, UK). Subtype-specific secondary antibodies were conjugated with TC or PE (Caltag Laboratories, Invitrogen). The samples were analysed on a FACS CALIBUR flow cytometer (Becton Dickinson), equipped with cell-Quest Pro software.  $2-10 \times 10^4$  viable cells gated in the forward and side scatter plot were analysed. Intracellular perforin labelling was performed with the perforin-FITC kit (clone  $\delta$ G9, IgG2b) and the Cytofix/Cytoperm and Permash solutions from BD Pharmingen.  $2 \times 10^5$  gated NK cells were analysed.

### 2.4. Carboxyfluorescein diacetate succinimidyl ester proliferation analysis

For cell proliferation studies, positively selected NK cells cultured for 20 h in the presence of rbIL-2 or rhIL-15 were incubated with 5  $\mu$ M carboxyfluorescein diacetate succinimidyl ester (CFDA-SE) (Molecular Probes, Invitrogen) in PBS with 0.2% bovine serum albumin (BSA) for 10 min at 37 °C; cold RPMI was added and they were then incubated for 5 min on ice. The cells were washed three times in RPMI and cultured in the presence of 100 U/mL rbIL-2 or 10 ng/mL rhIL-15 for 3–6 days. To obtain non-proliferating NK cells as controls, these cells were cultured with rbIL-2 at 1 U/mL [8] and rhIL-15 at 0.1 ng/mL.

### 2.5. Cytotoxicity assay

The cytotoxic activity of NK cells cultured with rbIL-2 or rhIL-15 was tested against a bovine kidney cell line (MDBK) and a murine tumour cell line

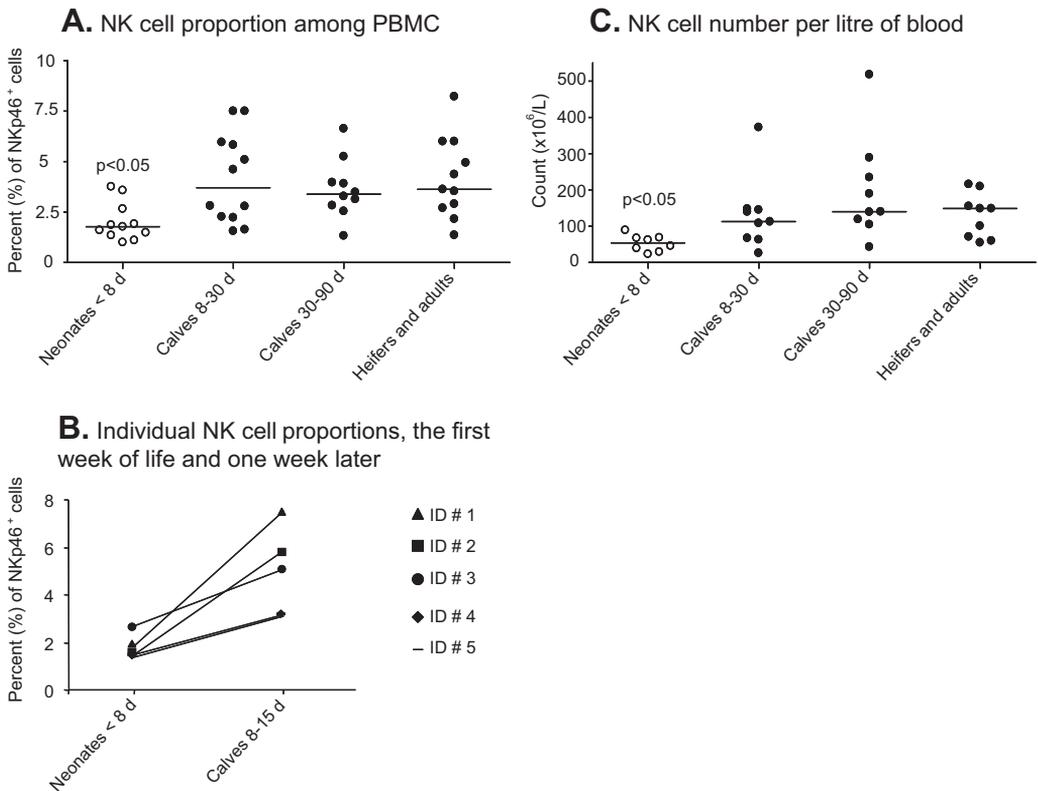
(P815) which expresses Fc $\gamma$ -receptors, allowing antibody linking for redirected lysis, in a flow cytometry assay adapted from Godoy-Ramirez et al. [28]. Briefly, target cells were labelled with 5  $\mu$ M CFDA-SE (Molecular Probes, Invitrogen) in PBS containing 0.2% BSA for 10 min. Then 5 mL cold RPMI was added and the samples incubated on ice for 5 min. They were washed three times in RPMI and immediately used in the cytotoxicity assay. For redirected lysis, the target cells were preincubated with 1  $\mu$ g/mL of mAb against NKp46 (AKS1) for 5 min.

