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Anatomical distribution of respiratory tract leukocyte cell subsets in neonatal calves

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ABSTRACT

Neonatal calves are highly susceptible to a number of diseases including those that infect via the mucosal surfaces of the respiratory and gastrointestinal tracts. In order to determine appropriate vaccine design and delivery systems, or to identify suitable immunostimulatory methods to combat these infections, a detailed understanding of the immune cell populations present at clinically relevant sites is key. Few studies have assessed the immune cell composition of the neonatal calf lung and comparisons with circulating immune cells in the blood are lacking. We describe immune cell populations present in the peripheral blood, bronchoalveolar lavage (BAL) fluid and lung tissue of young disease-free calves. Flow cytometric analysis revealed significant differences in cell subset distribution between the peripheral blood and respiratory tract, and between compartments within the respiratory tract. Notably, whereas $WC1^+$ γδ TCR + T lymphocytes dominate the peripheral blood, both the BAL fluid and lung tissue contained a high proportion of myeloid cells which expressed CD14 and CD172a (SIRPα). Very low numbers of tissue myeloid cells expressed MHC Class II in comparison to circulating myeloid cells in the blood. Respiratory tract tissues had low frequencies of CD4+ and CD8 + T lymphocytes, which were significantly lower than in the blood. Differences in the proportion of NKp46+ natural killer cells were also observed between tissue compartments. In order to target vaccines or immunostimulatory therapeutics appropriately, these differences in immune cell populations in tissue compartments should be taken into consideration.

1. Introduction

Bovine respiratory disease (BRD) is a major cause of calf mortality and morbidity in both the beef and dairy sectors globally (Johnson and Pendell, 2017). In addition to the economic impacts of the disease, it is a major driver of antibiotic use and poor welfare in cattle production (Fulton, 2009). The disease is multifactorial in nature, with a number of key viral and bacterial pathogens that interact with host factors such as nutrition and immune status to cause disease (Murray et al., 2016a). Whilst highly efficacious vaccines exist for specific respiratory pathogens such as Bovine Herpes Virus 1 (BHV-1), Bovine Respiratory Syncytial Virus (BRSV) and Parainfluenza Virus Type 3 (PI3), there are a number of important pathogens for which respiratory vaccines with demonstrated efficacy are not available e.g. *Mycoplasma bovis*, bovine coronavirus and *Pasteurella multocida* (Calcutt et al., 2018; Dabo et al., 2007; Ellis, 2019; Murray et al., 2016b). Furthermore, due to the structure of the cattle industry, it is often difficult to establish vaccinal immunity prior to the peak disease risk period. This is particularly true in neonatal dairy calves, which are often moved to calf rearing units at under one month of age. In these neonatal calves, colostral antibody transfer is variable, with a significant proportion of calves at increased risk of BRD due to failure of passive transfer (Beam et al., 2009; Cuttance et al., 2017; Todd et al., 2018). Conversely, neonatal calves that have received sufficient maternally derived antibodies (MDA) represent a different challenge, due to the interference of MDA in the establishment of vaccinal immunity (Ellis, 2001; Windeyer and Gamsjager, 2019).

Improving the passive transfer status of neonatal calves will reduce, but not abolish, BRD risk and hence the design of strategies to boost early-life immunity for resilience to BRD is urgently required. Vaccination is the mainstay of disease prevention in the young animal, providing continuing immunity as MDA wanes. Whilst mucosal

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vaccination can circumvent some degree of MDA interference to establish immune memory and protective cell mediated immunity, improvements in both the degree and duration of immune protection are required (reviewed by Osman et al., 2018). There is also increasing interest in the use of immunostimulants, both as novel vaccine adjuvants and to provide non-specific activation of innate immunity at times of increased disease risk (Ilg, 2017).

In order to determine appropriate vaccine design and delivery, or to identify appropriate immunostimulatory methods, a detailed understanding of the immune cell populations present at clinically relevant sites, including the respiratory tract, is key. This will enable targeted approaches. Developing effective strategies to boost early life immunity will also significantly impact on the use of antibiotics, thereby easing the threat of antimicrobial resistance (AMR).

