THE UNIVERSITY *of* EDINBURGH

Evaluating Natural Killer Cells' Role in Immunological Responses to the Bacillus Calmette-Guérin Vaccine for Bovine Tuberculosis

by

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Declaration

I, Kenedi Lynch, declare that this thesis has been composed by myself, the work within this thesis is my own, and I have not submitted this thesis for any other degree or professional qualification except as specified.

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I would like to take the time to thank multiple people who have supported me throughout this endeavor.

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1. Introduction

1.1 Human Tuberculosis

Tuberculosis (TB) is the leading cause of death due to infectious disease worldwide despite it being both curable and preventable, with approximately 10.6 million new human cases of TB reported and 1.6 million deaths from the disease in 2021 (*Global Tuberculosis Report 2021*, 2021). With the increasing prevalence of antibiotic resistance, multidrug-resistant TB is also on the rise, representing an even greater threat to human health. TB is caused by Mycobacterium spp. within the Mycobacterium tuberculosis complex (MTBC). Characterized by 99.9% similarity between species at the nucleotide level, the difference in these species lies within their preferred hosts (Brosch et al., 2002; Rodriguez-Campos et al., 2014). Two pathogens of major importance for human health within this complex are *M. tuberculosis* and *M. bovis*, which primarily cause disease within humans and cattle, respectively.

Human TB can be characterized in four different stages: early infection, early primary progressive (active), late primary progressive (active), and latent. The majority of individuals exposed to *M. tuberculosis* will not progress to active TB; progression to active TB only occurs in 5-10% of cases. Primary pulmonary TB can often be asymptomatic, and early signs of active infection are comprised of nonspecific symptoms such as fatigue, fever, chills, general malaise, and night sweats. As infection progresses, a cough will emerge; while this cough may be nonproductive at first (not producing sputum), it will eventually evolve into a productive cough that may or may not contain blood. At this point, patients have progressed into the late primary progressive stage and can begin to experience weight loss (also known as wasting), a classic symptom of the disease. Though pulmonary TB is most common, over 20% of patients (especially those who are immunocompromised) can develop extrapulmonary TB, in which the bacteria spread to other parts of the body, initiating multiorgan involvement. Patients can also develop latent TB, in which the infection is contained within calcified granulomas; this infection may never be activated within an individual's life, but is more likely to develop into active infection in situations where the immune system is compromised. TB is definitively diagnosed by identification of a pathogen from the MTBC in a diagnostic sample, usually sputum. Additional diagnostic tests such as chest radiographs to visualize infiltrates in the lungs and quantiFERON test to detect latent TB are also common techniques employed (Knechel, 2009).

1.2 Bovine Tuberculosis

In addition to the ramifications of TB concerning human health, bovine TB (bTB) is also a widespread problem, with an estimated 7.4% prevalence in livestock worldwide (International Livestock Research Institute, 2012). According to the British government, bTB is one of the most significant issues affecting animal health in the UK, with the disease costing taxpayers over £100 million every year. Over 27k cattle in the UK were slaughtered in 2020 alone in an attempt to stop the spread of spread (APHA Science, 2021). bTB is an increased problem in the UK compared to other developed countries due to the presence of Eurasian badgers (*Meles meles*) which act as a reservoir for infection. Although other countries have reservoirs of infection, badgers are a protected species in the UK, making their culling illegal (Buddle et al., 2011). There have been experimental and field vaccination trials to try and combat the spread of bTB from badgers to cattle, but they have shown variable efficacy (Siddiqui et al., 2012; Buddle et al., 2011).

Though bTB treatment with antibiotics is possible, the current response to cattle testing positive for the disease in the agriculture industry is culling due to concerns of the disease spreading between animals, the potential transmission and subsequent harm to human populations, and M. bovis' inherent resistance to pyrazinamide, which is frequently used to treat human infections caused by pathogens within the MTBC (Khairullah et al., 2024; De Jong et al., 2005; Palmer & Waters, 2011).

bTB does not only affect the agriculture industry; infection with M. bovis also represents a threat to human health, as it can zoonotically spread to humans through the consumption of unpasteurized dairy products or infected bovine tissues, prolonged contact with bacteria in the air, animal secretions, or animal carcasses (Khairullah et al., 2024; Bolaños et al., 2017). While the amount of human TB infections resulting from M. bovis due to ingesting contaminated milk has shrunk from 20-40% to less than 10% since the invention of pasteurization, the threat of disease incidence is still significant, especially in rural areas of low and middle-income countries (Cosivi et al., 1998). In 2019, there were an estimated 140k new cases of zoonotic TB caused by M. bovis specifically, with an estimated 11.4k deaths attributed to the disease (*Global Tuberculosis Report 2021*, 2021). Considering that TB can infect other hosts via other species in the MTBC, this is a conservative estimate for zoonotic TB incidence and mortality overall (Table 1).

Table 1. Estimated incidence and mortality due to zoonotic TB for WHO regions and globally, 2019 (WHO Global Tuberculosis report, 2019).

Estimated incidence and mortality due to zoonotic TB for WHO regions and globally, 2019[a,b]

WHO REGION	NUMBER OF INCIDENT CASES		NUMBER OF DEATHS	
	BEST ESTIMATE	UNCERTAINTY INTERVAL	BEST ESTIMATE	UNCERTAINTY INTERVAL
Africa	68 900	18 500–152 000	8 440	2 220–18 700
The Americas	870	236–1 910	42	11–92
Eastern Mediterranean	8 190	2 110–18 300	604	161–1 340
Europe	986	263–2 180	65	18–143
South-East Asia	43 400	11 200–96 900	2 020	548–4 440
Western Pacific	18 000	4 720–40 000	270	73–594
Global	140 000	69 800–235 000	11 400	4 470–21 600

[a] Estimates are derived from data on *Mycobacterium bovis*, the most common cause of zoonotic TB globally.
[b] Numbers shown to two significant figures if under 100 and to three significant figures otherwise.

Diagnosis of bTB is most often accomplished by routine testing rather than based on clinical signs alone, as symptoms of bTB tend to take months to years to develop and are fairly nonspecific and considered pathognomonic. Initial symptoms that can occur when there is infection at the lung level include persistent cough (hallmark symptom) weakness, low-grade fluctuating fever, noticeably enlarged lymph nodes, trachypnea (rapid, shallow breathing), and decreased appetite or anorexia/gradual emaciation(Khairullah et al., 2024; Ramos et al., 2015; Islam et al., 2021). Like human TB infections, bTB can remain dormant for years before being reactivated later in life due to factors like progressing age and increased immunological stress (Ramos et al., 2015).

The primary test used internationally for antemortem bTB detection at the herd level is the single intradermal test (SIT)/tuberculin skin test (also used in humans), in which subjects are given an intradermal injection with purified tuberculin protein (a protein extract from mycobacterial culture supernatant) and monitored for a delayed hypersensitivity reaction. If the animal has not been sensitized to the tuberculin antigens, presumably via infection, there is no significant inflammatory response observed. However, if the animal has been sensitized via infection, inflammation and swelling at the injection site will be observed, with the greatest effect being observed 48 to 72 hours post injection. Ancillary serial tests like the single intradermal comparative cervical tuberculin (SICCT) test or the in vitro IFN-γ blood test are used in order to screen individuals with inconclusive results to the SIT test (De La Rua-Domenech et al., 2006).

Despite the widespread use if TB diagnostics, it is widely believed that control of the disease cannot be achieved without the use of vaccines.

1.3 The BCG Vaccine

BCG is a live, attenuated vaccine created from the bacterium M. bovis used to protect against TB and other mycobacterial infections such as leprosy(Okafor et al., 2023). Despite being created over 100 years ago (first administered in 1921) and providing a variable range of efficacious protection against both bovine and human forms of TB, it is currently the only licensed vaccine for the disease, though there are other candidates currently being investigated for use in enhancing the protective effects of BCG (Chambers et al., 2014; Sachdeva & Chadha, 2024). The variability of the vaccine's protective effects can be attributed to multiple different factors, including but not limited to genetic factors and environmental factors such as geographical location, socioeconomic status, coinfections, variable substrain vaccine preparation, and more (Fine, 1995; Mahairas et al., 1996; Moliva et al., 2015). With its variable levels of protection, BCG is at best 80% effective, and at worst can provide no protection (Moliva et al., 2015). BCG has been shown to be more effective in neonates/infants than adults, and is generally only routinely administered in countries where the disease is considered endemic (Okafor et al., 2023). The mechanisms of immunity elicited by administration of the BCG vaccine are not fully understood. In order to develop an improved vaccine for TB that is more effective than the current BCG vaccine, it is important to understand and characterize the underlying mechanisms of the immune response that is induced by BCG and why it is more effective in some situations than others.

Because of the similarity of *M. tuberculosis* and *M. bovis* in both genome and pathology, cattle have become increasingly popular as a model to study TB-related issues such as pathology and immune responses to apply to knowledge of the disease in terms of human (as well as animal) health. Additionally, because of the risk of transmission and the effects bTB has on the agriculture industry and economy, the study of bTB holds an important place in the OneHealth sphere. Relevant studies suggest that BCG is most effective in both cattle and humans when administered to neonates; detailed understanding of the immune response to BCG in neonatal animals and humans could provide significant insight into the protective immune mechanisms that should be targeted for new TB vaccines using a One Health approach. Previous studies have hypothesized that BCG-induced protective immunity in neonates is related to innate immune

effector cells that predominate in early life, including natural killer (NK) cells (Kulberg, 2004; Ho, 2017 unpublished).

1.4 Natural Killer Cells

Although not completely understood, natural killer (NK) cells are an integral component of the immune response against infection. An innate immune component with adaptive characteristics (and therefore said to "bridge the gap" between these two arms of the immune system), these cells are considered as one of the first lines of defense against microbial pathogens due to their ability to induce relatively early and rapid immune responses when an infection is encountered. In the last few years, increasing evidence has emerged showing the involvement of NK cells in controlling mycobacterial infections, including TB, and that this is achieved via multiple mechanisms. These include enhancing T cell responses as well as cytokine production, cytotoxic granule production, and lysis of bacterial-infected cells. These likely underpin the immune responses stimulated by the BCG vaccine. Additionally, it has been shown that an overall strong NK cell response can improve protective immunity in a variety of diseases. In a study with Mycobacteria spp.-infected cattle, findings suggested that NK-like cells were vital for immunity induced by BCG administration (Hope et al., 2002). NK cells were responsive to Mycobacterial antigens even in non-infected animals, producing IFN-γ in response to ESAT-6 (MTBC) and MMP14 (M. avium). This is relevant to potential roles in the reported interference with IFN-γ tests in young animals, which are used as an ancillary test alongside the tuberculin skin test for TB diagnosis in humans and cattle (Olsen & Storset, 2001). In studies, when NK cells were depleted, the IFN-γ response to mycobacterial antigens ceased entirely (Olsen et al., 2005). Furthermore, in another separate study, isolated NK cells were shown to significantly reduce M. bovis replication inside autologous macrophages in a contact-dependent manner (Denis et al., 2007) suggesting the cytolytic activity of these cells is important for immunity. There is also a growing body of evidence that NK cell secretion of IFN-γ is central to BCG-induced protective immunity in cattle, humans, and primates (Murphy et al., 2023).

NK cells are not a prolific immune component of blood, with frequencies in cattle typically varying from 2 to 10% of all lymphocytes in healthy individuals (Boysen & Storset, 2009; Kulberg, 2004; Endsley et al., 2005), and they represent about 15% of all lymphocytes in humans (Cooper et al., 2001). NK cell levels are, in general, highest in number early in an individual's life. This is due to neonates not yet having a fully developed adaptive immune

system at this point in their development, leaving them reliant on innate immune measures such as the activity of NK cells (Boysen & Storset, 2009; Endsley et al., 2005; Kulberg, 2004; Kampen et al., 2006; Siddiqui et al., 2012). BCG is thought to be most effective in neonates because of these increased and highly functional innate responses from components like NK cells (Siddiqui et al., 2012).

Due to their relative scarcity as compared to other immune components, it may be difficult to harvest a significant and sufficient number of NK cells from bovine subjects to study their immune responses. This represents both a gap in scientific knowledge and a barrier to further, high-throughput investigations regarding immune responses involving NK cells. The ability to generate large numbers of NK cells in vitro could reduce the use of animals in experimentation, and subsequent associated costs, while retaining cattle as a suitable model. Human NK cells can be generated from embryonic stem cells (Kaneko, 2019) and although this has not yet been established in other species, it could be used as an in vitro model providing large quantities of NK cells to investigate their interactions with BCG either directly or indirectly through their interactions with other cells such as dendritic cells (Hamilton et al., 2016); such interactions have been shown to be important for activation of the adaptive immune response. Additional characterization of NK cells in BCG-vaccinated calves will provide important insights into their roles that can then also be investigated in humans.

NK cell function is regulated by different activating and inhibitory receptors. NKp46 is a well-studied activating receptor in both humans and cows that is used to identify NK cells due to the lack of its presence on other cell types (Storset et al., 2004). In humans, functional subsets of NK cells are defined based on expression of the cell surface marker CD56. In humans, CD56bright NK cells (which comprise approximately 10% of blood NK cells) express a high density of CD56, but low density of the Fcγ receptor III (FcγRIII; CD16). These CD56bright CD16dim NK cells are classified as primarily cytokine-releasing. Conversely, CD56dim NK cells have a high level of CD16 expression (CD56brightCD16dim) and are classified as the subset with higher cytotoxic abilities, though they are still able to release cytokines(Cooper et al., 2001) However, these human NK subsets are not perfectly comparable to bovine NK subsets (Boysen & Storset, 2009). bNK cells are CD56 negative but subsets can be functionally divided by the presence or absence of the cell marker CD2, with $NKp46^{+}CD2^{-}$ cells being generally more activated, better able to produce IFN-γ, and better able to replicate in IL-2 culture while retaining their cytotoxic

abilities. The $CD2^-$ NK cell subset, like CD56bright human NK cells predominate in lymphoid tissues where they interact with dendritic cells and T cells early post-antigen exposure. NK cells are also generally characterized by their lack of CD3 expression, differentiating NK cells from T-cells, which possess many of the same surface markers as NK cells (Boysen & Storset, 2009). However, emerging evidence has revealed a novel subset of bovine T cells that are $NKp46^+CD3^+$ and represent NKT-like cells. These cells demonstrated the ability to lyse autologous Theileria parva-infected cells in vitro, demonstrating their potential in pathogen control (Connelley et al., 2014). While previous studies identified bovine $NKp46^+CD3^+$ cells, these began expressing NKp46 after activation and were not detected pre-stimulation (Johnson et al., 2008; Toka et al., 2011). However, much is still unknown about this cell subset, leaving a gap in knowledge concerning their roles in infection control, including in regulating TB.

1.5 The Project

This project tested two hypotheses:

1. NK and NKT cells are associated with immune responses induced by BCG vaccination
2. Functional bNK cells can be grown from ESCs

Aims:

1. Characterize pre- and post-vaccination cell populations via flow cytometry assessing cell surface molecules and intracellular expression of cytokines or cytotoxic molecules
2. Investigate the use of bovine ESCs for culturing of NK cells
    a. Document the growth and progression (changes in morphology) of the bovine NK cell line (lymphoid lineage) as compared to bovine macrophages (myeloid lineage)
    b. Characterize cells to determine their properties
        i. Flow cytometric analyses
        ii. qPCR at days 4 and 6 to demonstrate hematopoietic potential and differentiation to NK cells

2 Materials and Methods

2.1 ESC Arm

2.1.1 Culturing and Maintenance of Stock Bovine ESCs

Bovine ESCs were generated from in vitro fertilized abattoir derived oocytes. IVF embryos were allowed to develop to E8-9 before derivation. Cells were derived, passaged, and maintained using the procedure outlined in Kinoshita et al., 2021. The cell lines used throughout these experiments were the R1, RI6, and Episc99.1 lines.

2.1.2 Culturing Bovine Macrophages

Bovine macrophages were cultured from ESCs using a modified version of the protocol found in Haideri et al., 2017, which is outlined in the supplementary material (Section 6.1). A preliminary macrophage differentiation trial was conducted to determine which bovine ESC line, RI6 or R1, would be more suitable for use in the first NK cell differentiations, which would be grown alongside a macrophage differentiation used as a positive control. Macrophage differentiation success was qualified by microscopy , but not quantified. Each cell line was plated at six different densities: five test conditions (0.25x104, 0.5x104, 1x104, 2x104, and, 4x104) and one control condition plated at normal passaging density (1x105). Production of macrophages was confirmed visually by brightfield microscopy; these cells were identified by large size and granularity before being transferred to Mix3 (Supplementary Table 3) for maturation and use in other projects in the Burdon Lab.

2.1.3 Producing Cytokines Used for Culturing Bovine NK Cells

In accordance with previous human NK cell differentiation protocols that informed our modified protocol, five cytokines were used to initially push ESC cells towards NK cell differentiation: IL-3, IL-7, IL-15, SCF, and FLT3L (Forrester, unpublished; Kaneko, 2019). Due to unavailability of bovine IL-3 and SCF, porcine versions of these two cytokines (shown in previous unpublished studies by the Hope Group to partially cross react with bovine) were used in reactions. Bovine IL-7, IL-15, and FLT3L were produced by the Roslin Immunological Toolbox for use in this study.