NK cells activated with IL-2 or IL-15 for 7–10 days were seeded in a 96-well micro plate with a constant number of target cells (50 000) over a range of effector:target (E:T) ratios (4:1, 2:1, 1:1, 0.5:1, 0.25:1 and 0.125:1). Cells were incubated in a total volume of 200  $\mu$ L RPMI supplemented with 10% FCS for 1.5 h in a 5% CO<sub>2</sub> atmosphere at 37 °C. Cells were then washed with PBS containing 0.2% BSA, and then incubated in the same buffer containing 3  $\mu$ M propidium iodide (PI) (Molecular Probes, Invitrogen) for 10 min at room temperature in the dark. Target cells incubated without NK cells and stained with PI served as controls to measure basal apoptosis. Cell samples were kept on ice until flow cytometry analysis. For each E:T ratio,  $2 \times 10^4$  target cells were acquired. The percentage of specific lysis was calculated as follows: percentage of necrotic target cells when co-cultured with NK cells minus percentage of spontaneous deaths of target cells.

### 2.6. IFN- $\gamma$ ELISA

To assess their IFN- $\gamma$  secretion, NK cells were pre-cultured for 7–10 days in the presence of 100 U/mL of rbIL-2 or 10 ng/mL of rhIL-15. Triplicates of  $10^5$  cells were then incubated for 20 h in the presence of various rhIL-12 concentrations (Immuno Tools, Germany) (0, 100, 500 and 1 000 pg/mL); rhIL-12 induces the secretion of IFN- $\gamma$  by bovine NK cells [20].

To assess the effect of cross-linking receptors in IFN- $\gamma$  production, NK cells were cultured as previously for 20 h in the absence or presence of 100 pg/mL rhIL-12 in 96-wells MaxiSorp plates (NUNC, Germany) pre-coated overnight in 0.05 M carbonate buffer (pH 9.6) with antibodies (anti-NKp46 (AKS1), anti-CD8 (38–65) or anti-CD2 (MUC2A)) to trigger the corresponding receptors. IFN- $\gamma$  production was measured in the supernatants by ELISA using MaxiSorp plates pre-coated overnight in PBS with anti-IFN- $\gamma$  mAb (CC330; IgG1, Serotec, Germany), then incubated for 1 h with a blocking buffer containing 0.05% tween, 1% BSA



**Figure 1.** Enumeration of NK cells from birth to adulthood. (A) The proportion of NKp46<sup>+</sup> cells among PBMC from cattle at different ages was determined by flow cytometry. (B) Total number of NKp46<sup>+</sup> cells per litre of blood. Individual values and medians are presented. Statistical analysis was carried out with a nonparametric Mann–Whitney test (*p* value is indicated above the scatter plot). (C) The proportion of NKp46<sup>+</sup> cells among PBMC from five animals (ID # 1 to 5) within the first two weeks of life was determined by flow cytometry.

and 5% sucrose in PBS. To detect IFN- $\gamma$  capture, a biotinylated anti-IFN- $\gamma$  mAb (CC302; IgG1, Serotec) was used. Concentrations were calculated from a standard dilution curve of purified bovine IFN- $\gamma$  (Perbio, Endogen, France).

**2.7. Statistical analyses**

Nonparametric Mann–Whitney tests and one-way Wilcoxon tests were used to compare data from neonates and calves in various conditions. We calculated the summary measure variable, Area Under Curve, using the trapezium rule as described by Altman [3] as the cytotoxicity assay involved a serial measurement of lysis at different E:T ratios.

**3. RESULTS**

**3.1. Circulating NK cell counts are lower in calves up to a week old than in older cattle**

We analysed the number of circulating NK cells in healthy cattle of different ages by labelling PBMC with the NK cell-specific marker NKp46. During the first week of life, the proportion of neonate NK cells among PBMC was less than half that for older calves and adult cattle (Fig. 1A). Moreover, for the same animal the NK cell proportion increased between the

first and the second week of life (Fig. 1B). As neonates have fewer blood PBMC than older calves and adults, they had, on average only a third of the NK cells of older calves and adults per litre of blood (Fig. 1C). Thus, the end of the first week of life appears to be a critical moment in NK cell ontogenesis. We therefore defined animals younger than 8 days as “neonates” and animals between 4 to 6 months old as “calves”.

