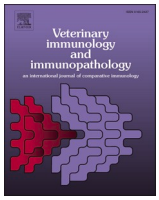
Bovine NK subsets in the afferent lymph and lymph nodes have distinct expression of naïve and activation-associated cell surface expressed molecules, and are differentially stimulated by BCG vaccination

Veterinary Immunology and Immunopathology 266 (2023) 110682

Contents lists available at ScienceDirect

Veterinary Immunology and Immunopathology

journal homepage: www.elsevier.com/locate/vetimm



Bovine NK subsets in the afferent lymph and lymph nodes have distinct expression of naïve and activation-associated cell surface expressed molecules, and are differentially stimulated by BCG vaccination

Andrew J. Hanton [a], Lindsey A. Waddell [a], Jayne C. Hope [a], Mark Gray [b], Zhiguang Wu [a,*]

[a] The Roslin Institute, University of Edinburgh, EH25 9RG, UK
[b] Royal (Dick) School of Veterinary Studies, University of Edinburgh, EH25 9RG, UK

ARTICLE INFO

Keywords:
Bovine tuberculosis
Bovine natural killer cells
BCG
Flow cytometry

ABSTRACT

Bovine natural killer (bNK) cells are heterogeneous cell populations defined by constitutive expression of the natural cytotoxicity receptor, NKp46 (CD335). Two major subsets of bNK cells, classified by differential expression of CD2, display divergent functions in innate immunity, and are hypothesised to contribute to adaptive immunity following vaccination. Here we characterised phenotypic variation of bNK cells within afferent lymph and lymph node (LN) tissues and between $CD2^+$ and $CD2^-$ bNK subsets, and report phenotypic changes induced by BCG vaccination. $CD2^-$ bNK cells, which dominate in the afferent lymph and LN, displayed lower expression of the activation marker CD25 within the LN, with $CD25^+$ cells being less than half as frequent as in afferent lymph. Furthermore, we found bNK cells had a lower expression of CD45RB, associated in cattle with naïve cell status, within LN compared to afferent lymph. Following BCG vaccination, bNK cells in afferent lymph draining the vaccination site showed increased $CD2^-CD25^+$ frequencies and increased expression of CD25 on $CD2^+$ bNK cells, although the frequency of these cells remained unchanged. In summary, we provide an overview of the phenotype of bNK cells within bovine lymphatic tissues, and provide an indication of how subsets may diverge following BCG vaccination.

1. Introduction

Bovine tuberculosis (bTB) is a respiratory disease of cattle caused by the intracellular bacterium *Mycobacterium bovis* (Domingo et al., 2014), causing significant economic and human and animal health concerns. Over 29,000 cattle were culled in England and Wales in the year to March 2023 due to bTB incidents while Scotland remains bTB-free (Defra, 2023). The Bacille Calmette-Guérin (BCG) vaccine is not routinely given to cattle in the UK as a result of confounding statutory diagnostic tests for bTB, although trials in cattle indicate high levels of protection (Hope et al., 2005; Nugent et al., 2018; Thom et al., 2012), and newly developed DIVA (differentiating infected from vaccinated animals) skin tests could circumvent this issue (Jones et al., 2022). A meta-analysis of studies performed in cattle demonstrated significant potential for BCG vaccination to impact bTB disease control (Srinivasan et al., 2021), although the vaccine does not induce sterile immunity. As such, understanding the molecular and cellular mechanisms underpinning bovine immunity to *M. bovis* is critical in developing improved, effective vaccines. Furthermore, cattle and humans share several elements of tuberculosis (TB) biology including immune responses and correlates of immunity. Therefore, cattle represent an effective one-health model of TB that is mutually beneficial for both veterinary and medical research into TB immunology and vaccine development (Buddle et al., 2005; Waters et al., 2014; Waters et al., 2011). This becomes particularly valuable as the BCG vaccine lacks high efficacy in adult humans, and as such novel vaccine development is similarly a priority in human TB eradication (Fogel, 2015).

One subset of innate immune cells implicated in BCG-induced immunity are natural killer (NK) cells, characterised by the expression of cytotoxic effector molecules and potent secretion of IFN-γ (Boysen and Storset, 2009). Upon stimulation with BCG, human NK cells undergo enhanced secretion of pro-inflammatory cytokines following vaccination compared to pre-vaccination (Kleinnijenhuis et al., 2014) and NK cells are implicated in immune responses to *M. tuberculosis* (Esin and Batoni, 2015). Bovine NK cells comprise up to 10% of total lymphocytes in peripheral blood and lymphatic tissues and can be defined by

* Corresponding author.
E-mail address: zhiguang.wu@roslin.ed.ac.uk (Z. Wu).

https://doi.org/10.1016/j.vetimm.2023.110682
Received 8 September 2023; Received in revised form 10 November 2023; Accepted 14 November 2023
Available online 19 November 2023

expression of the NK cell receptor NKp46 and a lack of the T cell marker CD3 ($NKp46^{+}CD3^{-}$) (Storset et al., 2004). Furthermore, bNK cells can be divided into two major subgroups: $NKp46^{+}CD3^{-}CD2^{+}$ and $NKp46^{+}CD3^{-}CD2^{-}$, the major populations within blood and lymphatic tissues, respectively (Boysen et al., 2008; Hamilton et al., 2017a). $CD2^{-}$ bNK cells have a higher expression of the IL-2 receptor α chain CD25, indicating a higher activation status and receptiveness to IL-2 signalling (Boysen et al., 2006). Indeed, $CD2^{-}$ bNK cells proliferate more rapidly in vitro and secrete more IFN-γ when cultured with IL-2 compared to $CD2^{+}$ bNK cells (Boysen et al., 2006; Hamilton et al., 2016). Critically, $CD2^{-}$ bNK cells are preferentially activated by BCG stimulated- or *M. bovis*-infected bovine dendritic cells (DCs) compared to $CD2^{+}$ bNK cells (Hamilton et al., 2016; Siddiqui and Hope, 2013), and are therefore implicated in responses to vaccination and infection.