The cDNA sequences (excluding stop codons) of bovine IL-7 (NCBI Reference Sequence: AF348422) and IL-15 (NCBI Reference Sequence: NM_174090), each containing a XhoI restriction site at the 5′ end and a BglII restriction site at the 3′ end, were synthesized by Integrated DNA Technologies (IDT) and cloned into the pFUSE-hG1Fc1 expression vector (InvivoGen, San Diego, USA) to generate recombinant proteins fused to a human IgG1 Fc tag at the C-terminus. Similarly, the cDNA sequence encoding the extracellular domain of bovine

FLT3L (AB051841), with a BsiWI restriction site at the 5′ end and an NheI site at the 3′ end, was synthesized by IDT and cloned into the pFUSEN-hG1Fc expression vector (InvivoGen) to produce a recombinant protein with an N-terminal human IgG1 Fc tag. Protein expression was performed using the Expi293™ Expression System Kit (Thermo Fisher Scientific) according to the manufacturer's protocol. Recombinant proteins were purified using a HiTrap Protein G HP column (Cytiva, Fisher Scientific, Loughborough, UK), and their identities were confirmed by mass spectrometry (MS) conducted by the Proteomics and Metabolomics Facility at the Roslin Institute, University of Edinburgh.

2.1.4 EB Trials and qPCR Primer Testing

In order to assess the best method for embryoid body (EB) formation before plating for NK cell differentiation, multiple methods for EB formation were tested using both the R1 and RI6 bovine ESC lines. The first method tested, referred to as single cell dispersion (SCD), entailed normal dispersion of cells to single cell suspensions using TrypLE during routine passaging procedure before splitting the passaged cells between two wells in a 6-well low-attachment plate and feeding with Mix 1a medium (Supplementary Table 4) for two days and Mix 1b medium (Supplementary Table 5) for two days (Kinoshita et al., 2021). The second method, referred to as multicell dispersion (MCD), entailed normal dispersion of cells to small clumps using a PBS/EDTA solution during routine passaging procedure before splitting the passaged cells between two wells in a 6-well low-attachment plate and feeding with Mix 1a and Mix 1b. The third method involved dispersing a set number of cells into a 96-well v-bottom plate (96wv) after routine passaging with TrypLE and feeding with NK Cell Differentiation medium. The fourth method entailed using a StemPro cutting tool in place of any type of chemical dispersion method to separate the cells into equivalently sized squares on the laminate plate cells were originally seeded on (Forrester, unpublished). In the first EB formation trial, the SCD and MCD methods were tested on the R1 line. In the second EB formation trail, SCD, MCD, StemPro, and 96wv methods were tested on the RI6 line. Due to poor EB yields from the StemPro and 96wv methods, these additional tests were not carried out on the R1 line.

EBs and cells from the EB trials were harvested at day 4, which is the point in the differentiation procedure where the EBs would normally be plated on gelatin and switched into the NK cell differentiation medium ((Forrester, unpublished); Section 6.3). These samples were then used to

validate three different primers for KDR and two different primers for TBXT, which are both early mesoendoderm markers (Kataoka et al., 1997; Technau, 2001).

In the first EB formation trial, samples were resuspended in PBS after passaging to lift them from the plate, frozen at -80 ℃. and defrosted at a later time for RNA extraction and cDNA conversion. In the second EB formation trial, RNA extraction and cDNA conversion were conducted directly after harvesting. RNA was extracted from samples using the Qiagen RNeasy Mini Kit (Qiagen #74104) and its corresponding Quick-Start Protocol CITATION. During the process of RNA purification, each sample was treated with RNase-free DNase to remove remaining traces of DNA. The concentration of RNA in each sample was quantified by Nanodrop. cDNA was subsequently stored at -20 ℃ until qPCR was conducted.

Novel SYBR primers were designed using the databases NCBI and Ensembl to obtain a transcript for the gene of interest. These transcripts were then entered into Primer3, which determined adequate primer sequences for use. Primer3 specifications were left to default settings apart from the following parameters, which were changed as follows: Primer length 19-21, 20 optimum; Tm - 58-62, 60 as optimum; GC content 40-60%, 50% optimum. After testing, primers were retroactively validated using bovine ESC cultured cells. Primer sequences are displayed in the supplementary materials (Section 6.5).

qPCR was run on the AriaMax Real-Time PCR Instrument (G8830A), using SYBR Green qPCR Master Mix (Low Rox) (Cat #HY-K0522, Lot #656320) in triplicate 20 µL reactions. Mastermix reaction components were composed of 10 µL of SYBR green + rox reference mix and 1 µL of forward and reverse primer, respectively. 12 µL of mastermix and 8 µL of cDNA were added to each well to total the 20 µL reaction. The primers for the housekeeping gene used, qphRR4, were previously validated in pig ESCs. qPCR for R1 samples was conducted using 300 ng of template cDNA while qPCR for RI6 samples was conducted using 200 ng of template cDNA due to lower RNA yields post-extraction according to Nanodrop readings. Cycling conditions used to determine Cq values were as follows: 95˚ C for 2 min, 95˚ C for 15 s, 60˚ C for 30, 95˚ C for 30s, 65˚ C for 30 s, 95˚ C for 30s, followed by a plate read. Samples containing no reverse transcriptase (RT) served as a negative control. Relative changes in expression for the specified gene targets was calculated using the 2-ΔΔCT method (Applied Biosystems, 2004).

2.1.5 Culturing Bovine NK Cells

EB formation was conducted using the SCD method. Wells of freshly passaged ESCs were split and seeded into two wells of a low-attachment 6-well plate and made up to 3mL/well of EB-Mix1a and incubated at 37ºC for two days. 500 uL of EB-MIX1b was then added to each well and incubated at 37ºC for another two days for a total of four days (Suppmenetary Table4; Supplementary Table 5).

After four days, EBs were collected and transferred into a 15 mL tube and allowed to settle by gravity (it was important not to spin them down so EBs were not disturbed) for 10 minutes. The old medium was then removed by pipette and gently replaced with 2 mL of NK Differentiation Medium, effectively serving as a wash step. EBs were allowed to settle for another 10 minutes before adding 2 mL per plate of EBs collected. This solution of resuspended EBs was transferred to a 10 cm dish. EBs were transferred to 0.1% gelatin plates (previously incubated at 37˚C for 30 minutes) filled with 3 mL of NK Differentiation Medium (Supplementary Table 6). Previous human protocols determined that each gelatin well should contain 14-16 EBs visually (Forrester, unpublished). However, due to difficulty retrieving individual EBs due to small size, the solution was mixed and evenly split via pipette into gelatin wells (generally 100-200 µL). For the first week only, the NK Differentiation Medium included IL-3. NK Differentiation Medium was changed every 5-7 days. Cells were allowed to grow for at 4 weeks. Growth was documented via pictures using brightfield microscopy.

For the first differentiation, cells were harvested every week for flow cytometry. Due to low cell number and negative staining for the first two weeks, in subsequent differentiations, flow cytometry was conducted beginning at week three. In total, four bNK differentiations were conducted.

2.1.6 Flow Cytometry

Cells were passaged for collection according to previously outlined methods (Kinoshita et al., 2021). Live cell count was determined using a trypan blue, then cells were resuspended in a blocking buffer of PBS with 5% normal goat serum (PBS/5% NGS). Monoclonal antibodies (mAbs) that were fluorescently labeled (Table 2) were diluted in PBS/5% NGS according to predetermined optimized concentrations and incubated on ice for 30 min. Cells were washed twice in PBS, then resuspended in PBS and stored at 4˚ C until flow cytometry was conducted

later that day. Sytox blue at a final dilution of 1:2000 was added as a viability dye before cells were run through the flow cytometer. The maximum possible number of events for each sample was collected, with variable numbers of events per sample due to variations with staining. Data was analysed using FlowJoTM v.10.10.0 software. Lymphocytes were selected based on FSC-A v. SSC-A profile, dead cells were excluded by sytox blue, and doublets were omitted based on FSC-A v. FSC-H. Gates were set according to PBMC controls (cells + viability dye) stained alongside test samples. Issues with the CD45 antibody resulted in removing it from the flow cytometry panel. The CD161 antibody which is thought to be a marker for early NK cell development, later developed in a separate unpublished project from the Hope Group, was added in later flow panels.

Antibody optimization was also carried out via flow cytometry. CD45 is a pan-leucocyte cell surface marker present on all cells of hematopoietic lineage, excluding erythrocytes, and therefore all PBMCs (Bembridge et al., 1993). To assess the presence or absence of CD45 on cultured ESC cells, a mouse anti-bovine CD45 antibody, clone CC1 (Bio-Rad MCA832GA), was optimized. The ability of CC1 to detect expression of CD45 on bovine cells was examined by flow cytometry. The mAb, which was directly conjugated, was tested at dilutions of 1:50, 1:100. 1:200, 1:400, 1:800, and 1:1600. The antibody was diluted in PBS/5% Normal Goat Serum (NGS). Flow cytometry was carried out on the BD LSR Fortessa, with a minimum of 10,000 events gathered for each dilution.

Table 2. Fluorophores and antibodies used in flow cytometric analysis of ESC cultures.

Marker	Conjugate	Clone	Laser Filter
NKp46	AF647	Gr13.1	R 670/14
CD45	FITC	CC1	B 530/30
CD161	AF568	1F2	YG 610/20
Sytox Blue	Violet	—	V 450/50

2.2 PBMC Arm

2.2.1 Animals and Sample Collection

Animals used for this project were British Holstein Friesen cows (*Bos taurus*) from 2024 and 2025 (n=4). Animals were obtained from the University of Edinburgh Farm and housed at the University of Edinburgh Large Animal Research Imaging Facility. All experimental protocols undertaken were authorized according to the UK Animals (Scientific Procedures) Act, 1986, and performed according to standards aligned with and approved by The Roslin Institute's Animal Welfare and Ethical Review Board.

PBMCs that were frozen at -155 ˚C from prior studies were used for this project. Availaible PBMCs were available from the following timepoints: day 0 (pre-vaccination), day 3, day 7, day 28, and day 42. Sample collection timeline is shown in Figure 1. Due to time constraints, two time points (day 0 and day 42) were analyzed here. Day 0 samples from the day of vaccination serve as a baseline measurement for comparison. Previous studies from the Hope Lab have shown an initial IFN-γ spike around days 7/8, a dip in BCG specific responses at day 28, and a second spike observed at day 42, with a hypothesis that NKT cell activity should also start to appear around day 42.

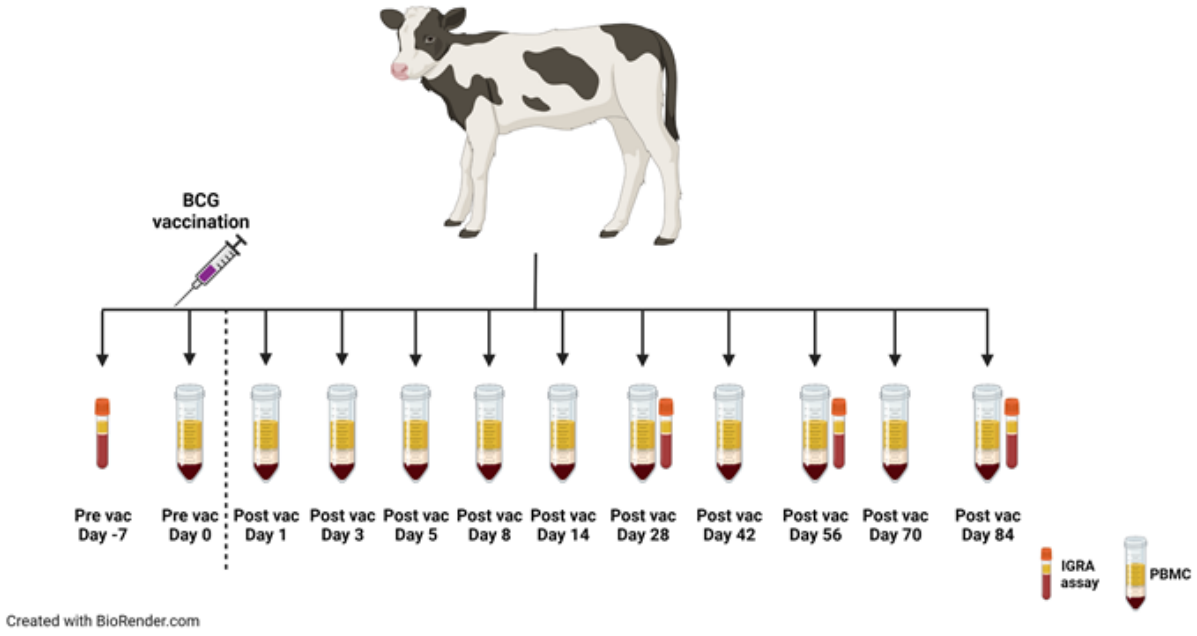

Figure 1. General PBMC sample acquisition timeline. Previous experiments collected PBMCs from multiple timepoints post-vaccination. In this particular study, timepoints from days 3, 7, 28, and 42 were collected and stored at -155 ˚C for later assay use. Figure courtesy of Lindsey Waddell from previous studies.

2.2.2 Flow Cytometry

Flow cytometry was conducted according to previous protocols outlined in Hanton et al., 2023 and modified as follows. Cryopreserved samples stored at -155 ˚C were thawed at 37˚C and washed with PBS. Live cell count was determined using trypan blue, then resuspended in a blocking buffer of PBS with 5% normal goat serum (PBS/5% NGS). For fluorescence minus one (FMO) controls, excess cells from one individual calf were used. Extracellular mAbs that were fluorescently labeled (Table 3) were diluted in PBS/5% NGS according to predetermined optimized concentrations from previous experiments in the Hope Group and incubated on ice for 30 min. Cells were washed with PBS twice then incubated with Zombie NIR as a viability dye at room temperature for 15 minutes. Cells were washed twice with PBS before fixation with 2% paraformaldehyde and stored at 4˚ C overnight. After incubation overnight, cells were permeabilized by incubating with BD Perm Buffer 2 (supplier) at room temperature for 15 minutes. Cells were washed twice with PBS before again being resuspended in a blocking buffer of PBS/5% NGS for 15 min on ice. Intracellular mAbs that were fluorescently labeled (Table 3) were diluted in PBS/5% NGS according to predetermined optimized concentrations and

incubated on ice for 30 min. Cells were washed twice in PBS, then resuspended in PBS and stored at 4˚ C until flow cytometry was conducted later that day. The maximum possible number of events for each sample was collected, with variable numbers of events per sample due to variations with staining. Data was analyzed using FlowJoTM v.10.10.0 software. Lymphocytes were selected based on FSC-A v. SSC-A profile, dead cells were excluded by Zombie dyes, and doublets were omitted based on FSC-A v. FSC-H. Gates were set using FMO controls (Supplementary Section 6.6).

Table 3. Antibodies and fluorophores used in flow cytometric analysis of pre- and post-BCG vaccination samples.

Marker	Conjugate	Clone	Laser Filter
Extracellular			
NKp46	AF647	Gr13.1	R 670/14
CD2	AF488	CC42	B 530/30
CD3	PB	MM1A	V 450/50
CD45RB	PB	CC76	V 450/50
CD62L	PB	CC32	V 450/50
live/dead	Zombie NIR	—	R 780/60
Intracellular			
IFN-γ	AF568	CC302	YG 610/20

Three different flow cytometric panels were run to assess different fluorophores in combination. The combination of fluorophores in each panel can be found below (Table 4).

Table 4. Flow cytometric panel components.

Panel #	Laser				
	V 450/50	B 530/30	YG 610/20	R 670/14	R 780/60
Panel 1	CD3 (PB)	CD2 (AF488)	IFN-γ (AF568)	NKp46 (AF647)	Zombie NIR
Panel 2	CD45RB (PB)	CD2 (AF488)	IFN-γ (AF568)	NKp46 (AF647)	Zombie NIR
Panel 3	CD62L (PB)	CD2 (AF488)	IFN-γ (AF568)	NKp46 (AF647)	Zombie NIR

2.3 Statistical Analysis

Statistical analysis was conducted using GraphPad Prism 10. Mean bNK cell proportions and mean fluorescence intensity (MFI) were compared between samples collected pre-vaccination (day 0) and post-vaccination (day 42) using paired t-tests. P-values were adjusted using the Bonferroni-Dunn correction to account for multiple tests conducted within the same sample (PBMC population). NK subset prevalence (Panel 1) within days was compared using one-way ANOVA. P -values were adjusted using the Geisser-Greenhouse correction.

3 Results

3.1 ESC Arm

3.1.1 Culturing Bovine Macrophages

Both the R1 and RI6 lines produced macrophages, providing confidence of their viability and robustness for use in these experiments. The RI6 line produced macrophages when initially seeded at the density of 4x104 while the R1 line produced macrophages at 1x104, 2x104, and 4x104. As the RI6 line visually appeared to produce more macrophages and less cellular debris as compared to R1, it was chosen for use in the first NK cell differentiation for the purpose of producing macrophages.