### **3.2. Following IL-2 or IL-15 stimulation, CD2<sup>+</sup>, CD8<sup>+</sup> and CD25<sup>+</sup> NK cells predominate in cultures from neonates as well as from calves**

IL-15 expanded  $\gamma\delta$  T-cells can include a population that expresses NKp46 [35]. We double labelled NKp46<sup>+</sup> cells with anti-NKp46 and anti-TcR1 antibodies and found that NKp46<sup>+</sup>/ $\gamma\delta$  T<sup>+</sup> cells represented less than 0.01% of PBMC and after 7 days culture, less than 0.3% of the population (Fig. 2A). Resting peripheral blood NK cells do not have the same phenotype as IL-2 expanded NK cells [9, 56]. For both neonate and calf 70–75% of freshly isolated NKp46<sup>+</sup> cells expressed CD2 but only 10–15% were CD8<sup>bright</sup> and about 25% expressed CD25 (Figs. 2B and 2C). Thus, the proportions of resting NK cells from neonates and calves expressing CD2, CD8 and CD25 were similar. After 6 days culture in the presence of rIL-2 or rIL-15, the percentage of neonate and calf NK cells expressing CD2 was reduced to half, but there was a 3–7 fold increase in the percentage of CD8<sup>+</sup> NK cells and a 2.5–3 fold increase in that of CD25<sup>+</sup> NK cells. Therefore, neonate and calf NK cells have similar patterns of expression of CD2, CD8 and CD25 when cultured in the presence of IL-2 or IL-15.

### **3.3. Neonate NK cells have a higher proliferation capacity when expanded with IL-2 or IL-15 and express more CD25**

NK cells were labelled the day after isolation with the cell division marker CFDA-SE, and expanded in the presence of rIL-2 or rIL-15, to explore their proliferation capacity. NK

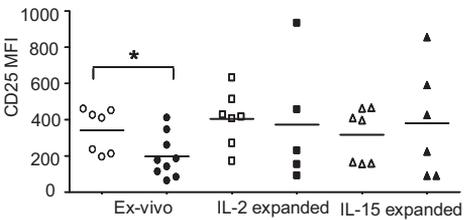
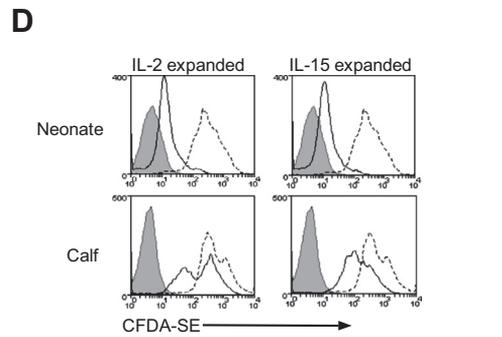
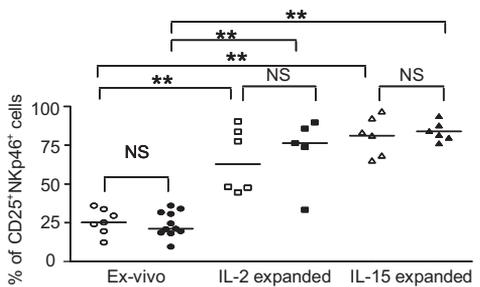
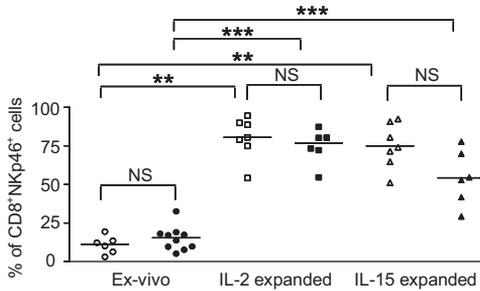
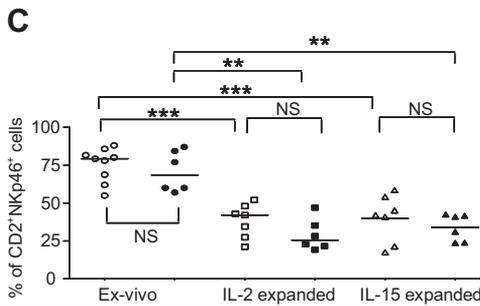
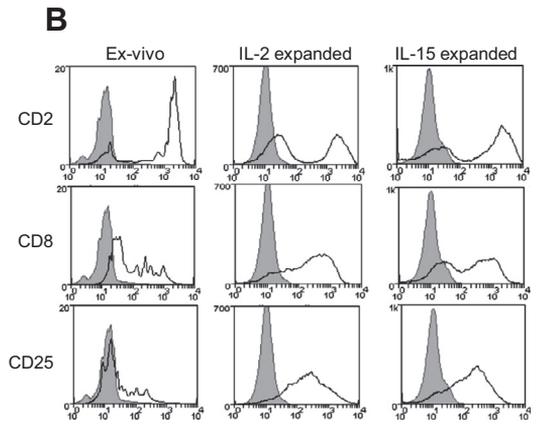
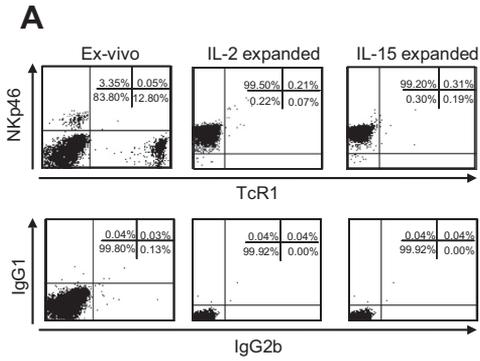
cells cultured with the minimal IL-2 or IL-15 concentrations compatible with cell survival were used as controls. After 3 days of expansion, neonate NK cells had proliferated more rapidly than those from older animals (Fig. 2D). After 6 days culture, the rates of proliferation of neonate and calf NK cells were similar (data not shown), suggesting that NK cells from older calves may need more time to start active proliferation. We analysed the mean fluorescence intensity (MFI) of the IL-2 receptor  $\alpha$ -chain (CD25) on resting NK cells to investigate the reasons for the difference in the onset of proliferation. The MFI of CD25 on neonate NKp46<sup>+</sup> cells in freshly isolated PBMC was slightly but significantly higher than on calf NKp46<sup>+</sup> cells (Fig. 2D). This difference was still clear after 3 days culture (data not shown), but after 6 days the level of expression of CD25 was similar on neonate and calf NK cells (Fig. 2D).