To date, few studies have assessed the immune cell composition of the neonatal calf lung. A number of studies have focused on the peripheral blood as a readily accessible source of cells, but tissue-specific differences exist that are key to providing immunity at local sites and as noted above, mucosal vaccination is likely to be most effective in the young calf. In the blood, significantly higher frequencies of innate effector cells including natural killer (NK) cells and sub-populations of gamma delta T cell receptor-bearing lymphocytes (γδ TCR +) are found in neonatal animals (Boysen and Storset, 2009; Guzman et al., 2014; Kampen et al., 2006). These populations are hypothesized to provide immunity to the young animal as the adaptive immune response develops, but little is known of their frequency or function in the respiratory tract.

Characterizing the cell populations present in compartments of the respiratory tract will aid the design and development of improved intervention strategies for diseases including BRD. This could significantly impact on early life immunity and provide large health and economic benefits to the cattle industry.

2. Materials and methods

2.1. Animals & sample collection

This study received ethical approval (study 17.18) from the Royal (Dick) School of Veterinary Studies (R(D)SVS) Veterinary Ethics Research Committee (VERC).

Six clinically healthy Holstein-Friesian male calves between the ages of 12–24 days were purchased from a commercial dairy facility. These animals were not vaccinated. The animals were housed on straw and fed 3 L of powdered milk replacer twice a day for 1–3 days. The calves were humanely euthanized by captive bolt and death was confirmed by auscultation. Immediately post-mortem, nasopharyngeal swabs were taken, and blood was collected into lithium heparin tubes from the jugular vein. Peripheral blood mononuclear cells (PBMC) were separated as previously described (Hamilton et al., 2016). Cells were frozen in Fetal Calf Serum (FCS, Thermo Fisher, USA) containing 10 % DMSO (Sigma-Aldrich) and stored at −155 °C prior to being characterized by flow cytometry.

The lungs of each calf were thoroughly inspected for lesions characteristic of BRD complex infection following the post-mortem lung evaluation methods of Leruste et al. (2012) before further processing. Post-mortem examination was carried out with the chest elevated during incision of the trachea to avoid blood contamination. The incised trachea was cleaned, and a funnel inserted prior to lavage to ensure bronchoalveolar lavage (BAL) fluid did not contact the site of the incision thereby minimizing blood contamination. In order to perform BAL, one liter of sterile saline was decanted into the lungs via the trachea, lungs were massaged for 1 min and the BAL fluid was transferred back into a sterile container, and subsequently processed as previously described (Kapetanovic et al., 2013). In summary, BAL fluid was passed through a 40 μm cell strainer (Falcon, Corning, USA) and where necessary, excess red blood cells were lysed.

Immediately following collection of BAL fluid, the left Cranioventral and right Caudodorsal lobes were removed from each set of lungs. Approximately 1 cm^3 of tissue was placed in sterile tubes and tissue resident cells extracted by maceration of the tissue and isolation using Lymphoprep (Price et al., 2010). Viable cells were stored as described above.

Nasal swabs, and samples of BAL fluid and cells, and lung tissue (n = 40) were subjected to a commercial multiplex qPCR for nine common bovine respiratory pathogens (AusDiagnostics, Chesham, UK). A single caudodorsal lung tissue sample from one calf tested positive for *Pasteurella multocida,* whilst a nasal swab from one calf tested positive for bovine parainfluenza virus type-3. All other samples tested negative (data not shown).

2.2. Flow cytometry

Cryopreserved cell samples were thawed and washed in RPMI-1640 (Sigma-Aldrich, USA) supplemented with 10 % FCS, 1% Glutamax (Invitrogen, Thermo Fisher, USA), 100 U /mL penicillin and 100 μg/mL streptomycin (Invitrogen, Thermo Fisher, USA). Viable cells were enumerated using a haemocytometer and resuspended in blocking buffer: PBS with 5% normal goat serum (MP Biomedicals, UK). Primary,

Table 1
Antibodies Used in Flow Cytometry.