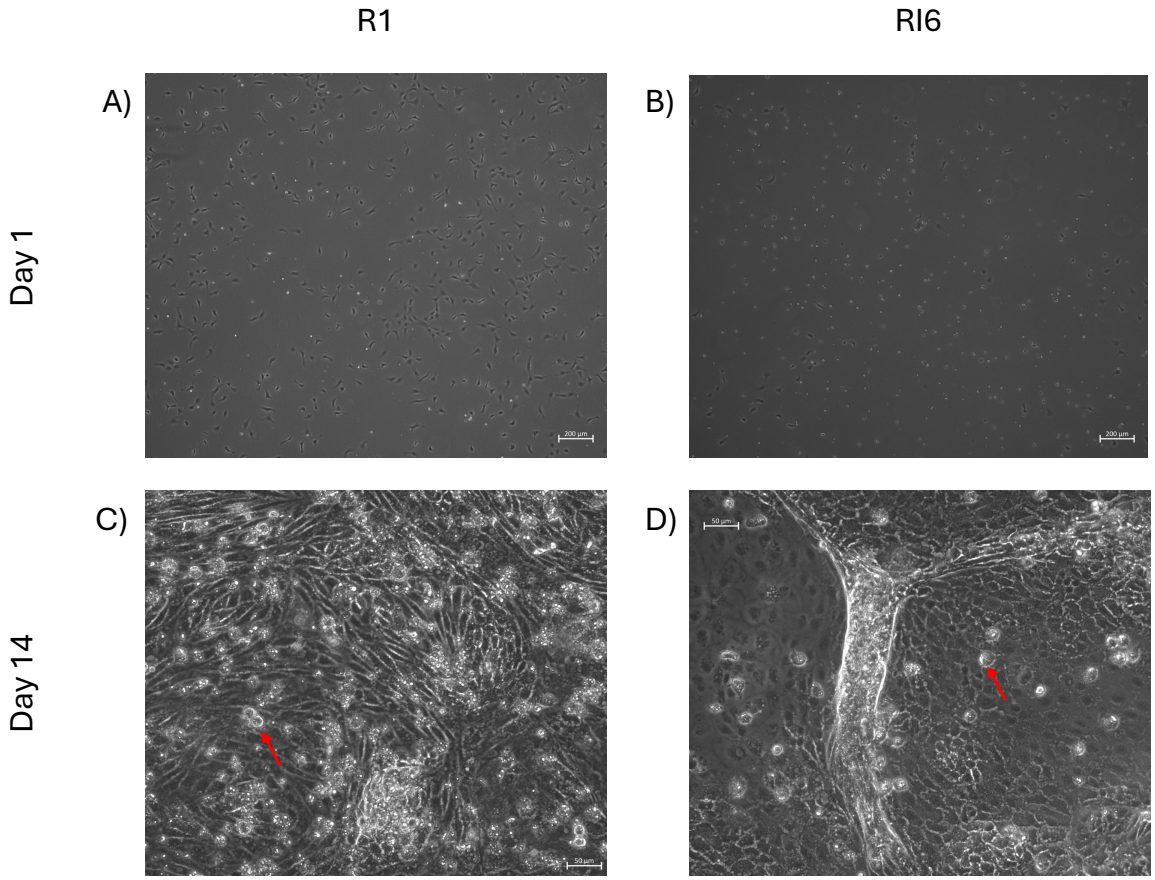

Figure 2. Macrophage differentiation trial 1 results. Red arrows indicate macrophages. A) R1 cells on day 1, originally plated at 4 x 104, and at 5x magnification. B) RI6 cells on day 1, originally plated at 4 x104, and at 5x magnification. C) R1 cells on day 14, originally plated at 4 x 104, and at 20x magnification. D) RI6 cells on day 14, originally plated at 4 x 104, and at 20x magnification.

3.1.2 Producing Cytokines Used for Culturing Bovine NK Cells

Bovine cytokines required for NK differentiation (IL-7, IL-15, and FLT3L) were produced in house by the Roslin Immunological Toolbox and analyzed by MS. EAKS Studio software (Bioinformatics Solutions Inc.) was used to analyze the MS data. For all three proteins, sequence coverage ranged between 97–98%, with exceptionally high confidence scores for protein identification (−10lgP), confirming the identity of the recombinant proteins.

3.1.3 Antibody Optimization

The CD45-FITC conjugated antibody was titrated on bovine PBMC, and staining was compared to unstained control cells. The concentration where there was clear separation of positive cells from unstained was selected as the optimal: this was shown to beat a dilution of 1:50.

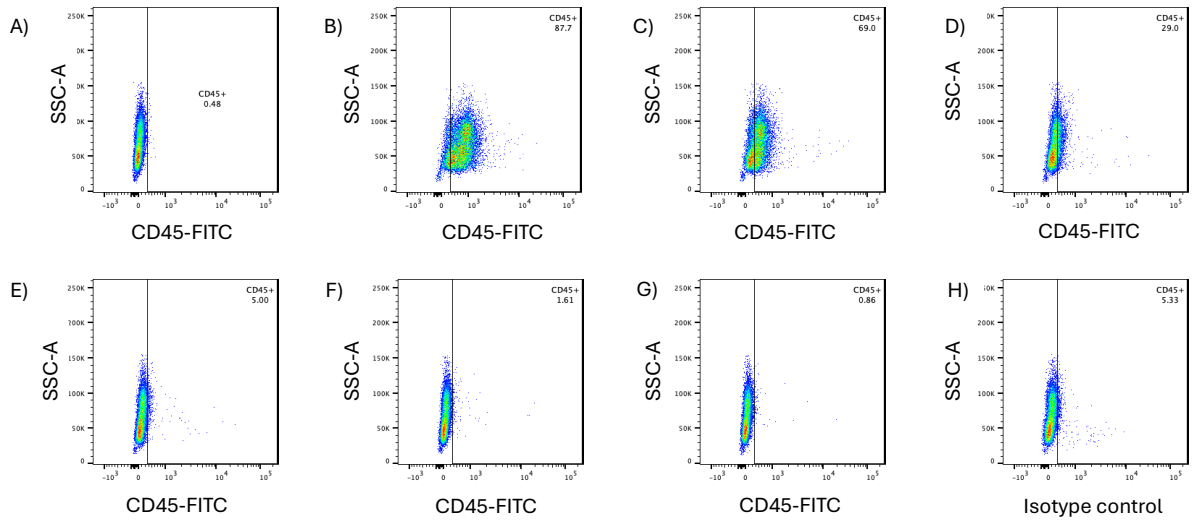

Figure 3. Flow cytometric analyses of CD45 antibody titration test. Titration was conducted using PBMCs. A control of cells only plus sytox blue (A) was titrated along with dilutions of CD45 at 1:50 (B), 1:100 (C), 1:200 (D). 1:400 (E), 1:800 (F), and 1:1600 (G). An IgG1 isotype control (H) was also titrated to ensure a lack of nonspecific binding. The dilution of 1:50 showed the best results, with near constitutive staining for CD45 (which should be seen in PBMCs). Cells were initially gated based on FSC-A v. SSC-A profile, doublets were omitted based on FSC-A v. FSC-H, and dead cells were excluded by sytox blue.

However, in subsequent experiments, the CD45 antibody failed to show staining constitutively across PBMCs despite multiple trials. For this reason, CD45 was removed from screening of

cells early in the differentiation process in favor of developing qPCR primers to measure gene expression of this molecule.

3.1.4 EB Trials and qPCR Primer Testing

For EB Formation Trial 1, which used the R1 line of ESCs, at day 4, visualization of EBs via brightfield microscopy revealed large EBs visible to the naked eye for the MCD condition larger in size than those produced by the SCD method (Figure 4). qPCR analysis with preliminary primers revealed significantly greater expression of both KDR and TBXT using the SCD method (Figure 5, Figure 6). qPCR analysis assessed expression of KDR and TBXT, as these genes are indicative of early mesoderm development, from which NK cells eventually develop (Kataoka et al., 1997; Technau, 2001). However, because these analyses were conducted after NK Cell Differentiation 1 was underway (started due to time constraints of the culturing process, which overall takes one month), the MCD method was used for subsequent experiments based on the visual quantification results which revealed large EBs.

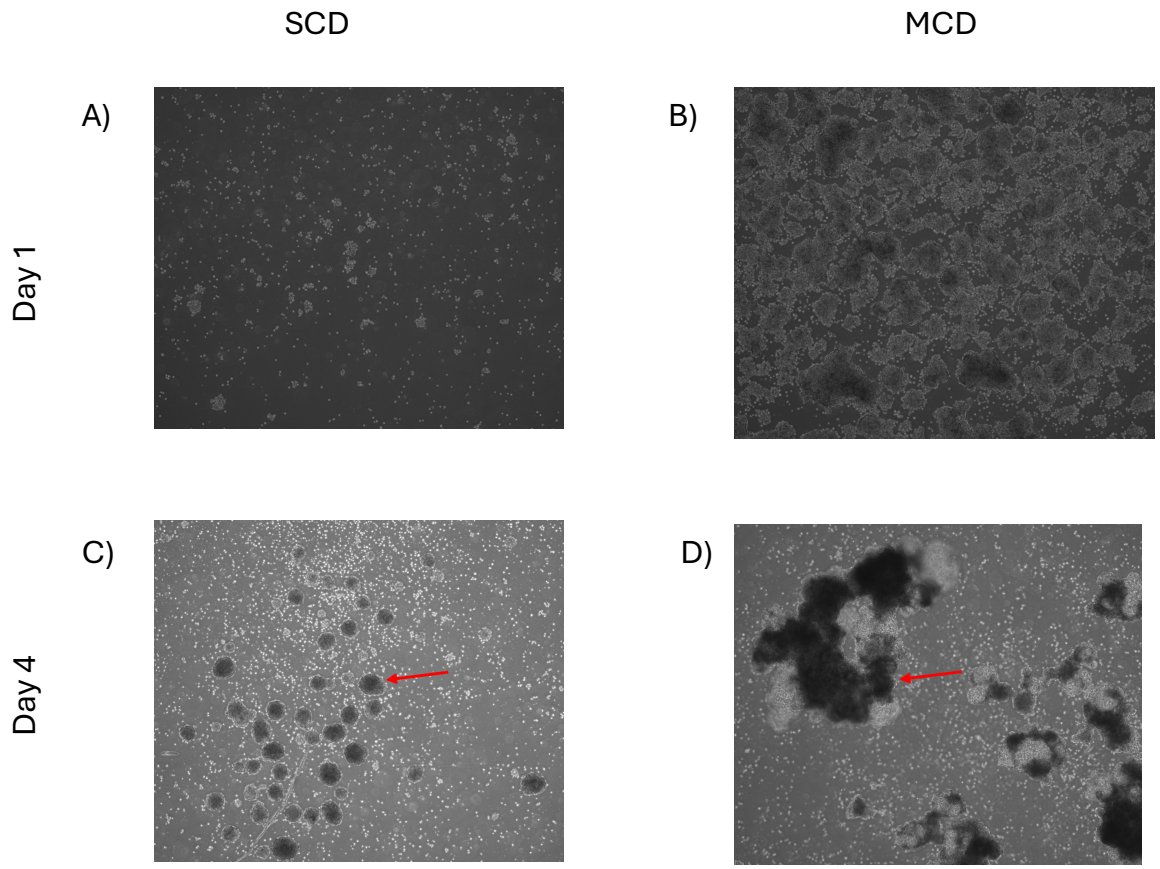

Figure 4. Brightfield microscopy images of EB Formation Trial 1 at day 1 and day 4. Red arrows represent EBs. A) SCD cells on day 1 at 5x magnification. B) MCD day 1 cells at 5x magnification. C) SCD cells on day 4 at 5x magnification. D) MCD cells on day 4 at 5x magnification.

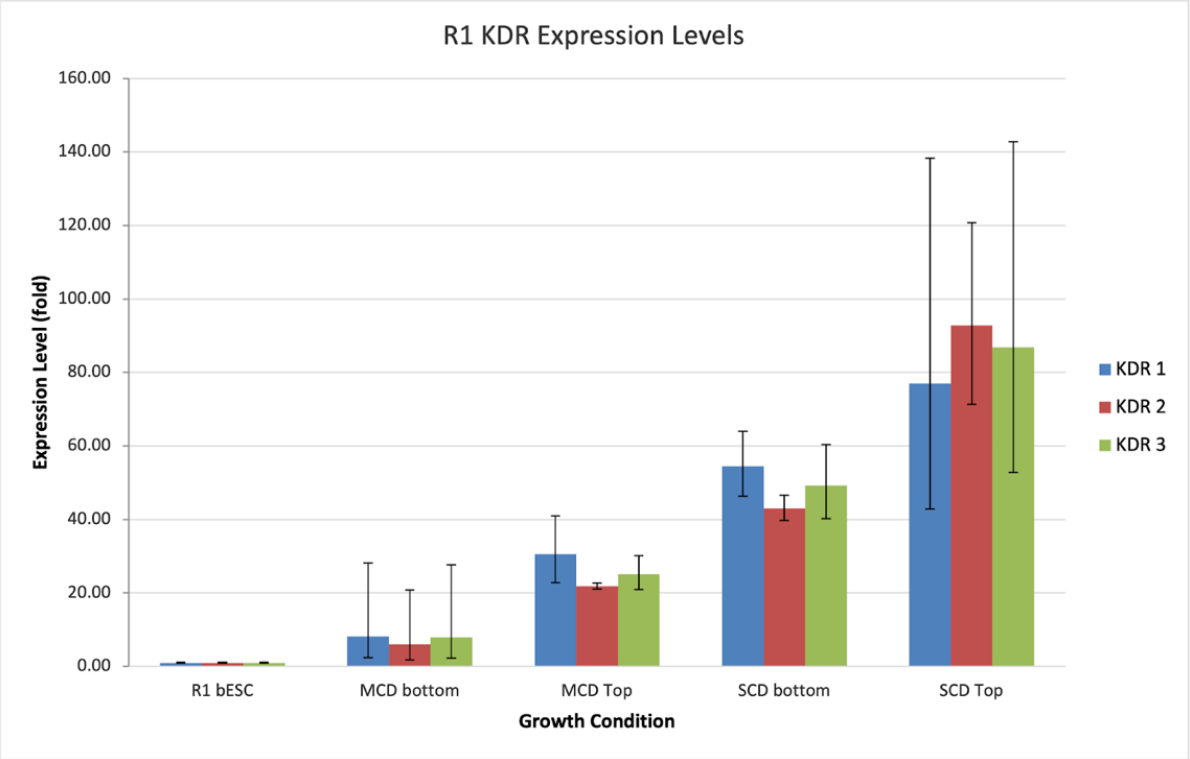

Figure 5. KDR expression levels in R1 cells from EB formation trials as measured via qPCR. SCD = single cell dispersion method, MCD = multicell dispersion method. Top and bottom designations represent which well cells were split into on the 6-well low attachment plate they were seeded on in Mix1a in order for form EBs. Fold values normalized to normally-passaged R1 bESCs (normal passaging methods found in Section 6.2).

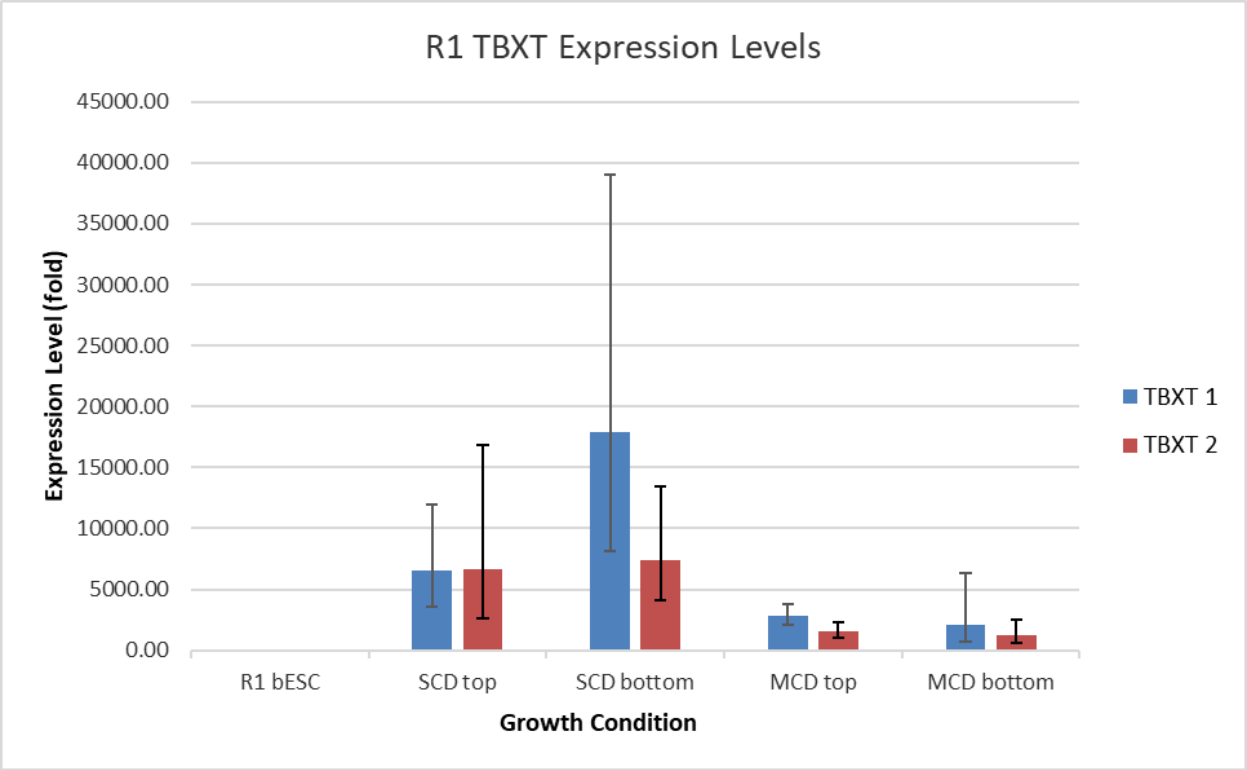

Figure 6. Expression levels of TBXT in R1 cells from EB formation trials as measured via qPCR. SCD = single cell dispersion method, MCD = multicell dispersion method. Top and bottom designations represent which well cells were split into on the 6-well low attachment plate they were seeded on in Mix1a in order for form EBs. Fold values normalized to normally-passaged R1 bESCs (normal passaging methods found in Supplementary Section 6.2).