Following BCG vaccination, antigen delivery to the draining lymph node (LN) is achieved by the trafficking of immune cells from the skin via the afferent lymphatics (Hope et al., 2012; Tozuka et al., 2016). In cattle, these draining immune cells include T cells, DCs, and bNK cells (Hamilton et al., 2017b; Lund et al., 2013; Marzo et al., 2022; Van Rhijn et al., 2007). Within afferent lymph draining the skin, $CD2^{-}$ bNK cells are predominant, and lymph bNK cells can be induced to express high levels of IFN-γ (Lund et al., 2013). Given the role of $CD2^{-}$ bNK cells in interactions with BCG exposed DCs following BCG vaccination, their predominance in lymphatic tissues, and the importance of IFN-γ for BCG induced immune protection (Hope and Vordermeier, 2005), bNK cells are a valuable focus of investigations into BCG vaccine-mediated immunity to bTB.

The development of a flow cytometry panel including NKp46, CD3 and CD2 represents a simple and highly effective mechanism for the phenotypic characterisation of bNK cells (Bryceson et al., 2010). In this study, we employed a surgical cannulation bovine model and a six-colour flow cytometry panel to characterise the cell surface phenotype of bNK cells in lymphatic tissues, and performed a comparative analysis of bNK cell phenotypes pre- and 24 h post-BCG vaccination. We demonstrate that BCG vaccination results in differential activation of afferent lymph bNK cell subsets.

2. Materials and methods

2.1. Animals and sample collection

Samples from eight male British Holstein Friesen cows (*Bos taurus*) between three and six months of age were used in this study. Animals were housed at the University of Edinburgh Langhill and Dryden Farms, and at the Large Animal Research Imaging Facility. All experimental protocols were authorised under the UK Animals (Scientific Procedures) Act, 1986, and performed according to Home Office guidelines with approval from The Roslin Institute's local Animal Welfare and Ethical Review Board.

Peripheral blood, afferent lymph and right pre-scapular lymph node (RPSLN) sample collection and preparation were conducted as described previously (Hamilton et al., 2017b; Hope et al., 2006). Briefly, peripheral blood samples were collected by jugular venepuncture into sodium heparin (Fisher Scientific, UK) prior to RPSLN removal and peripheral blood mononuclear cells (PBMCs) were separated by density gradient centrifugation using Lymphoprep™ (STEMCELL Technologies, UK). RPSLNs were removed surgically and processed to isolate mononuclear cells. Calves were allowed to recover from surgery and housed for a further 6–8 weeks. Following LN removal, afferent and efferent lymphatic vessels re-anastomose to form larger vessels receptive to cannulation (Hope et al., 2006). Two months after LN removal, pre-scapular lymphatic vessels were cannulated and 'pseudo-afferent' lymph fluid collected. Whole lymph was centrifuged at 400 x g for 5 min to pellet the cells. Calves were vaccinated with BCG Danish ($\sim 1 \times 10^{6}$ colony forming units; SSI, Denmark) subcutaneously 14 days post-cannulation (DPC). Peripheral blood, afferent lymph and RPSLN samples were cryopreserved in foetal bovine serum/10% DMSO (Sigma Aldrich) at − 155 °C until required.

2.2. Flow cytometry

Cryopreserved cell samples were thawed and washed in warm PBS. Viable cells were counted using a haemocytometer and resuspended in blocking buffer: either PBS with 1% bovine serum albumin (BSA) (Sigma-Aldrich) or PBS with 5% normal goat serum (Abcam). Fluorescent labelled monoclonal antibodies (mAbs) were diluted in blocking buffer to pre-determined optimal concentrations (Table 1). Cells were incubated with mAbs for 30 min on ice. Samples were pooled between calves for fluorescence minus one (FMO) control staining due to low number of cells. Cells were then washed with PBS three times. Following antibody staining, cells were incubated with Zombie yellow or NIR (BioLegend, USA) viability dye at room temperature for 15 min. Cells were then washed three times with PBS and fixed with 2.0% paraformaldehyde and stored at 4 °C prior to analysis with an LSRFortessa flow cytometer (BD Biosciences). A minimum of 50,000 events were collected. Data were analysed using FlowJo™ v10.8 Software (BD Life Sciences). Lymphocytes were selected based on the FSC-A v. SSC-A profile, dead cells were excluded by the Zombie dyes and doublets omitted based on signal processing (FSC-A/H). Isotype control antibodies were used to validate the absence of non-specific binding. Gates were set using FMO controls (Fig. 1).

2.3. Statistics

Statistical analysis was performed using GraphPad Prism 7.0 (GraphPad, US). Mean bNK cell proportions across biological replicates were compared between afferent lymphatic sample timepoints post-surgical cannulation by paired t-tests (two-tailed). Pre- and post-BCG vaccination analyses were similarly made by paired t-tests (two-tailed) to compare bNK cell proportions and mean fluorescence intensity (MFI). P-values were adjusted using the Bonferonni-Dunn method to account for multiple testing, and statistical significance of adjusted p-values was defined as follows: *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$.

3. Results and discussion

This study aimed to perform comparative analyses of bNK cells within naïve and post-BCG vaccination lymphatic tissues using a multicolour flow cytometry panel. The six-colour flow cytometric panel was

Table 1
Cell surface molecules targeted in experimentation.