Molecule	Clone	Species	Conjugate	Isotype	Optimized Conc.	Source
CD3	MM1A	Mouse anti-bovine	None	IgG1	1 μg/mL	Washington State MAB
CD4	CC30	Mouse anti-bovine	None	IgG1	5 μg /mL	Institute for Animal Health
CD8	CC63	Mouse anti-bovine	None	IgG2a	10 μg/mL	BioRad
CD14	Tuk4	Mouse anti-human	AF-647	IgG2a	1 μg/mL	BioRad
CD16	KD1	Mouse anti-human	FITC	IgG2a	0.5 μg/mL	BioRad
CD172a	CC149	Mouse anti-bovine	None	IgG2b	2.5 μg/mL	BioRad
MHC Class II	CC158	Mouse anti-bovine	None	IgG2a	Neat (culture sup.)	Institute for Animal Health
NKp46	Gr13.1	Mouse anti-ovine	None	IgG1	3 μg/mL	BioRad
WC1	CC15	Mouse anti-bovine	FITC	IgG2a	12.5 μg/mL	BioRad

Reactivity	Species	Isotype	Conjugate	Source
Anti-Mouse IgG1	Goat	IgG1	AF-647	Life Technologies
Anti-Mouse IgG2a	Goat	IgG2a	FITC	BioRad
Anti-Mouse IgG2b	Goat	IgG2b	PE	BioRad
Isotype control	Mouse	IgG2a	FITC	Miltenyi Biotech
Isotype control	Mouse	IgG2a	Unconjugated	BioRad
Isotype control	Mouse	IgG2a	AF-647	Biolegend
Isotype control	Mouse	IgG2b	Unconjugated	BioRad
Isotype control	Mouse	IgG1	Unconjugated	Southern Biotech

secondary, and isotype matched control antibodies (Table 1) were diluted in blocking buffer to pre-determined optimal concentrations and incubated for 30 − 60 min on ice. Cells were washed with PBS three times between each step. Zombie Violet (BioLegend, USA) viability dye was added at 1:1000 dilution in PBS and incubated at room temperature for 15 min. The Zombie Violet dye was removed by washing in PBS prior to fixation in 2% paraformaldehyde in PBS before analysis. Cell surface molecule expression was determined using a BD LSR Fortessa (Becton Dickinson and Company, USA) flow cytometer. For each sample, a minimum of 10,000 events were collected and data analyzed using FlowJo_v10 (Becton Dickinson and Company, USA).

Compensation was calculated using FlowJo and applied to all samples to account for spectral overlap of the fluorochromes utilized: Fluorescein isothiocyanate (FITC, B530/30 nm), Phycoerythrin (PE, B586/15 nm), and Alexa Fluor 647 (AF-647, R670/14 nm). Isotype and concentration-matched control antibodies were used to validate that there was no non-specific binding. Gates for analysis were set using an FMO control or unstained cells (+ Zombie Violet) where there was a clear distinction in the positive and negative populations.

2.3. Statistical analysis

All statistical analyses and graphics were carried out using R (v 3.6.3 (c) 2020 The R Foundation for Statistical Computing). Figures were drawn using the *ggplot2* (v3.2.1 Wickham (2016)) and *wesanderson* (v0.3.6 Ram and Wickham (2018)) packages. Statistical analysis of differences in cell subset percentages between the different sampled tissues (PBMC, BAL fluid, Cranioventral Lung, Caudodorsal Lung) were carried out in a two-stage process. Overall differences were established using linear mixed-effect models (LME) with calf ID entered as the random effect to account for samples coming from the same calves and duplicate samples, and Sample area as a fixed effect. If overall differences were found then *post hoc* pairwise Tukey comparisons were performed on the LME models. LME models were run using the *'lme4'* (v1.1-21 Bates et al. (2015)) and *'lmerTest'* (v3.1-1 Kuznetsova et al. (2017)) packages and the *post hoc* Tukey comparisons were run using the *'multcomp'* package (v1.4-12 Hothorn et al. (2008)). $P < 0.05$ was taken to indicate statistical significance throughout.