### **3.4. NKp46-mediated redirected cytotoxicity is higher in neonate NK cells**

We investigated the cytotoxicity of neonate and calf NK cells isolated and expanded for one week in the presence of rIL-2 or rIL-15; we performed a redirected lysis assay using anti-NKp46 antibody and P815 murine target cells, which bear an Fc receptor. Neonate NK cells expanded with IL-2 or IL-15 were clearly more cytotoxic than calf NK cells (Fig. 3A). In the presence of an isotype control, no lysis was observed, which indicated the specific involvement of the NKp46 receptor in this test. To investigate whether different expression levels of NKp46 might explain the difference between neonate and calf NK cells, we analysed the fluorescence intensity of this receptor, and found that neonate NK cells had a slight but significantly higher expression of NKp46 than NK cells from calves (Fig. 3B).

### **3.5. Neonate NK cells show increased direct cytotoxicity when cultured with IL-15**

To study NK cell cytotoxicity under more normal physiological conditions, we used a direct lysis assay with the bovine epithelial



kidney cell line MDBK. Under these conditions, neonate and calf NK cells expanded with rbIL-2 had similar cytotoxic activities. However, when expanded with rhIL-15, neonate NK cells killed MDBK target cells more efficiently than NK cells from calves (Fig. 4A). The reason for this difference was investigated by examining the intracellular perforin content of neonate NK cells to see if it was higher than that of calf NK cells, but there was no significant difference (Fig. 4B). Although NKp46 was expressed more on neonate NK cells than on calf NK cells, its expression was similar in IL-2 and IL-15 cultured cells (Fig. 3B); thus, the difference of NKp46 expression is not a likely explanation for the higher cytotoxicity of neonate cells.

### 3.6. Neonate NK cells can produce larger amounts of IFN- $\gamma$ than calf NK cells when stimulated with IL-12 and NKp46 in the presence of IL-15

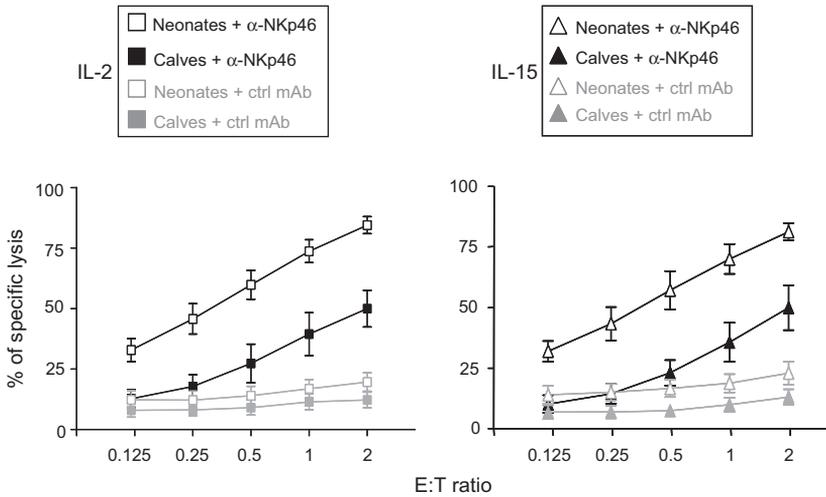
We assessed IFN- $\gamma$ -production using sorted NK cells from neonates and calves which were expanded in the presence of rbIL-2 or rhIL-15