Marker	Marker Description	mAb Clone Number	mAb Isotype	mAb Conjugate	Cytometer Laser and Filter
NKp46	bNK cell surface receptor	Gr13.1	IgG1	Pacific Blue (PB)	V450/50
CD2	Adhesion molecule; activation receptor on bNK and T Cells	CC42	IgG1	Fluorescein isothiocyanate (FITC)	B530/30
CD25	Interleukin-2 receptor α chain; activation marker	IL-A111	IgG1	Alexa fluor 568 (AF568)	YG610/20
CD3	T Cell marker	MM1A	IgG1	Alexa fluor 647 (AF647)	R670/14
CD45RB	Marker of naïve lymphocytes	CC76	IgG1	PE Cyanine 7 (PeCy7)	YG780/60

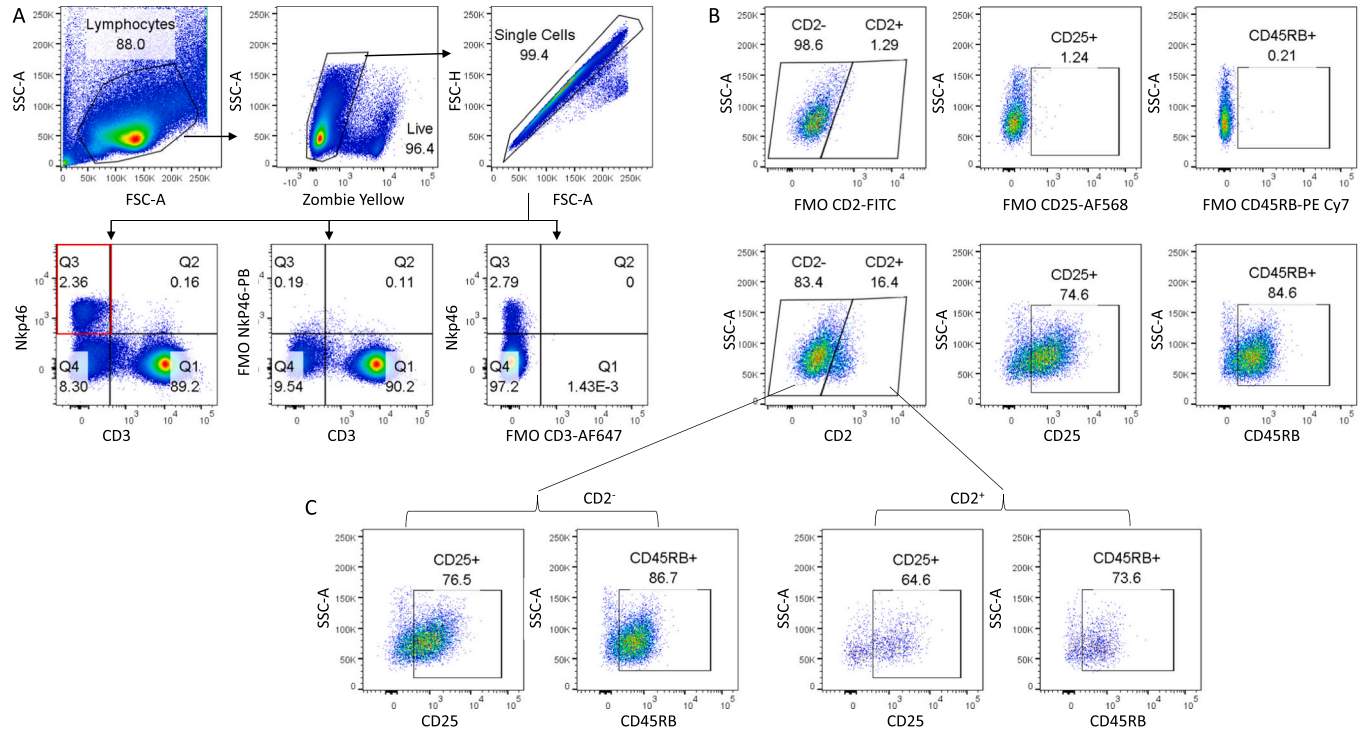


Fig. 1. Gating strategy for analysis of afferent lymph cells by flow cytometry. (A) Cells were first gated on their FSC-A/SSC-A properties to exclude debris (Lymphocytes). Non-viable cells were then excluded by gating cells negative for Zombie yellow viability dye (Live). Doublets were excluded by gating of FSC-A/FSC-H properties (Single cells). From the Single Cells gate, NK cells were identified as NKp46^{+}CD3^{-} (red box). FMOs for NKp46-Pacific Blue (NKp46-PB) and CD3-Alexa Fluor 647 (AF647) are shown on the right. (B) The expression of CD2, CD25 and CD45RB were assessed on the NK cells (NKp46^{+}CD3^{-} gate). The FMOs for CD2-FITC, CD25-AF568 and CD45RB-PE-Cy7 are shown above. (C) The expression of CD25 and CD45RB were assessed on CD2^{+} and CD2^{-} NK cell subsets. Plots show analysis from one BCG-naïve animal.

first developed using PBMCs from two BCG-naïve calves to optimise staining and gating strategies (Supplementary Figure 1). Cells were gated to exclude debris, dead cells and doublets. Gating of NKp46, CD2, CD25, CD3 and CD45RB was set on relative FMOs (Fig. 1).

The optimised flow panel was then utilised to characterise bNK cell phenotype in afferent lymphatic fluid at early and late timepoints post-surgical cannulation. Overall proportions of bNK cells were previously reported to remain unchanged following lymphatic cannulation (Marzo et al., 2022). Comparing early afferent lymph (EAL, collected two days post-cannulation (DPC)) and late afferent lymph (LAL, collected 15–18 DPC), a non-significant ($p > 0.05$) increase in NKp46^{+}CD3^{-} cells as a proportion of the total live lymphocytes was observed in LAL (Fig. 2A), which may be the result of absolute changes in other cell types following cannulation-induced inflammation. For example, bovine skin-dwelling γδ T cells migrate in increased numbers in the afferent lymph following inflammation: this was also demonstrated for B cells and neutrophils in humans, and γδ T cells and DCs in sheep (Egan et al., 1996; Geherin et al., 2012; Van Rhijn et al., 2007; Voisin and Nourshargh, 2019). The proportion of bNK cells with different phenotypes within afferent lymph remained similar between EAL and LAL, however the percentage of CD45RB^{+} bNK cells was significantly higher in LAL relative to EAL ($p = 0.045$) (Fig. 2A). Given this, bNK cell proportions and phenotypes remain largely unchanged following surgical cannulation.