3. Results and discussion

This study aimed to investigate the frequency of myeloid cells and lymphocytes in the respiratory tract of young, clinically healthy calves. Calves were shown to have minimal presence of typical BRD pathogens and no clinical signs of respiratory disease.

In order to characterize the mononuclear cell subsets from the peripheral blood, BAL and lung tissues, cells were stained with monoclonal antibodies specific for a range of cell surface molecules enabling detection of subsets of lymphocytes and myeloid cells. In each of the cell preparations, live cells were gated for further analysis based on Zombie Violet staining followed by exclusion of debris and doublets (Supplementary Fig. 1).

Within the PBMC, there was significant variability in the proportion of CD3+ T lymphocytes between animals (Fig. 1a). These T lymphocytes were largely WC1+ γδ TCR-expressing cells (Fig. 1b), with smaller proportions of cells expressing CD4 (Fig. 1c) and CD8 (Fig. 1d). These findings are largely in agreement with previously published data from Menge et al. (1999) and Wilson et al. (1996) who described relatively low proportions of CD4+ and CD8+ cells in the peripheral blood of young calves. The predominance of workshop cluster (WC)1+ γδ TCR-expressing T cells in the peripheral blood of young ruminants has been previously described, and the functional activity of these cells is hypothesized to contribute significantly to early life immunity in calves (Guzman et al., 2014). By contrast, only low numbers of T lymphocytes were present in the BAL fluid and lung tissue (Fig. 1). The majority of lung γδ TCR + T cells in young calves co-express the WC1 receptor and although the number of these cells in lung tissue was reported to be relatively low, it was significantly increased following intranasal BCG vaccination (Price et al. (2010) indicating an important role for these cells in immune protection. More recently Guerra-Maupome and McGill (2019) reported that the effector memory induced by aerosol vaccination with BCG was within the γδ TCR + compartment suggesting these cells are important targets for respiratory tract vaccination. There is also evidence that WC1+ γδ TCR + cells play a critical role in the early recognition of bovine respiratory syncytial virus (BRSV) (McGill and Sacco, 2016), an important causative agent of BRD in neonatal calves.

By contrast, the BAL fluid and lung tissues comprise mainly cells of the myeloid lineage which express CD14 (Fig. 2a) and CD172a (SIRPα (Fig. 2b). Within the BAL fluid and lung tissues, only very small proportions of cells expressing CD16 were observed, and the level of expression was low (Fig. 2c; Supplementary Fig. 2F–H). The presence of myeloid cell subsets with classical ($CD14^+CD16^{lo}$) and intermediate ($CD14^+CD16^+$) phenotype within the peripheral blood of cattle has been previously described (Corripio-Miyar et al., 2015). These broadly represent similar subsets of myeloid cells extensively described in humans and other species, which perform differential functions within immune responses. The BAL fluid cellular composition with respect to CD14, CD172a and CD16 was very similar to that of the cranioventral lung, whereas the caudodorsal lung tissue contained significantly fewer cells expressing CD14 and CD172a. These findings are in contrast to that reported by Pringle et al. (1988) who showed that there were no significant differences between BAL fluid collected from cranioventral and caudodorsal regions of the lung (Pringle et al., 1988). Detailed analysis of the myeloid cells within tissue compartments will likely reveal differences in phenotype, and subsets of myeloid cells in tissue compartments, that relate to diverse functions; these analyses were limited in the present (pilot) study. Further studies of the myeloid cell subsets, and their functions, are required to determine the significance of any difference between lung compartments in terms of response to infectious challenge or vaccination. The cranioventral lung region is the most likely to be colonized by pathogens that are inhaled into the lungs (Osman et al., 2018), therefore tissue differences between lung lobes could influence immune responses and therefore defining these carefully could have implications for disease control. Alternatively, the differences observed could reflect pathogen exposure in neonatal calves. Further studies are planned within our laboratory to examine this and the relative expression of CD14, CD16, CD172a on subsets of cells alongside other molecules including CSF1, CSF1R, ADGRE1 and CX3CR1.