for 7–10 days and further incubated for 24 h in the absence or presence of different concentrations of rhIL-12. Neonate and calf NK cells produced similar levels of IFN- $\gamma$  when cultured with rbIL-2 (Fig. 5A, left panel), but when NK cells were expanded in the presence of rhIL-15, the IL12 stimulation did not induce IFN- $\gamma$  production (Fig. 5A, right panel). However, we previously showed that the engagement of some receptors expressed at the surface of bovine NK cells may influence the level of IFN- $\gamma$  production [8]. We performed cross-linking assays with the corresponding antibodies, to clarify the involvement of NKp46, CD2 and CD8 receptors in the production of IFN- $\gamma$ . NK cells were cultured for 24 h in plastic wells pre-coated with NKp46, CD2 or CD8 mAb and IFN- $\gamma$  levels analysed by ELISA 24 h later. In the absence of IL-12 stimulation, only the triggering of the NKp46 receptor of IL-2-expanded NK cells resulted in a slight but significant increase in the production of IFN- $\gamma$  in all animals whatever their age. When the cells were stimulated with IL-12, the production of IFN- $\gamma$  varied according to the cytokine used to expand the cells and the age of the animals:

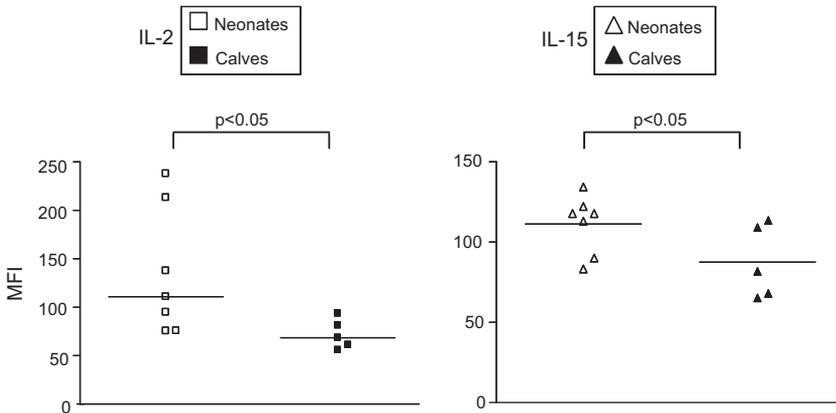


**Figure 2.** Phenotype and proliferation of peripheral blood NK cells from neonates and calves. (A) NK cells among PBMC defined as “Ex-vivo” and sorted NKp46<sup>+</sup> cells cultured for 6 days in the presence of rbIL-2 (“IL-2 expanded”) or rhIL-15 (“IL-15 expanded”) were double labelled with antibodies against NKp46 and TcR1 or with appropriate isotypes. The proportions of cells relative to the total cells in the culture are given. Data shown are from one representative neonate. (B) NK cells among PBMC and sorted NKp46<sup>+</sup> cells cultured for 6 days in the presence of rbIL-2 or rhIL-15 were double labelled with antibodies against NKp46 and one of the three markers, CD2, CD8 or CD25. Solid lines indicate CD2, CD8 or CD25 expression on NKp46<sup>+</sup> cells, shaded areas correspond to isotype control mAbs. Data shown are from one animal representative of seven neonates. (C) The percentages of neonate (open symbols) and calf (filled symbols) NKp46<sup>+</sup> cells expressing CD2, CD8 or CD25 were determined. Individual values and medians are presented. Statistical analyses were carried out with the nonparametric Mann–Whitney test. \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ , NS: Non significant. (D) NKp46<sup>+</sup> cells from neonate and calf PBMC were sorted, labelled or not with CFDA-SE the day after isolation and expanded for 3 days with rbIL-2 or rhIL-15. The intensity of CFDA-SE labelling was determined by flow cytometry. The solid black line corresponds to dividing NK cells. The broken line shows NK cells cultured in the presence of rbIL-2 or rhIL-15 concentrations which allow minimal proliferation. The filled grey area shows unlabelled expanded NK cells. Data shown are from one animal representative of four. The MFI of the CD25 marker on neonate (open symbols) and calf (filled symbols) NKp46<sup>+</sup> cells defined as in (A) and (B) were determined by flow cytometry. Statistical analyses were carried out with the nonparametric Mann–Whitney test. \*  $p < 0.05$ .