bNK cell phenotypes in the RPSLN were similarly studied (Fig. 2B). The proportion of bNK cells expressing CD25 was markedly lower in RPSLN (30.98%) versus either EAL (66.02%) or LAL (61.78%), reflecting a lower proportion of bNK cells with an active phenotype in this tissue. Bovine NK cells upregulate CD25 expression in response to stimulation with pro-inflammatory cytokines IL-12 and IL-15 (Endsley et al., 2006), a process shown in humans to be blocked by T regulatory cells (Gasteiger et al., 2013). As such, bNK CD25 expression may be lowered within the LN by immunological homoeostatic mechanisms mediated by other lymphocytes found within this tissue. Similarly, we observed lower expression of CD45RB in bNK cells found within the RPSLN (26.22%) compared to both EAL (43.52%) and LAL (52.03%). Previous research showed approximately half of bNK cells in peripheral blood express CD45RB (Graham et al., 2009), a finding replicated here (Supplementary Figure 1). In cattle, CD45RB corresponds with an immunologically naïve status, similar to CD45RA expressed by quiescent non-activated human NK cells (Krzywinska et al., 2016). The reduction in CD45RB expression in RPSLN bNK cells relative to those in afferent lymph may be the result of bNK cell maturation in the LN. Indeed, human NK cells are known to undergo maturation processes in LNs that involve the loss of CD45RA (Abel et al., 2018). In addition, CD2^{+} and CD2^{-} subsets of bNK were compared for the expression of CD25 and CD45RB within lymphatic tissues (Fig. 2C-H). The proportion of CD25^{+} cells was significantly lower in CD2^{-} subset than CD2^{+} subset in RPSLN ($p = 0.003$) but not EAL or LAL (Fig. 2C-E). The percentage of CD45RB^{+} population was higher in CD2^{-} subset relative to CD2^{+} subset in both LAL ($p = 0.016$) and RPSLN ($p = 0.015$) but not EAL (Fig. 2F-H). Collectively, these data reinforce the hypothesis of differential roles for CD2^{+} and CD2^{-} bNK cells, however functional characterisation is required to draw further conclusions.

Analysis of afferent lymph bNK cells prior to and 24 h post-BCG vaccination further characterised bNK cell phenotypes. The percentage of NKp46^{+}CD3^{-} bNK cells among total live lymphocytes did not change after BCG vaccination (Fig. 3A), and furthermore no significant changes in CD2^{+}, CD25^{+} or CD45RB^{+} frequencies were observed among bNK cells (Fig. 3B-D), reflecting previous findings that bNK cell activation does not induce changes in CD2 expression (Boysen et al., 2006). Despite CD25^{+} expression being non-significantly increased post-BCG in bNK cells, CD25 expression as determined by MFI was significantly ($p = 0.02$) higher following vaccination (Fig. 3F). This reflects previous