Interestingly, very few of the BAL fluid and tissue cells analyzed here expressed MHC Class II and the expression was of low intensity (Fig. 2d; Supplementary Fig. 2J–L). This is in contrast with the peripheral blood where greater numbers of cells expressed MHC Class II. Further analysis based on FSC-A and SSC-A characteristics revealed that in peripheral blood both monocytes and lymphocytes expressed MHC Class II with lower level expression observed on lymphocytes (Supplementary Fig. 2I). Previous work assessing the composition of BAL fluid in mice showed the percentage of MHC II + cells to be low at around 2% (van Rijt et al., 2004). Within the mononuclear BAL fluid cell population, cells with dendritic morphology were shown to express MHC Class II at a higher level (van Rijt et al., 2004). Other studies have suggested that interstitial macrophages have higher levels of MHC Class II and that cells at different differentiation or tissue migration stages show variable expression of this molecule (Franke-Ullmann et al., 1996). Resting myeloid cells such as those expected to be found in naïve animals are likely to have low level MHC Class II expression that may be upregulated upon inflammation (Moghaddami et al., 2005) but it remains to be determined whether this is the case in ruminants. Additional studies are required in naïve, and pathogen exposed/infected animals to determine whether MHC Class II is regulated or expressed by different cell subpopulations within the tissues.