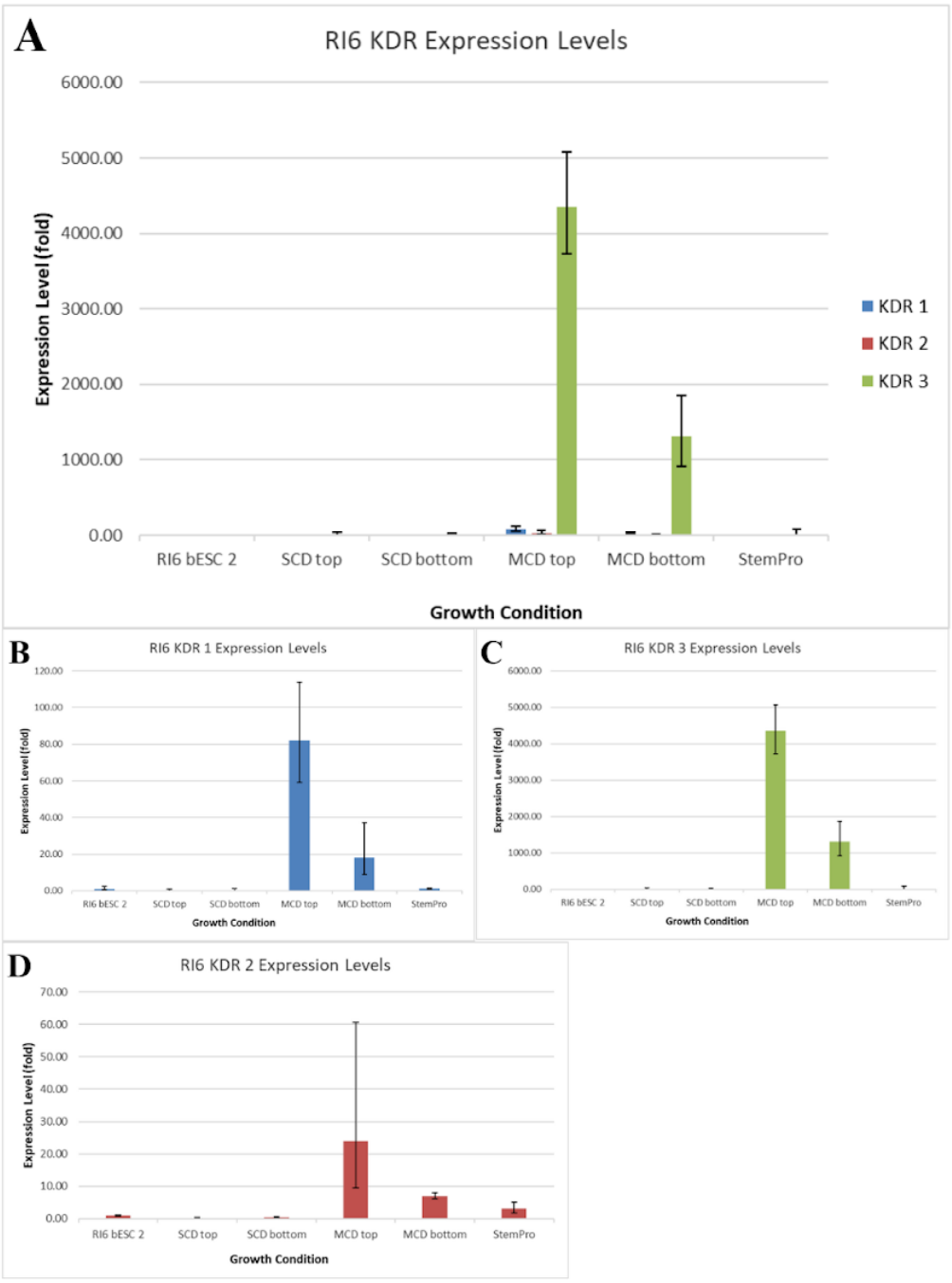

Figure 7. Expression levels of KDR in RI6 cells from EB formation trials as measured via qPCR. SCD = single cell dispersion method, MCD = multicell dispersion method. Top and bottom designations represent which well cells were split into on the 6-well low attachment plate they were seeded on in Mix1a in order for form EBs. Fold values normalized to normally-passaged RI6 bESCs (normal passaging methods found in Supplementary Section 6.2).

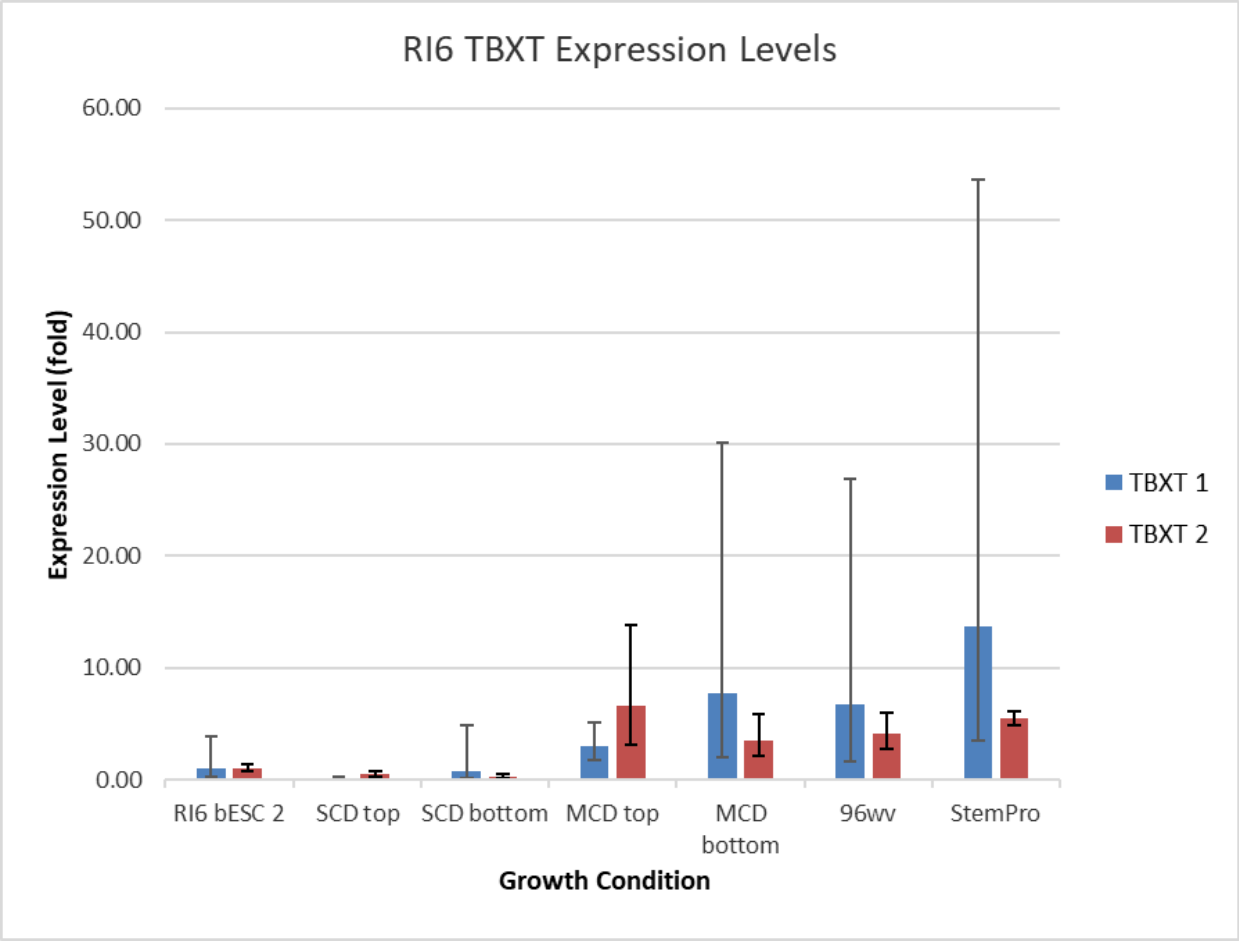

Figure 8. Expression levels of TBXT in RI6 cells from EB formation trials as measured via qPCR. SCD = single cell dispersion method, MCD = multicell dispersion method. Top and bottom designations represent which well cells were split into on the 6-well low attachment plate they were seeded on in Mix1a in order for form EBs. Fold values normalized to normally-passaged RI6 bESCs (normal passaging methods found in Supplementary Section 6.2).

Due to high levels of variation observed in qPCR and time constraints of the project, no further qPCR was conducted. Future efforts should focus on further validation of qPCR primers.

3.1.5 Culturing Bovine NK Cells

Days 20 and 27 flow cytometric analyses of the NK cell differentiation 1 revealed positive staining for NKp46, indicating the presence of NK cells (Figure 9). Under brightfield microscopy, some cells appeared to develop small, feet-like projections from their surface (Figure 10); these cells were assumed to be staining positive for NKp46, as this morphology was consistent with bNK morphology seen in previous experiments in the Hope Lab.

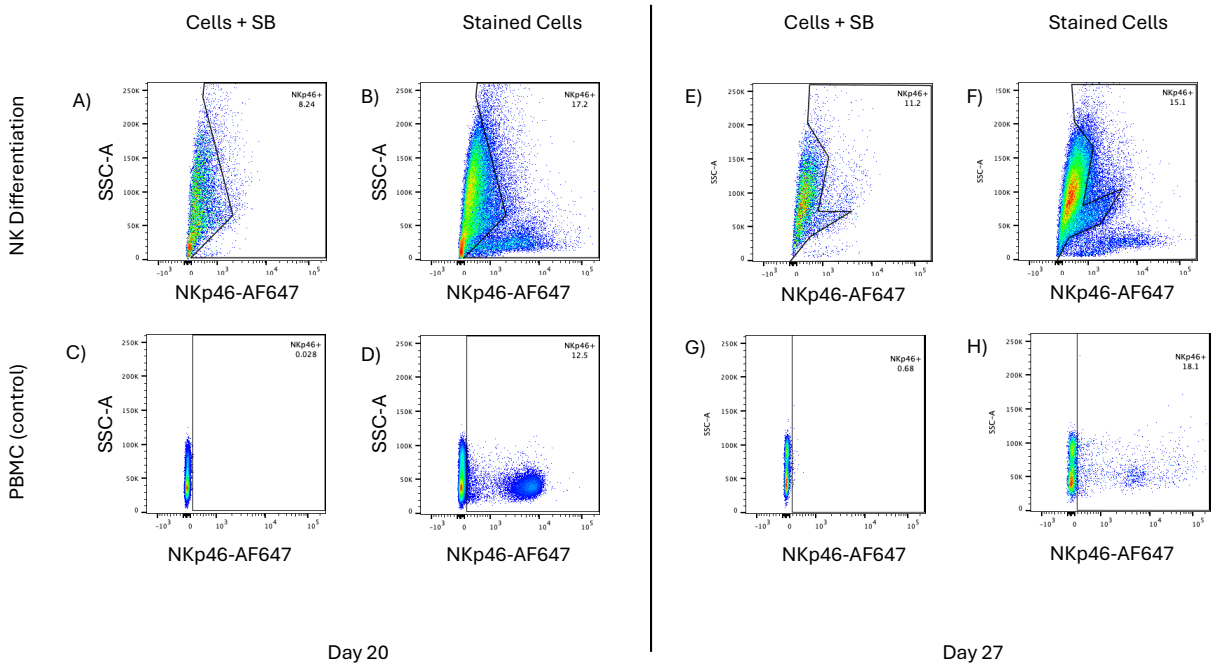

Figure 9. NK Cell Differentiation 1 flow cytometric analyses. PBMC controls (C, D, G, H) were used to demonstrate efficacy of fluorophores in staining alongside experimental NK cell differentiations (A, B, E, F). Cells were initially gated based on FSC-A vs. SSC-A profile, doublets were omitted based on FSC-A v. FSC-H, and dead cells were excluded by sytox blue. Gates were set according to controls (cells + sytox blue) stained alongside test samples.

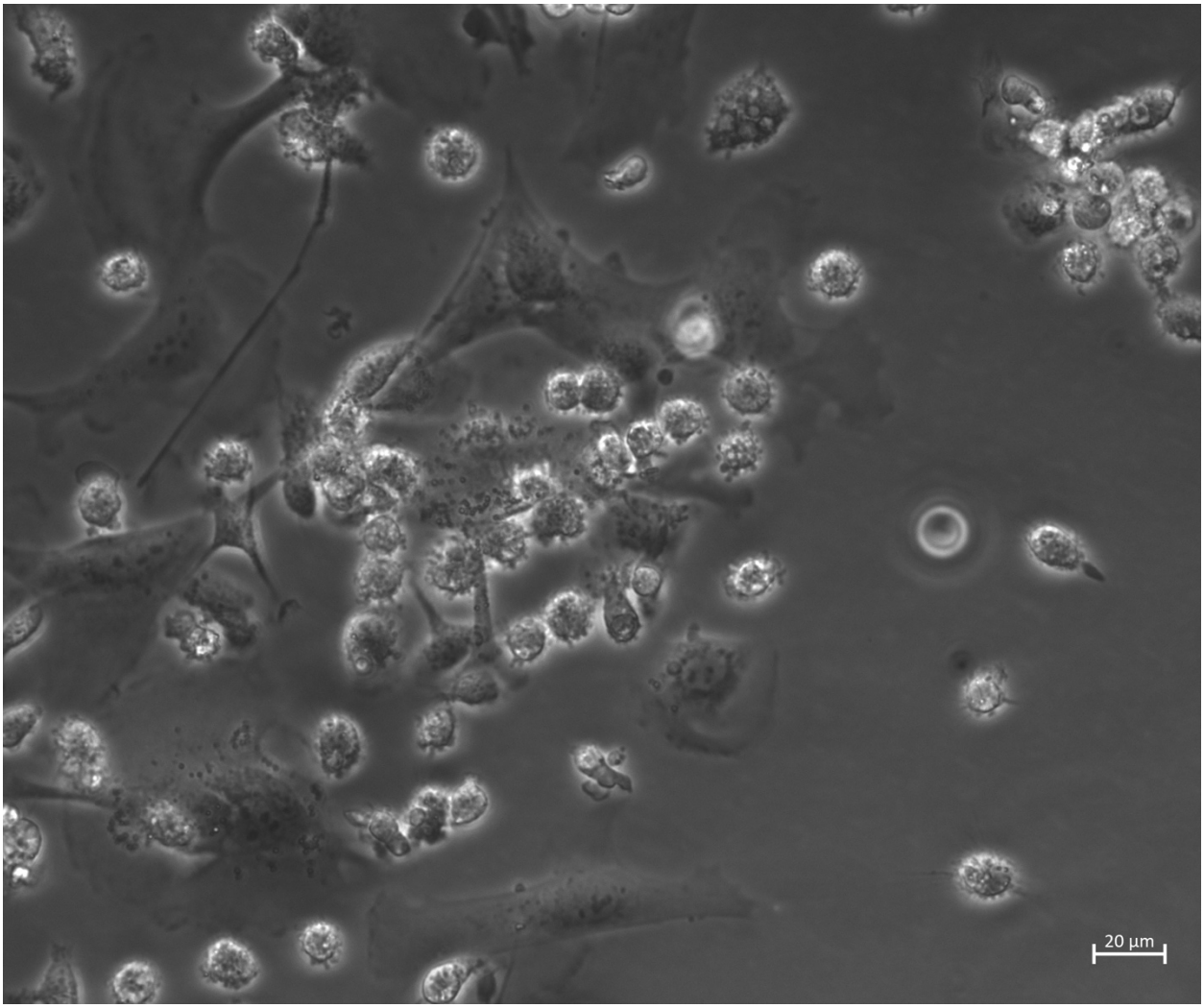

Figure 10. Brightfield microscopy images of NK Differentiation Trial 1 at day 15 at 40x magnification. Cells with feet like projections were suspected to be cultured NK cells.

However, in subsequent differentiations, neither NKp46 or CD161 – an early NK cell marker that was added to the panel in later experiments after a fluorophore was developed by the Hope Lab – showed positive staining, indicating a lack of NK cells present in the cell culture. Some differentiation cultures did not proliferate enough to undertake flow cytometry. Treatment with IL-2 in an attempt to stimulate additional proliferation by any NK cells that might have been present in the culture was seemingly unsuccessful. Additionally, some subsequent differentiations conducted seemed to produce morphologically similar cells to ones identified as potential NK cells in culture without staining positive for NKp46 via flow cytometry, possibly confounding positive results obtained in the first differentiation.

3.2 PBMC Arm

This part of the study aimed to undertake a comparative analysis between naive and post-BCG vaccinated bNK cells using a five-color flow cytometry panel. This panel was developed from previous unpublished experiments within the Hope Group and optimized on cells from naive calves to establish the optimal staining and gating strategies.