**A.** Redirected lysis of P815 murine cells incubated with the anti-NKp46 mAb



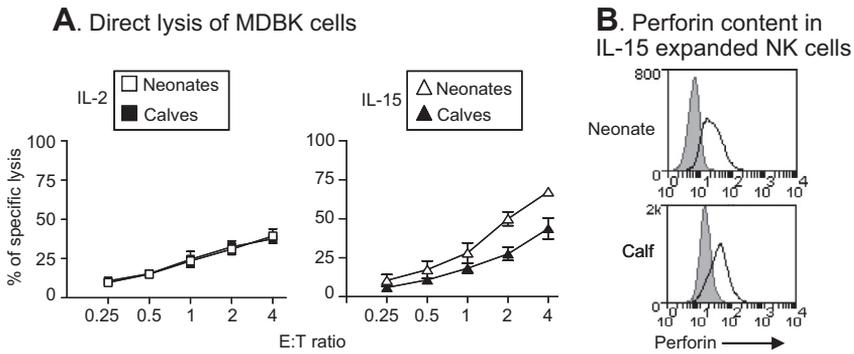
**B.** Expression of NKp46 on NK cells



**Figure 3.** NKp46 receptor mediated cytotoxicity by neonate and calf NK cells. NKp46<sup>+</sup> cells from peripheral blood of neonates less than 8 days and calves (6 months old) were sorted, cultured in the presence of rbIL-2 or rhIL-15 for 7–10 days and used in a flow cytometry cytotoxicity assay. (A) Redirected lysis of P815 murine target cells carrying an Fc $\gamma$  receptor by NK cells from neonates (open symbols) or from calves (filled symbols), in the presence of anti-NKp46 mAb (black lines) or with addition of the isotype control (grey lines). Data presented are means from 5 animals  $\pm$  SEM. Neonate and calf NK cell cytotoxicity were compared with the one-way Wilcoxon test:  $p < 0.01$ . (B) Level of expression of the NKp46 receptor after 7–10 days of culture in the presence of rbIL-2 or rhIL-15 (mean fluorescence intensity: MFI). Individual values and medians are presented. The nonparametric Mann–Whitney test ( $p$  value is indicated above the scatter plot) was used for statistical analysis.

in IL-2-expanded cultures, the cross-linking of the receptors NKp46, CD2 or CD8 for neonates and NKp46 for calves triggered IFN- $\gamma$

production (Fig. 5B, left panel). In IL-15-expanded cultures, the secretion of IFN- $\gamma$  was lower than with IL-2-expanded cells and an



**Figure 4.** Direct lysis of bovine epithelial cells (line MDBK) by NK cells. NKp46<sup>+</sup> cells from peripheral blood of neonates less than 8 days old, and calves (6 months old), were sorted and cultured in the presence of rb-IL2 (100 U/mL) or rh-IL15 (10 ng/mL) during 7–10 days and used in a flow cytometry cytotoxicity assay. (A) Direct lysis of MDBK target cells by NK cells from neonates (open symbols) or calves (filled symbols). Data represent means ± SEM (*n* = 3 for cells cultured with IL-2; *n* = 5 for cells cultured with IL-15). Statistical analysis is based on the one-way Wilcoxon test: *p* < 0.05 for IL-15 expanded NK cells. (B) Sorted NKp46<sup>+</sup> cells cultured for 6 days in the presence of rhIL-15 were labelled with anti-perforin mAb (solid lines) or with isotype control mAb (shaded area). Data shown are from one animal representative of three.