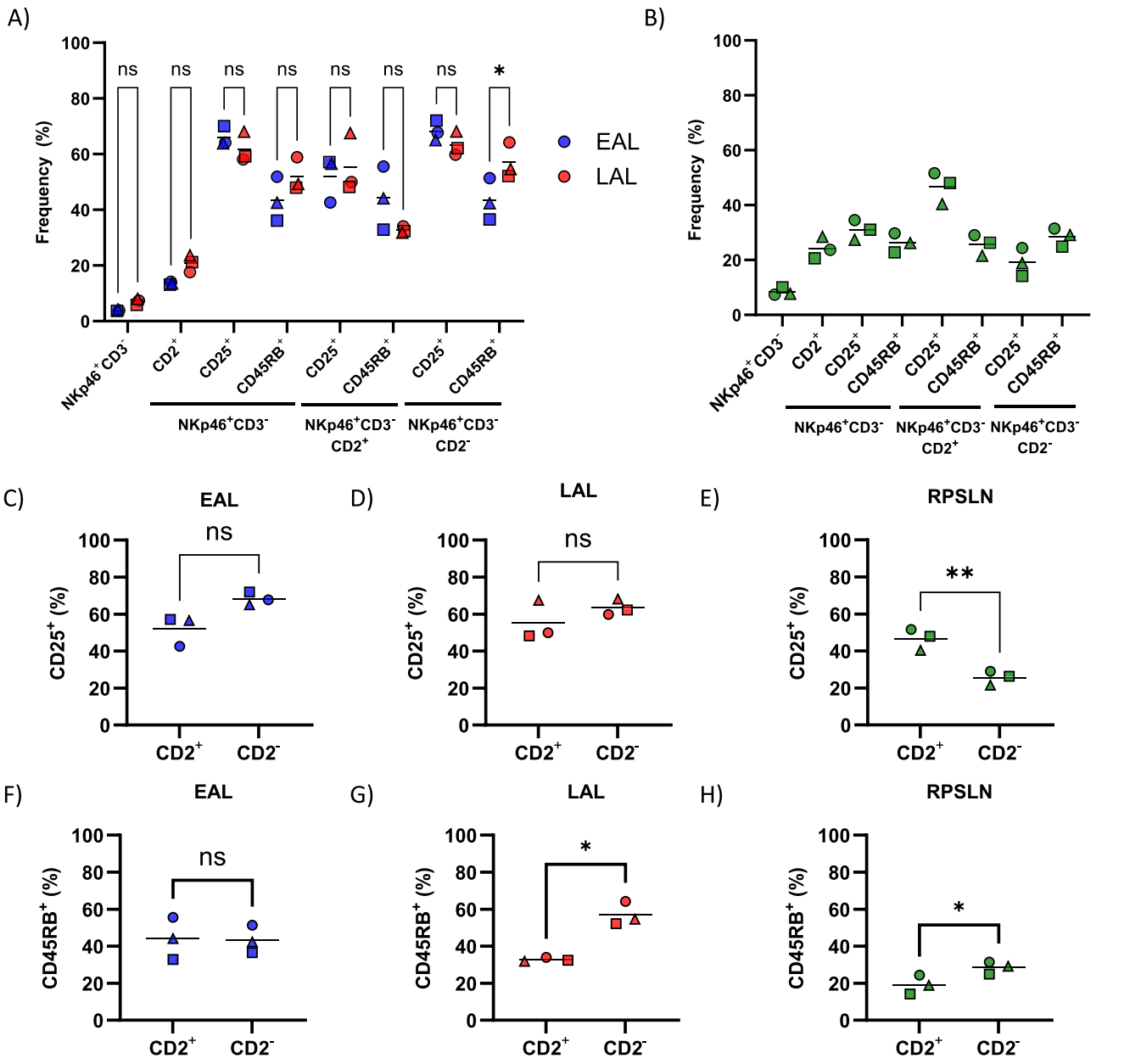


Fig. 2. Phenotypic proportions of bNK cells in lymphatic tissues. Cells were isolated from early afferent lymph (EAL), late afferent lymph (LAL) and right prescapular lymph node (RPSLN) of three calves and assessed by multicolour flow cytometry. Percentages of NKp46^{+}CD3^{-} bNK cells were compared between EAL and LAL (A). NKp46^{+}CD3^{-} cells were assessed for the expression of CD2, CD25 and CD45RB. bNK cells were similarly characterised in RPSLN (B). CD2^{+} and CD2^{-} bNK cell subsets from all three tissues were then compared for CD25^{+} and CD45RB^{+} proportions (C-H). Each symbol denotes an individual animal (n = 3 biological replicates). Values reflect cell proportions among total live lymphocytes and subsequent subpopulations.

findings regarding the activation of bNK cells following BCG vaccination. No significant differences in expression were observed for CD2 or CD45RB post-vaccination (Fig. 3E-G). Given the differential roles of CD2^{+} and CD2^{-} bNK cells, we interrogated these differences by examining CD25 and CD45RB expression among CD2^{+} and CD2^{-} bNK cells following BCG vaccination (Fig. 4). While no significant difference in CD25^{+} proportions was observed among CD2^{+} bNK cells (Fig. 4A), a significant ($p = 0.016$) increase in CD25^{+} proportions among CD2^{-} bNK cells was found (Fig. 4B), in agreement with the reported preferential activation of this subset by DCs following BCG stimulation (Hamilton et al., 2016). The CD2^{-} subset interacts most effectively with DCs, altering their ability to stimulate T cells and influencing Th1 bias through expression of IFN-γ (Siddiqui et al., 2012). Although the percentage of CD2^{+} cells expressing CD25 did not change following BCG vaccination (Fig. 4A), the MFI of CD25 was significantly increased ($p = 0.021$) (Fig. 4E), consistent with a high activation status and a potential role for CD2^{+} bNK cells following BCG vaccination. No change in CD25 MFI was observed among CD2^{-} bNK cells (Fig. 4F). No differences in CD45RB expression by either subset of NK cells were observed following BCG vaccination (Fig. 4C-D, G-H). Further analysis, including studies of the expression of CD45RO by bNK cells could provide further insight into the impact of BCG vaccination on activation and potential switch from naïve to memory state.

Analysis of the impact of BCG vaccination on the expression of IFN-γ by afferent lymph bNK cells could indicate specific roles in the induction of immune bias following BCG vaccination. Examination of the distribution of bNK cells in lymph nodes draining BCG vaccination sites indicates clustering in T cell areas and in proximity to DCs (Ho, Zifko et al., in preparation, data not shown) which could indicate cross-talk between these key cell types to influence the adaptive immune response