Initial optimization carried out within this project revealed a possible sample preservation issue. After thawing PBMC samples from -155 ˚C, there was a visually noticeable high level of cell death of PBMCs when counting on the hemocytometer using trypan blue. These cells were stained according to the flow cytometry protocol, which revealed selective death of monocytes. Figure 11 illustrates a normal PBMC profile comprised of both lymphocytes and monocytes alongside deficient samples found during optimization. This issue was also observed in the day 0 samples from the first BCG vaccination study. Since a deficit in day 0 (pre-vaccination) samples would not provide an accurate baseline for comparison with post-vaccination timepoints, a different sample pool was utilized moving forward. Cells were then gated to exclude debris, dead cells, and doublet clumps. Gating of individual fluorophores NKp46, CD2, CD3, CD45RB, IFN- γ, and CD62L were set on relative FMOs (Supplementary Section 6.6). These molecules were selected based on the literature, and previous data from the Hope group to identify NK cell subsets (Nkp46/CD2) and activation status (CD45RB/CD62L).

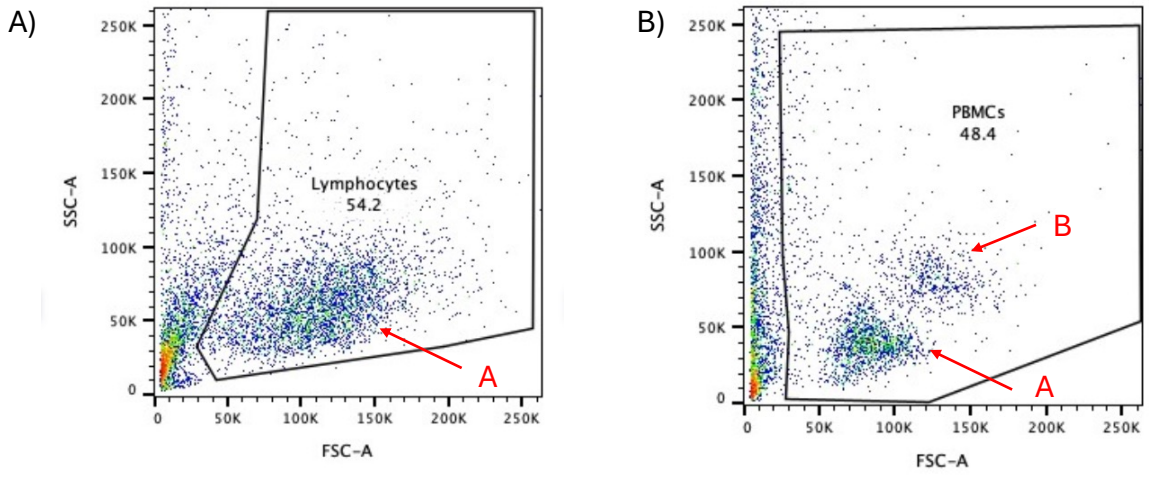

Figure 11. Abnormal vs normal PBMC profile. The letter A represents the lymphocyte population while the letter B represents the monocyte population. A normal PBMC profile

should contain both lymphocytes and monocytes, but no granulocytes, which were removed during the PBMC processing procedure. A) The first set of PBMC samples without monocytes. B) The second set of PBMC samples with the correct profile, which were used for further experimentation.

The optimized flow cytometry panel was then used to characterize bNK cells pre-BCG vaccination (day 0) and post-BCG vaccination (day 42). Day 42 was selected as the optimal time point based on previous studies that showed a peak of IFN- γ expression at this time point (Thom et al., 2012).

Overall, the phenotype of the lymphocytes did not differ significantly between day 0 to day 42 in terms of proportion (measured by percent of cells expressing specific molecules), or mean fluorescence intensity (MFI) for any of the measured markers for panel 1 ($CD2^+$, $CD3^+$, IFN- γ^+, and $NKp46^+$; Figure 12A, Figure 12D), panel 2 ($CD2^+$, $CD45RB^+$, IFN- γ^+, and $NKp46^+$; Figure 12B, Figure 12E), or panel 3 ($CD2^+$, $CD62L^+$, IFN- γ^+, and $NKp46^+$; Figure 12C, 12F). The proportion of bNK cell subsets also remained statistically similar between day 0 and day 42. Within day 0, the proportion of bNK cell subsets remained statistically similar to one another (Figure 13A). However, within day 42, the proportion of $NKp46^+CD2^-CD3^+$ cells were significantly higher than the proportion of $NKp46^+CD2^+$ ($p = 0.0498$; Figure 13B). Because of the way cells were gated into quadrants to look at two fluorophores (NKp46 and CD2) simultaneously, MFI could not be measured in this instance.

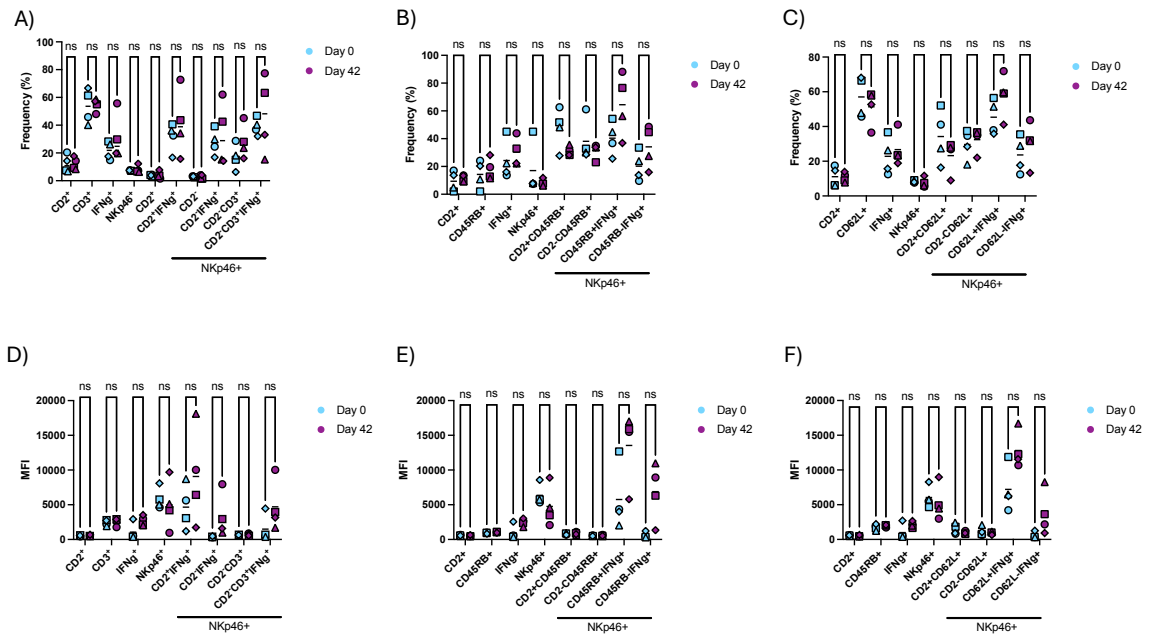

Figure 12. Phenotypic expression of lymphocytes from PBMCs. Cells were isolated from blood of four calves and assessed via flow cytometry (Panel 1 (A,D); Panel 2 (B, E); Panel 3 (C, F)) for the expression of $CD2^+$, $CD3^+$, IFN- γ^+, $NKp46^+$, $CD45RB^+$, and $CD62L^+$. Each symbol represents and individual animal (n = 4 biological replicates). The mean of all four values is displayed. Day 0 and day 42 samples were processed analyzed at separate times. Statistics were calculated via paired t-tests (two-tailed). Values are indicative of cell proportions (A-C) and MFI of the specified cells (D-F) from total live lymphocytes and resulting subpopulations.

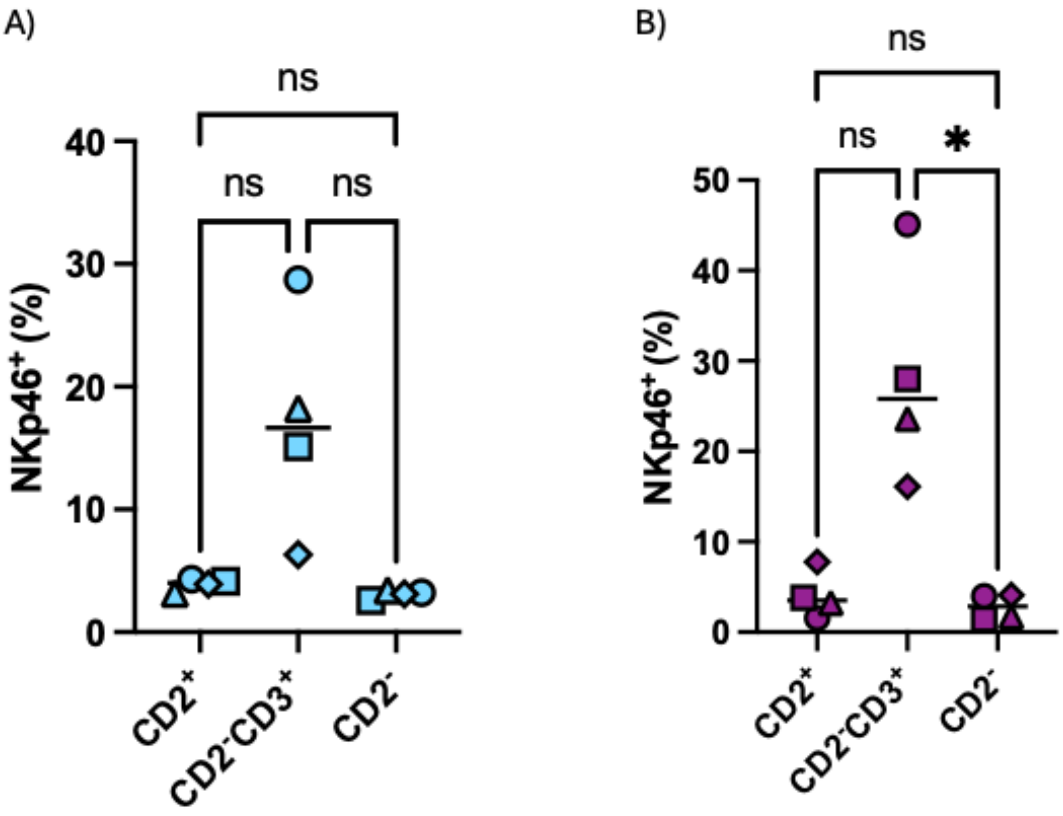

Figure 13. Comparison of NK cell subset prevalence within days. Cells were isolated as described in Figure 1 and assessed via flow cytometry (Panel 1) for expression (measured by percentage). Each symbol represents and individual animal (n = 4 biological replicates). The mean of all four values is displayed. Day 0 (A) and day 42 (B) samples were processed analyzed at separate times. Statistics were calculated via one-way ANOVA. Values are indicative of cell proportions from total live lymphocytes and resulting subpopulations.

Within day 0, the proportion of IFN- γ expression was significantly higher in the bNK cell subset $NKp46^{+}CD2\text{-}CD3^{+}$ than $NKp46^{+}CD2^{+}$ in terms of proportion ($p = 0.0112$), but not in terms of MFI (Figure 14A, Figure 14C). However, within day 42, $NKp46^{+}CD2\text{-}CD3^{+}$ IFN- γ expression was significantly higher than $NKp46^{+}CD2^{+}$ IFN- γ expression in terms of MFI ($p = 0.0421$), but not proportion (Figure 14B, Figure 14D).

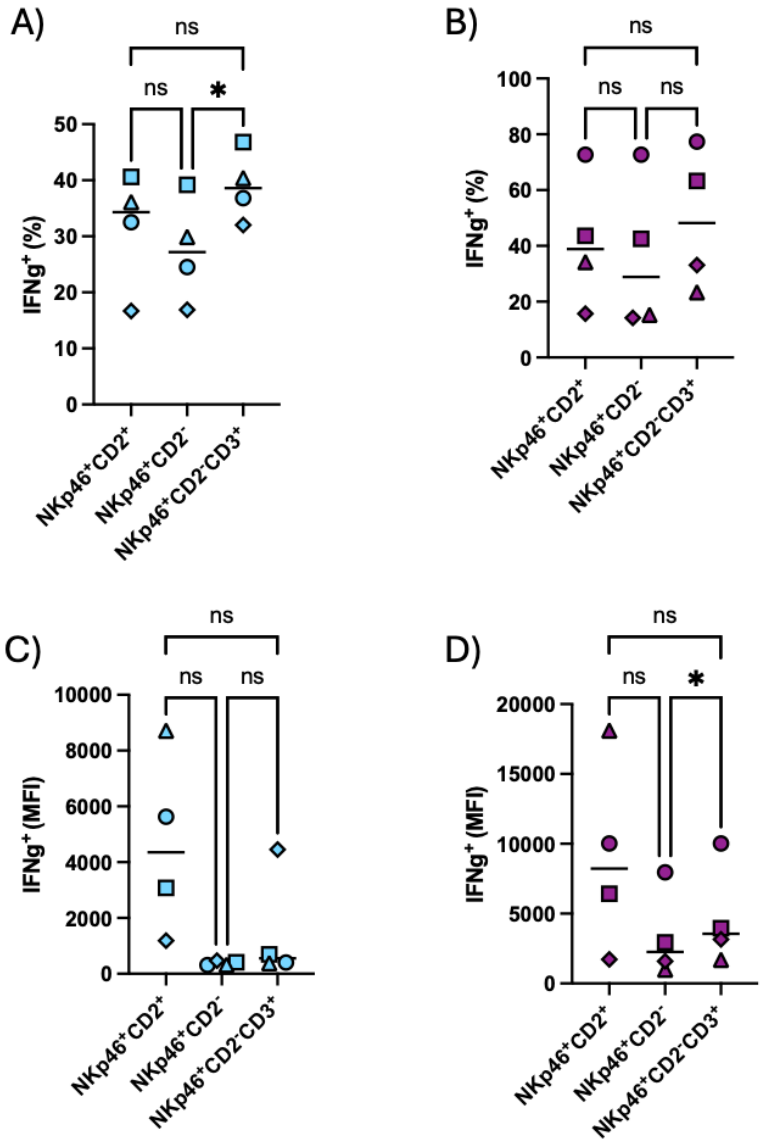

Figure 14. Comparison of IFN- γ expression levels by NK cell subsets within days. Cells were isolated as described for Figures 1-2 and assessed via flow cytometry (Panel 1) for expression and measured by percentage (A-B) and MFI (C-D). Each symbol represents and individual animal (n = 4 biological replicates). The mean of all four values is displayed. Day 0 (A, C) and day 42 (B, D) samples were processed analyzed at separate times. Statistics were calculated via one-way ANOVA. Values are indicative of expression levels from total live lymphocytes and resulting subpopulations.

The expression of the CD45RB, a marker of naïve bovine lymphocytes, and CD62L, thought to be important in NK cell homing to peripheral lymph nodes and therefore a marker of NK cell activation/differentiation status, were also compared between NK cell subsets $NKp46^{+}CD2^{+}$ and $NKp46^{+}CD2^{-}$ (Hanton et al., 2023; Boysen et al., 2008). Within both day 0 and day 42, the proportion of CD45RB expression was significantly higher in the $NKp46^{+}CD2^{+}$ bNK cell subset than in the $NKp46^{+}CD2^{-}$ subset in terms of MFI ($p = 0.0155$), but not in terms of proportion (Figure 15A, Figure 15B). Within day 0, there was no significant difference in expression levels of CD62L between NK subsets (Figure 15, E-F). However, within day 42, there was significantly higher expression of CD62L in terms of proportion (Figure 15G) but not MFI (Figure 15H).