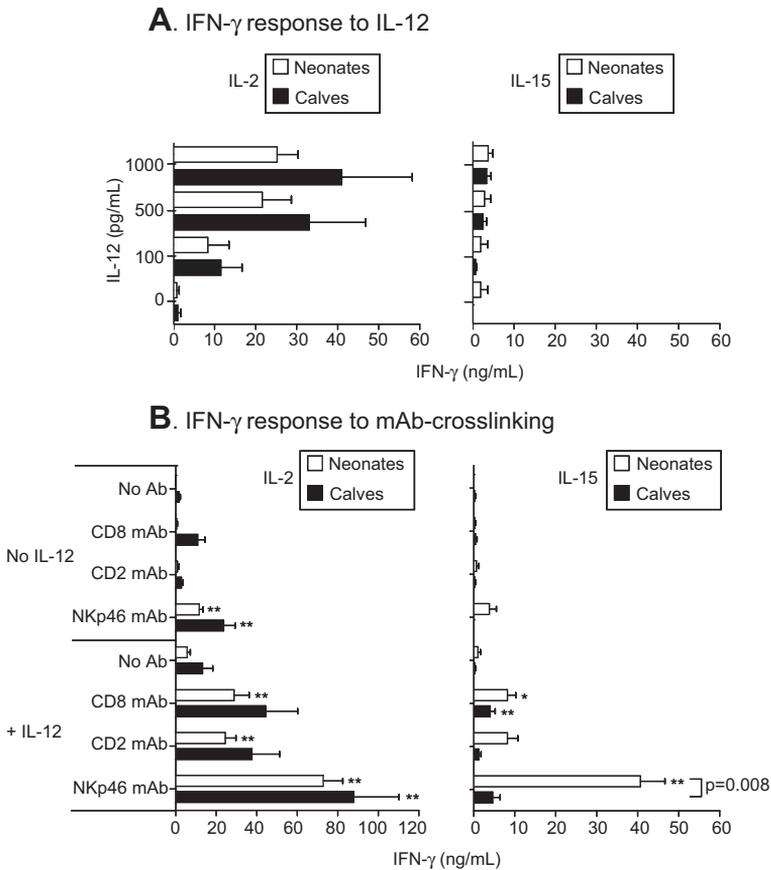
increased IFN- $\gamma$  production was mostly seen following cross-linking of the NKp46 receptor in NK cells from neonates (Fig. 5B, right panel).

**4. DISCUSSION**

We conducted a comparative analysis of blood NK cells from healthy neonates and calves, to clarify the peculiarities of neonatal immunity in cattle. We found that PBMC from neonates (less than 8 days old) had less than half the NK cells of older calves, heifers or adults. Moreover, there seemed to be a constant increase of the NK cell numbers during the first week of life, reaching a steady number from the second week. Therefore, in cattle, the end of the first week of life appears to be critical for NK cell ontogenesis. These data complete previous studies of Kulberg et al. (2004) and Kampen et al. (2006) [36, 39] for the neonatal period. There are no studies describing neonate NK cell blood proportions in other veterinary species but, in humans, most studies on cord blood show that there are more NK cells than in adult

peripheral blood [16, 21, 30]. However, some studies on peripheral blood of newborn infants describe a decreased proportion of NK cells compared to adult peripheral blood [18, 48].

We aimed to characterize neonate and calf NK cells, either freshly isolated or expanded in vitro. Resting, circulating neonate and calf NK cells shared a CD2<sup>+</sup>/CD25<sup>low</sup>/CD8<sup>-/low</sup> phenotype. After in vitro expansion of neonate and calf NK cells, the CD2<sup>+</sup> subset proliferated and the CD25 receptor was up-regulated as previously described [8, 56]. However, resting neonate NK cells, on a per cell basis, expressed slightly but significantly more of the activating receptor NKp46 (data not shown) and the IL-2 receptor CD25, suggesting that they might be able to respond more rapidly to activating stimuli than calf NK cells. Indeed, during the early stages of culture, NK cells from neonates proliferated more rapidly than those of calves, but both reached the same proliferation rate after 6 days culture. Our data are in line with those of Hope et al. (2002) who described a NK-like cell population in the blood from one-day old calves which proliferated more rapidly than that from older calves [32]. These results suggest



**Figure 5.** IFN- $\gamma$  secretion by neonate and calf NK cells. (A) IFN- $\gamma$  response of NK cells from neonates (open bars) and calves (filled bars) to rhIL-12. Sorted NKp46<sup>+</sup> cells were cultured in the presence of rhIL-2 or rhIL-15 for 7–10 days. IFN- $\gamma$  concentrations in the supernatants were analysed by ELISA after stimulation for 20 h with various concentrations of rhIL-12. Data shown are means  $\pm$  SEM of four individuals. (B) IFN- $\gamma$  response induced in NK cells by cross-linking of their NKp46, CD2 or CD8 receptors. RbIL-2 or rhIL-15 activated NK cells were seeded in plastic wells pre-coated with NKp46, CD2 or CD8 mAbs or without antibody, then incubated for 24 h in the presence or not of rhIL-12 (100 pg/mL). The concentration of IFN- $\gamma$  in the supernatants was analysed by ELISA. Data shown are means  $\pm$  SEM of five individuals. Statistical analyses were carried out with the nonparametric Mann–Whitney test. \*  $p < 0.05$ , \*\*  $p < 0.01$  is the comparison between IFN- $\gamma$  secretion in the presence or absence of antibody cross-linking.

that NK cells from neonates need less time to start active proliferation and this could partly be due to larger numbers of CD25 at their surface [15].