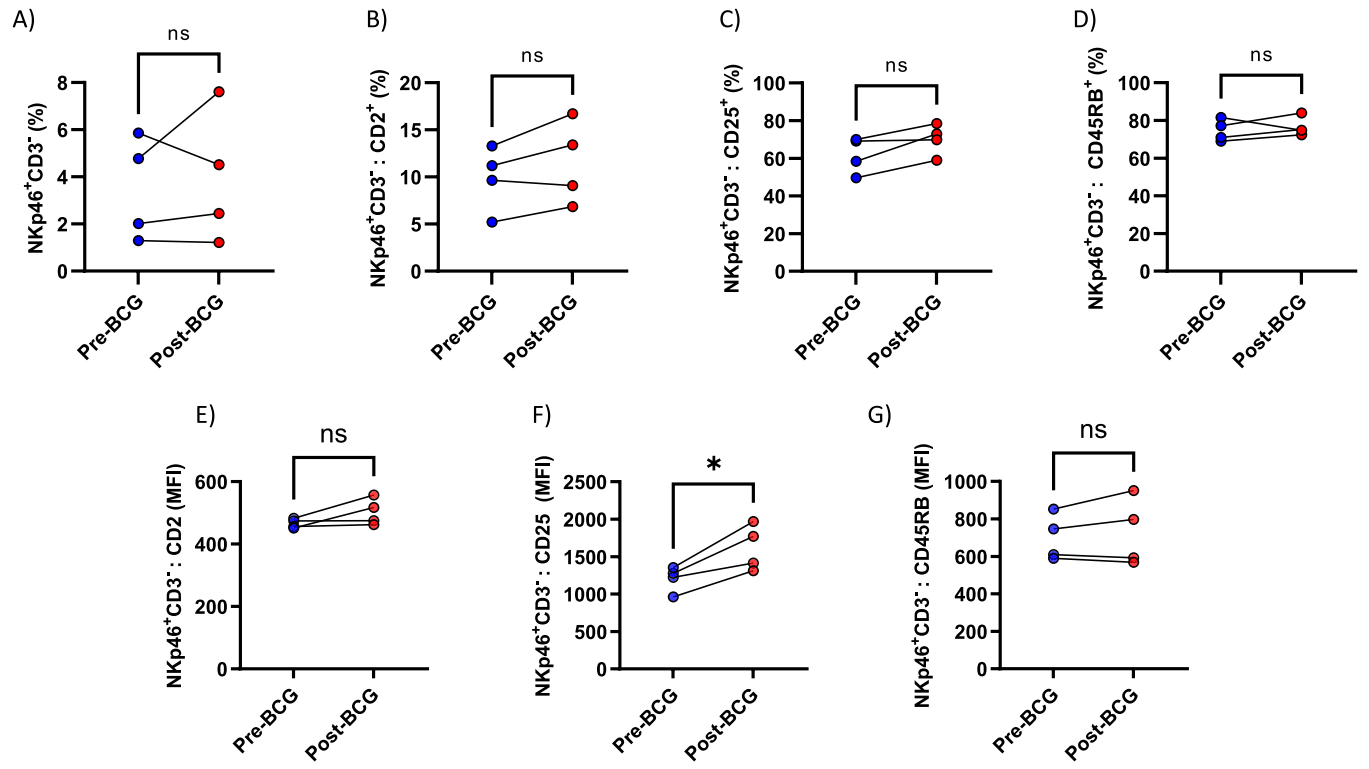


Fig. 3. Comparison of lymph bNK cell proportions from naïve and BCG vaccinated calves. Surgically cannulated calves were vaccinated with BCG directly above the cannulation site. Lymph collected immediately prior to (pre-BCG) and 24 h following vaccination (post-BCG) was assessed for the presence of NK cells ($NKp46^{+}CD3^{-}$, A) and their expression of CD2 (B, E), CD25 (C, F) and CD45RB (D, G). Expression was measured by both positive cell proportions (A-D) and MFI (E-G). Pre-BCG and post-BCG samples were processed and analysed simultaneously. Statistics were calculated by paired t-tests (two-tailed). Each symbol denotes an individual animal (n = 4 biological replicates). Values reflect cell proportions and MFIs among total live lymphocytes and subsequent subpopulations.

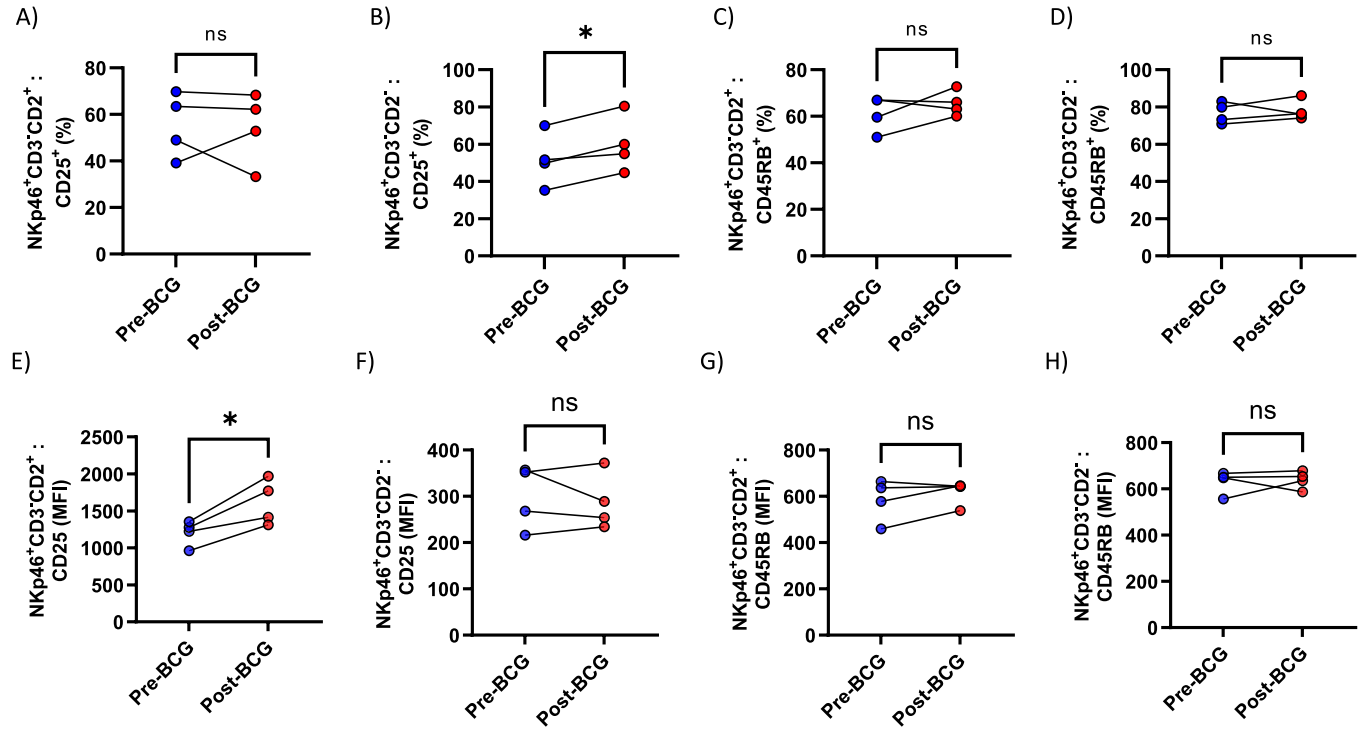


Fig. 4. Comparison of lymph $CD2^{+}$ and $CD2^{-}$ bNK cell subsets from naïve and BCG vaccinated calves. Cells were isolated as described for Figs. 1–3 and assessed by multicolour flow cytometry for the expression of CD25 (percentage (A, B), and MFI (E, F)) and CD45RB (Percentage (C, D) and MFI (G, H)). Pre-BCG and post-BCG samples were processed and analysed simultaneously. Statistics were calculated by paired t-tests (two-tailed). Each symbol denotes an individual animal (n = 4 biological replicates). Values reflect cell proportions and MFIs among total live lymphocytes and subsequent subpopulations.

(Fernandez et al., 2002; Horowitz et al., 2011). In addition, analysis of the cytolytic effector molecules granulysin and perforin, constitutively expressed by bNK cells, could provide further insights. $CD2^+$ and $CD2^-$ bNK cells in peripheral blood have been described as having equal cytotoxic capacity (Boysen et al., 2006; Endsley et al., 2006), however expression of granulysin and perforin increases following bNK cell activation (Endsley et al., 2006) and could have implications for anti-mycobacterial immunity (Denis et al., 2007).