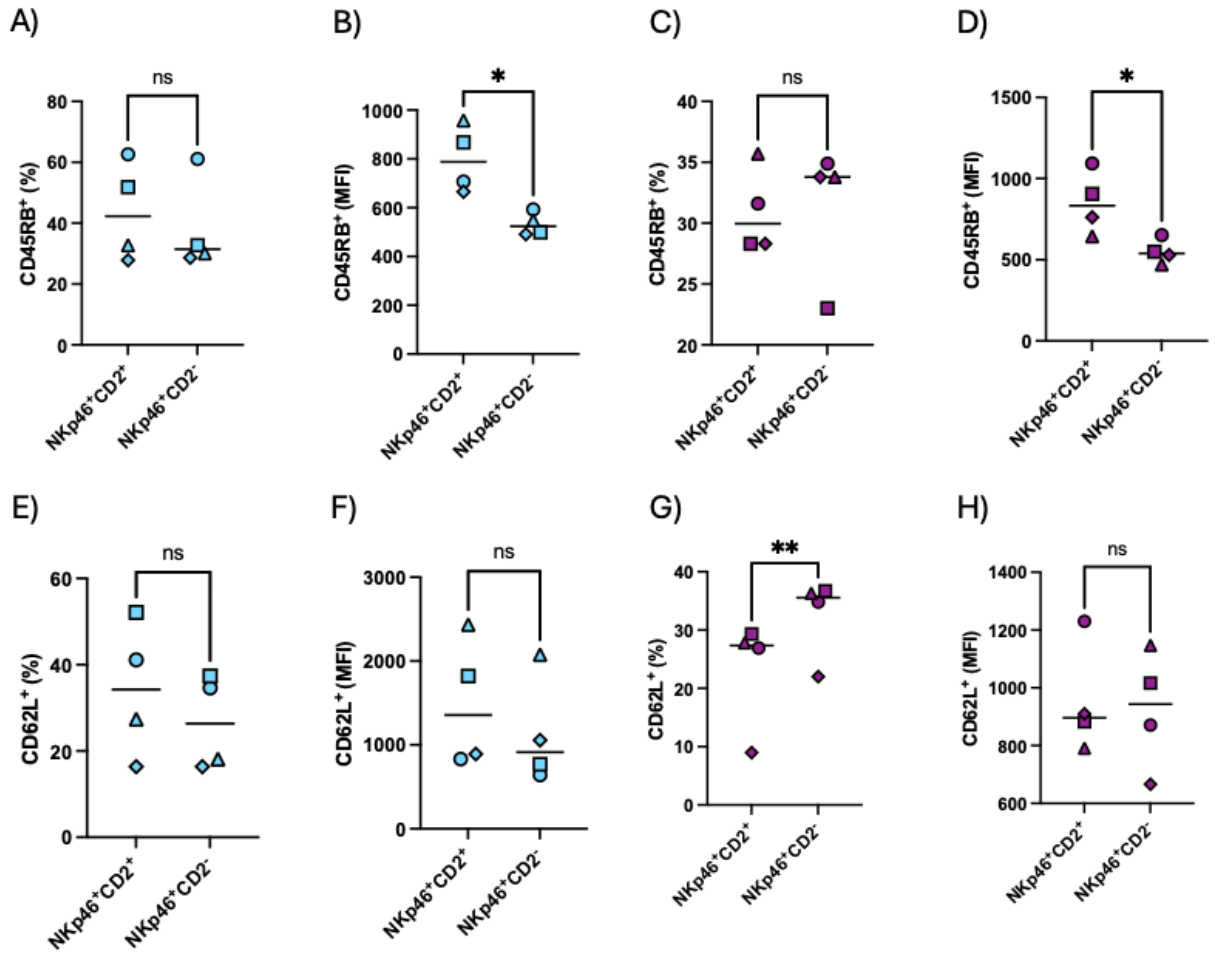

Figure 15. Comparison of CD45RB and CD62L expression levels by NK cell subset within days. Cells were isolated as described for Figures 1-3 and assessed via flow cytometry (Panel 2 (A-D); Panel 3 (E-H)) for expression and measured by percentage (A, C, E, G) and MFI (B, D, F, H). Each symbol represents and individual animal (n = 4 biological replicates). The mean of all four values is displayed. Day 0 (A, B, E, F) and day 42 (C, D, G, H) samples were processed analyzed at separate times. Statistics were calculated via paired t-tests (two-tailed). Values are indicative of cell proportions and MFI from total live lymphocytes and resulting subpopulations. Single asterisks indicate a p-value of less than 0.05 while double asterisks indicate a p-value of less than 0.01.

Within day 0, the proportion of IFN- γ expression was significantly higher in the bNK cell subset $NKp46^{+}CD2^{+}CD45RB^{+}$ than $NKp46^{+}CD2^{-}CD45RB^{+}$ in terms of proportion ($p = 0.0078$), but not in terms of MFI (Figure 16A, Figure 16B). Within day 42, $NKp46^{+}CD2^{+}CD45RB^{+}$ IFN- γ expression was significantly higher than $NKp46^{+}CD2^{-}CD45RB^{+}$ IFN- γ expression in terms of both proportion and MFI (Figure 16C, Figure 16D). Likewise, looking at IFN- γ expression in terms of NK cell subset and = CD62L expression, within day 0, IFN- γ expression was significantly higher in the bNK cell subset $NKp46^{+}CD2^{+}CD62L^{+}$ than $NKp46^{+}$CD2-$CD62L^{+}$ in terms of proportion ($p = 0.0008$) and MFI ($p = 0.0296$; Figure 16E, Figure 16F). Within day 42, IFN- γ expression was again found to be significantly higher in the bNK cell subset $NKp46^{+}CD2^{+}CD62L^{+}$ than $NKp46^{+}CD2^{-}CD62L^{+}$ in terms of proportion ($p = < 0.0001$) and MFI ($p = 0.0004$; Figure 16G, Figure 16H).

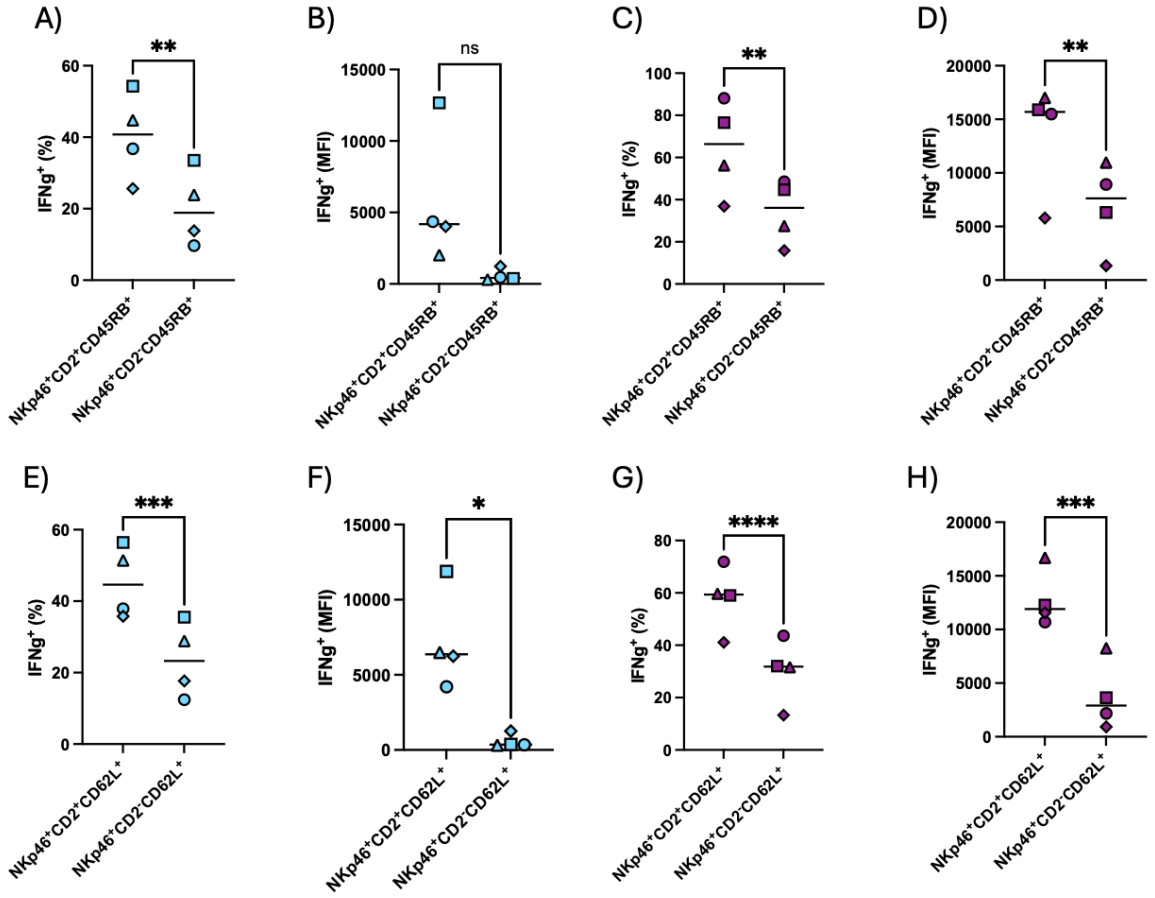

Figure 16. Comparison of IFN- γ expression levels by NK cell subset ($NKp46^+CD2^+$, $NKp46^+CD2^-$) and maturity marker (CD45RB, CD62L) expression. Cells were isolated as described for Figures 1-4 and assessed via flow cytometry (Panel 2 (A-D); Panel 3 (E-H)) for IFN- γ expression measured by percentage (A, C, E, G) and MFI (B, D, F, H). Each symbol represents and individual animal (n = 4 biological replicates). The mean of all four values is displayed. Day 0 (A, B, E, F) and day 42 (C, D, G, H) samples were processed analyzed at separate times. Statistics were calculated via paired t-tests (two-tailed). Values are indicative of cell proportions and MFI from total live lymphocytes and resulting subpopulations. Single asterisks indicate a p-value of less than 0.05, double asterisks a p-value of less than 0.01, triple asterisks a p-value of less than 0.001, and quadruple asterisks a p-value of less than 0.0001.

A complete list of cell proportions obtained via flow cytometry can be found in Supplementary Table 8.

4 Discussion

4.1 ESC Arm

Flow cytometric analyses from NK cell differentiation 1 showed positive staining for NKp46, indicating the presence of NK cells. However, for subsequent NK cell differentiations, staining for NKp46 and CD161, an early NK cell marker added at later stages of the experiment, was negative. Though NKp46 is considered the definitive marker for identifying bNK cells, the lack of replicability of the experiment combined with the lack of additional evidence towards NK cell identity via qPCR, positive staining for the early NK cell marker CD161, or other methods leaves conclusions of positive NK cell differentiation results weak at best. Additionally, the profile of cells that stained positively presented an atypical smear pattern that was not seen in PBMC controls. This could possibly be attributed to cells that were in the process of differentiating expressing differing quantities of NKp46. Additionally, the ideal concentration of antibody used for staining PBMCs could poorly correlate to use in cell culture, where there are a multitude of different cell types and morphologies present than pre-isolated PBMCs.

Differences in culture success could possibly be attributed to differences during the initial EB formation stage. Variability in the number and size of EBs formed may play a role in determining differentiation success. In NK cell differentiation 1, an excessive number of smaller sized EBs were initially plated than are specified in the protocol. In subsequent differentiations, EB formation was not as robust, resulting in fewer EBs being plated per well, though all were within the specified range of the protocol. Methodology also prevented the transfer of a precise number of EBs into each well upon initial plating, leading to approximate quantities being plated, leading to variability within plate to plate conditions. Future experimentation should explore the effect of initial EB deposition (in terms of both size and quanitity of EBs plated) on differentiation success.

4.2 PBMC Arm

I predicted that there would be significantly higher expression of multiple markers (IFN- γ, NKp46, CD62L) and NK cell subsets ($NKp46^{+}CD2^{-}$ and $NKp46^{+}CD2^{-}CD3^{+}$) on day 42 compared to day 0. Previous unpublished studies in the Hope Lab observed an initial BCG-specific spike of IFN- γ around day 7-8 post-vaccination and a secondary spike of IFN- γ around day 42, thought to be attributed to the NK cell response. However, results were not consistent

with this prediction based on previous results, as expression of these markers did not significantly differ between day 0 and day 42 in terms of proportion or MFI, indicating that there was not a significant difference between the two days in how many cells were expressing the marker or how much of the marker was being expressed per cell, respectively. A lack of significantly increased NKp46 and IFN- γ expression together indicates a lack of proliferation of NK cells in response to the vaccine as well as a lack of stimulation of NK cell response to secrete the pro-inflammatory cytokine. Likewise, a lack of increased CD62L and $NKp46^{+}CD2^{-}$ expression, respectively, indicates a lack of activation/differentiation of NK cells. Because this experiment only examined PBMCs collected from peripheral blood, it is possible that a different CD62L profile could have been observed in afferent lymph tissue as compared to peripheral blood. However, this was not tested in this study. Overall, these results could be explained by a lack of or low level of immune stimulation by the vaccine in these individuals as compared to individuals in previous studies. As previously discussed, efficacy of BCG is extremely variable, and a myriad of factors could have contributed to a decreased efficacy of immune stimulation in the chosen population of cows used for experimentation compared to individuals used in previous studies. Future experimentation should focus on examination of other timepoints from this sample group to determine if a true lack of immune stimulation is being observed. Lack of significantly increased expression of $NKp46^{+}CD2^{-}CD3^{+}$ could possibly be attributed to a lack of immune stimulation or not enough events being collected to show a significant effect, as the NKT subset has been shown in previous unpublished studies from the Hope Lab to occur in extremely small proportions.

Within day 42, a significantly higher proportion of $NKp46^{+}CD2\text{-}CD3^{+}$ cells were observed compared to $NKp46^{+}CD2^{-}$ cells, but the proportion of $NKp46^{+}CD2^{-}$ and $NKp46^{+}CD2^{+}$ cells did not significantly differ. With $NKp46^{+}CD2^{-}CD3^{+}$ cells being derived from an additional gating (the parent population being $NKp46^{+}CD2^{-}$), the increased proportion of cells does not correlate with the actual number of cells being measured. This is a limitation of the analysis program, FlowJo, which does not allow you to gauge what percentage a measured subset of cells is in terms of the parent population from which it was derived. As aforementioned, measuring expression via MFI for these populations was not feasible due to the gating strategy. As such, there is no way to accurately measure relative expression of how much of marker was being expressed per cell.

Within day 0, significantly higher expression of IFN- γ by $NKp46^{+}CD2^{-}CD3^{+}$ cells compared to $NKp46^{+}CD2^{-}$ cells in terms of proportion but not MFI was observed. Again, because of the gating strategy, this is not the most accurate representation in terms of number of actual number of cells expressing these markers. However, within day 42, IFN- γ expression was significantly higher in $NKp46^{+}CD2^{-}CD3^{+}$ cells compared to $NKp46^{+}CD2^{-}$ cells in terms of MFI, which indicates that upregulation of the cytokine's production occurred. Because the number of $NKp46^{+}CD2^{-}CD3^{+}$ cells is smaller than $NKp46^{+}CD2^{-}$ cells, this could point to NKT cells being a more powerful source of IFN- γ production than NK cells. This aligns with current literature about the time course of IFN- γ production in terms of different immune cells' responses to mycobacterial infection. NK cells act as early, innate responders to mycobacteria. In contrast, T-cells, as members of the adaptive immune system, require time to mount a response, but become the dominant producers of IFN- γ in later stages of immune responses. In this way, NKT cells could be occupying a unique niche of bridging the divide between innate and adaptive immune immunity in a similar way to γδ T cells (typically found in specific tissues rather than in circulation in the blood), which produce IFN earlier than helper T cells.

Within day 0, $NKp46^{+}CD2^{+}$ cells showed significantly higher expression of CD45RB than $NKp46^{+}CD2^{-}$ cells in terms of MFI, but not proportion. Once again, this can be interpreted as more CD45RB being expressed per cell rather than a higher number of cells expressing CD45RB, indicating differential upregulation of receptor expression. This could be indicative of the $NKp46^{+}CD2^{+}$ population being more naïve in a baseline state (before immunogenic stimulation) than $NKp46^{+}CD2^{-}$ cells. NK cells go through stages of progression into activation, though this process is poorly understood in comparison to in human models. $NKp46^{+}CD2^{-}$ cells are generally considered to be generally more activated, better able to produce IFN-γ, and better able to replicate in IL-2 culture while retaining their cytotoxic abilities than their counterparts. $NKp46^{+}CD2^{+}$ cells are thought to serve a more homeostatic role. Expression of CD62L did not significantly differ between these two NK cell subset populations within day 0. Within day 42, $NKp46^{+}CD2^{+}$ cells once again showed significantly higher expression of CD45RB than $NKp46^{+}CD2^{-}$ cells in terms of MFI, but not proportion. However, CD62L expression was significantly higher within the $NKp46^{+}CD2^{-}$ subset than the $NKp46^{+}CD2^{+}$ subset in terms of proportion, but not MFI. This could indicate the expansion of cells producing CD62L in the $NKp46^{+}CD2^{-}$ population, which could be a result of proliferation/expansion. CD62L is a marker thought to be important in NK cell homing to peripheral lymph nodes and therefore a marker of

NK cell activation/differentiation status. As such, this could be an indication of preferential response of the $NKp46^{+}CD2^{-}$ population post-BCG vaccination.

Lastly, within day 0, $NKp46^{+}CD2^{+}CD45RB^{+}$ cells showed significantly higher expression of IFN- γ than $NKp46^{+}CD2^{-}CD45RB^{+}$ cells in terms proportion but not MFI. Within day 42, $NKp46^{+}CD2^{+}CD45RB^{+}$ cells showed significantly higher expression of IFN- γ than $NKp46^{+}CD2^{-}CD45RB^{+}$ cells in terms of both proportion and MFI, indicating that the cell population was both expanded in a greater fashion and having upregulation of the cytokine's production. This contrasts with predicted outcomes, as $NKp46^{+}CD2^{+}CD45RB^{+}$ should, in theory, reflect a more naïve and generally less activated subset of NK cells. Within day 0, $NKp46^{+}CD2^{+}CD62L^{+}$ cells showed significantly higher expression of IFN- γ than $NKp46^{+}CD2^{-}CD62L^{+}$ cells in terms of both proportion and MFI, indicating that the $NKp46^{+}CD2^{+}CD62L^{+}$ population exhibits greater baseline/tonic expression of IFN- γ. Within day 42, $NKp46^{+}CD2^{+}CD62L^{+}$ cells also showed significantly higher expression of IFN- γ than $NKp46^{+}CD2^{-}CD62L^{+}$ cells in terms of both proportion and MFI. This once again contrasts predicted results, as the bNK $NKp46^{+}CD2^{-}$ subset is generally considered to be more activated and involved in IFN- γ secretion. In combination with expression of CD62L, which should be indicating progression towards activation/maturity, these results conflict with existing evidence about roles of bNK subsets. NK cells inherently display a tonic level of IFN- γ expression, though the production of the cytokine greatly increases when there is an immunological threat needing to be addressed. Tonic IFN- γ expression is thought to serve as a method for maintaining a ready state of surveillance in NK cells. In contrast to T-cells, which require time for activation and response mobilization, a small, basal level of IFN- γ expression along with pre-priming with mRNA allows NK cells to bypass gene transcription when immune mobilization is needed and directly translate mRNA into the cytokine, promoting rapid cytokine production (Mah & Cooper, 2016).