We assayed the cytotoxic properties of NK cells in an NKp46-mediated redirected lysis

and found that neonate NK cells were more cytotoxic than those of calves. Using bovine cells (MDBK line) as targets, we confirmed the higher cytotoxicity of neonate NK cells when expanded with IL-15 but not IL-2. Neonatal NK cells had a slightly higher

NKp46 density than those of calves in both expansion conditions, so this is unlikely to be the main cause of increased cytotoxicity since the IL-2-expanded NK cells killed MDBK equally well. The contribution of other receptors or molecules involved in cytotoxicity should be further investigated, as no difference in intracellular perforin was detected.

In addition to cytotoxicity, another important function of NK cells is to control intracellular pathogens by the production of large amounts of IFN- $\gamma$  [4, 7, 20, 29, 32, 37, 49, 50]. The dose-dependent IL-12 response of calf and neonate NK cells was strongly emphasized by NKp46 cross-linking and moderately by CD2 or CD8 cross-linking. The results obtained with NKp46 and CD2 are in agreement with our previous studies [7, 8], except that we obtained an IFN- $\gamma$  secretion even with CD8 cross-linking, possibly because another CD8 antibody was employed here, which apparently bound a reactive epitope. Thus, in addition to the NKp46 receptor, the CD2 and CD8 receptors apparently play a role in mediating effector functions of bovine NK cells, as described in humans [1, 54, 55, 60].

Finally we noted differences in the responses of NK cells depending on the cytokine used to expand them. IL-2 and IL-15 have several similar biological functions because their signalling occurs via shared IL-2/15R $\beta$  and  $\gamma$ -receptor subunits [12]. However, IL-15 can have other effects; besides its role in NK cell survival, IL-15, unlike IL-2, has anti-apoptotic properties [5, 24, 62] which we also observed. Our IL-15 cultures were more prolific and presented fewer dead cells than IL-2 cultures (data not shown). Neonate and calf NK cells responded differently depending on the cytokine used to expand them. When expanded with IL-15, but not with IL-2, neonate NK cells killed MDBK target cells more efficiently than those from calves did. This is in agreement with human data indicating that IL-15 can act more efficiently than IL-2: (1) on the cytotoxicity of cord blood NK cells in short- and long-term cultures [14, 44, 47], (2) on the restoration of the T lymphocyte cytotoxic function in cancer [13] and (3) for inducing cytotoxicity of intestinal intraepithelial lymphocytes [19]. Regarding IFN- $\gamma$

secretion, IL-2 expanded NK cells stimulated with IL-12 were able to produce IFN- $\gamma$ , while in IL-15 expanded cells the yield was very low for both neonates and calves. In humans, NK cells stimulated with rhIL-12 secreted IFN- $\gamma$  at similar levels whether expanded with rhIL-2 or rhIL-15 [12, 64]; the difference between these two species might be due to the use of heterologous rhIL-15. However, our data on proliferation and cytotoxicity do not support this hypothesis. Moreover, in IL-12/IL-15 stimulated NK cells, the IFN- $\gamma$  yield is still very low after the cross-linking of the CD2 or CD8 receptors, while the cross-linking of NKp46 led to a 4–5 fold increase in secretion of IFN- $\gamma$  by neonate NK cells compared with those of calves. This suggests a crucial role of NKp46 in the activation of neonate NK cells and confirms that they are fully functional. In humans, cord blood NK cells are less functional [23, 26, 33, 38] because mononuclear cells produce less cytokines [41, 51, 52], but when stimulated *in vitro* with IL-12, IL-15 or IL-2, their effector functions are similar to those of adult NK cells [23, 26, 33, 38]. In ruminants, this does not seem to be the case as in goats [58], neonate splenocytes produce much more IL-12 than adult splenocytes when stimulated with TLR ligands. Thus, an adequate *in vivo* cytokine environment produced by monocytes/macrophages might explain the fact that bovine neonate NK cells are already functional; IL-2 produced by T lymphocytes might be present at very low levels during the early stages of the immune response while IL-15 produced by monocytes/macrophages can readily activate NK cells. Therefore, a receptor repertoire modulated by the cytokine environment could partly explain the higher responsiveness of IL-15 expanded neonate NK cells.

In conclusion, although infectious diseases induce high rates of morbidity and mortality in farm animal neonates, their immune system is poorly studied. We present the first comparative study between neonate and calf NK cells. Although less numerous, neonate peripheral blood NK cells proliferate more rapidly, are fully functional and present a higher cytotoxicity and IFN- $\gamma$  response to IL-15 and NKp46 receptor stimulation.

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