In summary, this paper applied multicolour flow cytometry to the phenotypic characterisation of bNK cells, describing phenotypes within lymphatic tissues. Furthermore, this analysis reinforces the dominance of $CD2^-$ subset of bNK cells within lymphatic tissues and the preferential activation of this subset following BCG vaccination. This phenotypic analysis assessing multiple cell surface markers provides an insightful initial overview of lymphatic bNK cells, and provides a solid foundation for further work.

Funding

AJH was funded by a Wellcome Trust PhD programme grant 218471/Z/19/Z. LAW, JCH and ZW were funded by the Biotechnology and Biological Sciences Research Council (BBSRC) Institute Strategic Programme funding (BBS/E/RL/230002B, BBS/E/D/20002174, BB/CCG2270/1). The afferent lymphatic cannulation work was supported by BBSRC response mode funding (BB/P003958/1) and carried out at the Large Animal Research Imaging Facility (LARIF) at the University of Edinburgh which is funded by BBSRC Institute Strategic Programme funding (BBS/E/RL/23NB0003).

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

We gratefully acknowledge the staff at the LARIF at the University of Edinburgh for their expertise in handling cattle and assistance with sample collection and afferent lymphatic cannulation. We thank Dr Heather A. Mathie and Irene McGuinnes for processing samples and The Roslin Institute Bioimaging and Flow Cytometry Facility staff for training and guidance on flow cytometry.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.vetimm.2023.110682.

References

Abel, A.M., Yang, C., Thakar, M.S., Malarkannan, S., 2018. Natural killer cells: development, maturation, and clinical utilization. Front. Immunol. 9, 1869.
Boysen, P., Gunnes, G., Pende, D., Valheim, M., Storset, A.K., 2008. Natural killer cells in lymph nodes of healthy calves express CD16 and show both cytotoxic and cytokine-producing properties. Dev. Comp. Immunol. 32, 773–783.
Boysen, P., Olsen, I., Berg, I., Kulberg, S., Johansen, G.M., Storset, A.K., 2006. Bovine CD2-/NKp46+ cells are fully functional natural killer cells with a high activation status. BMC Immunol. 7, 10.
Boysen, P., Storset, A.K., 2009. Bovine natural killer cells. Vet. Immunol. Immunopathol. 130, 163–177.
Bryceson, Y.T., Fauriat, C., Nunes, J.M., Wood, S.M., Björkström, N.K., Long, E.O., Ljunggren, H.-G., 2010. Functional analysis of human NK cells by flow cytometry. Methods Mol. Biol. 612, 335–352.
Buddle, B.M., Skinner, M.A., Wedlock, D.N., de Lisle, G.W., Vordermeier, H.M., Glyn Hewinson, R., 2005. Cattle as a model for development of vaccines against human tuberculosis. Tuberculosis 85, 19–24.
Defra, 2023. Quarterly TB in Cattle in Great Britain Statistics Notice: March 2023.
Denis, M., Keen, D.L., Parlane, N.A., Storset, A.K., Buddle, B.M., 2007. Bovine natural killer cells restrict the replication of Mycobacterium bovis in bovine macrophages and enhance IL-12 release by infected macrophages. Tuberculosis 87, 53–62.
Domingo, M., Vidal, E., Marco, A., 2014. Pathology of bovine tuberculosis. Res. Vet. Sci. 97, S20–S29.
Egan, P.J., Kimpton, W., Seow, H.F., Bowles, V.M., Brandon, M.R., Nash, A.D., 1996. Inflammation-induced changes in the phenotype and cytokine profile of cells migrating through skin and afferent lymph. Immunology 89, 539–546.
Endsley, J.J., Endsley, M.A., Estes, D.M., 2006. Bovine natural killer cells acquire cytotoxic/effector activity following activation with IL-12/15 and reduce Mycobacterium bovis BCG in infected macrophages. J. Leukoc. Biol. 79, 71–79.
Esin, S., Batoni, G., 2015. Natural killer cells: a coherent model for their functional role in Mycobacterium tuberculosis infection. J. Innate Immun. 7, 11–24.
Fernandez, N.C., Flament, C., Crepineau, F., Angevin, E., Vivier, E., Zitvogel, L., 2002. Dendritic cells (DC) promote natural killer (NK) cell functions: dynamics of the human DC/NK cell cross talk. Eur. Cytokine Netw. 13, 17–27.
Fogel, N., 2015. Tuberculosis: a disease without boundaries. Tuberculosis 95, 527–531.
Gasteiger, G., Hemmers, S., Bos, P.D., Sun, J.C., Rudensky, A.Y., 2013. IL-2-dependent adaptive control of NK cell homeostasis. J. Exp. Med. 210, 1179–1187.
Geherin, S.A., Fintushel, S.R., Lee, M.H., Wilson, R.P., Patel, R.T., Alt, C., Young, A.J., Hay, J.B., Debes, G.F., 2012. The skin, a novel niche for recirculating B cells. J. Immunol. 188, 6027–6035.
Graham, E.M., Thom, M.L., Howard, C.J., Boysen, P., Storset, A.K., Sopp, P., Hope, J.C., 2009. Natural killer cell number and phenotype in bovine peripheral blood is influenced by age. Vet. Immunol. Immunopathol. 132, 101–108.
Hamilton, C.A., Mahan, S., Bell, C.R., Villarreal-Ramos, B., Charleston, B., Entrican, G., Hope, J.C., 2017a. Frequency and phenotype of natural killer cells and natural killer cell subsets in bovine lymphoid compartments and blood. Immunology 151, 89–97.
Hamilton, C.A., Mahan, S., Bell, C.R., Villarreal-Ramos, B., Charleston, B., Entrican, G., Hope, J.C., 2017b. Frequency and phenotype of natural killer cells and natural killer cell subsets in bovine lymphoid compartments and blood. Immunology 151, 89–97.
Hamilton, C.A., Mahan, S., Entrican, G., Hope, J.C., 2016. Interactions between natural killer cells and dendritic cells favour T helper1-type responses to BCG in calves. Vet. Res. 47, 85.
Hope, J.C., Guzman, E., Cubillos-Zapata, C., Stephens, S.A., Gilbert, S.C., Prentice, H., Sopp, P., Howard, C.J., Charleston, B., 2012. Migratory sub-populations of afferent lymphatic dendritic cells differ in their interactions with Mycobacterium bovis Bacille Calmette Guerin. Vaccine 30, 2357–2367.
Hope, J.C., Howard, C.J., Prentice, H., Charleston, B., 2006. Isolation and purification of afferent lymph dendritic cells that drain the skin of cattle. Nat. Protoc. 1, 982–987.
Hope, J.C., Thom, M.L., Villarreal-Ramos, B., Vordermeier, H.M., Hewinson, R.G., Howard, C.J., 2005. Vaccination of neonatal calves with Mycobacterium bovis BCG induces protection against intranasal challenge with virulent M. bovis. Clin. Exp. Immunol. 139, 48–56.
Hope, J.C., Vordermeier, H.M., 2005. Vaccines for bovine tuberculosis: current views and future prospects. Expert Rev. Vaccin. 4, 891–903.
Horowitz, A., Stegmann, K.A., Riley, E.M., 2011. Activation of natural killer cells during microbial infections. Front. Immunol. 2, 88.
Jones, G.J., Konold, T., Hurley, S., Holder, T., Steinbach, S., Coad, M., Neil Wedlock, D., Buddle, B.M., Singh, M., Martin Vordermeier, H., 2022. Test performance data demonstrates utility of a cattle DIVA skin test reagent (DST-F) compatible with BCG vaccination. Sci. Rep. 12, 12052.
Kleinnijenhuis, J., Quintin, J., Preijers, F., Joosten, L.A.B., Jacobs, C., Xavier, R.J., van der Meer, J.W.M., van Crevel, R., Netea, M.G., 2014. BCG-induced trained immunity in NK cells: role for non-specific protection to infection. Clin. Immunol. 155, 213–219.
Krzywinska, E., Cornillon, A., Allende-Vega, N., Vo, D.-N., Rene, C., Lu, Z.-Y., Pasero, C., Olive, D., Fegueux, N., Ceballos, P., Hicheri, Y., Sobecki, M., Rossi, J.-F., Cartron, G., Villalba, M., 2016. CD45 isoform profile identifies natural killer (NK) subsets with differential activity. PLoS One 11, e0150434.
Lund, H., Boysen, P., Hope, J.C., Sjurseth, S.K., Storset, A.K., 2013. Natural killer cells in afferent lymph express an activated phenotype and readily produce IFN-γ. Front. Immunol. 4, 395.
Marzo, S., Gray, M., Loh, W., Waddell, L.A., McGuinnes, C.I., Hope, J.C., Mathie, H.A., 2022. Characterisation of dendritic cell frequency and phenotype in bovine afferent lymph reveals kinetic changes in costimulatory molecule expression. Vet. Immunol. Immunopathol. 243, 110363.
Nugent, G., Yockney, I.J., Cross, M.L., Buddle, B.M., 2018. Low-dose BCG vaccination protects free-ranging cattle against naturally-acquired bovine tuberculosis. Vaccine 36, 7338–7344.
Siddiqui, N., Hope, J., 2013. Differential recruitment and activation of natural killer cell sub-populations by Mycobacterium bovis-infected dendritic cells. Eur. J. Immunol. 43, 159–169.
Siddiqui, N., Price, S., Hope, J., 2012. BCG vaccination of neonatal calves: potential roles for innate immune cells in the induction of protective immunity. Comp. Immunol. Microbiol Infect. Dis. 35, 219–226.
Srinivasan, S., Conlan, A.J.K., Easterling, L.A., Herrera, C., Dandapat, P., Veerasami, M., Ameni, G., Jindal, N., Raj, G.D., Wood, J., Juleff, N., Bakker, D., Vordermeier, M., Kapur, V., 2021. A meta-analysis of the effect of bacillus calmette-guerin vaccination against bovine tuberculosis: is perfect the enemy of good? Front. Vet. Sci. 8, 637580.
Storset, A.K., Kulberg, S., Berg, I., Boysen, P., Hope, J.C., Dissen, E., 2004. NKp46 defines a subset of bovine leukocytes with natural killer cell characteristics. Eur. J. Immunol. 34, 669–676.
Thom, M.L., McAulay, M., Vordermeier, H.M., Clifford, D., Hewinson, R.G., Villarreal-Ramos, B., Hope, J.C., 2012. Duration of immunity against Mycobacterium bovis following neonatal vaccination with bacillus Calmette-Guerin Danish: significant