Due to time constraints, further analyses were not undertaken. However, future analyses should focus on the comparison of $NKp46^{+}CD2^{+}CD45RB^{+}$ vs. $NKp46^{+}CD2^{+}CD62L^{+}$ subsets as well as $NKp46^{+}CD2^{-}CD45RB^{+}$ vs. $NKp46^{+}CD2^{-}CD62L^{+}$ subsets between days rather than just within days.

4.3 Limitations of the Study

4.3.1 ESC Arm

Due to high levels of variation observed in qPCR and time constraints of the project, qPCR primers were not effectively utilized throughout this project. Further validation of qPCR primers and use in measuring gene expression of markers specific to hematopoietic precursors (to very differentiation is proceeding through the correct developmental lineage) as well as NK cell-specific markers would provide more robust evidence for successful differentiation of bNK cells in culture.

Additionally, the flow cytometric markers CD45 and CD161 were not effectively utilized throughout this experiment, in part due to the development of the CD161 antibody after the first NK cell differentiation had already occurred. Re-optimization of the original antibody used or further optimization of a new pan CD45 antibody should be undertaken in the future for future assay use, and both CD45 and CD161 should be implemented in future panels from early stages.

4.3.2 PBMC Arm

Because of the statistical corrections used on conducted t-tests (Bonferroni-Dunn being a conservative adjustment method) in conjunction with the large number of comparisons conducted within the same datasets, the probability of type II errors (false-negatives) within the data is increased. In panels 1, 2, and 3 that compared expression of different cellular markers between days 0 and 42, the p-value before being adjusted suggested significantly higher expression of $NKp46^{+}CD2\text{-}CD3^{+}$ ($p = 0.018$, panel 1 (frequency)), IFN-γ ($p = .025$, panel 1 (MFI); $p = 0.027$, panel 2 (MFI)), and CD45RB ($p = 0.009$, panel 2 (MFI)) on day 42 than day 0. These findings would support rather than contrast the prediction of an increased NKT cell population and IFN-γ production at 42 days post-vaccination, which was made based on data from previous experiments. Additionally, it would indicate that there was some measure of immune response happening despite a lack of apparent NK cell proliferation. Likewise, because of the statistical corrections used on conducted ANOVAs (the Greenhouse-Geisser correction), the probability of type I errors (false positives) within the data is increased for those analyses.

Smearing in the V 450/50 channel was observed throughout this experiment. Ultimately, this was attributed to inherent autofluorescence of the cells, which previous projects have observed in

bovine models in this channel. Further optimization of staining protocols could be undertaken to minimize this issue alongside optimization of compensation to better minimize the effects of spectral overlap.

Additionally, gating methodology could be further optimized to better isolate the activity of NK cells. In analysis of day 42 samples, the appearance of the T-cell population was included in initial gating methods of singling out lymphocytes. While the effects of these cells should have hypothetically been filtered out of final results when gating specifically for cells expressing NKp46, reanalyzing data excluding these cells in the gating would give a more definitive results.

4.4 Future Directions

There are a variety of future experiments that could be undertaken to better elucidate the role of NK cells in inducing immunity from the BCG vaccine in a One Health context. Due to time constraints of the degree, further comparisons were unable to be undertaken in this project. However, future directions are broadly outlined as follows.

Data should be collected from additional timepoints (days 3, 7, and 28) to compare the immune response elicited at different stages post-vaccination. These procedures should also be replicated with another set of cows to determine if there was a lack of immune stimulation in this particular population. Data from this project should be incorporated with past data sets to get a larger idea of how BCG is stimulating NK cell-mediated immunity with a better statistical power. Additionally, T-cell subset contribution to overall IFN-γ production could be measured with T-cell specific flow cytometric markers and subsequently compared with NK cell IFN-γ production across timepoints to better elucidate the role of NKT cells' IFN-γ production in the grand scheme of immune stimulation in response to BCG administration.

Additionally, we have access to data and samples from collaborators at the Jenner Institute (University of Oxford) concerning human neonate samples vaccinated with BCG via injection. Flow cytometric analyses and comparisons of pre- and post-vaccination NK cell responses between analogous human and bovine neonates at a variety of timepoint could provide increased insight into differences in how immunity is elicited by the vaccine in humans and cattle. This

could also potentially highlight new avenues of investigation in terms of bovine NK cell immunity, which is not as well defined as in humans.

In terms of culturing bovine NK cells, the data from this project alone is not yet optimized to use this procedure as a source of bNK cells for further experimentation. Future directions should be focused on standardization and optimization of this protocol for reliable production of bNK cells. Additional tests that could be conducted to bolster evidence for successful bNK cell culture include phagocytosis assays and non-flow cytometric-based cytotoxicity assays using cultured cells. After optimization has been achieved, cultured bNK cells could be compared to ex-vivo bNK cells (the current source of NK cells for experimentation) via flow cytometry to determine similarity levels. Additionally, these cultured bNK cells could be compared to cultured human NK cells via flow cytometry to compare the relative presence and distribution of different cell markers. Other potential analyses that could be used to compare the population include cytotoxicity assays and proliferation analyses. These two populations could also be compared to ex-vivo human NK cells. Downstream experiments could entail testing cultured bNK cells' viability as an artificial source of NK cells for assay use in terms of stimulation and infection.

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6 Supplementary Material

6.1 Macrophage Differentiation Protocol

Macrophage differentiation of bovine ESCs.
Aim: To direct bovine ESCs out of the pluripotent state and initially into the haematopoietic state and ultimately into the myeloid lineage for the production of in vitro-derived macrophages.

Day 0
1) Harvest a single cell population of bovine ESCs as described in the 'Bovine ESC Passaging' protocol.
2) Resuspend the ESC cell suspension (which will also include feeder cells) in AFX+Ri (5M).
3) Count using a haemocytometer
4) Plate 5x104 cells in a 6-well (9.6cm2) in AFX+Ri. Gently rock to evenely disperse.
5) Incubate overnight at 38.5oC/7%CO2/5%O2.

Day 1 – Change to Mesoderm Induction Medium (Mix1)
6) Carefully aspirate the medium.
7) Add 3ml Mesoderm Induction Medium (Mix 1) to each well.
8) Incubate overnight at 38.5oC/7%CO2/5%O2.
9) Feed every day with Mix1.

Day 4 – Change to Macrophage Induction Medium (Mix2)
10) Carefully aspirate the Mix1 medium from the well(s).
11) Add 3ml Macrophage Induction Medium (Mix 2) to each well.
After a day or two the cells should start to take on a 'swirly appearance' with condensed areas of cells.

Day 7 onwards – Continue to feed every 3-4 days
12) Over the next few days feed with fresh Mix2 every 3-5 days.

Day 10-12 – Early signs of macrophage production.
23) Macrophages will start to appear. Production will increase and peak at around d25-35.
24) Harvest non-adherent macrophages every 3-5 days and feed remaining cells with fresh Mix2.
25) Centrifuge the macrophages at 600-1000g, 3 minutes and resuspend pellet in 1ml of Mix2 and count using a haemocytometer.
26) Macrophages can be matured by plating in Mix 3 for 3-5 days.

6.2 Macrophage Differentiation Protocol

Bovine ESC culture

Bovine ESCs are cultured at 38.5oC, 7% CO2 and 5% O2.
Avoid keeping the cells out of the incubator for longer than necessary.

Passaging Bovine ESCs cells
1. Aspirate medium.

2. Wash the well once with PBS (1ml for 6-well).
3. Add 0.5xTrypLE (500µL for 6-well) and incubate 3-5 min in incubator.
4. Check that the cells are rounded then disperse to single cell and check under microscope.
5. Transfer to a 1.5ml eppendorph tube.
6. Wash well with 1ml wash buffer the transfer to eppendorph (to recover as many cells as possible).
7. Centrifuge at 1200RPM, 4 minutes.
8. Remove supernatant and resuspend the pellet in 1ml bESCM+5µM Ri (Y-27632).
9. Count cells using haemocytometer.
10. Plate 0.5-1x105 cells on to a laminin-coated 6-well (9.6 cm^2) in a final volume of 3ml bESCM+Ri.
11. Feed the next day without Ri and feed every day thereafter (remove almost all the medium when feeding)
10. Passage every 3-4d.

6.3 NK Cell Differentiation Original Protocol

Protocol to Produce iPSC-Macrophages_Forrester Lab

[0] Preparation of reagents * Solution can be stored at -20C up to 1 year (Once thawed, it can be stored at 4C for 7 days)

Product	Company	Cat #	Storage
STEMPRO Kit	Invitrogen	#A1000701	Supplied as a kit with 3 components i) DMEM/F12 (1:1) >>> Aliquot to 50 mL and store at 4ºC ii) StemPro hESC supplement >>> Aliquot to 500 uL and store at -20ºC iii) 25% BSA >>> Aliquot to 5 mL and store at 4ºC
2-mercaptoehtanol (50 mM)	Invitrogen	#31350-010	Aliquot to 1 mL and store at 4ºC
PBS (Mg+Ca+)	Invitrogen	#14040091	Store at 4ºC
Human serum albumin (10%)	Irvine Scientific	#9988	Store at 4ºC
Human basic-FGF	Thermo Fisher	PHG0021	i) Add 100 uL of 10% Human Serum Albumin stock to 9.9 mL PBS (Mg+Ca+) ii) Add 1 mL of 0.1% HSA solution to 100 ug tube of hbFGF (100ug/mL) iii) Aliquot 9 mL of 0.1% HSA solution to 50 mL tube iv) Transfer the 1mL of 100ug/mL hbFGF sol. Into iii) v) Mix thoroughly by inversion (10ug/mL) vi) Aliquot the final 10ug/mL hbFGF to 200uL in cryotubes and sotre at -20ºC
CELLstart	Invitrogen	#A10142-01	Supplied in 2ml >>> aliquot to 200 uL and store at 4ºC
Rock Inhibitor (Y27632, 1mg)	Merck (Millipore)	#688000	Resuspend to 1mg/mL by PBS (Mg+Ca+) >>> Aliquot to 50 uL and store at -20ºC
BMP4	R&D	314-BP-010	Reconstitute with 0.1% HSA solution (25ug/mL) >>>Aliquot to 50uL in cryotubes and sotre at -20ºC
VEGF	R&D	293-VE-010	Reconstitute with 0.1% HSA solution (100ug/mL) >>>Aliquot to 50uL in cryotubes and sotre at -20ºC
SCF	Thermo Fisher	PHC2111	Reconstitute with 0.1% HSA solution (100ug/mL) >>>Aliquot to 50uL in cryotubes and sotre at -20ºC
Cell Culture Multi-well Plate, 6 WELL	Greiner bio-one	657970	RT
Gelatin	SIGMA	#G1890-500G	1% solution with PBS (with Ca/Mg) >>> Aliquote to 1 mL in cryo tube
DMEM	Gibco	10566016	4 ºC (Note: High glucose, GlutaMAX™ Supplement)
Ham's F-12	Gibco	31765035	4 ºC (Note: Nutrient Mix, GlutaMAX™ Supplement)
HI Human AB Serum	SIGMA	H3667-100ML	Aliquot to 4 mL and store at -20C
Pen/Strep (100x)	Gibco	10378016	4ºC
L-glutamine (200mM)	Gibco	25030081	4ºC
2-mercaptoehtanol (50 mM)	Invitrogen	#31350-010	Aliquot to 1 mL and store at 4C
Sodium Selenite	SIGMA	S5261-10G	Dissolve 5 mg sodium selenite in 10 ml in PBS (Ca/Mg free), filter sterilize, and store at 4 C
Ethanolamine >98% (16.6 M)	SIGMA*	E0135-100ML	Store at 4C * Original Protocol: MP Biomedicals:https://www.mpbio.com/uk/ethanolamine-acs-100-ml)
Ascorbic Acid	SIGMA	A8960	Dissolve 50 mg ascorbic acid 2-phosphate in 1 ml in PBS (Ca/Mg free), filter sterilize, and store at 4 C
IL-3 (100ug)	Peprotech	200-03	Reconstitute with 0.1% HSA solution (10 ug/mL) >>>Aliquot to 50uL in cryotubes and sotre at -20C
IL-7 (100ug)	Peprotech	200-07	Reconstitute with 0.1% HSA solution (100 ug/mL) >>>Aliquot to 50uL in cryotubes and sotre at -20C
IL-15 (250ug)	Peprotech	200-15	Reconstitute with 0.1% HSA solution (100 ug/mL) >>>Aliquot to 50uL in cryotubes and sotre at -20C
Flt3 Ligand (100ug)	Peprotech	300-19	Reconstitute with 0.1% HSA solution (50 ug/mL) >>>Aliquot to 50uL in cryotubes and sotre at -20C
IL2 (1mg)/CF	Peprotech	200-02	Reconstitute with 0.1% HSA solution (100ug/mL) >>>Aliquot to 50uL in cryotubes and sotre at -20C
IL-21 (10ug)	Peprotech	200-21	Reconstitute with 0.1% HSA solution (10 ug/mL) >>>Aliquot to 50uL in cryotubes and sotre at -20C

[1] Thaw & Culture Feeder-Free iPSC

1 Preparation of media

i) Add the appropriate volumes of the components of StemPro complete together to make 1x complete media sufficient for 1 week culture.

Component	Final Conc	Final volume of x1 Complete medium 100mL	50mL	25mL	15mL	10mL	Unit	
DMEM/F12*	x1	90.8	45.4	22.7	6.81	4.54	mL	
Stem Pro hES Suppl*	x1	2	1	0.5	0.15	0.1	mL	
25% BSA*	1.80%	7.2	3.6	1.8	0.54	0.36	mL	* STEMPRO Kit
2-ME (55mM)	0.1mM	*200*	*100*	*50*	*30*	*20*	*uL*	
hbFGF (10ug/mL)	20ng/mL	*200*	*100*	*50*	*15*	*10*	*uL*	

* Media without bFGF is used as **WASH MEDIA**
* Media with bFGF (daily add before use) is used as **CULTURE MEDIA**
* Media can be stored in the dark at 4C for up to 7 days.

ii) Aliquot **5 mL** of **WASH Media** and **4mL** of **CULTURE Media** (add hfFGF) into 50mL tubes >>> incubate at **37ºC (euilibrate in incubator)**

2 Preparation of plates

i) Dilute CELLstart sock solution to **1:50** by **PBS (Ca+Mg+)** >>>>>>>>

ii) Add the diluted CELL start to TC plates as required
* Make sure that the surface is evenly coated

iii) Incubate, **37C, 1hr**
* Plates should be used immediately (can be stored at 4C for 24hr)

Total (mL)	PBS (mL)	CELLStart (uL)
5	4.9	100
4	3.92	80
3	2.94	60
2	1.96	40
1	**0.98**	**20**

Well size	Area (cm2)	Diluted CELLstart(uL)
6-well	**9.6**	**750**
12-well	3.2	250
24-well	1.9	200

3 Thaw vial of cells by holding in palm of hand.
* Normally thaw 1 vial and seed in 1 well

5 Using **1 mL** of warmed **WASH MEDIA** gently resuspend in the vial.

6 Transfer the resuspended cells to the remaining **4 mL** of **WASH MEDIA** remaining in the 50ml tube.

7 Centrifuge @1000rpm for 5 min

8 Remove supernatant and resuspend in **1 mL CULTURE MEDIA + 6.6 uL Rock Inhibitor (Y27632: 1mg/ml stock)**

9 Aspirate CELLstart from the culture dish and immediately put **1 mL CULTURE MEDIA**, do not let dry!

10 Add **resuspended cells (1mL)** with the Rock inhibitor to coated well with media.

11 Gently shake dish for even distribution.

12 Incubate the cells at **37C** for **3 days**
* **Change the media every 24hr by replacing 1.5mL of medium**
* During weekends (Saturday), add **3 mL (double feed)** and change media on Monday. You can only get away with one 'double feed' per week.
* We are expecting 1-3 colonies max from that well (sometimes you even see more)
* If cells are too sparse, using the EZ tools to re-seed cells together

[2] Passage & Culture Feeder-Free iPSC

1 Prepare media and plates as descibed above
2 Replace **4 mL CULTURE MEDIA** on cells that are to be passaged **approx. 1 hour before.**
3 Mechanical detachment ot the cells
  i) Hold the EZ passaging (disposable) tool firmly
  ii) Place the cutting head in the well
  iii) Make confident horizontal movement left to right across the centre of the well
    * Apply only moderate downward pressure on the well.
    * Cut the entire well
    * Take care not to cut over areas you have already done.
  iv) Rotate the well 90 degrees and repeat the cutting.
4 Pipette the media in the well using a sterile pastette to dislodge the cut colonies.
5 Collect the dislodged colonies in the **4 mL CULTURE MEDIA** in a 50mL (or 15mL) tube
  * If you are cutting in a right way, you do not get massive colonies, and can use all colonies
  * If you find large unwanted colonies, wait 1-2min for the colonies to settle
6 Take required volume of cell suspension from the top half of 5) and seed them to new wells including **1 mL CULTURE MEDIA**
  * Normally 1:4 or 1:5 dilution is sufficient. Therefore from 1 well, 4 or 5 further wells can be seeded.
  * If you need just 1 well, then take 1mL (1:4) or 800uL (1:5) from the 4mL cell suspension and seed them
7 Label dishes with cell line and passage number.
8 Move the plate gently to disperse evenly the colonies.
9 **Change media every day**
  * Passage cells when it reach more than 50% confluence
  * **Normaly 1 week until cells become confluence** (depends on time, after a 3-4 passages they will reach confluence every 3-4 days.)
  * If they reach 100% confluency, cell differentiation ability will be negatively affected
  * In the second passage, some part of cells should be put into storage
  * For experiment, use cells after 2-3 pssages.