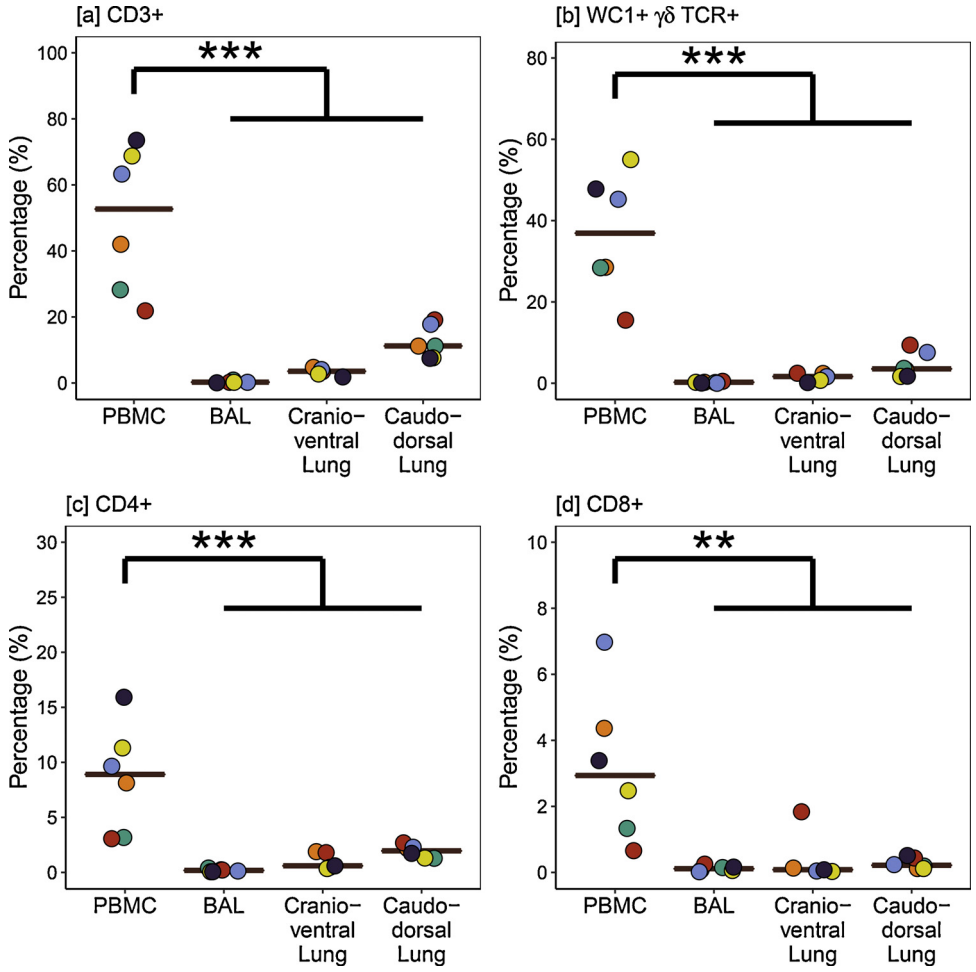

Fig. 1. Comparison of T lymphocyte populations in blood and respiratory tract of neonatal calves.
Cells were isolated from the peripheral blood (PBMC), by lung lavage (BAL) and from the cranioventral and caudodorsal lung lobes of six calves. These were assessed by flow cytometry for the expression of (a) CD3 (b) WC1 (c) CD4 (d) CD8. Mixed-effect and *post hoc* pairwise Tukey statistical analyses were carried out in R. *, **, *** denote p values of < 0.05, 0.01 and 0.001 respectively. Each symbol denotes an individual animal.

Natural killer (NK cells) were present in each of the samples assessed, although the frequency was very low in the BAL fluid (Fig. 3). Considerable variation between animals in NK cells was noted, but there were significant differences between the PBMC, lung lobes and the BAL fluid. Notably, the frequency of NK cells in the caudodorsal lung was significantly higher than all of the other sites ($p < 0.001$). Significant differences in the frequency and phenotype of NK cells in young calves when compared to adult cattle have previously been reported (Graham et al., 2009; Kampen et al., 2006). Kampen et al., reported that the frequency of peripheral blood NKp46 + cells was highly variable between individual animals and hypothesized that this was potentially due to transient sub-clinical infections affecting the recirculation of cells through blood and tissue sites (Kampen et al., 2006). In neonatal calves, alterations in the peripheral blood population of NK cells either between individuals or over time could also reflect colonization of the gut and respiratory tract in early life: differences were observed in the frequency of NK cells in gnotobiotic (specific pathogen free) and conventional newborn calves within the first days of life (Graham et al., 2009). There is an increasing body of evidence to suggest that NK cells are central to BCG vaccine induced immunity in neonatal calves and humans (Hamilton et al., 2016; Semple et al., 2011) suggesting important roles as vaccine targets for early life immunity. A number of studies have demonstrated that NK cells present at, or underlying, mucosal surfaces provide protective immunity via secretion of IL-22 that may be particularly important where viral infections are succeeded by secondary bacterial colonization (Barthelemy et al., 2018; Ivanov et al., 2013). This is a common feature of BRD and therefore developing strategies to target NK cell activation may hold promise. Further studies of the function and phenotype of mucosal NK cells, determining whether these express CD16, or exist as subsets defined by CD2 expression as reported for other tissues (Boysen and Storset, 2009) will be important in this context.

In summary, significant differences between cell populations in different tissues should be taken into account when designing vaccines or other intervention strategies to enable maximum induction of protective immunity. Further studies of naïve calves, and those vaccinated or exposed to pathogens at mucosal sites are ongoing and will further extend the findings reported here.

Declaration of Competing Interest

None.