protection against infection at 12, but not 24, months. Clin. Vaccin. Immunol. 19, 1254–1260.
Tozuka, M., Oka, T., Jounai, N., Egawa, G., Ishii, K.J., Kabashima, K., Takeshita, F., 2016. Efficient antigen delivery to the draining lymph nodes is a key component in the immunogenic pathway of the intradermal vaccine. J. Dermatol. Sci. 82, 38–45.
Van Rhijn, I., Rutten, V.P.M.G., Charleston, B., Smits, M., van Eden, W., Koets, A.P., 2007. Massive, sustained gammadelta T cell migration from the bovine skin in vivo. J. Leukoc. Biol. 81, 968–973.
Voisin, M.-B., Nourshargh, S., 2019. Neutrophil trafficking to lymphoid tissues: physiological and pathological implications. J. Pathol. 247, 662–671.
Waters, W.R., Maggioli, M.F., McGill, J.L., Lyashchenko, K.P., Palmer, M.V., 2014. Relevance of bovine tuberculosis research to the understanding of human disease: historical perspectives, approaches, and immunologic mechanisms. Vet. Immunol. Immunopathol. 159, 113–132.
Waters, W.R., Palmer, M.V., Thacker, T.C., Davis, W.C., Sreevatsan, S., Coussens, P., Meade, K.G., Hope, J.C., Estes, D.M., 2011. Tuberculosis immunity: opportunities from studies with cattle. Clin. Dev. Immunol. 2011, 768542.