[3] Cryopreservation

1 Remove 1.5 mL of medium from the cell culture
2 Add **2mL CULTURE media** (Stempro+hbFGF)
3 Add **4uL** of **ROCK Inhibitor**
4 Incubate **1hr at 37°C**
5 Cut cells with EZ Passaage and transfer to a universal 50mL (or 15mL) tube
6 Centrifuge @1200rpm for 3 min Centrifuge @1200rpm for 3 min
7 Aspirate Media
8 Resuspend in **1 mL Cryostor media** >>> Aliquot to two cryopreservation tube (500uL/tube)
  * Cryostor cell cryopreservation media: SIGMA, #C2874-100ML
9 Put vials in a chilled 'Mr. Frosty' and store at -80oC 24h
10 Move to -150°C

[4] Differentiation into EB

1 Preparation of Medium
  i) **EB-MIX 1a**

Component	Final Conc	**10mL**	Unit
DMEM/F12*	x1	9.08	mL
Stem Pro Suppl*	x1	0.2	mL
25% BSA*	1.80%	0.72	mL
BMP4 (25ug/mL)	20ng/mL	8	uL
VEGF (100ug/mL)	20ng/mL	2	uL
SCF (100ug/mL)	40ng/mL	4	uL

*STEMPRO Kit
* Cytokines are stable for 2 days at 37C

  ii) **EB-MIX 1b (6X)**

Component	Final Conc	**1.66mL**	Unit
DMEM/F12	x1	1.507	mL
Stem Pro Suppl	x1	0.033	mL
25% BSA	1.80%	0.120	mL
BMP4 (25ug/mL)	20ng/mL	8	uL
VEGF (100ug/mL)	20ng/mL	2	uL
SCF (100ug/mL)	40ng/mL	4	uL

* Cytokines in MIX1b are at the 6x concentration to that of MIX1a
>>> When 500ul of MIXb is added into 2.5ml MIXa in Step-8, cytokines for embryo formation is refeshed.

2 Replace CULTURE MEDIA in iPSCs to **4mL EB-MIX 1a** approx. 1 hour before passage
3 Mechanical detachment ot the cells
  i) Hold the EZ passaging (disposable) tool firmly
  ii) Place the cutting head in the well
  iii) Make confident horizontal movement left to right across the centre of the well
    * Apply only moderate downward pressure on the well.
    * Cut the entire well
    * Take care not to cut over areas you have already done.
  iv) Rotate the well 90 degrees and repeat the cutting.
4 Pipette the media in the well using a sterile pastette to dislodge the cut colonies.
5 Seed the cells into two wells of **low-attachment 6-well plate** (3mL/well)
6 Incubate, 37°C, **2 days**
7 Add **500 uL** of **EB-MIX1b** to each well
8 Incubate, 37°C, further **2 days** (total 4 days) >>> Differentiation into Monocytic cells or NK cells

[5] Differentiation of EBs into NK Cells (day4)

1 Preparation of plates
- i) Incubate GelatinStock Solution at 37-45 ºC until completely resolved
- ii) Dilute Gelatin with PBS (with Ca/Mg) at **1:10** (**0.1% Gelatin**)
- iii) Add **750 - 1000 uL** of **0.1% Gelatin** into each well
- iv) Incubate at **37ºC for 30 min**

2 Preparation of Medium

NK-Diff-Medium (1x)

Component	Final Conc	200 mL	20 mL	5 mL	Unit
DMEM	56.6%	111.2	11.12		mL
Ham's F-12	28.3%	56.6	5.66		mL
HI Human AB Serum	15%	30	3		mL
Pen/Strep (100X)	1%	2	0.2		mL
L-glutamine (200mM)	2 mM	2	0.2		mL
Ascorbic Acid (50 mg/mL)	20 ug/mL	80	8		uL
2-mercaptoehtanol (50 mM)	1 uM	4	0.4		uL
Sodium Selenite (50 ug/mL)*	5 ng/mL	20	2		uL
Ethanolamine (500 mM)**	50 uM	20	2		uL
IL-3 (10ug/mL)***	5 ng/mL		10	2.5	uL
SCF (100ug/mL)	20 ng/mL		4	1	uL
IL-7 (**100ug/mL**)	20 ng/mL		4	**1**	uL
IL-15 (100ug/mL)	10 ng/mL		2	0.5	uL
Flt3L (**50ug/mL**)	10 ng/mL		4	**1**	uL

*** SS original stock: 0.5mg/mL >>> Dilute 1/10 by DMEM/F12 (2:1) before use (=50 ug/mL)**
**** EthAm original stock: 16.6M >>> Dilute 1/33.2 by DMEM/F12 (2:1) before use (=500mM)**
***** IL-3: Add only first week**

3 Transfer **ALL** EBs into 15mL tube*
4 Let the cells settle by gravity (DO NOT spin, wait around **10 min**)
5 Remove old medium w/o interfering with EBs (by Pippetman) >>> Replace with **2 mL NK-Diff-Medium** gently
6 Let the cells settle by gravity (DO NOT spin, wait around **10 min**)
7 Remove medium w/o interfering with EBs >>> Add new **2 mL NK-Diff-Medium** gently
8 Take ALL Ebs* >>> Transfer to 6 or 10 cm dish
9 Remove gelatin from 6-well plate >>> Immediately add **3 mL NK-Diff-Medium** to the gelatin coated 6-well plate
10 Take 14-16 EBs (count roughtly by eyes) from [8] >>> Transfer to each well of the gelatin-coated plate
11 Change medium (**3 mL**) **every 5-7 days**
    * After 14 days, medium needs to be changed **every 3 days**
12 Continue medium changes for **3–4 weeks** >>> Check expression of NK cell makers (CD45+CD56+) by flow cytometry on suspension cells
    * It usually takes 4 weeks to obtain >90% CD45+CD56+ cells from spin EBs NK cells for most of ES or iPSC lines

* When dealling with EBs and iPS cells on passage, **use sterile pastette** to avoid damages on them
* You can see by naked eyes that EBs fall rapidly.

* Use **sterile pastette** to avoid damages on EB

6.4 Reagents Used

Supplementary Table 1. Mesoderm Induction Medium (Mix1)

Component	Amount
Stempro	10 mls
rhbFGF (20ng/ml)	10ml of 20mg/ml
rhBMP4 (50ng/ml)	10ml of 20mg/ml
rhVEGF (50ng/ml)	10ml of 50mg/ml (100ml sterile PBS, 10ml aliquots).
rhSCF (20ng/ml)	2ml of 100mg/ml (1ml H_2O, 50ml aliquots)

Supplementary Table 2. Macrophage Induction Medium (Mix2)

Component	Amount
X-Vivo 15	10mls
Pig M-CSF (100ng/ml)	10 ml of 100mg/ml
Pig IL3 (25ng/ml)	10 ml of 25mg/ml
Glutamax (2mM)	100 ml (200mM)
Penicillin/Streptomycin	100 ml

Mercaptoethanol (50nM)	10 ml (50mM)

Supplementary Table 3. Macrophage Maturation Medium (Mix3)

Component	Amount
X-Vivo 15	10 mls
Pig M-CSF (100ng/ml)	10 ml of 100mg/ml
Glutamax (2mM)	100 ml (200mM)
Penicillin/Streptomycin	100 ml

Supplementary Table 4. Mix 1a

Component	Final Concentration	Amount (10 mL)	Notes * Cytokines in MIX1b are at the 6x concentration to that of MIX1a
DMEM/F12*	x1	9.08 mL	*STEMPRO Kit
Stem Pro Suppl*	x1	0.2 mL	
25% BSA*	1.80%	0.72 mL	
BMP4 (25ug/mL)	20ng/mL	8 uL	
VEGF (100ug/mL)	20ng/mL	2 uL	
SCF (100ug/mL)	40ng/mL	4 uL	

Supplementary Table 5. Mix 1b

Component	Final Concentration	Amount (1.66 mL)	Notes * Cytokines in MIX1b are at the 6x concentration to that of MIX1a
DMEM/F12	x1	9.08 mL	>>> When 500ul of MIXb is added into 2.5ml MIXa in Step-8,
Stem Pro Suppl	x1	0.2 mL	cytokines for embryo formation is refeshed.
25% BSA	1.80%	0.72 mL	
BMP4 (25ug/mL)	20ng/mL	8 uL	

VEGF (100ug/mL)	20ng/mL	2 uL	
SCF (100ug/mL)	40ng/mL	4 uL	

Supplementary Table 6. NK Cell Differentiation Medium

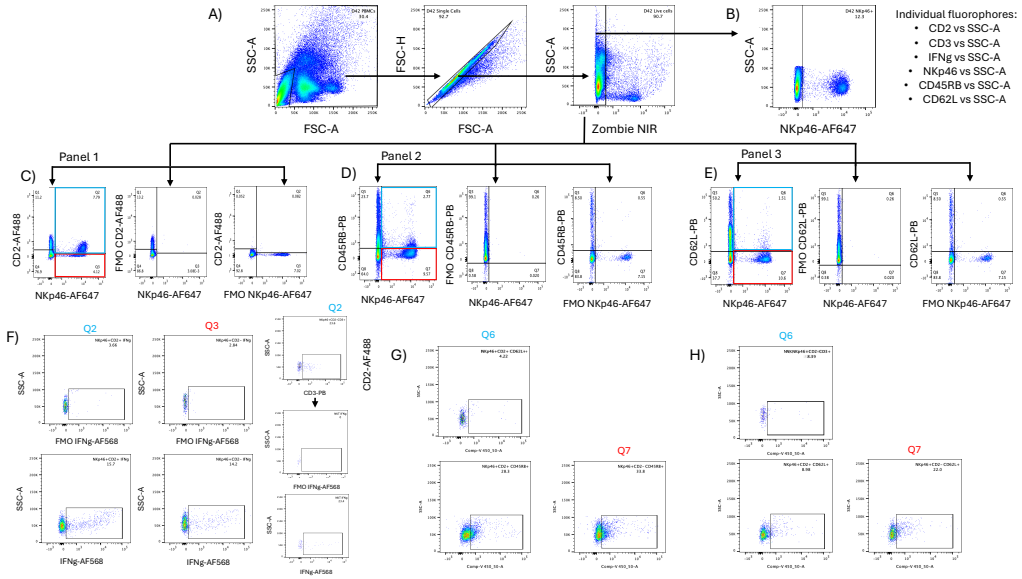
Component	Final Concentration	Amount (200 mL)	Notes
DMEM	56.60%	111.2 mL	
Ham's F-12	28.30%	56.6 mL	
HI Human AB Serum	15%	30 mL	
Pen/Strep (100X)	1%	2 mL	
L-glutamine (200mM)	2 mM	2 mL	
Ascorbic Acid (50 mg/mL)	20 ug/mL	80 uL	
2-mercaptoehtanol (50 mM)	1 uM	4 uL	
Sodium Selenite (50 ug/mL)*	5 ng/mL	20 uL	* SS original stock: 0.5mg/mL >>> Dilute 1/10 by DMEM/F12 (2:1) before use (=50 ug/mL)
Ethanolamine (500 mM)**	50 uM	20 uL	** EthAm original stock: 16.6M >>> Dilute 1/33.2 by DMEM/F12 (2:1) before use (=500mM)
IL-3 (10ug/mL)***	5 ng/mL	100 uL	*** IL-3: Add only first week
SCF (100ug/mL)	20 ng/mL	40 uL	
IL-7 (100ug/mL)	20 ng/mL	40 uL	
IL-15 (100ug/mL)	10 ng/mL	20 uL	
Flt3L (50ug/mL)	10 ng/mL	40 uL	

6.5 qPCR Primer Sequences

Supplementary Table 7. qPCR primer sequences. Primer sequences were designed using gene transcripts from the databases NCBI and Ensembl, which were then ran through Primer3 to determine viable primer sequences.

Name	Sequence
bovine_KDR_F1	GCATGGAAGAGGATTCGGGA
bovine_KDR_F2	ATTCGGGACTCTCTCTGCCT
bovine_KDR_F3	CCGGCCCGTCAGTGTAAAA
bovine_KDR_R1	TTTTACACTGACGGGCCGG
bovine_KDR_R2	CCGGCTCTTTCGCTTACTGT
bovine_KDR_R3	AGATGGGGCTAACTTGGTTCT
bovine_TBXT_F1	CACACGGCTGCGAAAGGTA
bovine_TBXT_F2	CGCATCGCAACAGTTCTCCA
bovine_TBXT_R1	CTGGTGTGGGCAGACATTCC
bovine_TBXT_R2	TGGGCAGACATTCCAAGGC

6.6 Flow Cytometry Gating Strategy



6.7 Flow Cytometry Quantitative Results

Supplementary Table 8. PBMC arm flow cytometry data.

	Calf 110737 Day 0		*Calf 110737 Day 42*		*Calf 210738 Day 0*		*Calf 210738 Day 42*		*Calf 410740 Day 0*		*Calf 410740 Day 42*		*Calf 510741 Day 0*		*Calf 510741 Day 42*	
	Percentage	**MFI**	**Percentage**	**MFI**	**Percentage**	**MFI**	**Percentage**	**MFI**	**Percentage**	**MFI**	**Percentage**	**MFI**	**Percentage**	**MFI**	**Percentage**	**MFI**
Panel 1																
CD2+	20.4	450	14.3	502	7.45	613	9.31	502	6.8	503	8.38	481	14.3	582	17.7	669
CD3+	45.9	2168	48	1774	61.4	2799	55	2928	40.1	1928	58.6	2810	66.7	2723	57.2	2894
IFNg+	14.9	503	55.7	2992	28.3	410	29.8	2160	26.2	380	19.8	2048	17.5	2922	19.8	3525
NKp46+	7.47	4623	7.23	954	7.16	5762	6.66	4211	7.69	5029	6.35	5103	7.48	8099	12.3	9685
NKp46+CD2-CD3+ (NKT)	28.7	620	45.1	857	15.1	694	28	521	18.2	627	23.6	868	6.31	634	16.1	555
Q2: NKp46+CD2+	4.3	-	1.57	-	4.16	-	3.83	-	3.14	-	3.21	-	3.9	-	7.79	-
Q3: NKp46+CD2-	3.22	-	3.96	-	2.57	-	1.42	-	3.41	-	1.72	-	3.12	-	4.12	-
NKp46+CD2+ IFNg	32.5	5628	72.7	10019	40.6	3077	43.6	6432	36.1	8716	34.2	18115	16.7	1188	15.7	1718
NKp46+CD2- IFNg	24.5	310	62	7957	39.2	414	42.5	2940	29.9	320	15.3	988	16.9	471	14.2	1591
NKT IFNg	36.8	407	77.4	10019	46.8	694	63.3	3959	40.4	383	23.4	1693	32	4453	33.1	3163
Panel 2																
CD2+	17	663	13.5	494	5.8	597	9.45	481	4.85	497	9.2	488	13.7	562	13.4	634
CD45RB+	24.1	984	19.5	1046	19.2	974	12.8	1112	10.8	927	11.5	1049	20.4	823	28.2	976
IFNg+	13.7	582	43.9	2629	37	402	32.9	2350	22.3	352	22.2	1784	16	2558	22.2	3028
NKp46+	7.45	5347	6.87	2080	8.34	5787	7.71	3490	8.01	5737	6.18	4585	7.74	8581	11.8	8893
NKp46+CD2+CD45RB+	62.6	707	31.6	1093	51.8	868	28.3	905	48.2	958	35.7	644	27.8	665	28.3	762
NKp46+CD2-CD45RB+	61.1	593	34.9	652	32.8	499	23	549	30.1	549	33.8	471	28.6	491	33.8	529
NKp46+CD45RB+ IFNg	36.8	4352	88.1	15491	54.3	12669	76.5	15893	44.8	2021	56.3	17006	25.6	4016	36.9	5787
NKp46+CD45RB- IFNg	9.65	460	48.5	8933	33.5	380	44.7	6308	23.9	306	27.5	10970	13.8	1225	15.9	1342
Panel 3																
CD2+	17.5	630	11.8	451	6.25	574	8.94	477	6.08	491	7.78	436	14.6	593	13.9	639
CD62L+	45.7	1881	36.5	1718	66.4	1591	58.4	2064	47.8	1249	57.8	2033	68.1	2206	52.7	2076
IFNg+	12.5	478	41.1	2236	36.7	403	23.5	1661	26.3	381	23.3	1991	15.7	2707	18.9	2660
NKp46+	7.73	5509	5.39	2987	9.12	4662	7.48	4903	8.83	5737	6.01	4453	7.99	8281	11.6	8973
NKp46+CD2+CD62L+	41.1	830	26.9	1230	52.1	1822	29.3	882	27.4	2433	27.8	791	16.4	890	8.98	910
NKp46+CD2-CD62L+	34.6	641	34.8	871	37.4	769	36.7	1016	18.1	2076	36.3	1147	28.4	1057	22	666
NKp46+CD62L+ IFNg	37.9	4202	71.9	10699	56.4	11881	59	12268	51.4	6488	59.7	16691	35.8	6240	41.1	11560
NKp46+CD62L- IFNg	12.4	341	43.6	2173	35.5	370	32	3641	28.9	322	31.6	8262	17.7	1258	13.3	922