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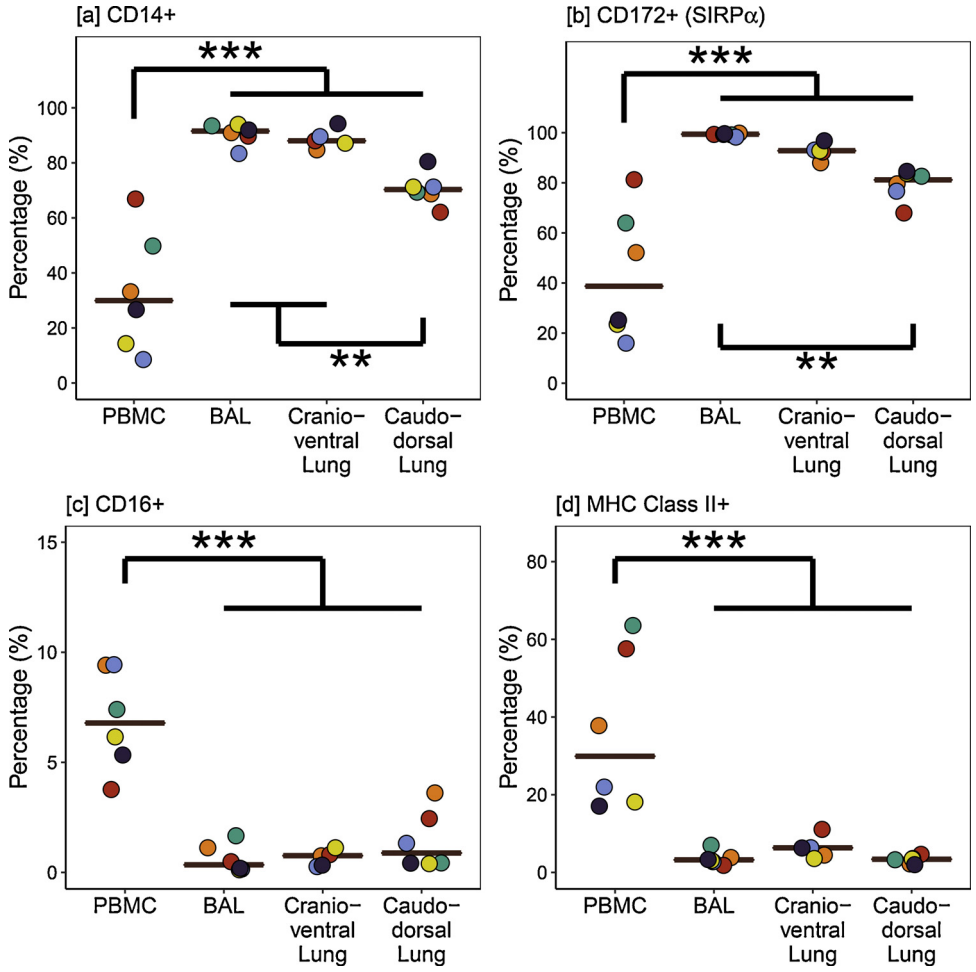

Fig. 2. Comparison of cell populations in blood and respiratory tract of neonatal calves.
Cells were isolated from the peripheral blood (PBMC), by lung lavage (BAL) and from the cranioventral and caudodorsal lung lobes of six calves. These were assessed by flow cytometry for the expression of (a) CD14 (b) CD172a (SIRPα) (c) CD16 (d) MHC Class II. Mixed-effect and *post hoc* pairwise Tukey statistical analyses were carried out in R. *, **, *** denote p values of < 0.05, 0.01 and 0.001 respectively. Each symbol denotes an individual animal.

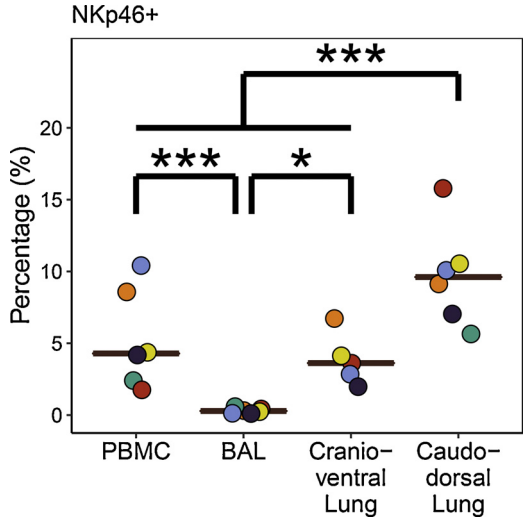

Fig. 3. Comparison of NK cells in blood and respiratory tract of neonatal calves.
Cells were isolated from the peripheral blood (PBMC), by lung lavage (BAL) and from the cranioventral and caudodorsal lung lobes of six calves. These were assessed by flow cytometry for the expression of NKp46. Mixed-effect and *post hoc* pairwise Tukey statistical analyses were carried out in R. *, **, *** denote p values of < 0.05, 0.01 and 0.001 respectively. Each symbol denotes an individual animal.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.vetimm.2020.110090.

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